Development of Liquid Chromatographic and Mass Spectrometric Methods for the Identification of Chlorinated Triacylglycerols in Natural Oils

by

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DEDICATION

To my beloved city Benghazi (Elaseah)

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ABSTRACT

There is a growing concern about halogenated organic compounds, in particular organochlorines, entering the food chain and causing adverse health effects to humans. Known persistent halogenated anthropogenic substances, such as PCBs and DDT, account for only 10-15% of the total. The remaining 85-90% may contain chlorinated triacylglycerols (Cl-TAGs) which have been investigated to some extent using gas chromatography (GC). Progress in this research area is rather limited due to the lack of appropriate reference samples and reliable methods.

The main objective of this thesis project was to develop analytical methods for the characterization of Cl-TAGs in natural oils, particularly corn and salmon oils. Since no certified Cl-TAGs were commercially available, our attention was first focused on their synthesis. Four new Cl-TAGs were synthesized using enzyme-catalyzed and carbodiimide-mediated coupling reactions. They were then characterized by ESI(+)/MS and NMR techniques.

An untreated commercially available corn oil sample was studied extensively and 25 natural TAGs were identified in it by eight methods developed in this thesis. These methods are: GC/MS, both off-line and on-line 2D Ag-HPLC and RP-HPLC, off-line 2D RP-HPLC/GC/MS, off-line 2D Ag-HPLC/ESI(+)/MS, off-line 2D RP-HPLC/ESI(+)/MS, and a new off-line 3-D Ag-HPLC/ESI(+)/MS/GC/MS. In addition, 10 oxidized TAGs were identified in the corn oil sample by an off-line RP-HPLC/ESI(+)/MS method. These oxidized TAGs have not yet been reported in the literature. The corn oil was then selected as a representative oil. It was spiked with four Cl-TAGs synthesized in this thesis and then studied using the off-line RP-HPLC/ESI(+)/MS method.

Among the eight methods developed to study corn oil only two, namely GC/MS and RP-HPLC/ESI(+)/MS, were applied to characterize TAGs in raw farmed salmon skin and tissue oils. The off-line RP-HPLC/ESI(+)/MS method was successfully used to identify 88 TAGs in the salmon skin oil and 81 TAGs in the salmon tissue oil. This is first time TAGs in raw farmed salmon skin and tissue oils have been directly identified. Attempts were also made to identify Cl-TAGs in the salmon oil. Only four TAGs, so far unknown, were identified using the off-line RP-HPLC/ESI(+)/MS method. Doubly unsaturated fatty acids of C16 and C18 (*e.g.,* 3 and 4) were detected in the CID spectra but not by GC/MS. No information on these fatty acids is available in the literature.

LIST OF ABBREVIATIONS USED

APCI/MS	atmospheric pressure chemical
AED	atomic emission detector
ALL	TAG-C20:0/C18:2/C18:2
ALO	TAG-C20:0/C18:2/C18:1
ALP	TAG-C20:0/C18:2/C16:0
ESI/MS	ionization mass spectrometry
BF_3-MeOH	boron trifluoride-methanol
C16:0	plamitic acid
C18:0	stearic acid, octadecanoic acid
C18:1	oleic acid, octadecenoic acid
C18:2	linoleic acid, octadecadienoic acid
C14:0	myristic acid, tetradecanoic acid
CID	collision- induced dissociation
CI	chemical ionisation
Cl_TAG	chlorinated triacylglcerol
ClFAME	chlorinated fatty acid methyl ester
Cl-FA	chlorinated fatty acid
DAG-C16:0	dipalmitin
DB	double bond
DDT	dichlorodiphenyltrichloroethane
DHA	docosahexaenoic acid
DUSR	dalhousie university SLOWPOKE-2 reactor
ECD	electron capture detector
EI	electron ionization
ESI	electrospray ionization
ELCD	electrolytic conductivity detector
ELSD	evaporative light scattering detector
EOX	extractable organic halogen
EPA	eicosapentaenoic acid

EPS	Canadian Environmental Protection Service
FFA	free fatty acid
FAME	fatty acid methyl ester
FABE	fatty acid butyl ester
FAEE	fatty acid ethyl ester
FID	flame ionization detection
LLE	liquid/liquid extraction
LLL	TAG-C18:2/C18:2/C18:2
LLLn	TAG-C18:2/C18:2/C18:3
LLO	TAG-C18:2/C18:2/C18:1
LLS	TAG-C18:2/C18:2/C18:0
LOLn	TAG-C18:2/C18:1/C18:3
LnOLn	TAG-C18:3/C18:1/C18:3
LOO	TAG-C18:2/C18:1/C18:1
LSS	TAG-C18:2/C18:0/C18:0
GC	gas chromatography
HCH	hexachlorocyclohexanes
HPLC	high performance liquid chromatography
HTGC	high temperature gas chromatography
IARC	International Agency for Research on Cancer
INAA	instrumental neutron activation analysis
m/z	mass/charge ratio
MS	mass spectrometry
MAG-C18:1	1-oleoyl-glycerol
NAA	neutron activation analysis
NICI	negative ion chemical ionization
OC	organohalogen compounds
OCl	organochlorine compounds
OBr	organobromine compounds
OOO	TAG-C18:1/C18:1/C18:1
OLnO	TAG-C18:1/C18:3/C18:1
OLnLn	TAG-C18:1/C18:3/C18:3
PBBs	polybrominated biphenyls

PCB	polychlorinated biphenyl
PCAs	polychlorinated alkanes
PBDEs	polybrominated diphenyl ethers
PBBs	polybrominated biphenyls
PCDD/Fs	polychlorinated dibenzo-p-dioxins and polychlorinated furans
PICI	positive ion chemical ionzation
PLE	pressurized liquid extraction
PLs	phospholipids
PLL	TAG-C16:0/C18:2/C18:2
PLO	TAG-C16:0/C18:2/C18:1
POP	TAG-C16:0/C18:1/C16:0
POO	TAG-C16:0/C18:1/C18:1
PUFAME	polyunsaturated fatty acid methyl ester
RP-LC	reversed phase liquid chromatography
SIM	selective ion monitoring
SPE	solid-phase extraction
SFE	super critical fluid extraction
SLO	TAG-C18:0/C18:2/C18:1
SOS	TAG-C18:0/C18:1/C18:0
SOO	TAG-C18:0/C18:1/C18:1
TAG	triacylglycerols
TAG-C16:0	glyceryl tripalmitate
TAG-C18:1	glyceryl trioleate
TAG-C18:2	glyceryl trilinoleate
TAG-C18:0	glyceryl tristearate
TAG-C8:0	glyceryl trioctanotate
TOF	time-of-flight
TIC	total ion current
TLC	thin layer chromatography
u	atomic mass unit
USEPA	United States Environmental Protection Agency
USLE	Ultrasonic solid-liquid extraction

UV	ultraviolet
XFAs	halogenated fatty acids
XSD	halogen sensitive detector

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CHAPTER 1

Introduction

Organohalogen compounds (OCs) are organic compounds where the carbon atom is covalently bonded with a halogen atom. The term extractable organohalogen (EOX) is used when the OCs are extracted from a sample with an organic solvent. Sometimes terms such as organochlorine (OCl) and organobromine (OBr) compounds are used to designate the particular halogen atom under consideration. The OCl and extractable OCl (EOCl) compounds in marine samples have recently been investigated extensively. In this thesis emphasis has been placed on the characterization of EOCl compounds in natural oil samples.

The OCs in the hydrosphere, biosphere and atmosphere are widely considered as major environmental contaminants because of their toxicity and carcinogenicity. Examples of typical OCls in the environment include: polychlorinated biphenyls (PCBs), polychlorinated alkanes (PCAs), polychlorinated dibenzo-*p*-dioxins and polychlorinated furans (PCDD/Fs), dichlorodiphenyltrichloroethane (DDT), and hexachlorocyclohexanes (HCH). Several OBrs such as polybrominated diphenyl ethers (PBDEs), polybrominated biphenyls (PBBs), and halogenated fatty acids (XFAs), are also considered as environmental pollutants.

Detection, identification and measurement of EOX compounds are of much interest to analytical and environmental chemists as well as to toxicologists. The EOCl compounds account for most of the extractable organohalogens (EOX) in marine fish; only 10-15% of the EOCl has so far been identified [Kleijn *et al*., 2001]. The remaining 85-90% EOCl in fish lipids might contain chlorinated fatty acids (Cl-FA) which was investigated using gas chromatography (GC) with halogen-selective or electrolytic conductivity detector [Wèsen *et al.,* 1992; Bjorn *et al*., 1998; Mu *et al.,* 1996].

Because of their chemical structures, OCs persist in the environment for a long time and are classified in the group of persistent organic pollutants. Since OCs are lipophilic, they build up in fatty tissues and can accumulate in humans from fat-containing foods such as meat, fish and milk. The OCs have been found in most of the aquatic organisms, mainly in the storage compartments or membrane lipids [Endo *et al*.; 2011]. The incorporation of OCs into the membrane lipids can induce changes in membrane structures and may have severe effects on the membrane fluidity [Bratberg *et al*., 2013]. Several *in vitro* experiments have shown the effects of polynuclear aromatic

hydrocarbons (PAHs) [Engelke *et al*., 1996; Korchowiec *et al*., 2008], lindane [Suwalsky *et al*., 1998], DDT [Antunes-Madeira and Madeira, 1990; Bonora *et al*., 2008] and PCBs [Tan *et al*., 2004; Campbell *et a*l., 2008] on membrane fluidity.

Over the last few decades the scientists have focused on the effects of OCs in the environment and their contribution to several diseases. Some OCs have been regulated by agencies such as the United States Environmental Protection Agency (USEPA), the Canadian Environmental Protection Service (EPS), and the International Agency for Research on Cancer (IARC). The OCs can be found in every region of the world: close to home in our pet dogs and cats [Mizukawa *et al*., 2015], far from home in the Arctic polar bear [Sonne *et al*., 2005; 2012], and in our body [Cohen, 2016].

Many diseases are associated with OCs. For example, increased levels of thyroid hormones in humans have been correlated with high levels of PCBs and dioxin [Maervoet *et al.,* 2007]. Type 2 diabetes in rats has been detected with high serum levels of PCBs and dioxin [Airaksinen *et al*., 2011; Lind 2012; Thayer *et al*., 2012]. The interaction of OCs with human hormone systems and their effects on cognitive, endocrine and immune functions have been reported [Crinnion, 2011]. Mocareli *et al.* [2000] and Ryan *et al*. [2002] showed that higher prenatal dioxin levels in mothers resulted in significantly more girls than boys being born. Most recently, Dwan *et al*. [2013] concluded that exposure to organochlorine pesticides in pregnant women may be important for fetal growth restriction and size of a baby.

In addition to their toxicity some OCs are considered carcinogenic. The relations between PCB exposure and breast and thyroid cancers [Recio-Vega *et al.*, 2011; Helmfrid *et al*., 2012] and between DDT and breast cancer have been reported [Barbara Cohn, 2011]. Lung and liver cancers have been associated with exposure to dioxins such as TCDD [Cole *et al*., 2003; Steenland *et al*., 2004; Warner *et al*., 2002]. Adverse health effects including reproductive [Meeker *et al*., 2011], developmental [Park *et al*., 2010], immunologic [Glynn *et al*., 2008], and neurologic [Faroon *et al.*, 2000] have been correlated with high levels of PCBs and dioxins in human blood. In addition, PBDE and their metabolized hydroxylated PBDEs (OH-PBDEs) and methoxylated PBDEs (MeO-PBDEs) have been reported to disrupt sex hormone and cause neurotoxicity [Gutleb *et al*., 2010; Ucan-Marin *et al*., 2010; Eguchi *et al.,* 2012]. The Cl-FAs have strong inhibitory effects on cell growth and can be carcinogenic [Hmtmark *et al.,* 1998, 1999; DeAngelo *et al.* 1996; Ewald, 1999]. The exposure to toxic mixtures of OCs and their effects on organs and physiology of the body are covered in a book by Zeliger [2011].

Recently, Robertson and Hansen [2015] reviewed the recent advances in environmental toxicology and the health effects of OCs.

It is evident from the above discussions that anthropogenic OCs are abundant in the environment. The OCl compounds are of particular interest because of their stability, toxicity and carcinogenicity. It is therefore necessary to develop methods to detect, identify and measure OCls in a given sample. A detailed literature survey of the work reported so far has been reviewed in Chapter 2. Briefly, the OCls are classified to two groups depending on their source: natural or anthropogenic sometimes called unnatural. Most of the anthropogenic OCls are synthetic and found in the environment in significant amounts largely through leakage, disposal, evaporation and emission from industrial and agricultural activities [Tolosa *et al*., 2010]. The natural OCls, on the other hand, are produced naturally, as the name implies, by mostly marine and some non-marine but natural species [Gribble *et al*., 2015].

Food is the main source of OCls in humans [Aylward *et al*., 2002]. Among different food groups, seafood in general and salmon in particular, has recently become very popular in many countries for a variety of reasons [Yagi *et al*., 2015]. Salmon is an important source of omega-3 and omega-6 polyunsaturated fatty acids (PUFAs), namely eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), which play a preventive role in cardiovascular diseases and other health problems [von Schacky, 2009; and Lavie *et al*., 2009, Swanson *et al.*, 2012, Hara *et al*., 2013; Nishizaki *et al*., 2014; Yagi *et al*., 2015].

Salmon (*Salmo salar*) is the common name for several species of fish of the family Salmonidae (*e.g.* Atlantic salmon, Pacific salmon) while other species in the family are called trout. Salmon species are naturally available in the wild and are also raised in salmon hatcheries (called farmed salmon). Salmon stocks are found in both Atlantic and Pacific Oceans, as well as in the Great Lakes (North America) and other land-locked rivers; and about CAD11.2 billion per year of salmon is consumed by Canadian population groups [statistics Canada, web, 2103]. Canada is the fourth-largest producer of farmed salmon in the world behind Norway, Chile and UK. British Columbia, New Brunswick, and Newfoundland and Labrador produced almost 116,000 tonnes of farmed salmon representing 95% of total salmon produced in Canada in 2013. Atlantic salmon is Canada's top aquaculture export in 2013. Almost all commercially available Atlantic salmon is farmed. Therefore, it can be concluded that farmed salmon is a very important fisheries industry for not only Atlantic Canada but also the whole country.

In 1975, Lunde and coworkers [Lunde and Steinnes, 1975; Lunde *et al.*, 1976] used neutron activation analysis (NAA) to measure EOCl content of some marine fish oils and found that their levels were 1.5 to 5 times higher than that of PCBs or DDTs determined by gas chromatography (GC) and that 5 to 50% of the chlorine remained after treatment with concentrated sulphuric acid. It has also been reported in a Canadian project by Newsome *et al.*, [1993], using NAA and GC, that between 74% and 99% of EOCl in several species of fish cannot be accounted for by PCBs and chlorinated pesticides. In a literature review, Boyd [1993] concluded that about 85% of chlorine in fish and 95% of chlorine in the sediments cannot be accounted for by known OCs. Several studies have then reported relatively high levels of OCls in farmed salmon [Hites *et al*., 2004a, b, c; Hamilton *et al*., Mu *et al*., 2004; King *et al*., 2006; Nøstbakken *et al*., 2015]. The high levels of OCls coupled to increased global consumption of farmed salmon have raised public health concerns [Shaw *et al*., 2008]. A study of Atlantic salmon from different locations has shown different OCl profiles [Svendsen *et al*., 2008]. The salmon is exposed to OCls mostly through its food and also by direct contact with polluted water [Scott, 2001]. We have calculated that a normal portion of salmon (about 85 g) should be eaten once every 6 months if its OCl level is 10-15% but once every 10 years if the level is 85-90%, considering adverse health effects. So it is very important to study OCls, in particular TAGs, Cl-TAGs and CL-FA, in farmed Atlantic salmon.

Farmed Atlantic salmon (*Salmo salar*) was selected in this thesis for studying its OCls for several reasons. Its commercial and potential health risk importances are stated above. This salmon species can live for more than 5 years thereby significantly increasing potential bioaccumulation of OCls. Canadians generally consume large amounts of salmon. Finally, there is very little information reported in the literature on OCls in Atlantic salmon.

In order to detect, identify and measure OCls in salmon two main features need to be considered, namely separation and detection. Separation can involve simple extraction followed by transesterification, GC and/or HPLC. A detailed literature survey of these techniques is given in Chapter 2.

One of the most important steps in separation is the method of extraction of OCl from its matrix. The best extraction procedure should quantitatively extract lipids and lipophilic pollutants without contamination and changing the composition of the original components. Soxhlet extraction is the traditional method to obtain EOCl from biological samples with good efficiency as well as recovery. However, this method is time-

consuming and requires large amount of organic solvents. Extraction using a Polytron is preferable for fish muscles because it homogenizes and sonicates the sample simultaneously resulting in better extraction (Newsome, 1993). It is also necessary to select a chlorine-free solvent system so that the chlorine from solvent does not interfere with the OCls in the sample. Several solvent systems of different compositions have been evaluated by our group [Newsome *et al.*, 1993; Kiceniuk, 1998; Bottaro, 1999; Labrada, 2003; Bahroun, 2007]; these include: ethyl acetate/methanol/hexane/water, acetone/hexane, acetone/toluene, acetone/petroleum ether, acetone/cyclohexane, isopropanol/hexane, and ethyl acetate/hexane. All of these solvent syetems were tested by our group for contaminants such as chlorine, bromine and iodine, and procedural blanks were estimated. The solvent system which gives the minimum procedural blank, highest lipid extraction efficiency and concentration of EOCl was found to be acetone/hexane (1:1 v/v) [Bottaro, 1999]; therefore, it was selected for extraction of OCl from the farmed salmon sample in this thesis. Several extraction techniques have been developed and reviewed [Tang, 2013]. The extract may need to be cleaned. Washing of the extract is important to ensure that no inorganic chloride ions are transferred from the solvents. Bottaro [1999] used sea salt to demonstrate the efficacy of her washing method involving two steps: one with 0.9% KNO_3 and 0.1% $(NH_4)_2HPO_4$, and the other at pH 2 with sulfuric acid, and concluded that no added Cl could be detected by NAA after washing.

The detection of Cl in OCl compounds was considered the second important item for the characterization of OCl in TAGs and Cl-FA. Neutron activation analysis is a well-established analytical technique which allows simultaneous and qualitative as well as quantitative determination of many elements in various matrices with high precision and accuracy. It has been extensively used for the measurement of total amount of EOCl in biological and environmental samples. Previous work in the Chatt group using instrumental neutron activation analysis (INAA) has shown that northern pink shrimp contained approximately 160 µg Cl [Bottaro, 1999], cod brain had 50 µg chlorine [Labrada, 2003], and salmon muscle had 30 µg chlorine [Bahroun, 2007] per gram of lipid. The nature of the detection problem can be best illustrated by a simple calculation. If one assumes that the lipid is pure glyceryl tripalmitate, the molecular weight would be 807 g mol^{-1}. One gram of lipid would correspond to 1.24 mmoles and 50 µg of chlorine would correspond to 1.41 µmoles of chlorine or about 1 atom of chlorine per 1000 molecules of lipid or 1 atom of chlorine per 3000 fatty acid molecules. This value is

below the detection limits of INAA under the experimental conditions used. Moreover, the Dalhousie University SLOWPOKE-2 reactor (DUSR) facility was decommissioned in 2011. For this reasons, INAA was not applied in the present thesis. Anpther disadvantage of NAA is that it does not provide any information on the compounds present in the sample. In order to get this kind of information several techniques need to be applied, such as NMR and MS.

The Chatt group [Bottaro, 1999; Labrada, 2003; Bahroun, 2007] further investigated the possible structures of OCl compunds using both NMR and MS. The ^{1}H and ^{13}C NMR spectra were dominated by the signals from triacylglycerols which agreed with TLC and MS results. In addition, MS/MS spectra of the samples provided some information about the composition of the triacylglycerides present in the samples. The biggest advantage of their studies was the identification of some chlorine-containing compounds in TAGs. However, individual Cl-TAGs could not be identified. This was perhaps due to the complexity of the samples which required further separation by GC and HPLC and poor sensitivity of the older MS instruments used.

Highly efficient methods are needed to separate OCls. The most widely used method is GC because most of the OCls are non-polar, thermally stable and relatively volatile that can be determined by GC [van Leeuwen and de Boer, 2008]. However, due to the poor volatility of some compounds, derivatization steps aimed to produce more volatile products are required to improve the sensitivity of subsequent GC analysis. However, loss of sample and formation of additional interfering compounds and metabolites can take place during the derivatization step. For this reason, GC is partially substituted with LC for some OCls. Several GC and HPLC methods were developed in this thesis for the separation of OCls. The most common detection technique used for studying OCls is MS often coupled with either GC or HPLC. GC/MS is most widely employed using electron ionization (EI) or chemical ionization (CI) modes [van Leeuwen and de Boer, 2008].

During the last 10 years, HPLC/MS has gained popularity due to its high sensitivity and increased scope of the determination of non-volatile and thermally labile molecules without derivatization [Lebedev, 2012; Tang, 2013]. Both electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) are being used extensively in OCls [Lebedev, 2012; Hird *et al*., 2014]. The HPLC/ESI(+)/MS methods were developed in this thesis.

The overall objective of this thesis was to characterize extractable organochlorine compounds (EOCls) in a farmed salmon sample. In order to attain the overall objective, a series of smaller objectives given below had to be met first.

The first set of objectives was to develop methods for the characterization of FAs in samples by GC/MS. Commercially available corn, canola, grape seed, and salmon oils were selected for this work. The samples were first converted to their fatty acid methyl esters (FAMEs) using the BF_3-MeOH method. For the identification of the fatty acids in the oil samples; GC/MS methods were then developed (Chapter 3).

The second set of objectives was to develop reversed phase HPLC (RP-HPLC) methods for the separation of TAGs according to their partition number (PN) in the above oils, and to develop silver phase HPLC (Ag-HPLC) according to the number of double bonds (DB) in them (Chapter 3).

The third set of objectives was to identify an oil sample which could be spiked with Cl-TAGs. The corn oil was chosen as the representative oil for studying Cl-TAGs because it has been characterized more than any other vegetable oils by several researchers and has well-documented TAGs. Seven two-dimensional separation/ detection methods were developed and optimized for the characterization of the TAGs in corn oil. These methods included: RP-HPLC and Ag-HPLC (both on-line and off-line), RP-HPLC/GC/MS, RP-HPLC/ESI(+)/MS and Ag-HPLC/ESI(+)/MS. In addition, one three-dimensional detection method, namely Ag-HPLC/GC/MS/ESI(+)/MS, was developed (Chapter 4).

In order to identify Cl-TAGs in farmed salmon sample it was necessary to have Cl-TAG standards of known composition and structures. However, no such standard was commercially available. The fourth set of objectives was therefore to synthesize 6 new Cl-TAGs by enzymatic and chemo-enzymatic methods and to carry out a characterization of their structures using NMR and ESI(+)/MS. The corn oil was then spiked with these Cl-TAG standards and studied both qualitatively and quantitatively. The methods developed were then applied to identify TAGs in farmed salmon skin and tissue oils. Attempts were also made to identify Cl-TAGs in the farmed salmon oil sample using ESI(+)/MS (Chapter 5).

CHAPTER 2

Literature survey

This chapter gives a brief review of the literature on organohalogen compounds (OCs) in environmental samples. There are diverse types of OC but, of necessity, we have restricted this review to chlorinated fatty acids (Cl-FA) in the environment and their characterization methods. One of the objectives of this thesis was the synthesis of chlorinated triacylglycerols (Cl-TAG); therefore, the literature on the synthetic methods for the preparation of triacylglycerols and their identification and characterization methods are described here.

2.1. Classification of organohalogen compounds (OCs)

Many organohalogen compounds (OCs) have been detected in the environment for several decades following their extensive use in the 20th century up to restrictions and bans in most countries in the 1970s and 1980s. These can be classified in two ways. The most appropriate one is to group them in terms of the halogen atoms such as, chlorine, bromine, and iodine. Another classification method is to group them depending on their original sources whether natural or anthropogenic.

2.1.1. Anthropogenic sources of OC

The anthropogenic sources of OC have recently been the subject of intense research. Almost all OCs in use today are synthetic. Both industrial and agricultural sources have contributed significant amounts to the environment through leakage, disposal and evaporation [Zhao *et al*., 2015]. The synthetic OCs, particularly polyhalogen compounds, are used as pesticides, cleaning solvents, aerosol propellants, refrigerants, polymers, and so on.

Approximately 30,000 tons of PCBs were released to air, 60,000 tons to fresh and coastal waters, and 300,000 tons to dumps and landfills [HSDB, 2003] before PCB manufacturing was banned in the U.S. in 1970. The concentrations of PCBs in human serum samples were reported to depend on their concentrations in foods consumed in Norway [Rylander *et al*., 2009], Spain [Porta *et al*., 2010], UK [Thomas *et al*., 2006], Sweden [Schecter *et al.,* 2010], and Canada [Haines and Murray, 2012]. Also PCB levels varied from country to country, *e.g.* their levels in a Swedish population group were higher than that of American [Department of Health and Human Services, Centers

for Disease Control and Prevention, 2009] and Canadian groups [Haines and Murray, 2012]. This trend may be explained by the historical usage of PCBs in these countries.

Many of the chlorinated hydrocarbons such as DDT, Chlordane, and Lindane have been used extensively as insecticides, and now are partially banned because of growing concern about their extreme chemical and thermal stability (*e.g.* non-polar and low volatility). Once the OCs are introduced to the environment they remain there for years or even decades. When the OCs enter the hydrosphere, they are taken up by organisms and fish. The OCs accumulate in the fatty tissue of these organisms and can be transferred to humans through the food chain. A strong association therefore exists between the fat intake from fish and OCs in the human body. This relationship has been observed in many populations [Deutch *et al* ., 2004; Bergkvist *et al*., 2008, Brauner *et al*., 2011, Gasull *et al.,*2011; Rylander *et al*., 2009]. In addition, they are persistent and can be transported globally in the environments which are cited as reasons for their presence in Northern Canada and in Arctic polar bears, as both are far from point sources of PCB contamination [Fiedler, 1996]. The existence of OC contamination in the Antarctic ecosystems was first reported in 1966, and since then it has been growing [George and Frear, 1966]. The Arctic has been reported to be the final sink for persistent organic pollutants [Salvado *et al*., 2016; Pernilla Carlsson, 2013]. The literature on the types of anthropogenic OCs and their possible sources is presented in Table 2.1.

Table 2.1. A literature survey on the type of anthropogenic OCs and their sources.

Category	Common compounds	Source	Reference
Halogentated Biphenyls	PCBs	Manufacturing facilities and equipment such as hydraulic fluid, brake fluid, recycled oil, paper ink Leakage from PCB-filled transformers and capacitors	Wolska *et al*.,2012
	PBBs	Municipal and industrial solid waste (Landfills)	Alaee *et al.,* 2003
	PBBs	Flame retardants production waste incineration fossil fuel combustion Incineration of municipal hazardous waste sewage sludge) Bleaching process of paper and pulp	Harrad, 1996
Halogenated fatty acids	Cl-FA Br-FA	Pulp mill effluent Bleaching and disinfection of foodstuffs Disinfection in municipal sewage plants and water	LeBel *et al*., 1997
Halogenated pesticides/ herbicides	DDT, Dieldrin, Chloradane	Wood preservatives Pesticide formulations	Jorgenson, 2000
Halogenated aromatics	Chlorinated phenol	Municipal incinerators Electrochemical industry waste products Domestic and water waste Fluid in heat transfer Wood manufacture as anti-stain	Igbinosa *et al*., 2013
Halogenated alkanes	Chlorinated paraffin halogenated methane and ethane	Solvents and chemical in industry and chemical manufactures formulations in products such as paints, sealants, plastics and metalworking fluids	Zhoa *et al* ., 2013
Halogenated polycyclic aromatics	Chlorinated terphenyls, chlorinated naphthalene	Indoor-outdoor air Emission sources include vehicle exhaust, evaporated gasoline, cigarette smoke, moth and pest repellants, and deodorizers (e.g., diaperpail and toilet	Batterman *et al*., 2012
Halogenated heterocyclic compounds	Chlorinated /brominated thiophenes	Incineration Bleaching process in pulp and paper manufactures Car exhaust	Sinkkonen, 1997

2.1.2. Natural Sources of OCs

Many of the OCs found in the environment are believed to be produced in nature. The first review of the naturally occurring OCs published in 1968 cited about 50 compounds [Fowden, 1968]. A review published in 1973 documented more than 200 OCs [Siudan and DeBerriands, 1973]. Gribble [1992] reported more than 2000 naturally occurring OCs and the number is still growing. Recently, he published an extensive summary of his previous surveys in a book [Gribble, 2010). Most recently the same author [Gribble *et al*., 2015] listed nearly 5000 OCs from different marine and non-marine sources.

It has been suggested that the main reason for the natural production of OCs is the defence against predators. For example, epibatidine and chlorinated alkaloids isolated from the Ecuadorian poison frog (*Epipedobates tricolor*) [Richard *et al*., 2010]. Another example is the worm (*Notomastus lobatus*) which lives in deep ocean sediments and has high concentration of bromophenols in its tail; since it lives head down in the sediment, its tail will be the first defence against a potential predator [Yoon KS ,1994]. Other OCs have been reported to show some anti-fungal properties; a spectacular example of it is the Japanese lily *Lilium maximowiczii* that produces seven chlorinated fungicides in response to attack by the pathogenic fungus *Fusarium oxysporum* [Monde *et al*., 1998].

There are many examples of positive and beneficial effects of OCs on organisms, and excellent reviews on this topic are available [Smith, 2004; Nawman, 2003, 1999]. For example, hormones thyroxine and triiodothyronnine are the organoiodine compounds that are produced by the thyroid gland which control metabolism in our body [Lyn Patrick, 2008]. Some OCs produced naturally by marine species are shown to have great potential for the treatment of human diseases [Mayer and Hamann, 2004; Smith, 2004], against cancer [Provencio *et al*., 2009,), inflammation [Keyzer *et al*., 2005; Jaswir and Monsur, 2011], and malaria [El Sayed *et al*., 1996].

It has often been traditionally difficult to determine whether given OCs are actually natural or anthropogenic and to attain some understanding of their carbon source. The ^{14}C radiocarbon analysis is of major assistance in this problem. Reddy and his colleagues [Reddy *et al*., 2002, 2004] suggested a possibility of distinguishing the anthropogenic OCs (low or no^{14}C content) from the naturally occurring ones (with high^{14}C content). By mid-1990s, improvements of both MS and GC allowed these

techniques to measure ^{14}C concentrations in OCs of recent origin [Woudneh *et al*., 2006].

2.2. Methods of solvent extraction

The extraction of OCs is one of the most challenging tasks in analytical chemistry. A number of extraction techniques has been developed and applied successfully for the separation of OCs from a variety of matrices. Solid-liquid extraction (SLE) such as in soxhlet has been widely used for extraction of the OCs from solid samples (*e.g*., sediment and biota). Since it requires relatively large volumes of solvents and is time-consuming, it is considered as environment-unfriendly.

Another solid-liquid extraction technique is pressurized liquid extraction (PLE) [Toledo-Neira *et al*., 2013]. The lyophilized (freeze-dried) sample is placed in a container filled with solvents then high pressure and temperature are applied. The increases in pressure and temperature significantly speed up the extraction without sacrificing extraction efficiency. Super critical fluid extraction (SFE) has also been used as a solid-liquid extraction technique [Calvosa and Lagalante, 2010]. The SFE offers a greener extraction procedure by using non-toxic gases (*e.g*., carbon dioxide) instead of organic solvents.

Ultrasonic solid-liquid extraction (USLE) is another option for fast extraction of solid samples using a high-energy ultrasound. A solid sample is dispersed and disaggregated into very fine pieces in the extracting solvent and leads to increase in the samples contact with the solvent and thus facilitate the extraction. In most cases, the extraction could be finished within minutes to half-an-hour. The lower solvent requirement is one of the advantages [Martinez-Moral and Tena, 2013].

The liquid/liquid extraction (LLE) technique is widely used. Because large volumes of extracting solvents are generally needed in LLE, different microextraction techniques have recently been developed. Microextraction is achieved by using a small amount of non-polar solvents finely dispersed with a suspension of a single drop formed on a needle tip in a water sample [Rezaei *et al*., 2008]. The dispersion is step assisted by stirring, sonication, or vortex mixing.

The solid-phase extraction (SPE) is well known as a traditional extraction method for OCs in water samples using non-polar or ion-exchange absorbents [Zeng *et al*., 2012]. Various types of solid phase extraction (SPE) cartridges used include C18, silica and ion exchange materials.

The liquid-solid extraction can be carried out using Soxhlet, shake-flask, a homogenization and sonication. The homogenization and sonication using Polytron proved to be much more effective in extracting OCs, probably because a Polytron homogenizes the sample, both mechanically and sonically. Reviews of extraction techniques for environmental samples have been published [Tang, 2013]. In this thesis homogenization and sonication extraction procedure was used by applying a Polytron apparatus. Two members of our research group Bahroun [2007) and Bottraro [1999) have applied this technique successfully to extract OCs from Salmon and shrimp samples, respectively.

2.3. Methods of analysis

Analytical chemists have been working for over 40 years to develop a wide range of analytical methodologies for studying environmental pollutants (*e.g.* organohalogen compounds) and monitoring their presence in the environment. GC was one of the first chromatographic techniques to be developed and the most widely used for the analysis of OCs in samples such as water, soils, sludge, air, fish, *etc*. [Oros *et al*., 2012]. Most organohalogen contaminants are non-polar, thermally stable and relatively volatile that can easily be determined by GC. In addition, GC has also been applied to the analysis of non-thermal stable and polar compounds after derivatization. The popularity of GC is mainly because of its very high selectivity and resolution. GC/MS is most widely used, mainly using electron ionization (EI) and chemical ionization (CI) methods [van Leeuwen and de Boer, 2008].

One important feature of EI lies in its high reproducibility. A number of electron ionization mass spectral libraries are commercially available, such as the Wiley library with 275000 spectra and the NIST library with 230000 spectra, which makes mass identification in EI rather easy [van Leeuwen and de Boer, 2008]. Moreover, in recent years HPLC coupled to tandem MS has become a powerful tool for separation and detection of many OCs by allowing the determination of non-volatile and thermally labile molecules without derivatization. It has thus become a preferable method for the analysis of OCs [Tang, 2013]. Because of these advantages, HPLC/MS and GC/MS were both used in this thesis work.

A large number of papers has been published on the analysis of OCs in environmental samples by GC/MS. Selected examples of recent review articles include the analyses of vegetation [Beceiro-Gonzalez *et al*., 2012], soil [Tadeo *et al*.; 2012],

sewage sludge [Tadeo *et al*.; 2010], water [Tankiewicz *et al*.; 2011], food [Sharma *et al.*, 2010], air [Kosikowska *et al*.; 2010], and human blood [Salihovic *et al*., 2013]. In these articles an overview and current status of sample pretreatment, extraction, cleanup, chromatographic separation, detection methods, quality control, and method validation are discussed. Further development in the analysis of OCs is mostly focused on the reduction of sample handling time. The need for fast and on-site analysis of OCs is increasing rapidly whenever health and safety are at risk. That has led to a continuing effort to make GCs smaller and more portable. Reviews covering the application of portable MS to the analysis of OCs in environmental samples have been [Eckenrode, 2001].

2.4. Chlorinated Fatty Acids (Cl-FA)

Extractable organochlorine compounds (EOCl) account for most of the extractable organohalogens (EOX) in marine environment; only 10-15% of EOCl has so far been identified [Kleijn *et al*., 2001]. The remaining EOCl in fish lipids might be chlorinated fatty acids (Cl-FA). Because of several treatment and clean-up steps applied in the past for measuring OCl to fish samples, it is possible that some chlorinated fatty acids (Cl-FA) so far went unnoticed.

The chlorinated fatty acids (Cl-FA) were confirmed experimentally by Wesen *et al.* (1992, 1995) in eel lipids using GC with electrolytic conductivity detector. They found that the hydrophilic lipids of eel had a high concentration of EOCl (>1200 µg/g lipid) and that chlorine was associated with Cl-FA represented up to 90% of the EOX. This eel sample was used to develop methods for the identification of Cl-FA in fish with lower levels of EOCl. Literature on the subject of Cl-FA in marine environment identifies pulp and paper industry as the main anthropogenic source where large amounts of chlorine are used for bleaching. Agricultural pesticides and combustion of waste materials also contribute.

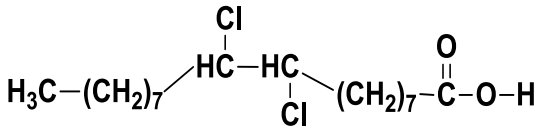
Cl-FAs have been found in both bleached flour and food [Heikes *et al*., 1992, 1993]. The flour manufactures in the western countries have applied Cl bleaching process in order to improve baking characteristics [Gough *et al*., 1978]. Many studies involving bleached flour detected mono-, di-, tri- and tetra-chlorinated fatty acids depending on the chlorine level. Several chlorinated octadecanoic acid (C18) were detected in US food products containing bleached flour. Moreover, trace amount has been detected in meat and poultry after treatment with chlorinated water to counteract

bacterial contamination. Cl-FA has also been detected in drinking, sewage and swimming pool water samples, and in household cleaning products [Jolley *et al.,* 1975; Gibson *et al*., 1986].

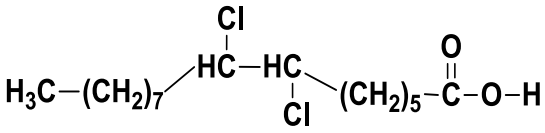
A number of chlorinating agents is used in pulp and paper industry [Komosuwelack *et al*., 1983a, b, and c]. About 3.5 million metric tonnes of chlorine was used in 1996 [for the world production of approximately 50 million tonnes bleached pulp, which in turn released 300-9500 tonnes of dichlorostearic acid ($C14:Cl_2$) to the environment [Johnston *et al*., 1996]. Canadian mills alone are estimated to use over 610 000 tonnes of chlorine annually to produce over 10 million tonnes of bleached pulp and over a million tonnes of OCs is released to the aquatic environment [The Canadian Environmental Protection Act (CEPA), 1991].

The Cl-FAs found in the aquatic environment and food chain raised an increasing concern because of their toxicity and bioaccumulation [Björn *et al*., 1998a; Ewald, 1999; Spickett, 2007]. They tend to be passively stored in the lipids of exposed animals, and cannot to be recognized in cell membrane as undesirable metabolites by organisms (xenobiotic). When present in phospholipids, modified properties of membranes have been reported [Ewald, 1999; Håkansson *et al*., 1991]. Also, Cl-FAs caused apoptotic effects in human cell lines [Lystad *et al*., 2001] and growth inhibition [Björn *et al*., 1998a; Høstmark *et al*., 1999]. Cl-FAs produce moderate toxicity to rat [Cunningham, 1980] and fish [Leach and Thakore, 1977] but high toxicity to sea urchin sperm cells [Cherr *et al*., 1987].

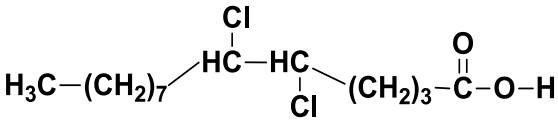
The common procedure for the identification of Cl-FAs is the extraction of lipids followed by transesterification, preconcentration, and finally detection by GC. Using these steps three most common Cl-FAs in fish have been studied in detail (Table 2.2). These Cl-FAs shown in Fig. 2.1 are: 9,10-dichlorooctadecanoic acid (also known as 9,10-dichlorostearic acid), 7,8-dichlorohexadecanoic acid (also known as 7,8-dichloropalmitic acid), and 5,6-dichlorotetradecanoicacid (also known as 5,6-dichloromyristic acid) [Björn *et al*., 1998b; King *et al*., 2006; Milley *et al.,* 1997; Mu *et al*., 1996a; Mu *et al*., 1996b; Mu *et al*., 1997b; Wesén *et al.,*1992; Wesén *et al.,* 1995a; Wesén, 1995b; Zhuang *et al.,* 2003; Zhuang *et al*., 2006] and pentafluorobenzyl [Zhuang *et a*l., 2004].



9,10-dichlorooctadecanoic acid



7,8-dichlorohexadecanoic acid



5,6-dichloromyristic acid

Fig 2.1. Three common chlorinated fatty acids.

The Cl-FAs in the extracted lipids were transesterified to their methylesters. The methylation procedure using BF_3-MeOH involves breaking the glycerol backbone of TAG and converting the fatty acids to fatty acid methylesters (FAME). The advantages of this procedure include easy methylation and identification by comparison with a variety of commercially available FAME standards. However, the disadvantage is that no detailed information can be obtained about the particular location of Cl-FA in the biological base structure (*i.e*., TAG and PL).

Since the concentrations of Cl-FAs are very low, several pre-concentration methods have been used prior to their detection. One such method involves complexation with silver ions to remove unsaturated fatty acids followed by co-crystallization with urea to selectively remove straight chain saturated fatty acids leaving Cl-FAs in solution. Another pre-concentration method involves solid-phase extraction (SPE) to isolate the Cl-FAME from the rest of the un-chlorinated fatty acids. Although the pre-concentration methods allow the use of small sample volumes and improve their detection and identification, these methods have disadvantages because they are lengthy, labor intensive, and expensive due to large amounts of materials used (chemicals, chromatography solvents, SPE cartridges, *etc*.). To the best of our knowledge, the detection of Cl-FAs in the real fish samples since 1990 until today were obtained only after transesterification followed by pre-concentration [Wesen *et al*.,1992; Brown and Järlskog, 2015].

Even with pre-concentration it is not always possible to assay Cl-FAs by most commonly used chromatographic detectors. Detectors highly specific for chlorine have been successfully developed for this reason and used; they are: electron capture detector (ECD), atomic emission detector (AED), mass spectrometer (MS), the electrolytic conductivity detector (ELCD), and the halogen specific detector (XSD). A review of the literature on the above aspects of Cl-FAs are presented Table 2.2.

Table 2.2. A literature survey of separation and determination methods for Cl-FAs in fish and water.

Type of sample	Analytical technique	Type of Cl-FA	pre-concentra methods	References
Drinking water	FAB/GC MS	$C18:Cl_2:OH_2$ C16:Cl:OH		Gibson (1986)
Eel (*Anguillaanguilla*)	GC-ELCD	$C18:Cl_2$	Ag^+ & urea	Wesen *et al.,* (1992)
Fresh water fish (white sucker) (*Catostomus Commersoni*)	GC-XSD GC- MS (NICI)	$C18:Cl_2$ $C16:Cl_2$ $C14:Cl_2$	Bio-Beads SX3	Zhwang *et al.,* (2004)
Fresh water fish (white sucker) (*Catostomus Commersoni*)	GC/MS	DMOX $C14:Cl_2$	Gel permeatio n HPLC	Zhwang *et al.,* (2003)
American lobster (*Homarusa mericanus*)	GC/MS (NICI)	$C14:Cl_2$	Sephadex LH-20	Milley *et al.,* (1997)
Eel*(Anguill aanguilla)*	GC/FID	$C14Cl_4$ $C14Cl_2$ $C16Cl_2$ $C18Cl_2$	Ag^+ Urea	Mu *et al.,* (1996a)
Eel *(Anguillaanguilla)* Flounder *(Platichthys Flesus)* Atlantic salmon (*Salmo Salar)* Perch (*Perca Fluviatilis)* Pacific salmon (*Oncorhynchus Nerka)*	GC/FID GC/ELCD GC/MS (PICI)	$C14Cl_2$ $C16Cl_2$ $C18Cl_2$	Ag^+, Urea, TLC	Mu *et al* .,(1996)
Eel *(Anguillaanguilla)*	GC/MS (EI)	$C18:Cl_2$ $C14:Cl_2$	SPE-NH_2 aminopropyl column	Akesson *et al.,* (2004)
Chinook salmon (*Oncorhynchus tshawytscha*)	GC/MS GC/XSD	$C16Cl_2$ $C18Cl_2$ $C14Cl_2$	SPE-NH_2 aminopro pl column	King *et al.,* (2006)
perch(*Perca fluviatilis*), Lavaret (*Coregonous lavaretus*) Stickleback(*Gaster osteus aculatus*).	GC/XSD	$C18Cl_4$	SPE-NH_2 aminopro pl column	Brown and Järlskog (2015)
harbour porpoise (*Phocoena phocoena*) harbour seal (*Phoca vitulina)*	GC/ELD GC/MS (PICI)	$C16Cl_2$ $C18Cl_2$	Ag^+, Urea, TLC	Clark, 2003

2.5. Triacylglycerols (TAG)

This thesis project involved the identification of Cl-TAGs in natural oils. No Cl-TAG standard was commercially available. It was then necessary to synthesize CI-TAGs and characterize them. The literature on this topic is reviewed below.

2.5.1. Synthesis of Triacylglycerol

Triacylglycerols can be synthesized by enzymatic, chemical, and chemo-enzymatic methods. Mono- and diacyl-glycerols are usually obtained as intermediates during the synthesis. The methods for syntheses of mono-, di-, and tri-acylglycerols have been reviewed [Smith *et al*., 1972; Bhati *et al*., 1980; Jensen *et al*., 1995; Sonnet *et al*., 1999].

2.5.1.1. Enzymatic Method

Lipases (*triacylglycerol hydrolases* E.C. 3.1.1.3) are well known enzymes for the hydrolysis catalysis of TAG to glycerol and fatty acids (FAs). Since the lipase was discovered by Eberle in 1834 and identified by Bernard in 1856 in the pancreas, it became one of the most important classes of enzyme. The use of lipases has been increasing in the oil and fat industry [Hou and Shimad, 2009]. Enzymatic methods using lipase offer several advantages over the chemical methods. Firstly, the lipases are generally used in an immobilized form which improves their stability at different temperatures, pHs, and in organic solvents. Secondly, the immobilized form allows for easy recovery and re-use of the lipase catalyst. Thirdly, the lipases offer the potential of positional selectivity and stereo-selectivity. Lipase region-selectivity provides the ability to distinguish between the acyl chain positions (*i.e. sn*-1,3 and *sn*-2) of the TAG structure. This has been used to incorporate health benefiting fatty acids (*e.g.,* eicosapentaenoic acid (EPA-C20:5) and docosahexaenoic acid (DHA-C22:6) into conventional TAG [Tsuneo Yamane, 2005; Harald Breivik, 2007]. Recently, enzymatic methods of synthesis have been reviewed by Soumanou *et al.* [2013]. Two lipase enzymes, namely, 1,3-*Rhizomucor miehei* and *Candida Antarctica*, were used in this thesis for the syntheses of Cl-TAGs (Section 5.2.3).

2.5.1.2. Chemo-enzymatic method

The chemo-enzymatic method consisting of two steps is generally used to synthesize a specific type of structured TAGs [Halldorsson *et al.,* 2003; Carlos *et al*.,

2010]. In the first step, lipase 1,3-(*Rhizomucor miehei)* and the vinyl ester of a FA react to produce diacylglycerols with the FA at the end-positions (*sn*-1,3) of glycerol. It is then followed by chemical introduction of the fatty acid into the remaining mid-position (*sn*-2) using 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDCl) and 4-dimethylaminopyridine (DMAP). The chemo-enzymatic method has been used to synthesize structured TAGs containing eicosapentaenoic acid (EPA-C20:5) and docosahexaenoic acid (DHA-C22:6) at the *sn*-1,3- positions of the glycerol of a TAG molecule [Haraldsson *et al*., 2000]. The chemo-enzymatic was applied to synthesize Cl-TAGs in this thesis work (Section 5.2.3).

2.5.1.3. Chemical method

The chemical method was first reported by Jensen *et al*. [1976]. He described the preparation of several TAGs by reaction of glycerol with an appropriate acid chloride at 80°C. Another chemical method involved multi-step combination of protection and de-protection of the hydroxyl groups of the glycerol [Fraser *et al.*, 2007; Andrews *et al*., 2008]. This method required chemical coupling agents, namely 1,1-dicylclohexyl-carbodiimide (DCC) and 4-dimethylaminopyridine (DMAP), which permitted the fatty acids to be acylated to hydroxyl groups of glycerol back bone [1987, Lie Ken Jie and Lam, 1995a, 1995b, 1995c]. The usefulness of several protective groups have been reviewed [Gunstone *et al*., 2007]. 1,2-acetonide (Isopropylidene group $Me_2C=$) is a wildly used protective group, and is best removed under acidic conditions. Several acids (acetic acid, HCl, CF_3COOH, H_2SO_4, *p*-toluenesulfonic acid, and acidic exchange resins) and solvents (MeOH, EtOH, *i*-PrOH, *t*-BuOH, water, THF, and hexane) were tested to hydrolysis of the protective group [Yu *et al*., 2003]. The acidic resin with a sulphonic acid functionality (Amberlyst 15, wet) and EtOH was most efficient. The chemical method described above was applied successfully for synthesis of Cl-TAGs in this thesis work (Section 5.2.3).

2.5.2. Identification of TAG

The structural characterization of TAGs is necessary to identify the length of the fatty acid chain, degree of unsaturation, location of double bonds, and position of the fatty acids on the glycerol backbone (position 1 or 3 *vs.* position 2) [Hsu and Turk, 1997]. The identification methods for TAG are covered in books like "*Lipid Analysis-Isolation, Separation, Identification and Lipidomic Analysis*" by Christie and Han [2010] and "*The lipid Handbook*" by Gunstone, Harwood and Dijkstra [2007].

The analysis of the TAG can be done using one of the following procedures: (1) direct analysis of the TAG by GC, MS or NMR; (2) separation of the TAG using HPLC followed by saponification, transesterification, and detection of their fatty acids by GC (method commonly known as 2D- HPLC/GC); and (3) separation of the TAG using HPLC followed by MS (also known as 2D-HPLC/MS).

2.5.2.1. Nuclear Magnetic Resonance spectroscopy

Nuclear Magnetic Resonance spectroscopy (NMR) has become a universal and powerful technique for the characterization of most organic compounds. ^{1}H- and ^{13}C-NMR spectroscopy are applied as a characterization technique in lipid chemistry. As early as 1960, Johnson and Shoolery were the first to determine the saturated and unsaturated fatty acids of TAG in natural fats using NMR. Although this technique was applied to reveal structural details of TAG, the application was limited by the broadening of signals and required long experimental time to obtain a spectrum with suitable signal-to-noise ratio. Significant deviation of ^{13}C NMR spectra for a number of saturated and unsaturated fatty acids of TAG have been reported by Lie Ken Jie and colleagues [1992, 1995a, 1995b, 1996]. ^{1}H NMR spectroscopy has been a crucial technique for monitoring progress of a reaction. ^{1}H-NMR spectra of various protons of the glycerol in TAG have been described in the literature [Lie Ken and Lam, 1995]. The application of NMR in vegetable and fish oils have been studied and by reviewed by Gunstone and coworkers [1991; 1995; 2009]. In this thesis, the ^{1}H and ^{13}C NMR methods were used to characterize and improve the purity of Cl-TAG standards synthesized, and also to identify Cl-TAGs in the framed salmon skin and tissue oils.

2.5.2.2. Mass Spectrometry

The use of MS technique for the characterization of lipid mixtures has become a better alternative than NMR due to its higher sensitivity. It is being used increasingly with atmospheric-pressure chemical ionization (APCI) or electrospray (ESI) ionization.

The APCI and ESI ionization techniques have been employed to analyze TAG at the molecular species level using considerably gentler conditions than GC/MS, and also in conjunction with liquid chromatography [Christia and Han, 2010]. Furthermore, these techniques were applied to provide composition of acyl chain of the fatty acid and their regiospecific location in TAG [Byrdwell *et al*., 2005].

Atmospheric pressure chemical ionization MS (APCI/MS) has proven to be a very valuable technique for the analysis of lipids from a variety of classes. It is a soft

ionization technique and produce some degree of fragmentation that is useful for structural characterization. The ionization process of APCI consiss of vaporization with heated desolvarion followed by corona discharge needle carrying a high voltage that generates molecular ion at the atmospheric pressure.

The ionization process of APCI produces protonated TAG molecules and other fragments which lead to the identification of each class of molecules. Unlike ESI, APCI requires no buffers in solution in order to produce efficient fragmentation [Byrdwell *et al*., 2001]. The APCI/MS technique has been applied to the analysis of TAG in vegetable oils [Laakso and Voutilainen 1996; Mottram *et al*., 2001].

Electrospray ionization (ESI) was developed by Fenn and coworkers at Yale University [1985]. Improvements in commercial ESI sources, such as electrospray [Bruins *et al.* 1987] allows higher effluent flow rates than previously used. These improvements in ESI caused it to gain more widespread usage. The ESI technique is based on ion formation in solution. Some molecules are particularly amenable to ESI because they are either ionic (*e.g*. phospholipids) or contain ionizable groups (*e.g.* amino acids). Other molecules such as TAGs are neutral and do not form ionized molecules under ESI.

TAGs require the use of an ionic reagent that can produce an adduct with TAG molecules. The ionic reagents are cations such as ammonium or an alkali metal (sodium or lithium) in the acetate form. The ESI has been used for the analysis of a mixture of TAGs introduced directly by a syringe pump to MS. Spectra obtained using ESI contains only molecular ions with no fragmentation. Tandem mass spectrometry $(MS)^n$ provides additional structural information necessary to uniquely identify TAG species using Collision- induced dissociation (CID) [Neff and Byrdwell, 1995].

Analysis of TAG by ESI/MS without chromatographic separation was first demonstrated by Duffin *et al*., [1991] by infusing TAGs directly into the ESI interface where TAGs containing unsaturated fatty acid produced better signals than the saturated ones. Hsu and Turk (1999) added ammonium or alkali metal ions (Li, Na, Cs) to TAG to form precursor ions and applied collision activated dissociation (CID) to study the product ions by MS. The fragmentation mechanism of the lithiated adducts $[M+Li]^+$ of TAG obtained by tandem mass spectrometry could be used to differentiate regioisomers of TAG [Hsu *et al*., 2010]. The ESI has been used extensively for the analysis of TAG [Cubero Herrera *et al*., 2010, 2012; Hsu and Turk, 2010; Leskinen *et al*., 2008]. The application of

ESI/MS for TAG analysis in edible oils has been reviewed by Rosaria Cozzolino and Beatrice De Giulio [2011].

The ESI(+)/MS was applied in this thesis to identify the structures of all TAGs in corn oil, salmon skin as well as tissues oils, and oxidized TAGs in corn and salmon oils. In addition, ESI(+)/MS was used to characterize Cl-TAGs in standards synthesized in this thesis.

2.5.2.3. Gas Chromatography

High-temperature gas chromatography in conjunction with MS (HTGC/MS) was used in the 1970s to analyze for TAGs [Muruta *et al.,* 1973; Grob, 1979]. The main problem of HTGC/MS lies in the separation of TAGs according to their degree of unsaturation and low bleeding of the column at high temperatures. Recently, Ruiz-Samblas *et al*. [2012] applied HTGC/MS to characterize TAG profiles of 10 olive oil samples. A more convenient method will be to convert the TAG to a low molecular weight, volatile derivative such as FAME then detect by GC.

Methods of methylation of FFA can be done using either a base or an acid catalyst. Ackman [1998] reported that boron trifluoride in methanol (BF_3-MeOH) would be sufficient to convert all FFA of fish oils to their methyl esters. Also he investigated the potential problems created by the alkali transesterification step of the official methods, including BF_3-MeOH treatment. Ackman concluded that alkali transesterification was not necessary and the oils can be analyzed directly using BF_3-MeOH. The results reported in the literature showed that the BF_3-MeOH method is applicable to both food and biological samples for preventing artificial isomerization and formation of byproducts [Igarashi *et al*., 2004]. The BF_3-MeOH method was used to convert the TAG of the oil samples to their FAME and detected by GC/MS in the work reported in this thesis.

2.5.2.4. High Pressure Liquid Chromatography

The HPLC technique is most extensively used for the separation of TAGs in oils and fats [Leo Nollet and Fidel Toldra, 2015]. The elution order of TAGs depends on their partition number (PN). The partition number is defined as the combined number of carbons (CN) minus twice the number of double bonds (DB) present in the three fatty acids (PN=CN-2DB). Two HPLC separation approaches are commonly used. One of them is based on separation of PN (as in reversed phase chromatography) and the other on the degree of unsaturation of TAGs (as in TLC or HPLC argentation chromatography)

Christia and Han [2010]. The separation of TAG has been covered in detail in the book entitled “HPLC of Acyl Lipids” by Lin and McKeon [2005]. Reversed phase HPLC methods have been developed in this thesis for the separation of TAGs.

Silver ion chromatography is useful for TAG analysis, and has been applied to different molecular species of lipids [Dobson *et al*.; 1995, Nikolova-Damyanova, 2003]. Christie *et al*. [1987] was the first to prepare an Ag-HPLC column. Laakso and Voutilainen *et al*. [1996] used an Ag-HPLC method to separate TAGs in seed oils. Ag-HPLC has been used extensively in this thesis for the separation of TABs in oils.

Although the use of mono-dimensional chromatographic techniques such as RP-HPLC and Ag-HPLC can often provide useful information of TAG profiles in lipids, a more complete characterization can be obtained by multidimensional chromatography. The coupling of two forms of chromatography can be done either off-line or on-line.

The off-line technique consists of separate collection of various fractions from the first method and then injection to the second method. A good example of this would be the HPLC separation of TAGs in the first stage and then analysis by another technique such as GC, HPLC/GC or HPLC/MS. Both off-line HPLC/GC, HPLC/HPLC, and HPLC/MS methods have been developed in this thesis for the separation of TAGs in oils.

The on-line technique could be either “heart-cutting” or “comprehensive” type. In the on-line heart-cut chromatography, the centre of a peak is cut using a sampling valve and transferred to a second chromatographic column. On the other hand, in the on-line comprehensive chromatography a long chromatographic column or slow gradient is used in the first dimension and a short chromatographic column or rapid gradient in the second dimension. The “heart-cutting” technique coupled to reversed phase argentation chromatography (Ag-HPLC/RP-HPLC) using a multiport sampling valve has been developed and applied in this thesis.

Several multidimensional chromatographic methods for the analysis of TAGs have been reported in the literature: Ag-LC/RP-LC [Lasskso and Christtie 1991], RP-LC/Ag-LC [Dugo *et al*., 2004; Petersson *et al*., 1994), Ag-LC/RP-LC/GC [Laakso *et al*., 1992] and RP-LC/ GC [Gresti *et al*., 1993]. An off-line three dimensional method has been described by Nikolova-Damyanova and Christie [1990].

The on-line comprehensive two-dimensional (2D) chromatographic methods in combination with MS have been applied to TAGs in foods [Mondello *et al*., 2005]. Other reports include the analysis of many edible oils using offline Ag-HPLC/APCI/MS and RP-HPLC/APCI/MS [Wei *et al*., 2015], of olive oil by off-line Ag-HPLC/GC/FID [Janssen *et*

al., 2003], and of rice oil and donkey milk by off-line RP-HPLC/Ag-HPLC/APCI/MS [Dugo *et al*., 2004; Dugo *et al*., 2006a] as well as corn oil by the same method [Elbert *et al.,* 2008].

For TAG analysis, the applicability of GC/GC is limited. There are currently no high-temperature GC columns available for comprehensive GC/GC. A comprehensive on-line HPLC/GC method has been described in literature for the analysis of volatile compounds in aqueous samples (Quigley *et al*, 2000). This method cannot be applied to non-volatile and high-molecular mass compounds such as TAGs in oil and fats. However, off-line LC/GC methods can be conveniently used, especially for large volume injection and fast GC set-ups [Janssen *et al.*, 2003]. An off-line HPLC/GC method was applied in this thesis for the analysis of TAGs in the corn oil sample.

The off-line approach is comparatively simple but has several limitations. It is time-consuming, difficult to automate, and characterized by low analytical reproducibility. Moreover, sample contamination or formation of artifacts can occur. Both on-line methods, namely “heart-cutting” or “comprehensive”, offer the advantages of ease of automation and greater reproducibility in a shorter analysis time but they are more difficult and expensive to operate.

CHAPTER 3

Separation and identification of free fatty acids in oils by GC/MS and separation of triacylglycerols by HPLC

3.1. Objectives

The main objectives of the work reported in this chapter were to optimize methods for the separation and identification of free fatty acids in four oil samples, namely corn, grapeseed, canola and salmon, by methyl esterification followed by gas chromatography/mass spectrometry (GC/MS). Another objective was the separation of triacylglycerols (TAGs) in the same oils by reversed-phase-high performance liquid chromatography (RP-HPLC) and silver ion-high performance liquid chromatography (Ag-HPLC).

3.2. Introduction

The major components of vegetable oils are TAGs which can be separated by techniques such as GC and LC. Most of the GC methods require transesterification of TAG in order to improve the detection of fatty acids (FA) in them. The GC methods require high-temperature columns because of the low volatility of TAGs [Ruiz-Samblás *et al*., 2012]. A more convenient approach will be to convert the TAG to a low molecular-weight, volatile derivative such as a fatty acid methyl ester (FAME) using either a base (such as NaOH or KOH in MeOH) or an acid (such as BF_3, HCl or H_2SO_4 in MeOH). The parameters affecting transesterification of TAG are reported to be the reaction time, temperature, type of catalyst and its concentration, and molar ratios of the reactants [Lewis *et al*., 2000; Park *et al*., 2001]. The BF_3-MeOH method was originally developed by Metcalfe and coworkers in 1961 and described in detail in 1966 [Metcalfe *et al.,* 1961; Metcalfe *et al.,* 1966]. This method was modified by Ackman [Ackman, 1998] where he converted free fatty acids (FFA) of fish oils to methyl ester. Ackman also investigated the potential problems encountered by the alkali transesterification in the first step of this method. He showed that this step could not be used for oils, such as raw fish and some vegetable oils, containing high amounts of FFA which remain unconverted. Ackman and his group [Joseph and Ackman, 1992] then recommended that the first alkali

transesterification step was not necessary and the oils can be directly analyzed using BF_3-MeOH. His method has been officially recommended by both the Association of Official Analytical Chemists (AOAC) and the American Oil Chemists' Society (AOCS) in 1989.

Igarashi and coworkers [Igarashi *et al.,* 2004] compared three acid-catalyzed methods (BF_3, HCl or H_2SO_4 in MeOH) and three base-catalyzed methods (MeONa, TMG/MeOH, and $TMSN_2CH_3$/MeOH) for the methyl ester preparation of conjugated dienoic and trienoic fatty acids in food and biological samples. The results indicated that the BF_3-MeOH method was applicable to both types of sample for preventing artificial isomerization and formation of byproducts. The BF_3-MeOH method was therefore selected for the present work.

Since 1970s several studies were done on the analysis of FFA in animal fats and oils using GC/MS with electron ionization GC/EI-MS [Muruta *et al*., 1973] and chemical ionization GC/CI-MS [Muruta, 1977]. The analysis of vegetable oil derivatives such as FAME by GC/MS has also been known in the food industry [Petrović *et al*., 2010]. Analysis of FAME by GC with different detectors including MS has recently been reviewed [Dolowy and Pyka, 2015] and MS has been identified as the most sensitive detector. The GC/MS technique is one of the most powerful tools for the identification of mono- and poly-unsaturated fatty acids and is also suitable for the localization of double bonds in very long-chain fatty acid esters in biological samples [Brondz, 2002; Seppänen-Laakso and Hiltunen, 2002; and Ruiz-Rodriguez *et al.*, 2010]. Mass spectrometry was selected in this thesis as the detection technique due to the ease of identification of observed compounds by comparing the mass spectra available in commercial libraries, such as Wiley library of 275 000 spectra and the NIST library of 230 000 spectra [van Leeuwen and de Boer, 2008].

An efficient separation of TAGs in vegetable oils has been achieved using different HPLC techniques, such as RP-HPLC [Cunha and Oliveira 2006; Holĉapek *et al*., 2005], TLC [Christie, 1992], and Ag-HPLC [Momchilova and Damyanova, 2012]. In addition, comprehensive two-dimensional chromatographic methods have been applied to achieve selectivity and peak capacities [Dugo 2006; van der Klift 2008]. Christie and coworker reviewed all LC techniques in an excellent series of books entitled Advances in Lipid Methodology [Christie, 1992 and 2003] and in the last edition of the major Lipid Analysis [Christie and Han, 2010].

The LC technique is most extensively applied to the separation of TAGs in oils and fats [Lin and McKeno, 2005]. Two LC separation methods have been found to be most useful for this purpose. The first method involves complexation/interaction of silver ions Ag(I) with the double bond (DB) of the TAGs known as Ag-HPLC-argentation chromatography; the higher the number of DB, the longer is the retention time [Adlof, 1997]. The second method consists of hydrophobic interaction of long chain fatty acids with (C18) in RP-HPLC and is based on the partition number (PN) of the TAGs; a higher PN value leads to increased retention times [Holĉapek *et. al.,* 2003]. The partition number is defined as the combined number (CN) of carbon atoms of the three fatty acids minus twice the number of double bonds (DB) present in the three fatty acids (PN = CN-2DB).

The efficient separation of TAGs in oil samples was successfully achieved using both RP-HPLC and Ag-HPLC techniques in this thesis.

3.3 Experimental

3.3.1 Chemicals

Boron trifluoride in methanol (14% BF_3) was purchased from Sigma Aldrich. Fatty acids, namely caprilyic acid (C8:0), myristic acid (C14:0), stearic acid (C18:0), oleic acid (C18:1), and linoleic acid (C18:2), were purchased from Eastman Organic Chemical. A Supelco 37-component fatty acid methyl ester mixture (10 mg mL^{-1} in CH_2Cl_2) typically containing individual fatty acids in either 2,4 or 6 wt% was purchased from Supelco. The $AgNO_3$ was purchased from the Nicolas chemical company Limited.

The commercial oil samples (Manufacturer) used in this thesis, namely corn (Mazola), canola (Crisco), grape seed (Borges), and salmon (PC Compliments, 100 mg liqui-gels), were obtained from a local supermarket. The HPLC grade dichloromethane, hexane, ethanol, and acetonitrile were purchased from Fisher Scientific.

3.3.2 Instrument operation conditions

3.3.2.1 GC/MS

The GC/MS experiments were done using a HP 6890 GC equipped with a HP 5937 mass selective detector and a HP 6890 autosampler/injector. The separations were done using a 5% diphenyl/95% dimethylsilane stationary phase (Supelco PTE-5 fused silica capillary 30 m X 0.025 mm id and 0.25 µm film thickness). The

chromatographic conditions used were: spilt injections (1:0, 10:1, 50:1, or 100:1 split depending on the sample concentrations), He carrier gas at 1 mL min^{-1} and a temperature gradient starting at 100°C (no delay), a ramp rate of 10°C min^{-1} to 320°C and a hold of 5 min. The injection port and transfer line were both at 260°C, the ionization source at 230°C and the quadrupole at 150°C. Mass spectral conditions were from 40 to 550 *m/z* at a rate of 2.3 scans per s. A solvent delay of 3 min was applied between injection and start of the data acquisition.

3.3.2.2 RP-HPLC

The RP-HPLC experiments were done using an Agilent1100 HPLC, a 1100 binary pump, a 1100 series diode array detector, and a Rheodyne 7260 injection valve fitted with a 20-µL sample loop. The separations were carried out using two Supelcosil LC-PAH (25 cm x 4.6 mm X 3 µ particles) columns coupled in series. A two solvent gradient was used at a flow rate of 1 mL min^{-1} and the applied linear gradient was 100% acetonitrile (solvent A) to 100% isopropanol (solvent B) over a period of either 50 min ("slow gradient") or 35 min ("rapid gradient"). The diode array detector was set to monitor 210, 230, and 254 nm as well as acquire spectra from 200 to 400 nm. All chromatographic experiments were done at room temperature.

3.3.3 Procedures

3.3.3.1 Preparation of Ag-HPLC columns

An Agilent 1100 HPLC using a Ag-column was prepared in-house using Supelcosil LC-SCX column (25 cm X 4.6 mm) employing the following steps. A blank column was flushed with distilled water for 1 h; then $AgNO_3$ was introduced to the column using two methods. The first method consisted of injection of 250 µL of 1 M $AgNO_3$ directly onto the column; this resulted in immediate bleeding of Ag^+ ions from the column and gave the column a short life-time; consequently, this method was not pursued. In the second method $AgNO_3$ was pumped on to the column for 1 h followed by a water wash for 1 h; no Ag^+ ions were detected in the wash using NaCl indicating the original SCX column was converted to the desired Ag-column. Prior to use for gradient elution the column was washed with MeOH for 1 h. Normal phase silver-ion high performance liquid chromatography (Ag-HPLC) was applied to corn oil and salmon oil using two solvent gradients with a flow rate of 1 mL min^{-1} and the diode array was set at 215 nm. A mixture of MeOH (solvent A) and 20% acetonitrile (ACN) in MeOH (solvent B)

was used as the mobile phase in three following gradient modes 100:100, 50:50, and 80:20.

3.3.3.2 Calibration curve for fatty acid ethyl ester (FAEE)

The experiments were performed to investigate the potential of using the ethyl esters of the fatty acids as internal standards. A stock solution of FFAs was prepared by weighing accurately about 100 mg of each of the fatty acids (C14:0, C18:0, C18:1, C18:2) into a 100-mL volumetric flask and diluting to the mark with dichloromethane (CH_2Cl_2).

One (1.0) g of the fatty acid stock solution was mixed with 8 mL ethanol (EtOH) and 2 mL H_2SO_4 acid, and heated at 80°C for 15 min. The mixture was extracted with about 10 mL of hexane, and the extract was dried over sodium sulphate (Na_2SO_4) and evaporated to dryness in a rotary evaporator. The residue (ethyl esters of the fatty acids) was dissolved in a small amount of CH_2Cl_2, transferred to a 50-mL volumetric flask and diluted to the mark with CH_2Cl_2. This primary stock solution of fatty acid ethyl esters (FAEE) had a concentration of 15 μg mL^{-1}.

A secondary stock solution was prepared by taking 1.0 g of the primary stock solution and diluting it with 20.0 g with CH_2Cl_2. A similar procedure was used to prepare a separate stock solution of caprylic acid (C8:0) ethyl ester for use as an internal standard either for calibration purposes or for recovery experiments. This stock solution had a concentration of 1.4 μg mL^{-1}.

The above two stock solutions were used to prepare a series of standard solutions as shown in Table 3.1. The solvent used in both cases was CH_2Cl_2. The standards were then analyzed by GC/MS using the conditions described earlier in the experimental section 3.3.3.1.

Table 3.1 the amounts of secondary stock solution, solvent, and internal standard for calibration curve of FAEE.

Stock solution of FAEE (g)	Solvent (g)	Internal standard of C8:0 ethyl ester (g)	Concn. μg mL^{-1}
1.00	0	0.250	0.608
1.00	1.00	0.250	0.338
0.50	1.50	0.250	0.168
0.10	1.75	0.250	0.036
0.05	1.95	0.250	0.0168
0.00	2.00	0.250	0

3.3.3.3 Calibration curve of the standard mixtures of FAME

The Supelco standard mixture (10 mg mL^{-1}) was transferred to a 25-mL volumetric flask and diluted to the mark with hexane. This stock solution corresponded to 0.4 mg mL^{-1} of total fatty acid. Concentrations of the individual fatty acids in it were 2, 4, or 6% of the total concentration; *e.g.* at 2% the concentration was 8.0 μg mL^{-1}. This stock solution was used to prepare standard solutions of FAA covering the range 0.031-4 μg mL^{-1} by dilution.

3.3.3.4 Esterification procedures for FFA and oils using BF_3-MeOH

3.3.3.4.1 Esterification of FFA using BF_3-MeOH

The reaction was carried out using a 1:1 mixture of the FFA stock solution (containing about 100 mg of each of the fatty acids (C14:0, C18:0, C18:1, C18:2) in 100 mL DCM) and BF_3-MeOH heated for 5 min at 80°C. After cooling, 2 mL of saturated NaCl solution was added followed by the extraction of the methyl ester formed by 2 mL hexane. The vial was immediately closed and shaken vigorously for 2 min. The top layer was removed with a pipette after the two layers had separated, and dried with sodium sulphate. After filtration, 1 μL of the extract was injected onto the GC/MS column. The formation of the derivatives was studied as a function of reaction time using 10, 20, 30, 40, 50, and 60 min.

3.3.3.4.2 Esterification of oil samples using BF_3-MeOH

Stock solutions of each oil sample, namely corn, canola, grape seed, and salmon, were prepared by weighing 100 mg and diluted to 100 mL with DCM in a volumetric flask. Then 1.00 g of each stock solution was transesterified using the BF_3-MeOH procedure. The mass ratio of BF_3-MeOH to TAG was 2:1 and the samples were heated at 80°C and the reaction time was 15 min. The reaction mixture was treated in the same manner as the free fatty acids.

3.3.3.5 Preparation of oil samples for HPLC analysis

Stock solutions of each oil sample, namely corn, canola, grape seed, grape seed, and salmon, were prepared by weighing about 0.3 g of the oil, 5.0 g of ACN, 10.0 g of isopropanol, and 3.0 g of hexane. Exactly 20 µL of each stock solution was injected onto the coupled PAH columns and separated using a slow gradient.

3.4 Results and Discussion

At the beginning, this thesis project involved the characterization of FAEE, FAME, and oils by GC/MS. In addition, TAGs of oils were separated using HPLC. The results are discussed below.

3.4.1 GC/MS separation of FAEE

The preliminary experiments were done using H_2SO_4 as the catalyst with EtOH to form the ethyl ester derivatives which were considered to be useful as internal standards for the methyl ester derivatives. Fig 3.1 A and B show the GC/MS chromatograms of a 5-component mixture of ethyl esters of C8:0, C14:0 C18:0, C18:1 and C18:2.

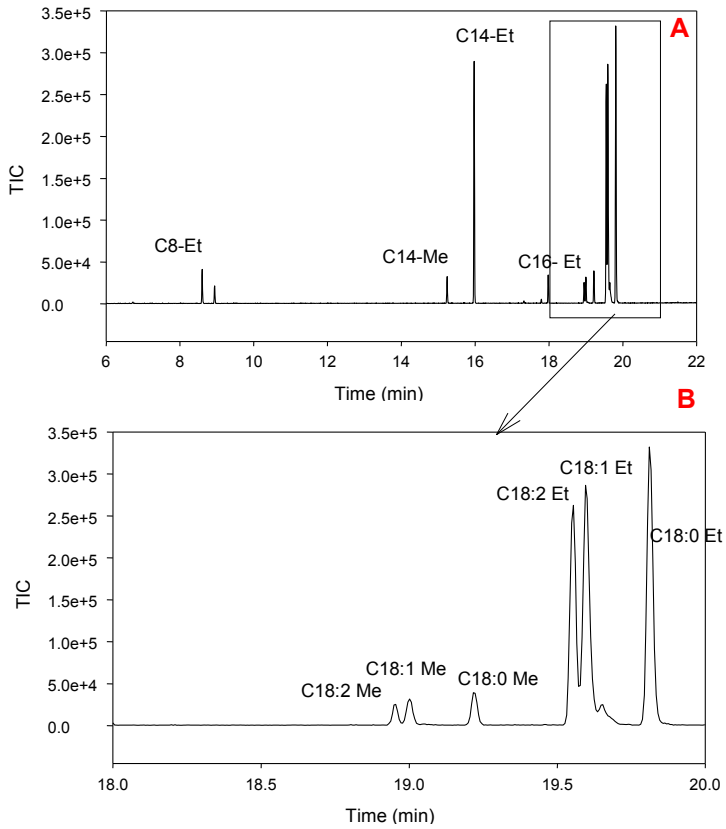


Fig 3.1 (A) GC/MS chromatogram of a mixture of standard fatty acid ethyl esters; (B) the expanded region of 18.0-20.0 min.

The above chromatograms show the presence of methyl esters because of MeOH impurity in the EtOH solvent used. The C16:0 (methyl ester) peak resulted from an impurity in the C18:0 stock solution. However, the separation of the methyl and ethyl esters suggested that the ethyl esters could be used as internal standards.

Figs. 3.2 and 3.3 show the EI-mass spectra of the methyl and ethyl esters of C14:0, respectively. Both esters show a small molecular ion (*m/z* 256 for ethyl and *m/z* 242 for the methyl). The principal ions in the mass spectra of the saturated fatty acids (C8:0, C14:0, C16:0, and C18:0) are *m/z* 74 and 87 for the methyl ester and *m/z* 88 and 101 for the ethyl ester, respectively. The base peak at *m/z* 74 results from a McLafferty rearrangement and the peak at *m/z* 87 is the radical cation $[CH_2CH_2COOCH_3]^{\cdot+}$. The loss of a radical methoxy group gives the peak at *m/z* 211 and is characteristic of methyl esters of saturated fatty acids [Christie, 1989].

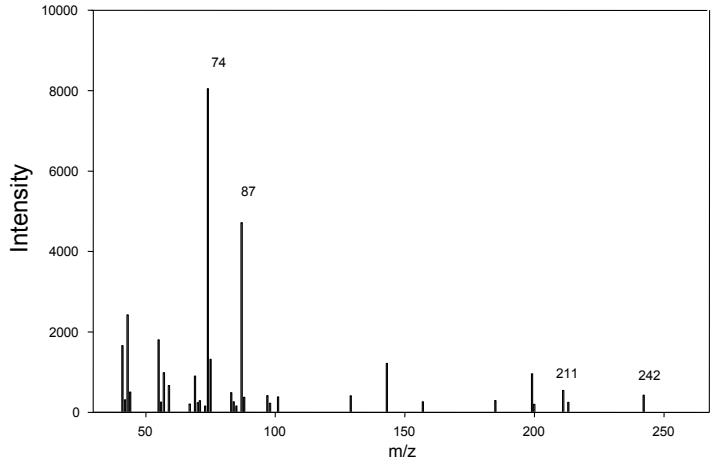


Fig 3.2. Mass spectrum of the methyl ester of C14:0.

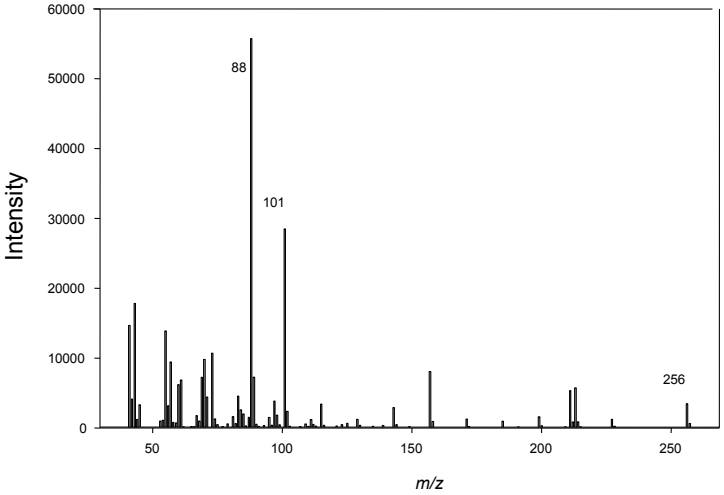


Fig 3.3. EI mass spectrum of the ethyl ester of C14:0.

The pattern of fragmantion in the mass spectra also depended on the number of double bonds. The mass spectra of the methyl esters of C18:0, C18:1 and C18:2 with different fragmentation patterns are shown in Figs. 3.4A, 3.4B and 3.4C, respectively. The saturated compounds show a loss of 35 u; and the monounsaturated shows loss of 34 u; and doubly unsaturated shows loss of 31 u which are not evident in polyunsaturated fatty acids (*e.g.*, C18:3) [Christie, 1989].

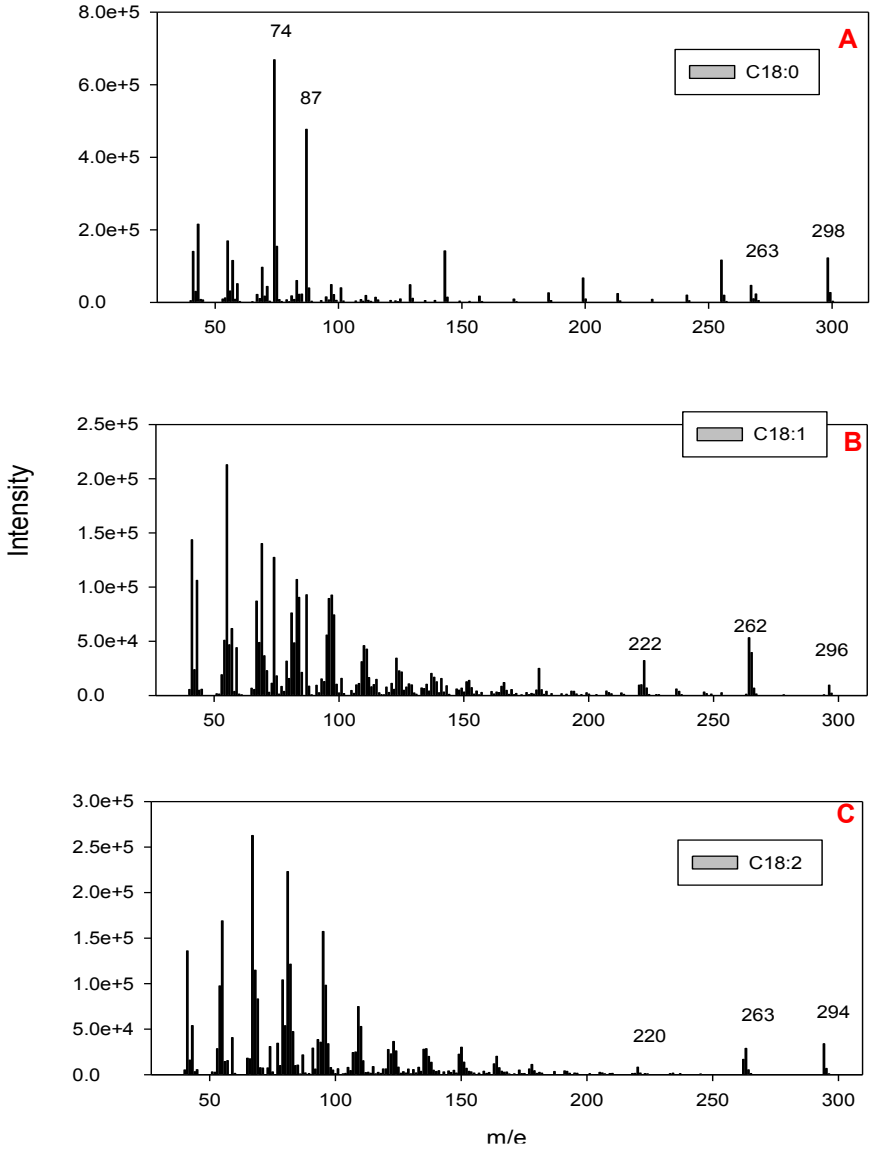


Fig 3.4. Comparison of mass spectra of the methyl esters of (A) C18:0; (B) C18:1; (C) C18:2.

The calibration curves of FAEE of C14:0, C18:2, C18:1, and C18:0 were prepared using TIC for the chromatographic peak areas given by the “Chemstation” program and are shown in Fig 3.5. The ethyl esters have very similar responses and the calibration is linear from the blank to 0.5 μg mL^{-1}. The calibration curve using C8:0 as an internal standard was not linear at higher concentrations of fatty acid methyl esters shown in Fig.3.6.

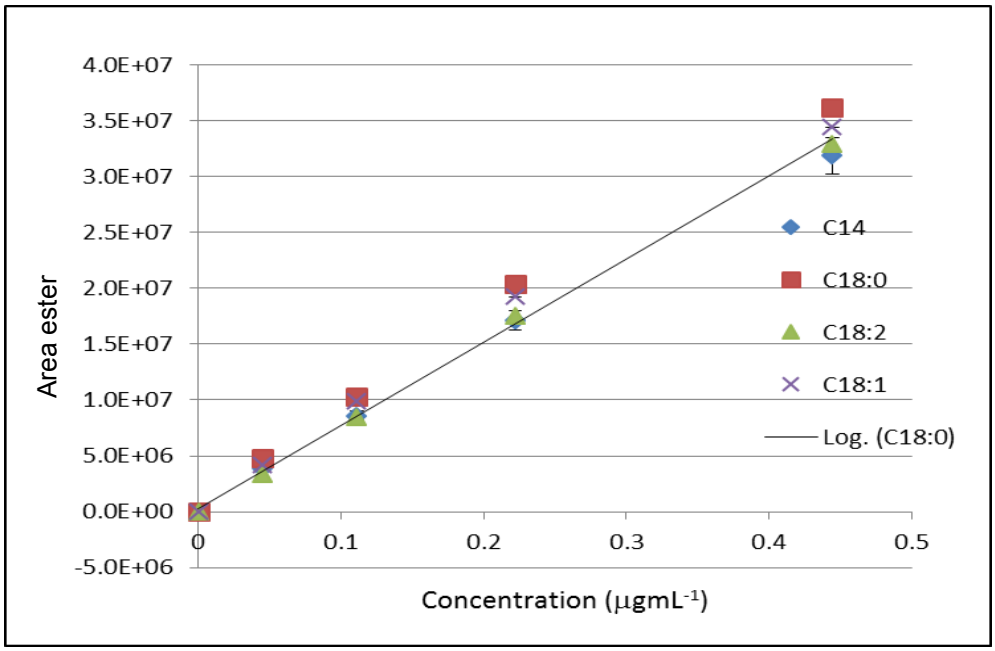


Fig 3.5. Calibration curve for the ethyl esters of C14:0, C18:2, C18:1, and C18:0.

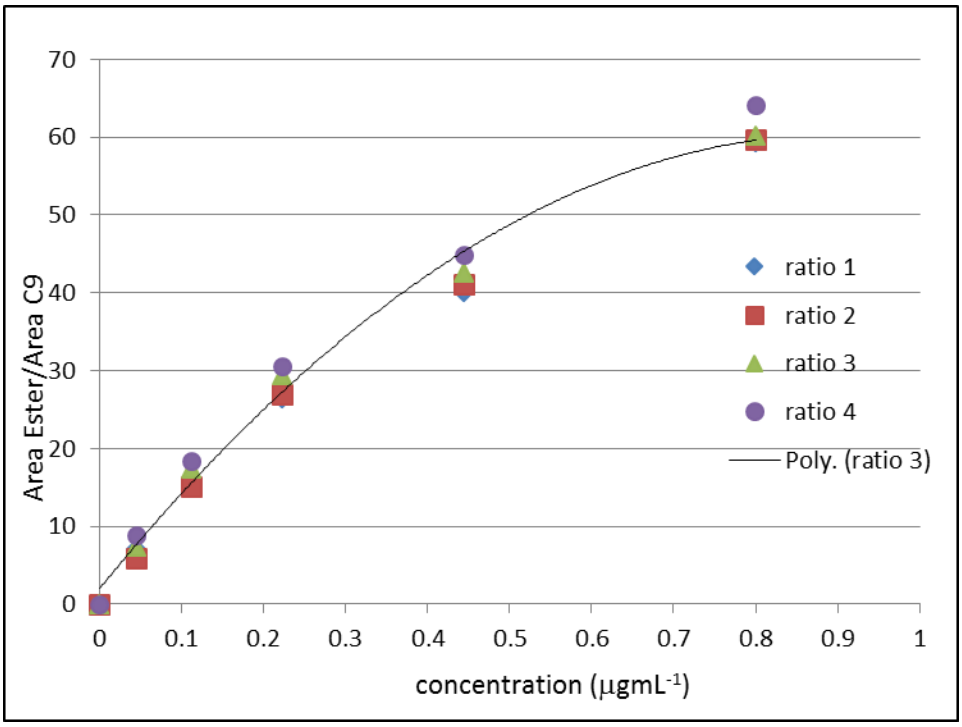


Fig 3.6. Calibration curve for the ethyl esters using C8 as internal standard.

3.4.2 GC/MS separation of FAME of the standard mixture

The second study was performed using BF_3 in methanol as the catalyst to form the methyl ester derivatives. Fig. 3.7A shows the total chromatogram for the FAME using the commercial Supelco standard mixture. This mixture consists of 37 FAME and the GC/MS method identified 32 of them using the NIST database supplied with the Chemstation program.

A number of small peaks are also observed, arising from other FAMEs present in the mixture. One notable feature in the chromatograms of FAME standard mixture is the occurrence of FAME isomers of the unsaturated species for the C20:X series as shown in Fig. 3.7B**.**

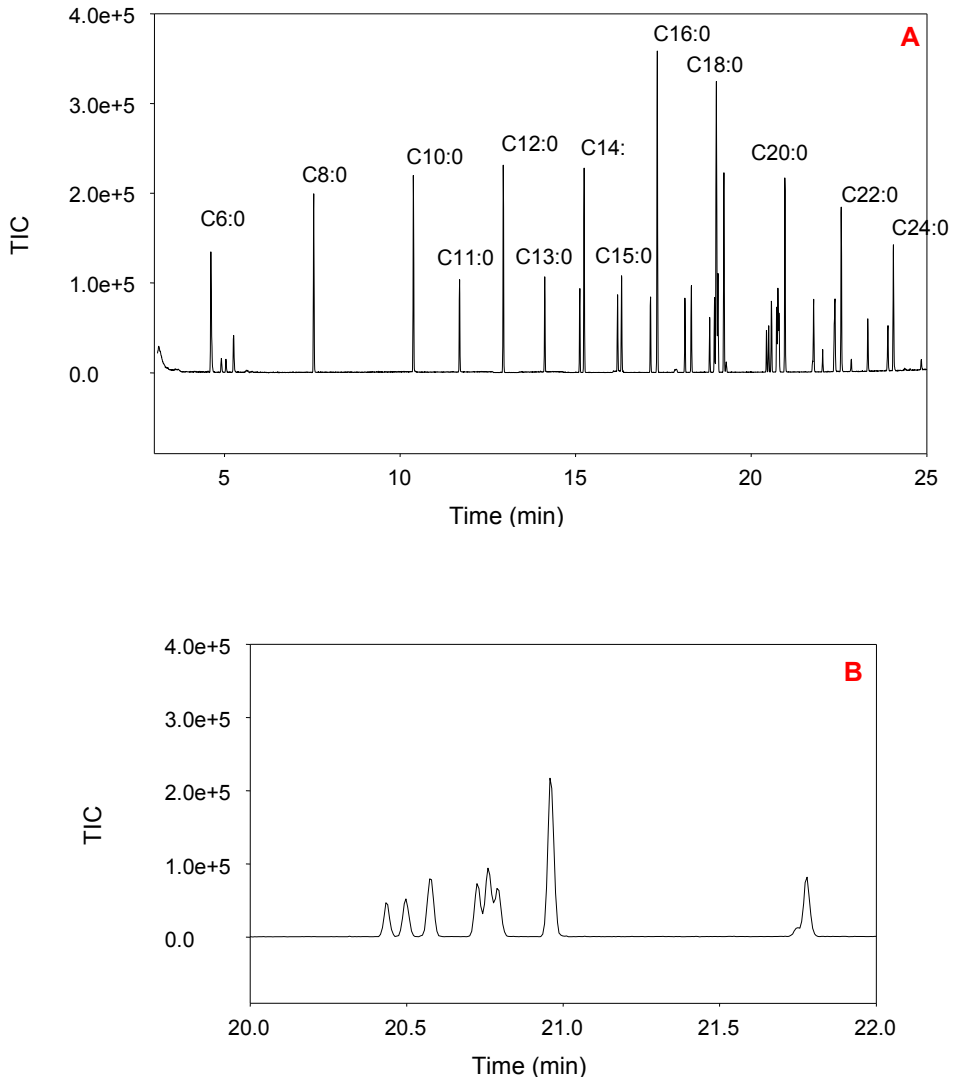


Fig 3.7 (A) GC/MS chromatogram of methyl ester standard solutions; (B) expanded region (20.0-22.0 min).

The calibration curve using C14:0, C18:0, and C16:0 of the FAME standard mixture is shown in Fig 3.8A. It is evident that the methyl esters of C14:0 and C18:0 have very similar responses and the calibration curve is linear. On the other hand, the methyl ester of C16:0 shows a different response. The reason for this is the FAME standard mixture consists of different FAME concentrations. The methyl ester of C16:0 has high concentration (6%) whereas the methyl ester of C14:0 and C18:0 have relative concentrations of 4%. The calibration curve of the FAME standard mixture was prepared over the range 0.016-4 μgmL^{-1} by dilution. Fig. 3.8B shows the calibration at the low end of this range.

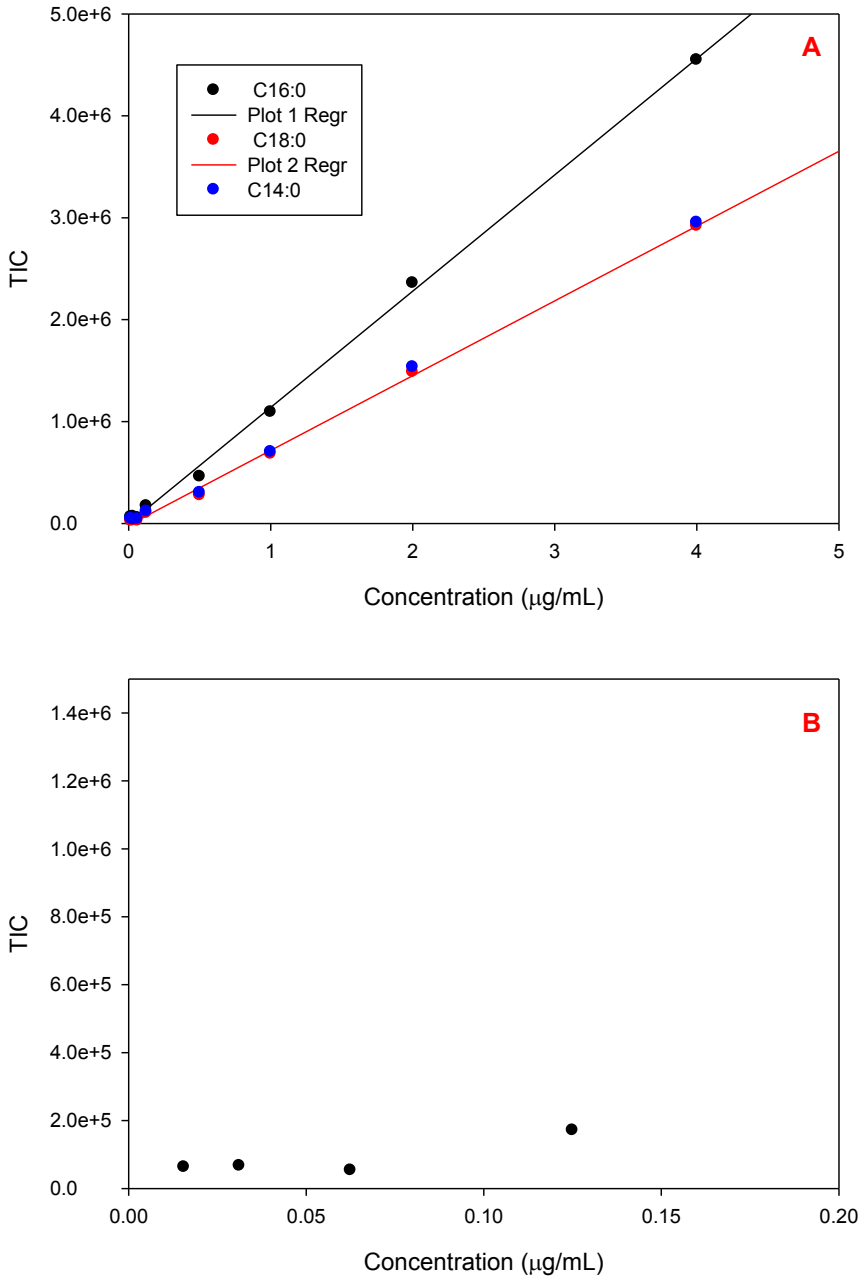


Fig 3.8 (A) Calibration curves for the methyl ester standard solutions; (B) expanded region (0.00-0.20 μg mL^{-1}).

The lowest concentration that gave a significant response was obtained at 0.125 μg mL^{-1} and only a few FAME peaks were identified as shown in Fig. 3.9. The reasons

for the similar responses for the standards below 0.125 μg mL^{-1} could be cross contamination of the samples by the GC syringe or possible problems with the liner in the injection port.

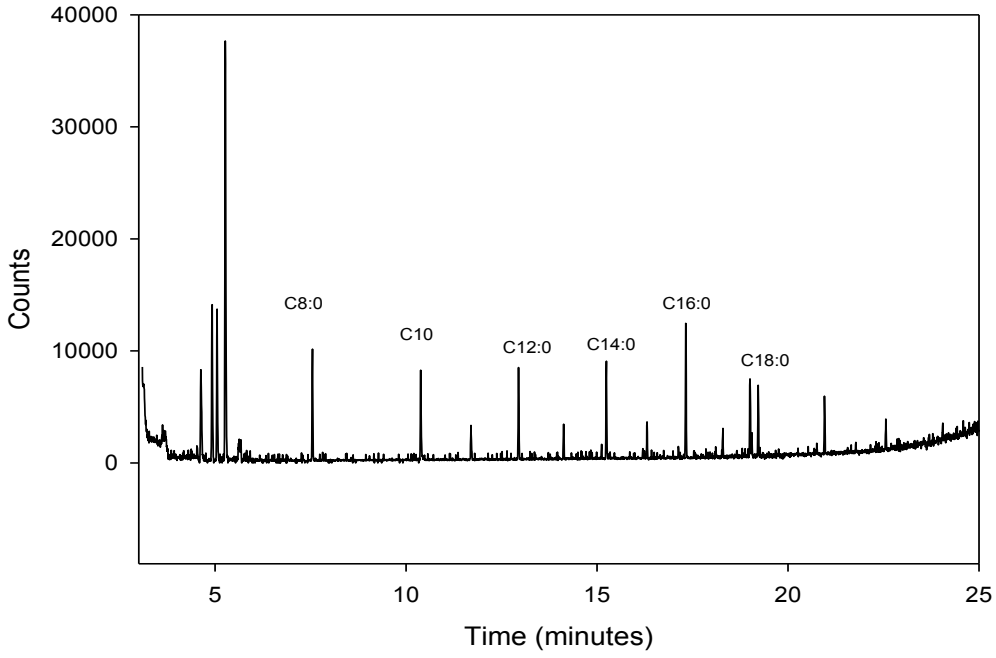


Fig 3.9. The GC/MS chromatogram for the lowest concentration (0.125 μg mL^{-1}) of standard mixture.

3.4.3 GC/MS separation of FAME of FFA

The first study of the BF_3-MeOH method was to determine the effect of reaction time on the formation of the derivatives. The effect of reaction time is graphically shown in Fig 3.10. In this study, the FAME derivatives were formed using BF_3-MeOH and the solutions were spiked with a C8:0 ethyl ester as an internal standard. The experiment was done using C14:0, C18:2, C18:1, and C18:0 but only the C14:0 and C18:1 data are shown in Fig. 3.10. The peak areas for the internal standard spikes are relatively constant but do show a relative standard deviation of ±8.7%. The methyl ester derivatives for C14:0 and C18:1 give a reasonable response for the first 20 min but fall off with time over the next 40 min. Similar results were found for C18:2 and C18:0.

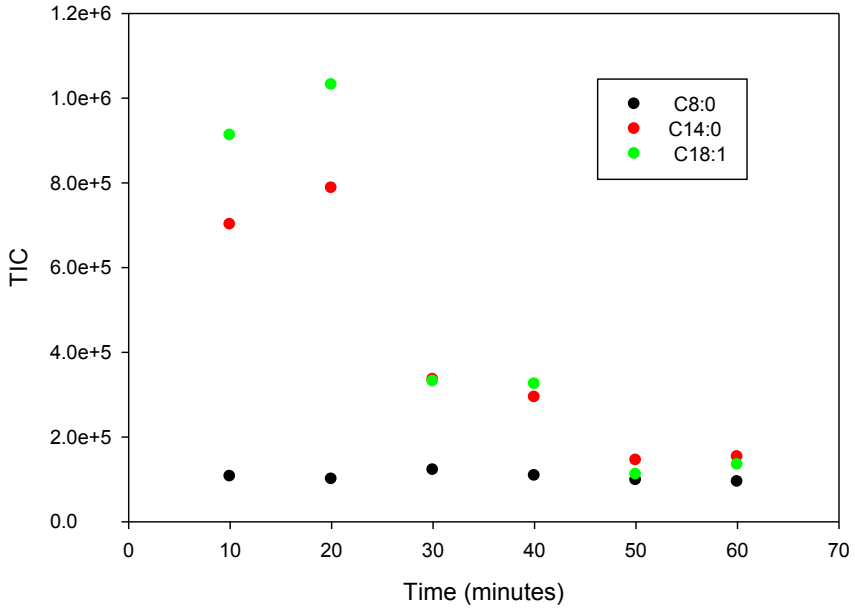


Fig 3.10. Time study for the formation of the methyl esters using BF_3-MeOH.

The entire procedure has already been given above (Section 3.3.3.4.1). Repeated GC/MS measurements of a single sample over two days showed RSD of ±10-16% for C14:0, C18:2, C18:1, and C18:0. However, when the ratio C18:X/C14:0 was used the RSD decreased to ±3-6% suggesting that there may be some reproducibility problem with the injection. This problem was solved by replacing the injection port and the septum. The septum was reported to be good for approximately 100 injections to provide easy, repeatable and reliable access for the syringe needle to inject the sample [McMaster, 2008].

3.4.4 GC/MS separation of FAME of oil samples

The BF_3-MeOH procedure was applied to the oil samples to convert them to their methyl ester derivatives which were then analyzed by GC/MS. Fig. 3.11 shows the methyl ester chromatogram for the corn oil sample.

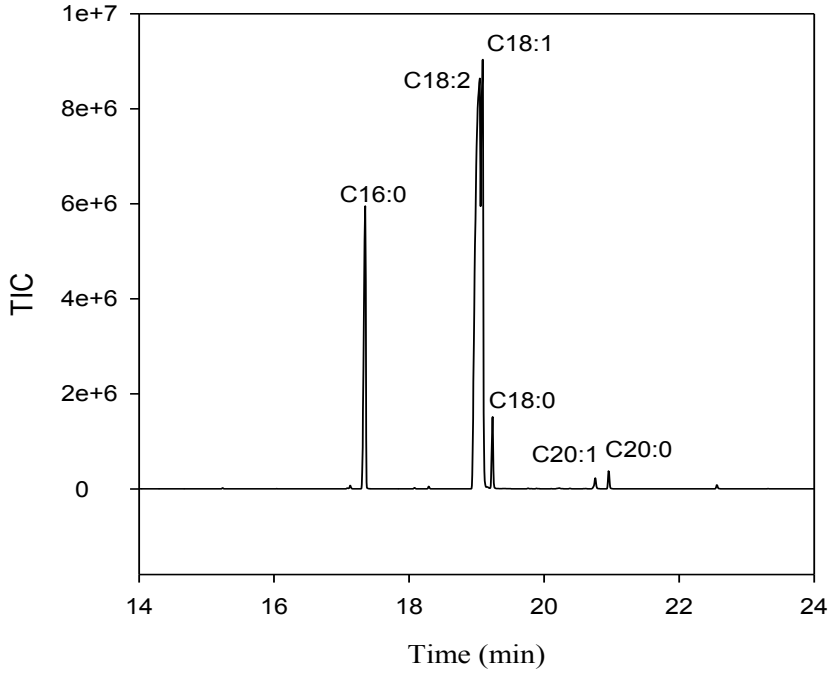


Fig 3.11. GC/MS chromatogram of corn oil sample derivative using the BF_3-MeOH procedure.

The fatty acid compositions of the 4 oils was prepared using TIC for the chromatographic peak areas given by the "Chemstation" program obtained and are shown in Table 3.2. From the GC-MS data of corn, grape seed and canola oils, it can be seen that the predominant fatty acids present are C18:1, C18:2, and C16:0 with small amounts of C18:0, C20:1 and C20:0. The salmon oil gave numerous of polyunsaturated fatty acids (which will be covered with more details in chapter 6). The accuracy of the data of the fatty acid composition in the oils reported in the literature has improved through the years as modern techniques such as GC-MS and HPLC became available [Chow, 2008]. Even then, the data vary because of differences in measuring techniques and errors among the analytical laboratories, and also due to different location, climate, soil, and the maturity of the oil seed. The percent composition of the fatty acids in the corn and grape seed oils given in Table 3.2 show the presence of C16:0, C18:0, C18:1, and C18:2. Canola oil had the same fatty acids with a minor amount of C16:1. Our results generally agree with the literature data suggesting that the predominant fatty acids present in vegetable oils are saturated and unsaturated with 14 to 22 carbon atoms (even number of carbons) in length with 0-3 double bonds [Xia and Larock, 2010].

Table 3.2. Comparison of FAME in the oil samples and the standards.

Type of oil	C16:1	C16:0	C18:2	C18:1	C18:0	C20:1	C20:	C20:5	C22:0	C22:6	Sum
Corn	-	15.5	54.3	25.6	2.9	0.5	0.6	-	-	-	99.4
Corn [a]		10	59.6	25.4	2	-	-	-	-	-	97
Grape seed	-	10.8	61.4	19	6.3	-	-	-	-	-	97.5
Grape seed [a]		7	72	16	4	-	-	-	-	-	99
Canola	0.3	6.6	21.5	61.1	3.1	2.1	0.9	-	0.5	-	92.7
Canola [a]		4	21	61	2	-	-	-	-	-	88
Salmon	5.3	11.3	12.4	26	2.7	8	1	9.6	-	11	87.3
Salmon [a]	5.8	13	16.1	27.4	3.5	8.7	-	4.6	-	7.1	86.2

[a] Ching Kuang Chow. Fatty acids in food and their health implication, CRC press, (2008).

3.4.5 Separation of TAGs in oils by RP-HPLC

The TAGs in oil samples were separated according to their partition numbers using RP-HPLC with slow gradient and the chromatograms are shown in Figs 3.12 to 3.15. The RP-HPLC was set up to separate the TAG components using C8 reversed-phase column with ACN and isopropanol as eluents and UV detection at 210 nm. All oil samples were separated to their TAG components within 50 min.

The separation of the salmon oil with good reproducibility (n=2) are shown in Fig 3.13. The chromatogram also shows several peaks between 20 to 30 min compared to other oil samples whose peaks start to appear after 30 min (Figs 3.12, 3.14, and 3.15). This is because the salmon oil contains TAGs with highly unsaturated fatty acids while others do not. The TAG chromatograms of the corn and grape seed oils agree reasonably with previous studies [Zab and Murkovic, 2010] and contain a few peaks compared to the TAG chromatograms in the salmon and the canola oils [Beermann *et al*., 2007].

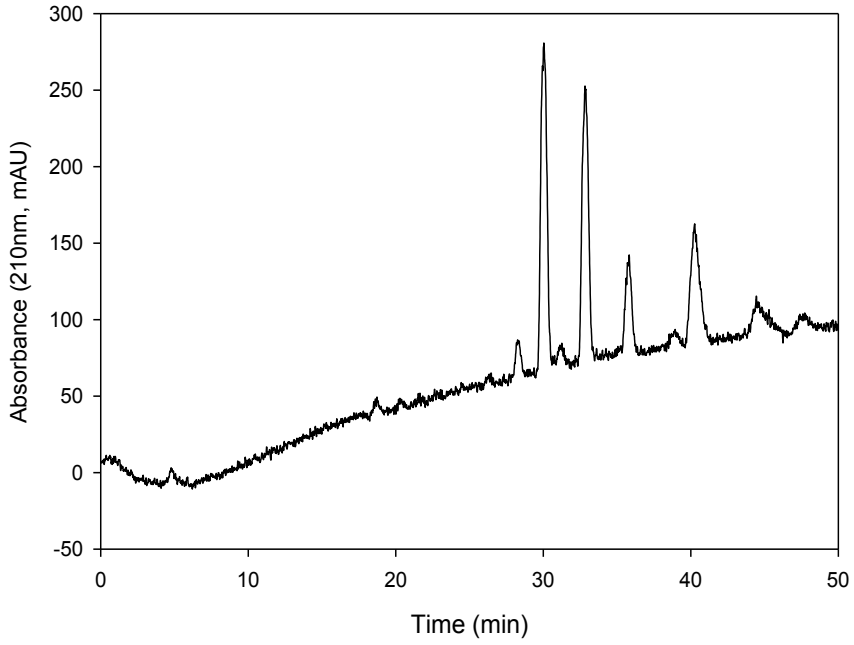


Fig 3.12. RP-HPLC of corn oil.

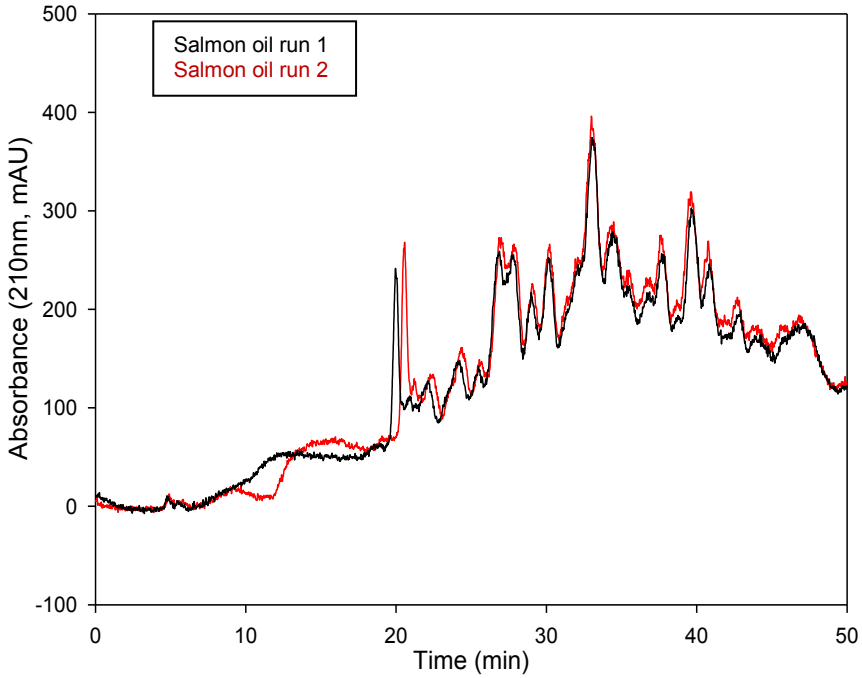


Fig 3.13. RP-HPLC of salmon oil.

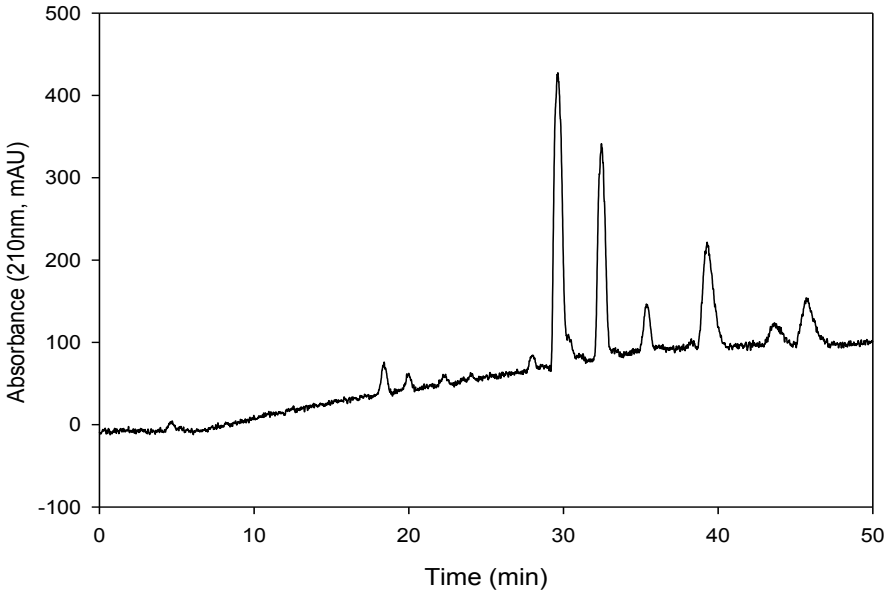


Fig 3.14. RP-HPLC of grape seed oil.

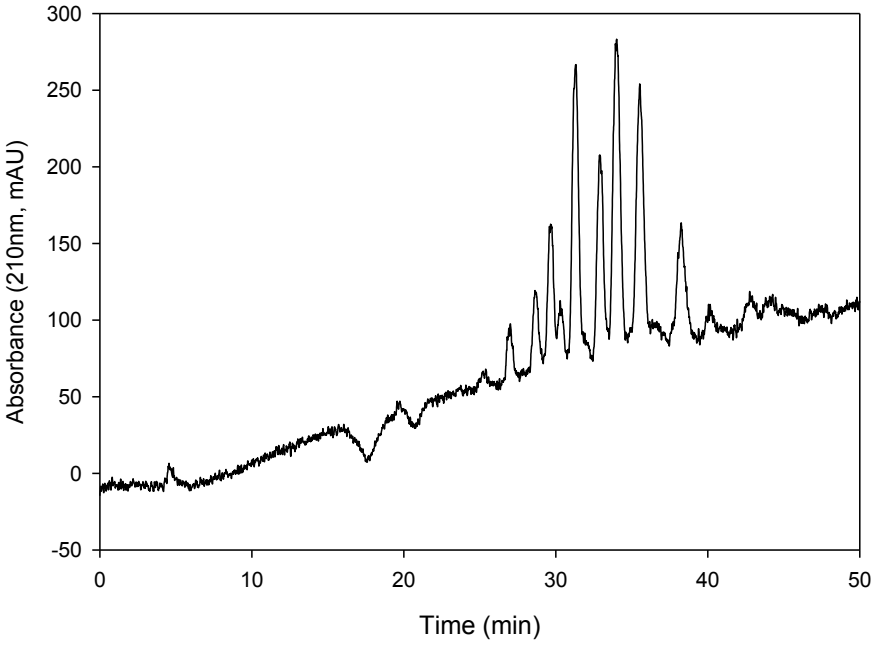


Fig 3.15. RP-HPLC of canola oil.

3.4.6 Separation of TAGs in oils by Ag-HPLC

3.4.6.1 Optimization of separation of TAGs in corn and salmon oils

The Ag-HPLC technique used to separate the TAGs in corn and salmon oil depended on the number double bonds in them. Three different gradients as given in section 3.3.3.1 were tested. It can be seen in Figs. (3.16A-C and 3.17A-C) the separation was achieved in less than 15 min for the corn oil and 20 min for the salmon oil. The better separation was obtained with the gradient mixture containing MeOH and MeOH with 20% ACN in a 100:50 ratio. A baseline separation between each TAG in the corn oil with certain number of double bonds was achieved and agreed with the published literature [van der Klift *et al*., 2008; Zab and Murkovic, 2010; Zab and Murkovic, 2013]. However, the salmon oil showed broad peaks (Figs. 3.16A, 3.16B and 3.16C) indicating co-elution of several TAGs.

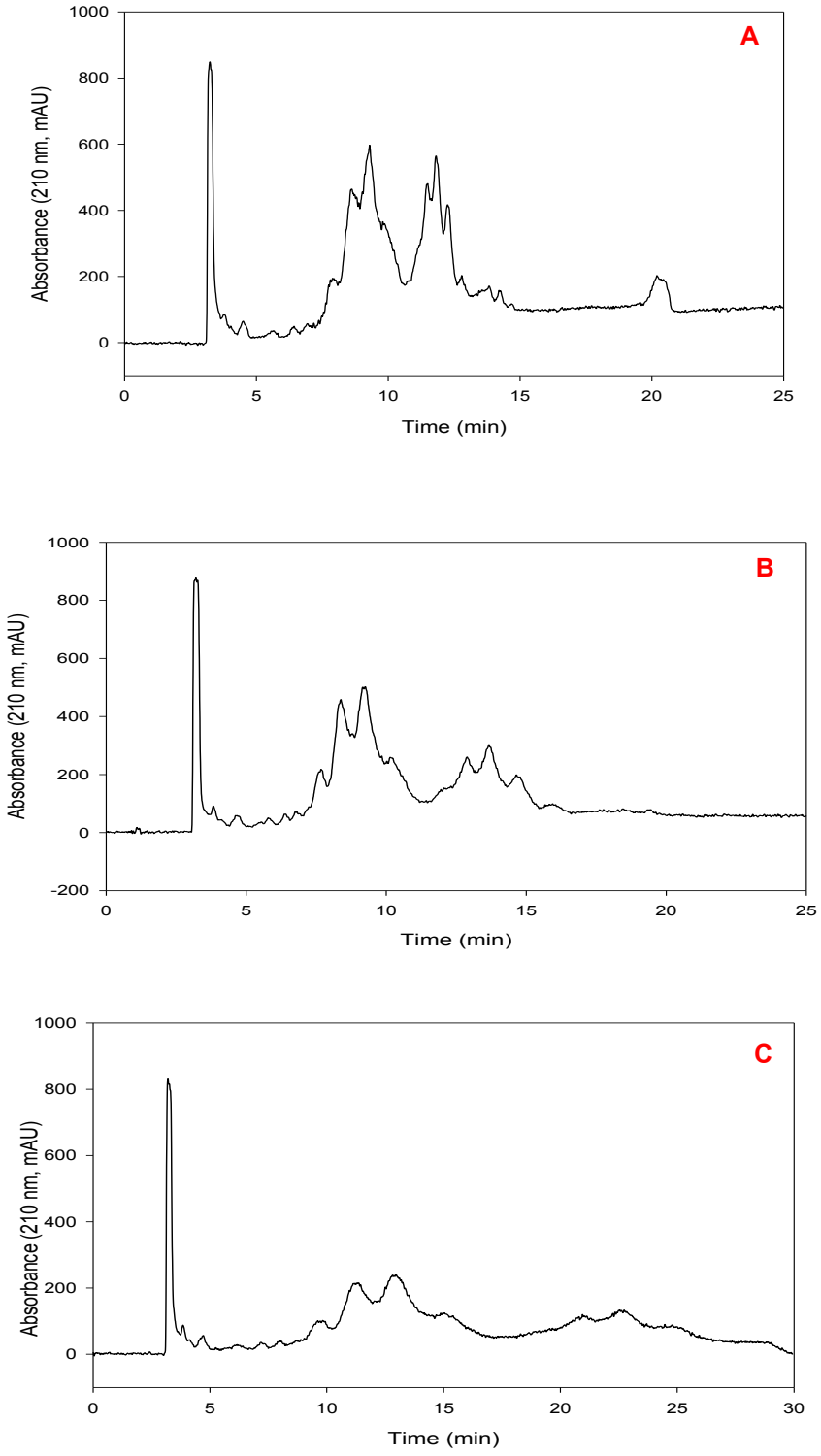


Fig 3.16. Separation of salmon oil by three Ag-HPLC gradients mixture of MeOH/ MeOH and 20% ACN: (A) 100:100; (B) 100:50; (C) 80:20.

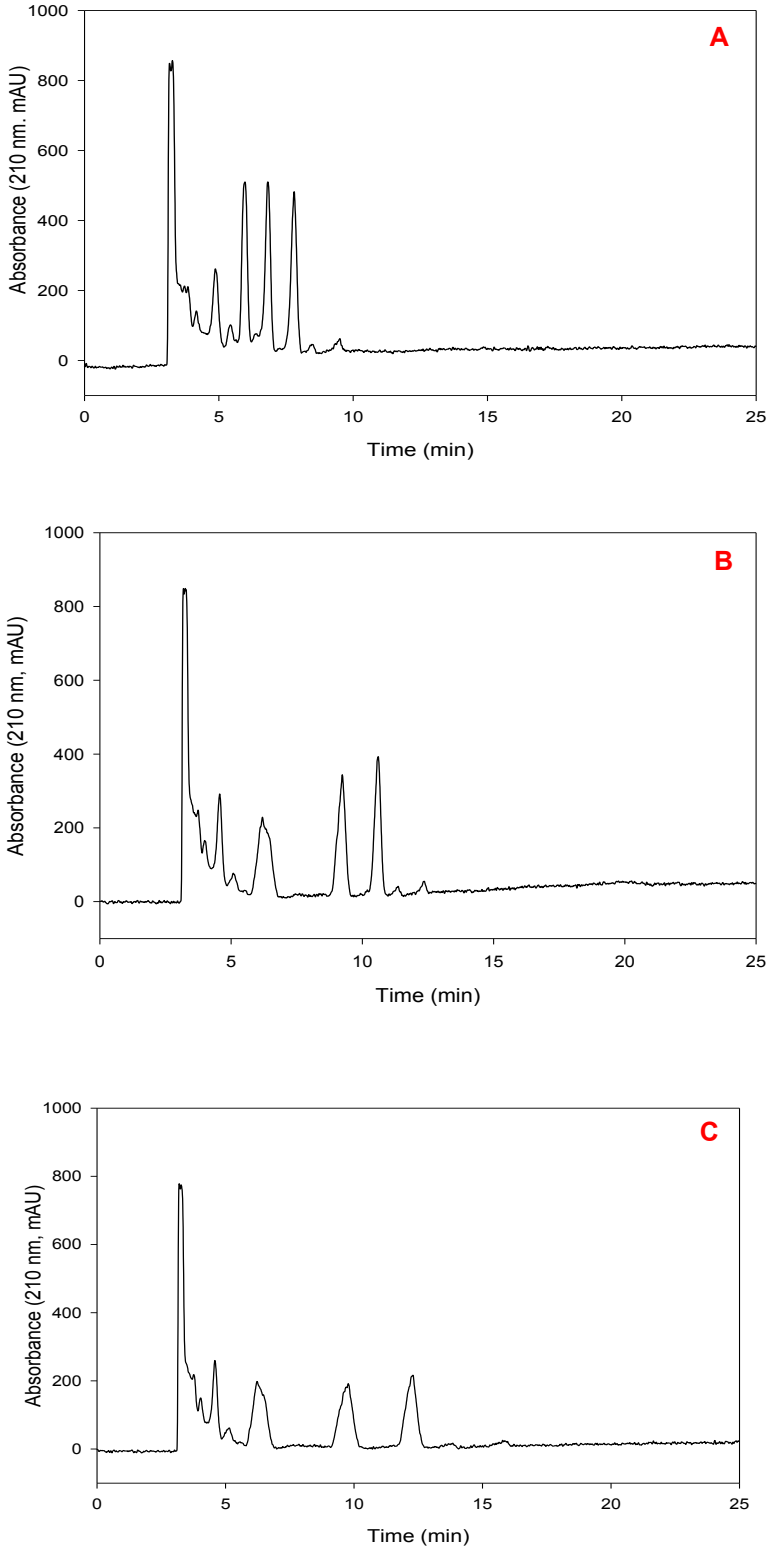


Fig 3.17. Separation of corn oil by three Ag-HPLC gradients mixture of MeOH/ MeOH and 20% ACN: (A) 100:100; (B) 100:50; (C) 80:20.

3.4.6.2. Separation of grape seed and canola oils by Ag-HPLC

The chosen gradient [MeOH (solvent A) and MeOH with 20% ACN (solvent B) with ratio of 100:50] was used to separate rest of the oil samples as shown in Figs. 3.18 and 3.19. The TAGs in grape seed and canola oils were well resolved and the canola oil showed more TAG peaks than the grape seed because the former contained more unsaturated fatty acids as confirmed by GC/MS.

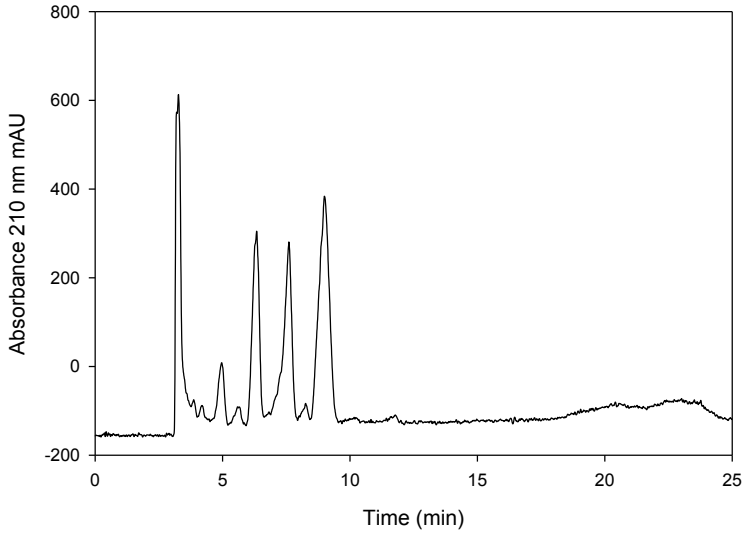


Fig 3.18. Ag-HPLC chromatograms of grape seed using gradients mode [MeOH and MeOH with 20% of ACN with ratio of 100:50].

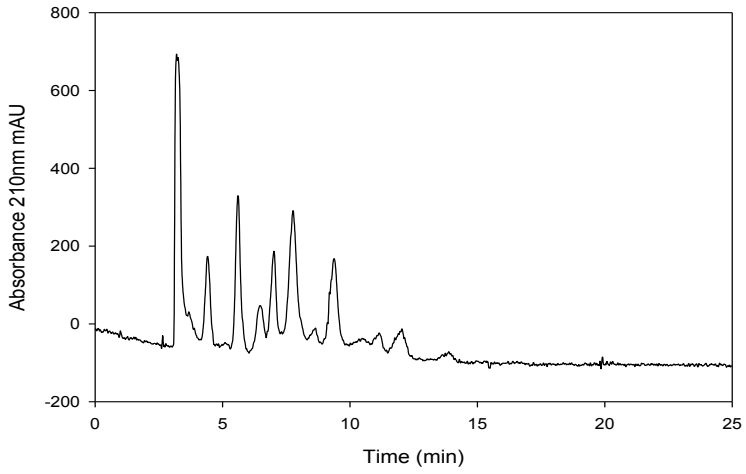


Fig 3.19. Ag-HPLC chromatograms of grape seed using gradients mode [MeOH and 20% ACN in MeOH with ratio of 100:50 at UV 215 nm].

3.5. Conclusions

The BF_3-MeOH method was applied to convert all free fatty acids and triacylglycerols to methyl esters; the method worked well. The FAMEs in TAGs were identified by GC-MS. The TAGs of the corn, canola, grape-seed, and salmon oils were resolved by two liquid chromatographic methods, namely RP-HPLC and Ag-HPLC.

The TAGs in the corn oil were resolved according to their PN and DB using RP-HPLC and Ag-HPLC. Their chromatograms were less complicated than the rest of the oils and a few TAG peaks were observed. Therefore, the corn oil was chosen as the model oil in this thesis for spiking it with chlorinated TAGs.

After the corn oil was chosen, the major challenge was the identification of every single peak in the RP-HPLC and Ag-HPLC chromatograms (Figs.3.12 and 3.17B). It became an extremely complex task because of the presence of many different fatty acids with diverse physical and chemical properties. This problem is dealt with in the next chapter.

CHAPTER 4

Identification of triacylgcerols in corn oil

4.1. Objectives

The overall objective of this thesis, as pointed out in the Introduction, is to investigate triacylglycerols (TAGs) and chlorinated TAGs (Cl-TAGs) in real salmon oil. It has been mentioned in Chapter 1 that not much work has been reported in the literature on these topics. Moreover, our preliminary results of salmon oil presented in Chapter 3 using Ag-HPLC and RP-HPLC show very complicated chromatograms containing a large number of unidentified TAGs. It was felt necessary to find an oil with known Cl-TAGs for identification the above TAGs by comparison. However, no such oil was available. The next step was then to find oil which could be spiked with Cl-TAGs. Among the three vegetable oils investigated in Chapter 3, corn oil was chosen as the representative oil for studying Cl-TAGs because it has been characterized more than any other oils and has well-documented TAGs. It was still necessary to characterize the corn oil being used in our laboratory for TAGs prior to spiking it with Cl-TAGs. The optimization of two-dimensional separation systems for the identification of TAGs in the corn oil sample was done using RP-HPLC/GC/MS, RP-HPLC and Ag-HPLC (both on-line and off-line), RP-HPLC/ESI(+)/MS and Ag-HPLC/ESI(+)/MS, and are described in detail in this chapter.

4.2. Introduction

Oil is a complex mixture of TAGs. They are composed of a glycerol backbone esterified with saturated or unsaturated fatty acids or a mixture of both. Thus for the structural characterization of TAGs one needs to know the exact chain length of the fatty acid, degrees of unsaturation, locations of double bonds, and positions of the fatty acids on the glycerol backbone (position 1 or 3 *vs.* position 2) [Hsu and Turk, 1997; 2008]. Furthermore, each TAG species may be differentiated by stereospecific or regiospecific isomers [Dobson *et al*., 1995].

Conventional separation of TAG mixtures containing saturated and unsaturated fatty acids can be done by either the total number of carbon atoms in the acyl chains by RP-HPLC (*i.e.,* A higher PN value leads to increased retention times) or the degree of unsaturation by Ag-HPLC (*i.e.,* the higher the number of DB, the longer is the retention

time) [Christie and Han, 2010]. However, samples containing TAGs with the same PN (*e.g.,* PLO and OLO) can be adequately separated by Ag-HPLC (PLO: DB=3; OLO: DB=4). On the other hand, RP-HPLC can be used to separate TAGs with the same number of DB (*e.g.*, POO and SOO) according to their PN (48 and 50) which cannot be accomplished by Ag-HPLC [Holčapek *et. al.,* 2003]. In such cases, a combination of these two techniques using two-dimensional chromatography has been developed.

A complete characterization of the TAG fraction of an oil/fat would require the separation of all individual TAGs followed by quantification of the separated species. To the best of author's knowledge, no single analytical tool exists that has the separation capacity to accomplish it. A combination of different analytical techniques generally allows detailed analysis, for example, coupling chromatography with mass spectrometry. Multidimensional chromatography can provide a more complete characterization of TAGs. The coupling of two forms of chromatography can be done in three ways: off-line, on-line "heart cut", and on-line comprehensive which have already been described in detail in Chapter 2 (Section 2.3.2.4). Two of these multidimensional techniques namely, off-line and on-line "heart cut" were used to identify the TAG components of corn oil in this thesis work.

An improved on-line comprehensive HPLC system for the analysis of TAGs of corn oil has been reported by Elbert *et al.,* (2008) using Ag-HPLC column with slow gradient in the first-dimension and a fast isocratic reversed-phase separation in the second-dimension. Detection was done by UV at 210 nm, evaporative light scattering and (+) atmospheric pressure chemical ionization mass spectrometry (APCI/MS) with the latter giving the best results. However, in terms of peak overlap, the development of the multidimensional Ag-HPLC/RP-HPLC/APCI-MS system showed a remarkable increase in peak capacity and was helpful for the qualitative and quantitative determination of TAGs. Recently, 18 TAGs were identified in a corn oil sample by applying off-line two dimension methods using RP-HPL/Ag-HPLC/APCI/MS; in addition, the TAGs data were analyzed by principal component analysis (PCA) [Wei *et al*., 2015]. For these reasons, the 2D off-line and on-line Ag-HPLC/ RP-HPLC coupled with ESI(+)/MS methods were applied to identify the TAGs in corn oil sample analyzed in this thesis.

Methods for the analysis of TAGs in corn oil using RP-HPLC-ELSD/FID [Neff *et al*., 1999], RP-HPLC/APCI/MS [Holčapek *et al*., 2003], RP-HPLC/APCI/MS/GC/FID [Byrdwell *et al*., 2001], and RP-HPLC/FID [Carvalho *et al*., 2012] have been reported in the literature. None of these researchers used ESI(+)/MS as the detection technique.

While our research on TAGs in corn oil using Ag-HPLC/ RP-HPLC coupled with ESI(+)/MS was in progress Zeb and coworkers [2010; 2013; and 2015] published reports on the oxidation of TAGs in corn oil.

4.3. Experimental

4.3.1. Chemicals

Various triglycerides namely, dipalmitin (DAG-C16:0, 99%), glycerol tripalmitate (TAG-C16:0, 99%), glycerol tristearate (TAG-C18:0, 99%), 1-oleoylglycerol (MAG-C18:1, 99%), glycerol trioleate (TAG-C18:1, 99%), glycerol trilinoleate (TGA-C18:2, 99%), and glycerol trioctanoate (TGA-C8:0, 99%) were purchased from Sigma. A standard mixture of triglycerides (MAG-C18:1, DAG-C16:0, TAG-C18:0, TAG-C16:0, TAG-C18:2, TAG-C8:0 and TAG-C18:1 was prepared by dissolving equal amounts in hexane.

4.3.2. Instruments and operating conditions

4.3.2.1. RP-HPLC

The second RP-HPLC system, namely Shimadzu LC-10AD model, was operated using an Ascentis Express C18 column (3 cm × 7 mm filled with 1.5 µm particles). A mixture of MeOH and isopropanol (70:30 v/v) were used as the mobile phase in isocratic mode with a flow rate of 3 mL min^{-1}.

4.3.2.2. ESI/MS

The ESI-MS and ESI-MS/MS experiments were done using a Thermo-Finnigan LCQ DUO ion trap mass spectrometer running Xcalibur software and equipped with an ESI probe set at 3.7 kV. Nitrogen was used as the source gas and the capillary temperature was maintained at 200°C. Tandem mass spectra (MS/MS) were acquired using helium collision gas at collision-induced dissociation (CID) energies given in arbitrary units (%). Sample solutions were introduced into the ion source of the mass spectrometer by flow injection in MeOH (syringe pump, 1.2 mL h^{-1}) via a Rheodyne 7725 injection valve.

4.4. Results and discussion

The corn oil sample was diluted with hexane and directly injected onto an HPLC column without any purification. Separation and detection of TAGs was achieved using various 2D methods off-line, namely RP-HPLC/GC/MS, Ag-HPLC/RP-HPLC, Ag-HPLC/ ESI(+)/MS, and RP-HPLC/ESI(+)/MS. Derivatization was done only for the RP-

HPLC/GC/MS method. Only Ag-HPLC/RP-HPLC method was used both on-line and off-line. Results obtained using these methods are discussed below.

4.4.1. Off-line 2D RP-HPLC/GC/MS derivatization of triglyceride mixture and corn oil

The off-line 2D RP-HPLC/GC/MS technique was first applied to a commercially available mixture of triglycerides and then to the corn oil sample. The sensitivity of the GC/MS method used in the second dimension could be improved either by evaporative pre-concentration of the HPLC fractions (suitable for small number of fractions) [Jassen *et al*., 2003] or by changing the GC at a spilt ratio of 50:1 rather than the typical 100:1. A ratio of 50:1 was used in the work reported in this chapter.

4.4.1.1. Off-line 2D RP-HPLC/GC/MS derivatization method for the commercial triglyceride mixture

The triglyceride mixture described in Section 4.3.1 gave 4 peaks by the RP-HPLC method using a rapid gradient (Supelcosil LC-PAH (25 cm x 4.6 mm X 3 μ particles) with 100% ACN (solvent A) and 100% isopropanol (solvent B) over 35 min ("rapid gradient") at a flow rate of 1 mL min^{-1}) as shown in Fig. 4.1. It should be noted that the rise in baseline in the chromatogram associated with the solvent and perhaps because the column is new. However, a shift was still observed even though the blank was run prior to the sample.

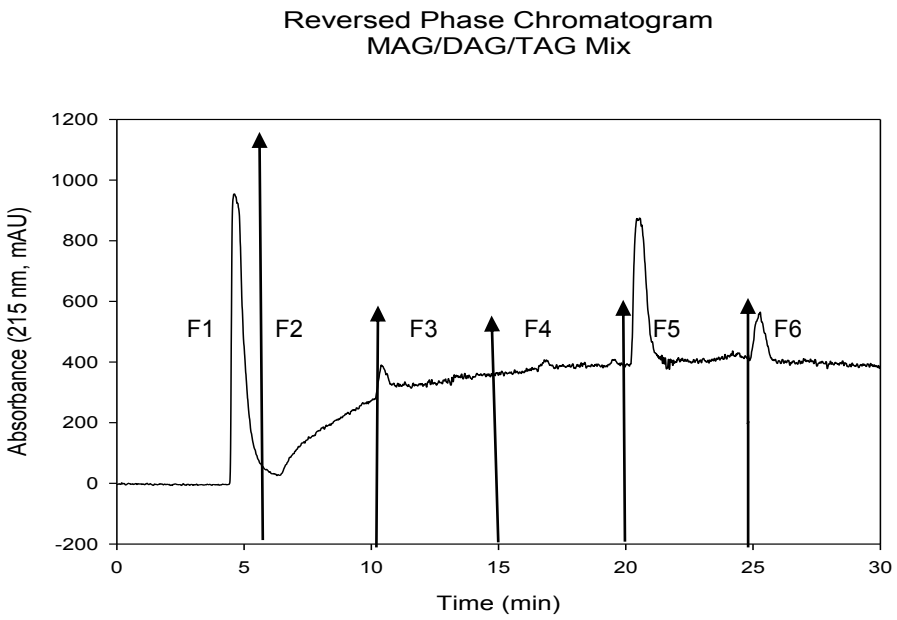


Fig 4.1. Collection of 6 fractions of triglyceride mixture by the RP-HPLC method.

Six RP-HPLC fractions, each of 5 min duration, were collected and converted to their methyl esters using the BF_3-MeOH procedure. The FAME and the isopropyl esters of the fatty acids (FA-isopropanol) in these fractions were identified by retention times and mass spectra. The isopropyl esters are produced by the reaction of isopropanol in the eluent with the fatty acids. For example, F5 gave FAME and FA-isopropanol of C18:2 as shown in Fig 4.2. The results of the GC/MS of each fraction (F) are presented in Table 4.1. It has been reported in the literature that the sensitivity of the GC/MS method used in the second dimension could be improved either by evaporative pre-concentration of the HPLC fractions (suitable for small number of fractions) [Jassen *et al.*, 2003] or by changing the GC at a spilt ratio of 50:1 rather than the typical 100:1. A ratio of 50:1 was used in the work reported in this chapter.

Table 4.1 The fractions of the triglycerides mixture depend on the time.

F #	Time (min)	Results (FAME / FA-isopro)
1	0-5	Nothing
2	5-10	C8:0
3	10-15	C8 & C18:1
4	15-20	C16
5	20-25	C18:2
6	25-30	C18:1

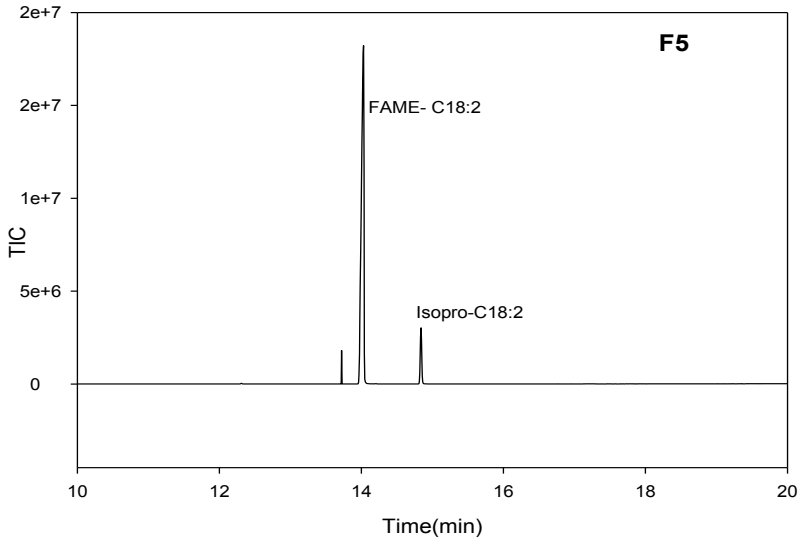


Fig 4.2. GC/MS chromatogram of F5 of RP-HPLC separation of the triglyceride mixture (GC/MS condition showed in chapter 3 section 3.3.2.1).

It is evident from Table 4.1 that tristearate (TAG-C18:0) was not detected. If present above the detection limit, would elute after the TAG-C16:0 because of its higher partition number; perhaps it could have been identified with a longer elution time. In order to identify each TAG of corn oil, a comparison of its retention times and mass spectra with the standard mixture was carried out.

4.4.1.2. Off-line 2D RP-HPLC/GC/MS derivatization method for the corn oil

The triacylglycerol species present in the corn oil sample were detected by a 2D RP-HPLC/GC/MS method with rapid gradient (using two Supelcosil LC-PAH (25 cm x

4.6 mm X 3 μ particles) columns coupled in series with linear gradient was 100% ACN (solvent A) to 100% isopropanol (solvent B) over 35 min ("rapid gradient") at a flow rate of 1 mL min^{-1}). Seven fractions were collected as shown in Fig. 4.3 chromatogram.

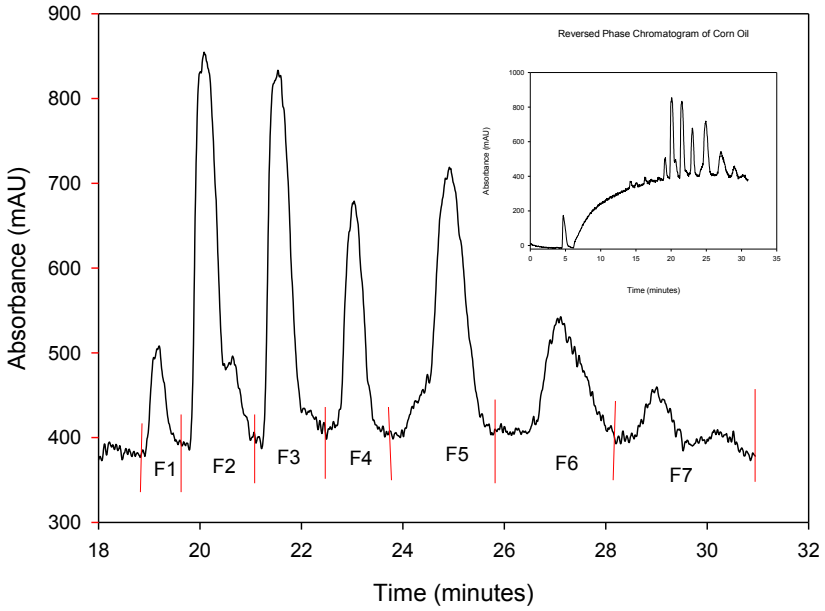


Fig 4.3. The RP-HPLC chromatogram of the corn oil fractions collected for GC/MS (the insert shows the complete chromatogram).

These fractions were converted to their methyl esters using the BF_3-MeOH method. Each FAME in these fractions was identified by GC/MS and the results are summarized in Table 4.2. These results are similar to those reported by Chow (2008).

Table 4.2. Fatty acids found in the different fractions of corn oil by GC/MS.

F #	Time (min)	Results (FAME)
1	19.3	C16:0, C18:1
2	20.2	C18:2
3	21.7	C18:2, C18:1
4	23.3	C18:2, C18:1
5	26.3	C16:0, C18:2, C8:1
6	27.5	C16:0, C18:2, C8:1
7	29.7	C16:0, C18:2, C8:1, C18:0, C20:1

The GC/MS analysis (Table 4.2) showed that F2 contained only C18:2 fatty acid. The retention time of F2 (Fig 4.3) was similar to that of the TAG-C18:2 commercial standard (Table 4.1), suggesting that TAG-C18:2 is a component of corn oil. The GC/MS chromatogram of F2 gave the FAME-C18:2 as shown in Fig.4.4.

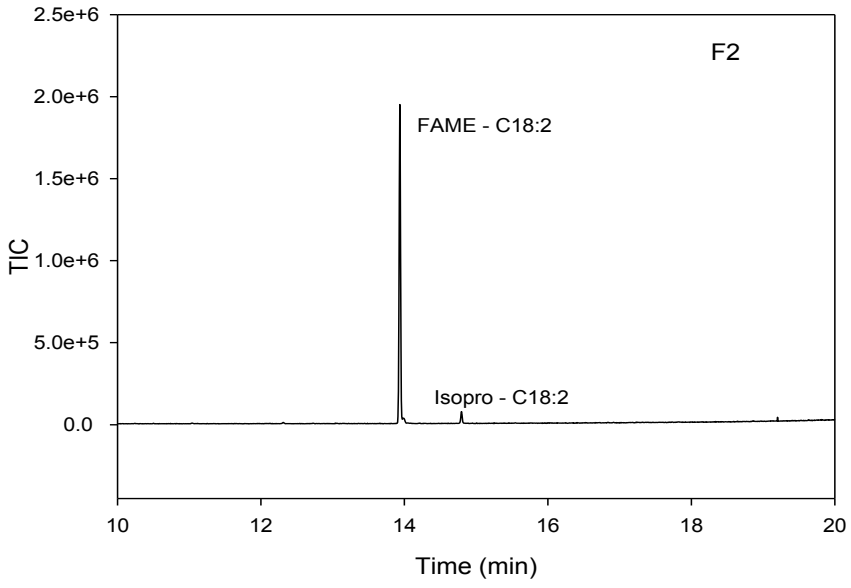


Fig 4.4. GC/MS chromatogram of F2 of the RP-HPLC separation of corn oil.

From the data given in Table 4.2 it is clear that the fatty acid compositions of each fraction derived from the GC/MS data are in excellent agreement with the elution order and the estimated PN of the TAGs. A higher PN value leads to increased retention times. As expected, F2 with three C18:2 (TAG-C18:2) and PN of 42 eluted before F3 containing two C18:2 and one C18:1(TAG-C18:2/C18:2/C18:1) with a PN of 44, and F4 with two of C18:1 and one of C18:2 (TAG-C18:1/C18:1/C18:2) with a PN of 46, shown in a separate Fig 4.5. The fractions 5, 6, and 7 are broader in the RP-HPLC chromatograms (Fig. 4.3) suggesting the presence of more than one TAG. Consequently, another detection technique, such as ESI/MS, is needed to identify type of TAGs in these fractions.

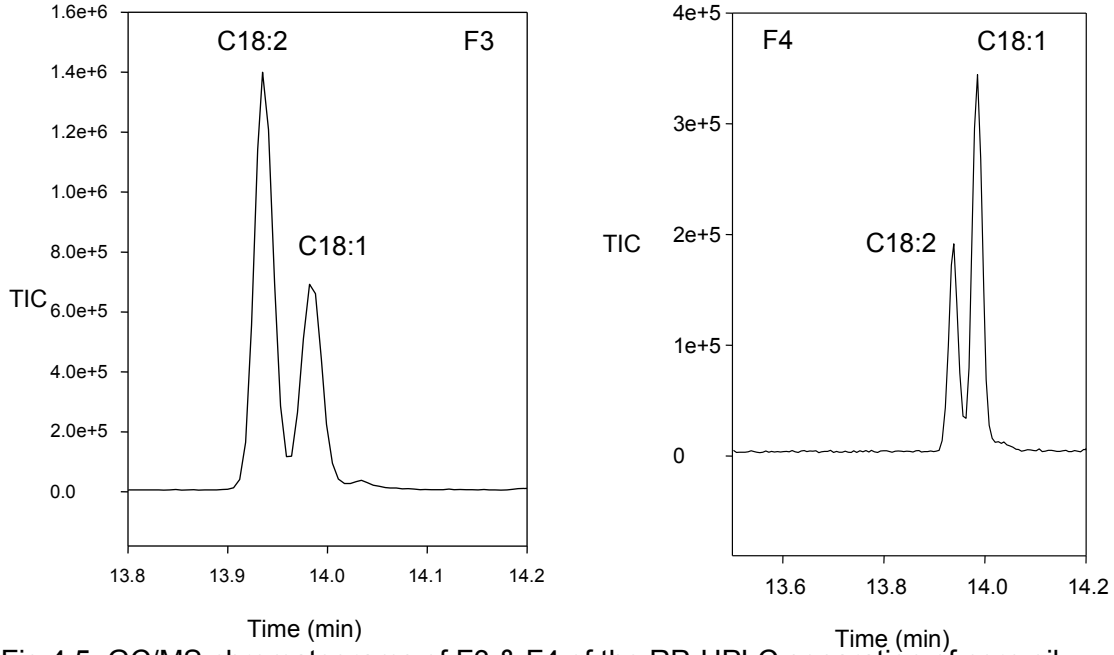


Fig 4.5. GC/MS chromatograms of F3 & F4 of the RP-HPLC separation of corn oil.

4.4.2. On-line and off-line Ag-HPLC/RP-HPLC methods for the separation of TAGs in corn oil

The 2D HPLC (Ag-HPLC/RP-HPLC) systems in both off-line and on-line modes were applied to the separation of TAGs in the corn oil for improved sensitivity and are herein described. In the first dimension of the off-line Ag-HPLC/RP-HPLC the TAGs of the corn oil sample were separated in gradient mode (chapter 3, section 3.3.2.2) and fractions collected. In the second dimension these fractions were run in isocratic mode to

improve sensitivity. The retention times in the second dimension was kept as short as possible with a high flow rate to avoid excessive dilution of the sample [Dugo *et al*., 2006; Horvath *et al*., 2009]. The fact that the separation could be carried out under isocratic conditions was considered especially important so that no time was lost for re-equilibration of the column prior to the next injection. Vanderheyden *et al.* [2013] studied different columns with different particle sizes in an isocratic mode and showed that for very fast separations with relative low plate numbers packed columns with small particles are required. For these reasons, a short C18 column (Ascentis Express C18 column, 3 cm × 7 mm filled with 1.5 µm particles) as the second dimension (RP-HPLC) in an isocratic mobile phase of MeOH-*iso*propanol (70:30) at a flow rate of 3 mL min^{-1} was used in this thesis.

4.4.2.1. Effect of eluent volume ratio for the separation of TAG in corn and salmon oils using RP-HPLC in isocratic mode

The TAGs in corn and salmon oil samples were separated using an isocratic RP-HPLC method at a flow rate (3 mL min^{-1}) and two eluent ratios (100:100 and 70:30 v/v of MeOH/isopropanol). The quality of separation was quite good even at this high flow rate as shown in Fig. 4.6 A and B and Fig. 4.7 A and B. Since a fast separation was needed for the on-line 2D method for TAGs in corn oil, the lower eluent volume ratio (70:30) was selected as it gave less than 10 min elution time. The same ratio was also applied to the off-line method for corn oil. It is evident from Fig. 4.7B that the 100:100 eluent ratio gave a better separation for salmon oil TAGs and was consequently used.

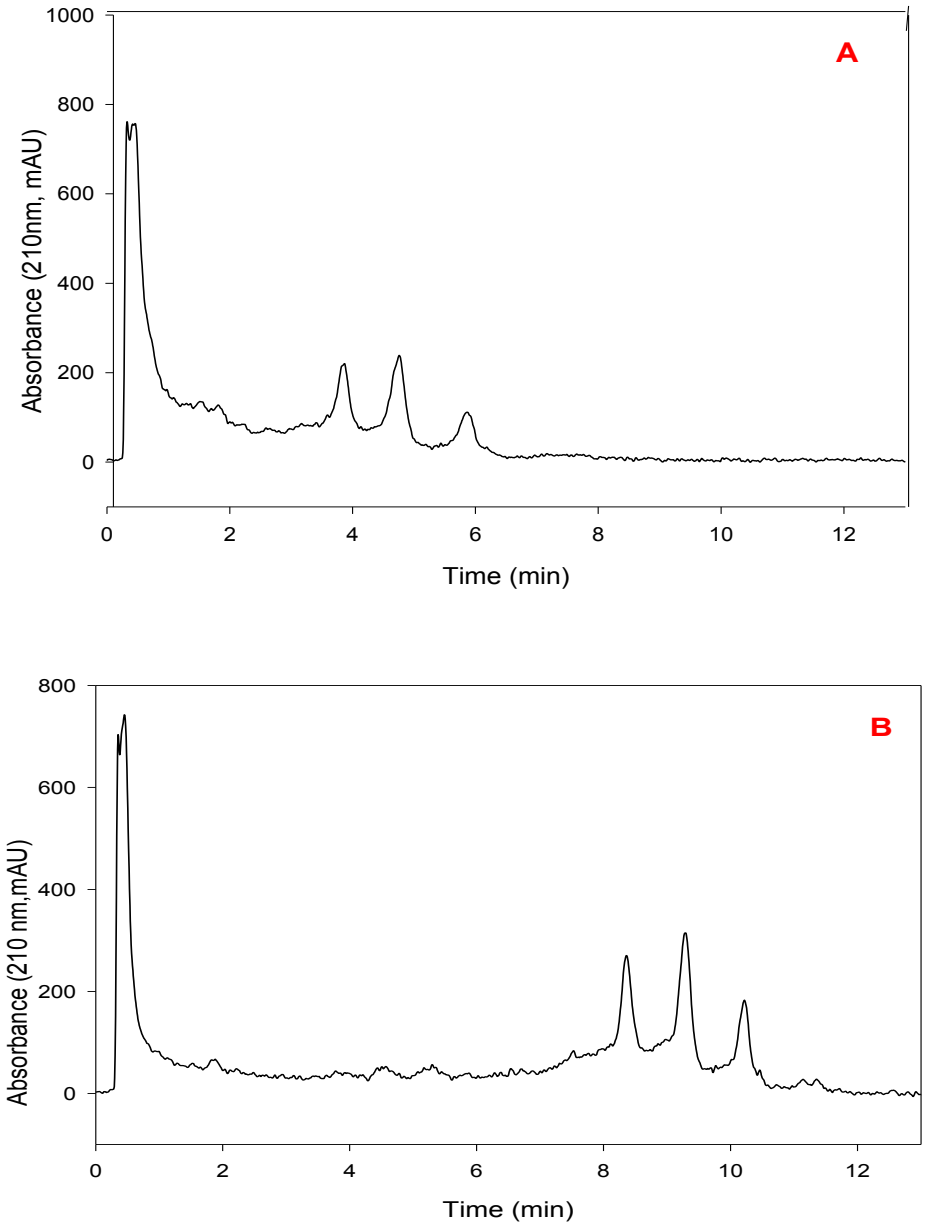


Fig 4.6. RP-HPLC isocratic separation of corn oil using MeOH:isopropanol: (A) 70:30; (B) 100:100 (v/v)

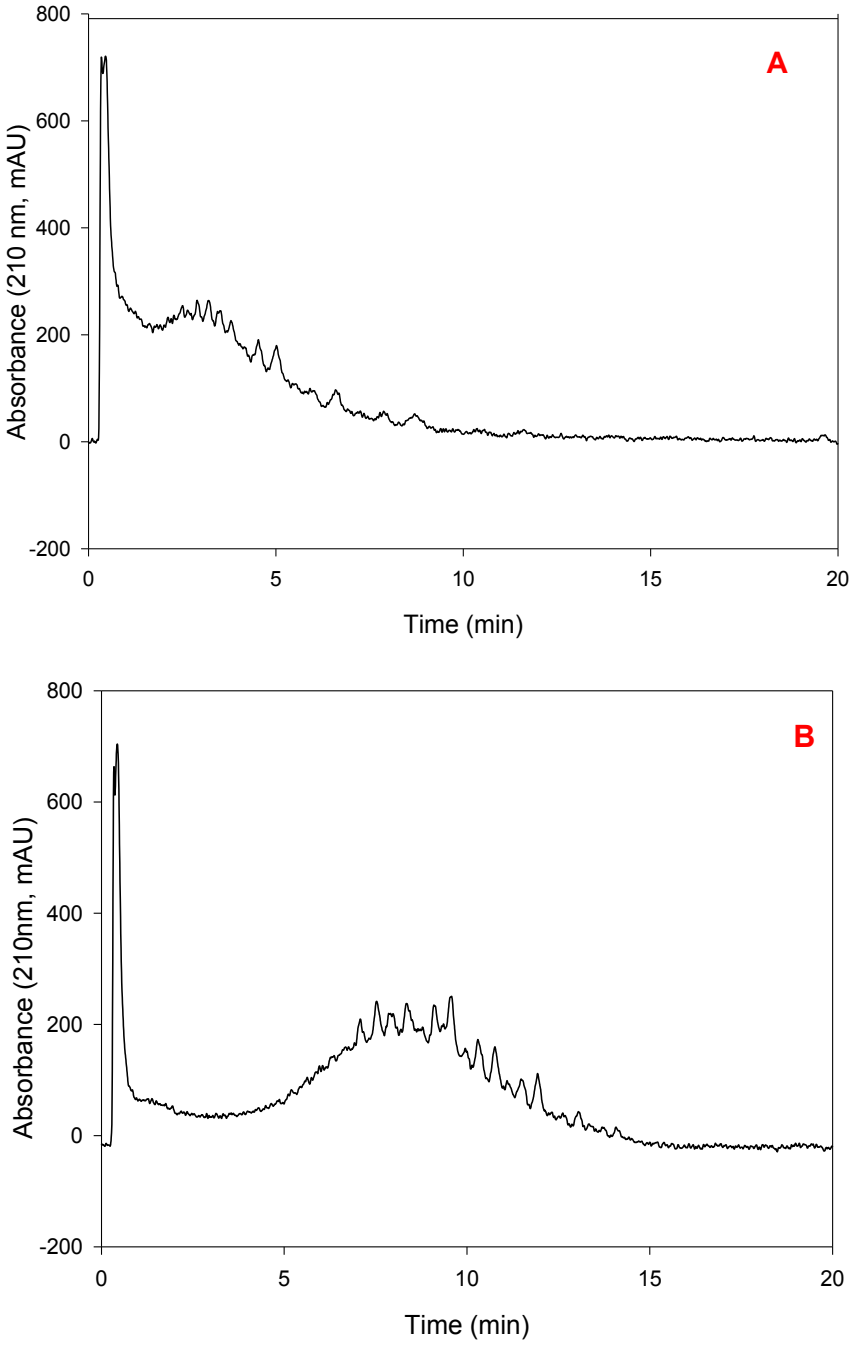


Fig 4.7. RP-HPLC isocratic separation of salmon oil using MeOH:isopropanol (A) 70:30; (B) 100:100 (v/v).

4.4.2.2. Off-line 2D Ag-HPLC/RP-HPLC method for the separation of the TAGs in corn oil

The corn oil sample was fractionated by the off-line 2D Ag-HPLC/RP-HPLC method for TAGs. In the first dimension 6 fractions were collected (Ag-HPLC column gradient mode with a mixture of MeOH and MeOH in 20% ACN (100:50)) as shown in Fig 4.8. These fractions were injected onto the second dimension of the C18 column (RP-HPLC isocratic mode with isopropanol and MeOH (70:30)). The sums of these fractions are shown in Fig 4.9 (black line).

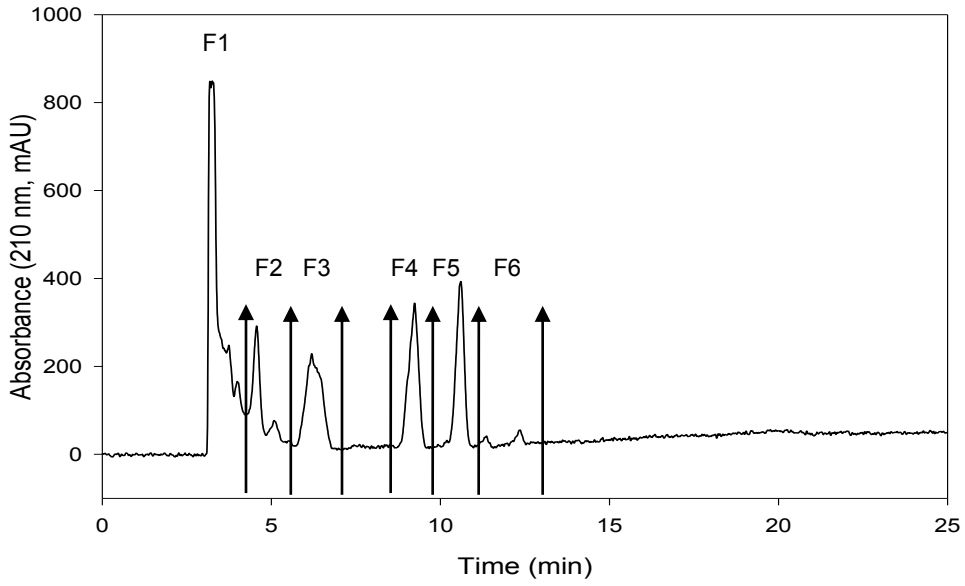


Fig 4.8. Collection of 6 fractions of corn oil sample from the first dimension (Ag^+ column gradient mode with a mixture of MeOH and MeOH in 20% ACN (100:50) at a flow rate of 1 mL min^{-1}).

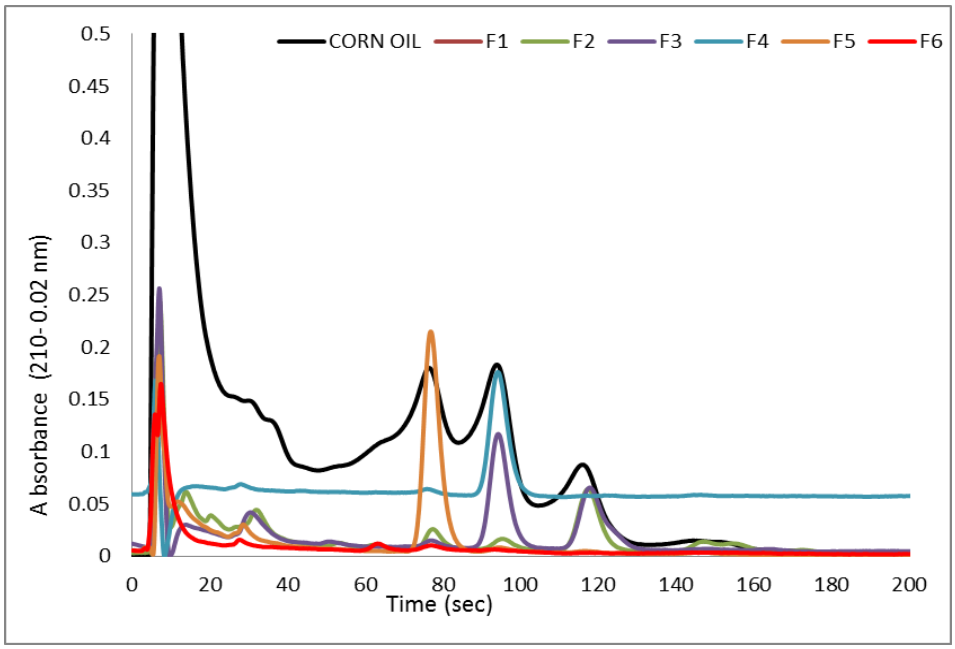


Fig 4.9. Separation of 6 fractions of the corn oil in second dimension RP-HPLC (C18 column (Ascentis Express C18 column, 3 cm × 7 mm filled with 1.5 µm particles) as the second dimension (RP-HPLC) in an isocratic mobile phase of MeOH-*iso*-propanol (70:30) at a flow rate of 3 mL min^{-1}

The chromatograms in Fig.4.9 are better viewed as two separate figures, namely Fig. 4.10 A and B. The Fig.4.10A shows the first three fractions (F1-F2-F3) separated to 4 TAGs with the same number of double bonds but different partition numbers. The Fig. 4.10B presents F4 and F5 fractions separated into one single TAG of each. The TAG in the F6 fraction appears to be below the detection limit of the method.

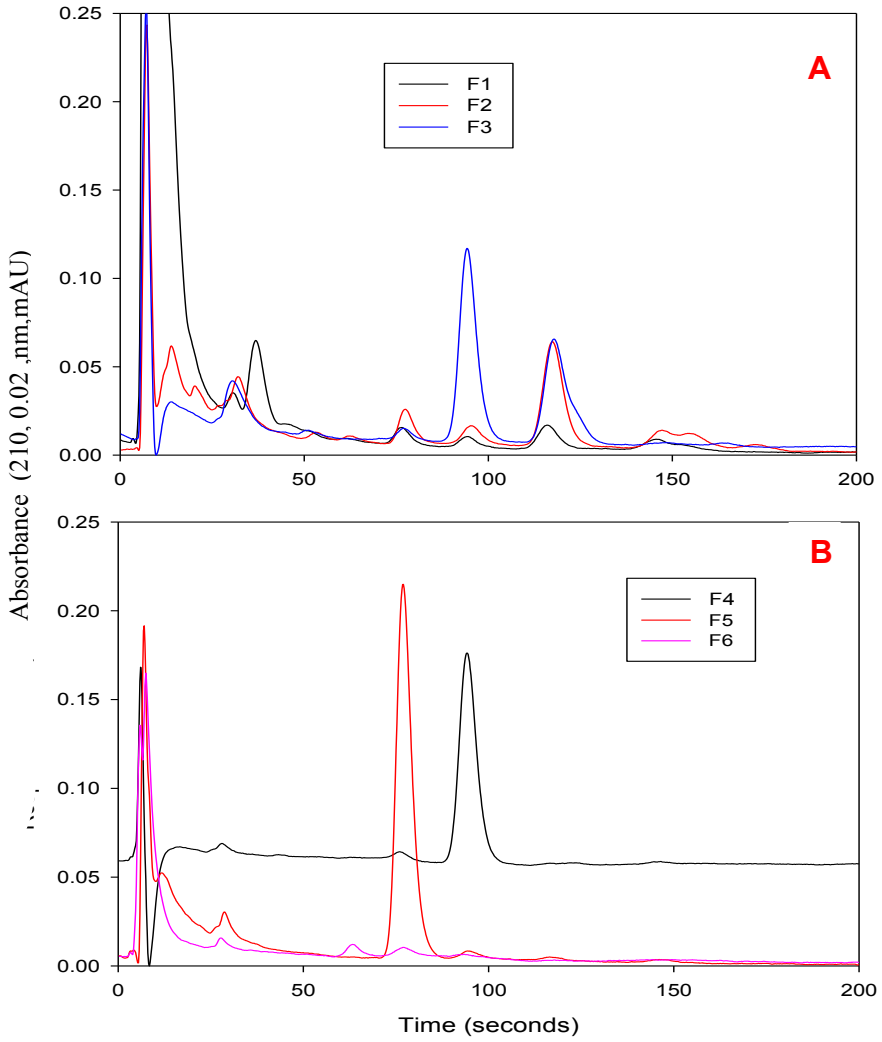


Fig 4.10. The TAG fractions separated in second dimension RP-HPLC (Fig 4.9.); (A) F1, F2, F3; (B) F4, F5, F6.

The separation of the above 6 fractions using 2D Ag-HPLC/RP-HPLC off-line method permitted a greater differentiation between several TAGs in corn oil that contained the same number of double bonds but different partition numbers.

4.4.2.3. On-line 2D Ag-HPLC/RP-HPLC method for separation of TAGs in corn oil

The on-line 2D Ag-HPLC/RP-HPLC method was carried out without any fractionation but using the same columns used in the off-line method. The 2D Ag-HPLC separation was applied using the “heart-cutting” technique using a 6-port valve equipped with one sample loop that allowed transfer from the first dimension column of Ag-HPLC to the fast second column of RP-HPLC. The 2D set-up is shown in Fig 4.11.

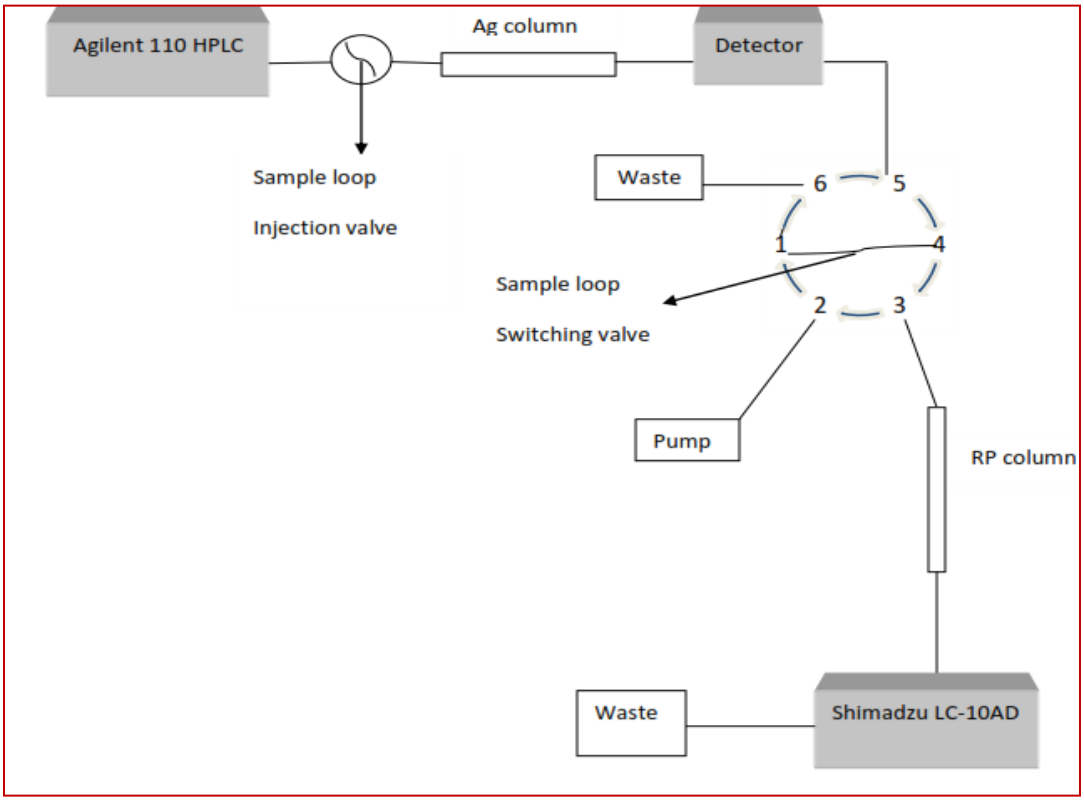


Fig 4.11. The set-up for the on-line 2D Ag-HPLC/RP-HPLC method

An on-line cut of the corn oil peak number 2 in Ag-HPLC is shown in Fig. 4.12A and the chromatograms of the 6 cut positions separated in RP-HPLC are given in Fig 4.12B. The chromatogram of each cut position was relative to different TAGs that eluted according to increasing PN. A preliminary investigation of 6 TAGs cut from peak 2 indicated the presence of (ALO), (SLO/SOL), (OOO), and (POL/PLO) with PNs of 50, 48, 48, and 46, respectively, with the same number of DB (3). The prediction of these TAGs in F2 was in the accordance with the one reported by Elbert *et al.,* (2008) using

Ag-HPLC column with slow gradient in the first-dimension and a fast isocratic RP-HPLC separation in the second-dimension; detection was done using (+) atmospheric pressure chemical ionization mass spectrometry (APCI+/MS). These abbreviations stand for the three fatty acids linked to the glycerol backbone of the TAGs where P is for palmitic acid (C16:0), S for stearic acid (C18:0), O for oleic acid (C18:1), L for linoleic acid (C18:2), Ln for linolenic acid (C18:3) and A for arachidic acid (C20:0).

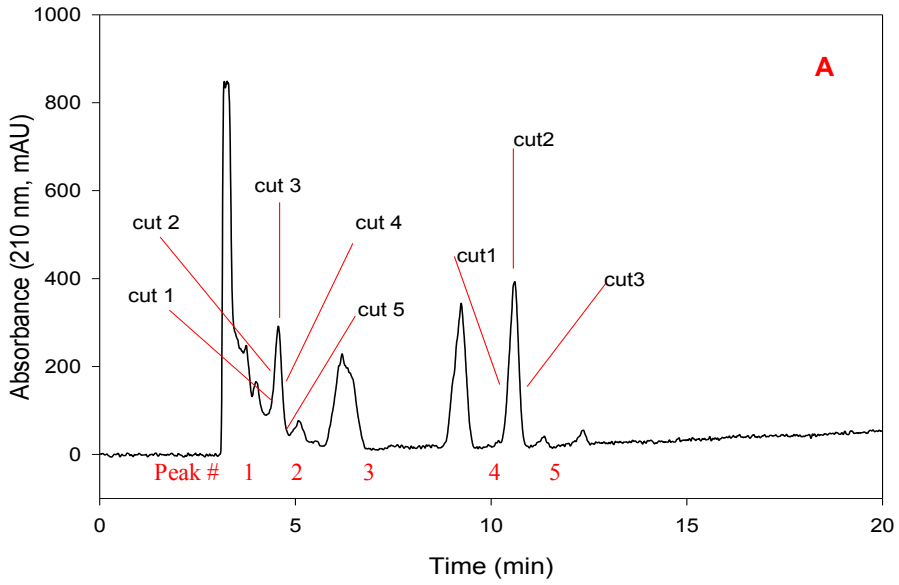


Fig. 4.12A Different cutting positions of peak 2 and 5 in Ag-HPLC.

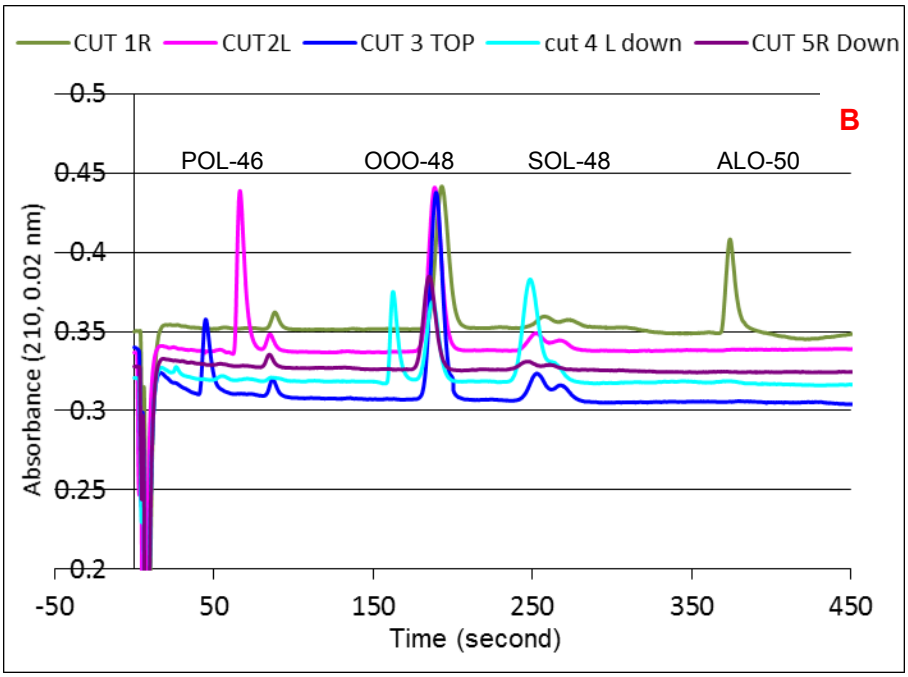


Fig 4.12B Chromatograms of the 6 cut positions of peak 2 separated in RP-HPLC on-line method.

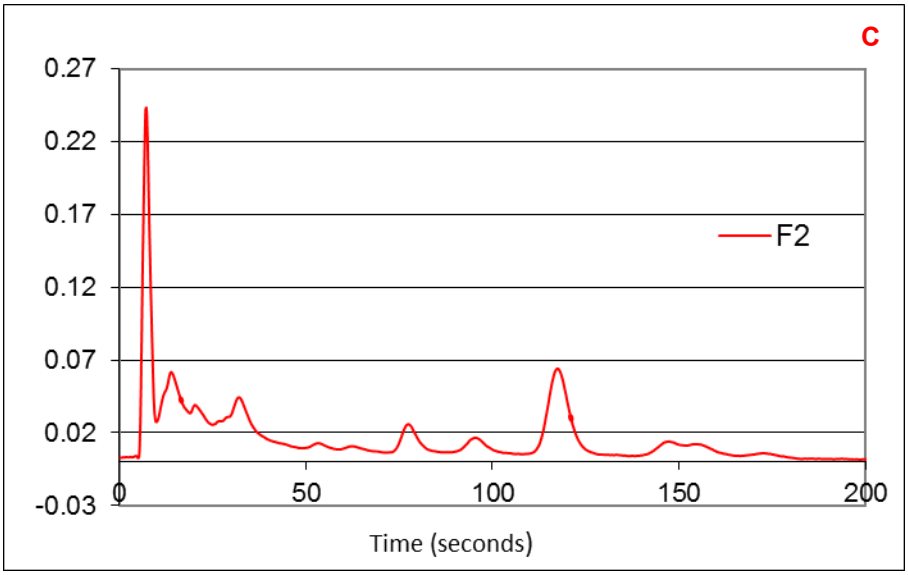


Fig 4.12C Chromatogram of F2 (Fig 4.8) separated in second dimension RP-HPLC (Fig 4.9.) off-line method.

A comparison of the results obtained by the two separation methods (2D off-line and on-line Ag-HPLC/RP-HPLC) was carried out. The result of peak 2 cutting using the 2D on-line method agreed with those of the off-line method for F2 shown in Fig 4.10A

and simplified in Fig 12C. In both 2D methods peak 2 gave 4 TAGs. In another case, cutting of peak 5 (Fig. 4.12A) in three different positions also gave the same results because of overlapped cutting positions (Fig, 4.13). The TAGs in RP-HPLC could be LLL (DB:PN 6:42) and OLLn (DB:PN 6:42). These results agree with the 2D off-line of F5 shown in Fig. 4.10B.

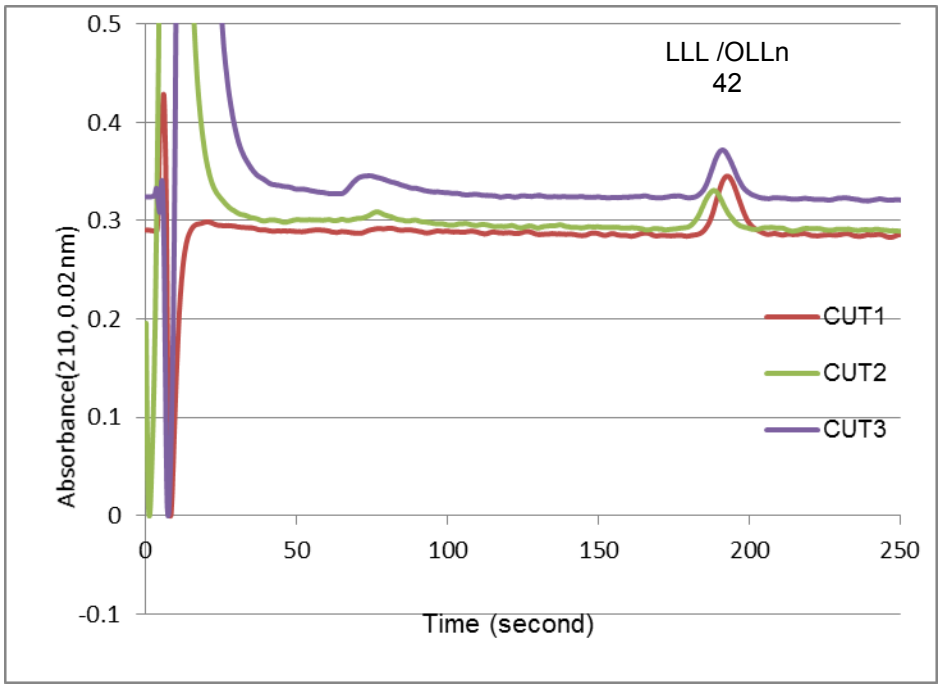


Fig 4.13. Chromatograms of the 3 cutting positions of peak 5 separated in RP-HPLC.

4.4.3. Off-line 3D Ag-HPLC/ESI(+)/MS/GC-MS for identification of TAGs in corn oil

The corn oil sample was separated into 10 fractions by the Ag-HPLC method (Fig 4.14) in the first dimension then subjected to ESI mass spectrometry as second dimension. The collected fractions were also converted to their methyl esters using the BF_3-MeOH method and the FAMEs in these fractions were determined by GC/MS as third dimension. The results are presented in Table 4.3. It is important to note that the Ag-HPLC chromatogram shown here (Fig 4.14) was the first run of a corn oil sample through the Ag^+ column; therefore, the separation pattern of the corn oil peaks in the Ag-HPLC chromatogram shown here (Fig 4.14) is slightly different from the Ag-HPLC chromatograms shown in Figs 4.8 and 4.12A. The fatty acids in each TAG fraction were

identified by GC/MS as 3D. The elution time (retention time) of each peak in the first dimension Ag-HPLC coeluated with the number of double bonds. Longer elution time on the Ag^+ phase was observed with increasing unsaturation. The TAGs present in each fraction were detected by combing the fatty acids identification to match the masses of the TAGs measured by ESI(+)/MS.

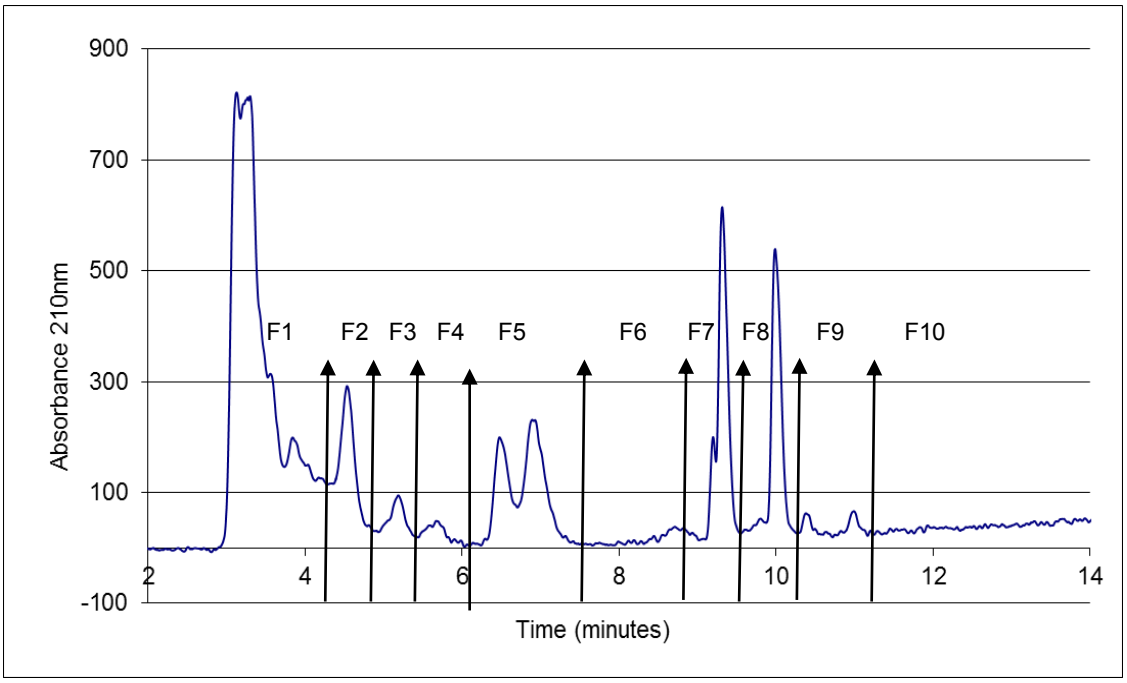


Fig 4.14. Collection of 10 fractions of corn oil sample from the first dimension (Ag^+ column gradient mode with a mixture of MeOH and MeOH in 20% ACN (100:50) at a flow rate of 1 mL min^{-1}.

Table 4.3. Peak identification of corn oil TAGs using 3D off-line Ag-HPLC/ESI(+)/MS/GC-MS

F#	M	[M+ $^{107/109}$Ag] $^{+}$ [M+Na]$^{+}$	GC-MS				Identified TAG	DB	PN
			C16:0	C18:2	C18:1	C18:0			
F2	856.7	963.7(965.6)	7	25	51	8	POL SLO OOO	3 3 3	46 48 48
	884.7	991.7(993.6)							
F3	[a]								
F4	[a]								
F5	882.7	989.7(991.6)	12	52	35	6	LOO SLL PLL	4 4 4	46 46 44
	854.7	961.7(963.6)							
F6	[a]								
F7	880.7	903.9	3	62	36	4	LLO	5	44
F8	878.7	901.8	-	85	-	-	LLL	6	42
F9	876.7	899.7	-				LLLn	7	40

[a]: no peak detected. P (C16:0); S (C18:0); O (C18:1); L (C18:2); Ln (C18:3); A (C20:0)

In F1-F6, the TAGs were detected as sliver adducts $[M+Ag]^{+}$ instead of the more common sodium adducts $[M+Na]^{+}$ observed in F7-F9. The sliver adducts were recognized from the patterns in the isotopic clusters (Fig 4.15). The ^{107}Ag and ^{109}Ag isotopes have natural abundance of 51.83 and 48.17%, respectively; leading to sliver adduct ions of similar abundance and separation by 2 *m/z* units. For example, in fraction 5, the two silver adduct $[M+Ag]^{+}$ ions at *m/z* 961.7(963.6) and 989.7(991.6) (Fig 4.15A, B) were assigned to PLL and SLL/LOO consistent with the detection of the fatty acids P, L, O, and S when F5 was converted to the FAMEs.

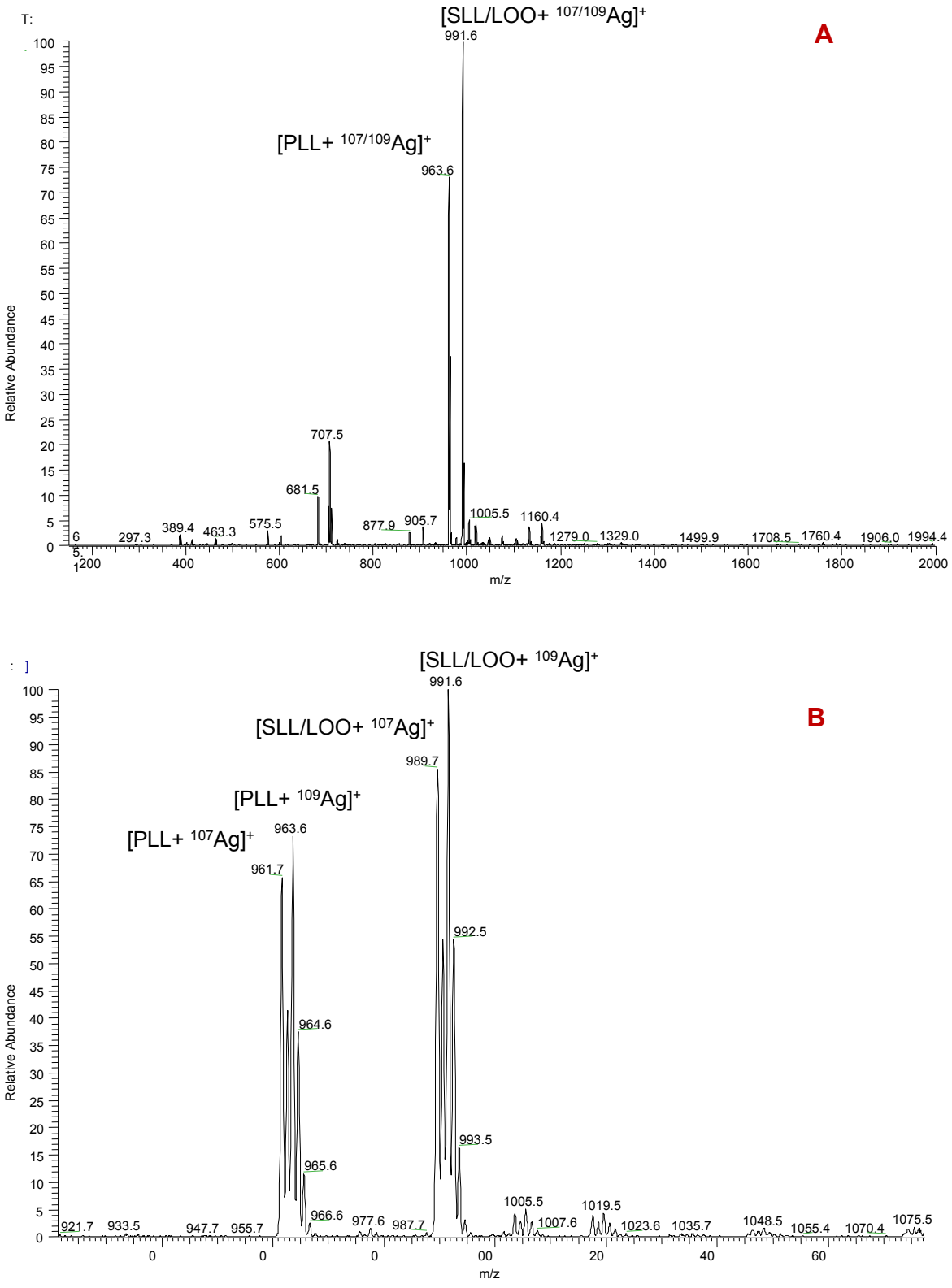


Fig 4.15 The ESI mass spectrum of (A) F5; (B) Expanded range *m/z* (920-1080).

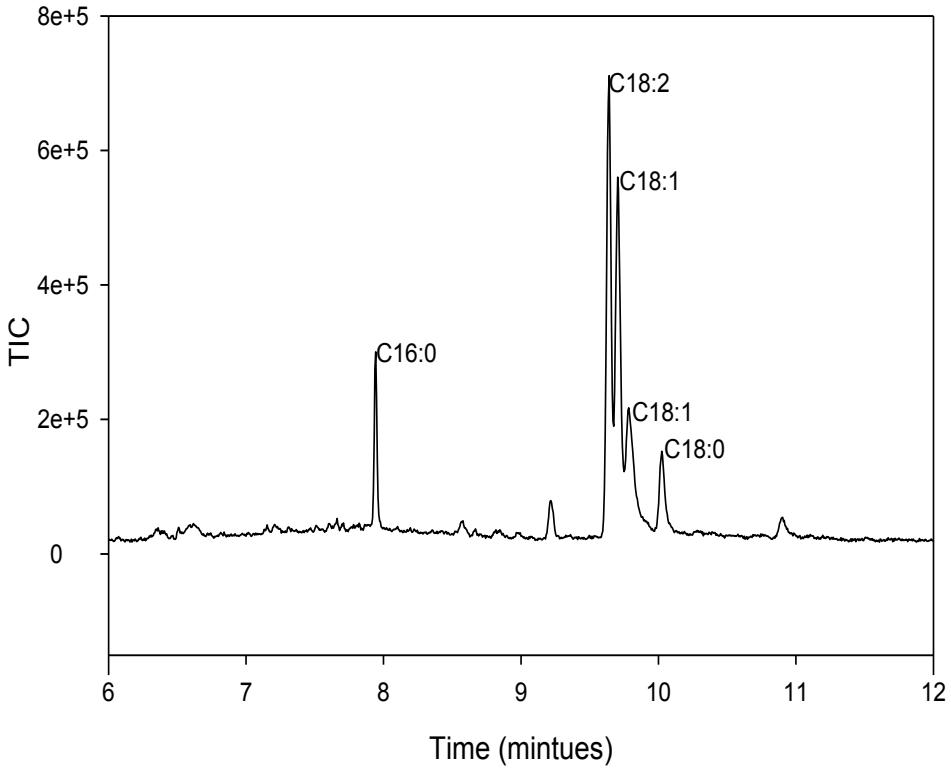


Fig 4.16. The GC/MS chromatogram of the FAME of F5.

FAME of C18:1 in the above Fig 4.16 gave two closely eluting peaks (retention times of 9.6 and 9.7 min; ratio 1:5) were observed upon GC of the methyl esters in accord with the *erythro-threo* order noted previously [Björn *et al*., 1998b; Mu *et al*., 1996a; Wesén *et al*., 1995a; Zhuang *et al*., 2003]. Similar results were found in the FAMEs commercial Supelco standard mixture (section 3.4.2, Fig 3.7).

4.4.4. Off-line 2D Ag-HPLC/ESI/MS and RP-HPLC/ESI/MS for identification of TAGs in corn oil

Although the corn oil TAGs were sufficiently separated by their PN and DB using RP-HPLC and Ag-HPLC, respectively, many peaks could still result from overlapping of two or more TAGs. Additional structural information can be obtained using tandem mass spectrometry (MS/MS) by collision-induced dissociation (CID); this fragmentation behavior is characteristic of sodiated TAG $[M+Na]^+$ ions and the assignments of the acyl groups (fatty acids) in the TAG were based on the mass difference between the precursor $[M+Na]^+$ ion and the product ions $[M+Na\text{-}RCOOH]^+$ and $[M+Na\text{-}RCOO\text{-}Na]^+$ [Herrera *et al*., 2010; Segall *et al*., 2004]. The off-line 2D Ag-HPLC/ESI/MS and RP-HPLC/ESI/MS methods were successfully used to identify 24 TAGs in corn oil with their fatty acid substitution, and are described here.

4.4.4.1. Off-line 2D Ag-HPLC/ESI/MS for identification of the TAGs in corn oil

The identification of the TAGs in the corn oil using 2D Ag-HPLC/ESI(+)/MS and the RP-HPLC/ESI(+)/MS methods were done in a three-step process. First, the separated TAGs were detected as sodiated ions $[M+Na]^+$, allowing a calculation of molecular weight of each TAG. Each $[M+Na]^+$ adduct was then fragmented by CID. Loss of a fatty acid and its sodium salt are typically observed upon CID of the sodiated ions of TAGs [Herrera *et al*., 2010; Hsu and Turk, 2010; Segall *et al*., 2004]. In this way, the acyl chains in the TAG were identified. It was then necessary to compare the MS/MS results with the elution time (retention time) of each peak in the first dimension Ag-HPLC. In the third step, these two sets of data and the Ag-HPLC retention times were compared with those reported in the literature [Byrdwell *et al*., 2001; Jakab *et al*., 2002; Holĉapek *et al*., 2003; Elbert *et al.,* 2008; Zeb *et al*., 2010; 2013; and 2015]. The peaks still unidentified were labeled as unknown. These steps were applied to the 6 fractions collected from Ag-HPLC separation of corn oil (Fig. 4.17) and 16 TAGs were identified by ESI(+)/MS. It can then be concluded that the identification by inspecting the first- and second dimension results is valuable and the TAGs with more double bonds are more strongly retained on the Ag^+ phase.

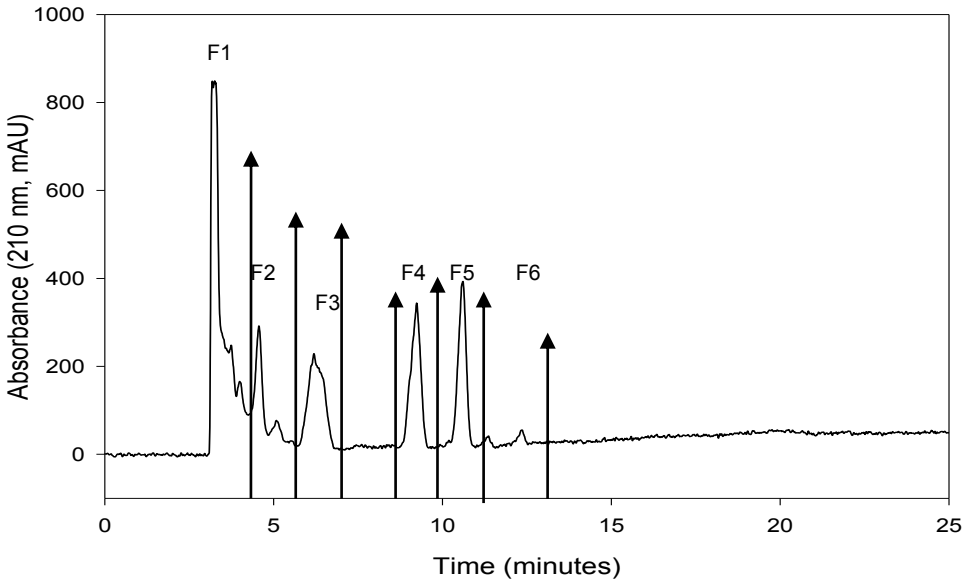


Fig 4.17. Collection of 6 fractions of corn oil sample from the first dimension (Ag-HPLC)

The results obtained for corn oil in this study (Table 4.4) compared with the literature data [van der Klift *et al*., 2008] show general agreement. On the average, 15-25 TAGs were identified in the literature depending on the region, environmental conditions, and the extraction methods used for the corn oil.

Table 4.4. Peak identification of corn oil TAGs using 2D off-line Ag-HPLC/ESI(+)/MS

F#	TAG	M	$[M+ Na]^+$	$[M+ Na-RCOOH]^+$ $[M+ Na-RCOO-Na]^+$	Loss	DB	PN
F1	PLP	830.5	853.8	597.3 575.4 573.4 551.5	P P-Na L L-Na	2	46
	POO	858.7	881.9	625.5 599.5 603.6 577.4	P O P-Na O-Na	2	48
	SLO	884.7	907.8	627.5 605.4 625.5 603.6 623.1 601.5	L L-Na O O-Na S S-Na	3	48
F2	PLO	856.7	879.9	623.4 599.4 597.5 621.4 601.4 577.5 575.5 599.5	P L O S P-Na L-Na O-Na S-Na	3	46
	OOO	884.7	907.8	625.4 603.4	O O-Na	3	48
	SLO	884.7	907.8	627.5 605.4 625.5 603.6 623.1 601.5	L L-Na O O-Na S S-Na	3	48
	ALO	912.6	935.7	655.5 653.2 623.3 633.5 631.3	L O A L-Na O-	3	48

				601.2	Na A- Na		
F3	LOO SLL	882.7	905.7	625.3 623.5 621.4 603.5 601.4 599.5	L O S L-Na O- Na S- Na	4	46
	PLL	854.7	877.7	621.3 597.4 599.4 575.4	P L P- Na L-Na	4	46
F4	LLO OLnO	880.7	903.7	625.1 623.3 621.5 603.5 601.5 599.4	Ln L O Ln- Na L-Na O- Na	5	44
F5	LLL OLLn	878.7	901.8	623.3 601.6 621.4 599.5 619.5 597.5	Ln Ln- Na L L-Na O O- Na	6	42
F6	LLLn LnOLn	876.7 876.7	899.8 899.6	621.3 619.4 616.3 599.5 597.5 595.4	Ln L O Ln- Na L-Na O- Na	7	40

P (C16:0); S (C18:0); O (C18:1); L (C18:2); Ln (C18:3); A (C20:0)

The ESI mass spectrum of F3 (Fig. 4.18) shows two sodium $[M+Na]^+$ ions at *m/z* 877.9 and 905.8, indicating the presence of two TAGs. These ions are well resolved and, assuming the formation of sodium $[M+Na]^+$ ions upon ESI, correspond to PLL and OLO, respectively. MS/MS of the ion at *m/z* 877.9 (Fig 4.19) gave major product ions at

m/z 621.3 and 597.4 along with lower intensity ions at *m/z* 599.4 and 575.4. These fragmentations correspond to the losses of P and L as fatty acids and their sodium salts.

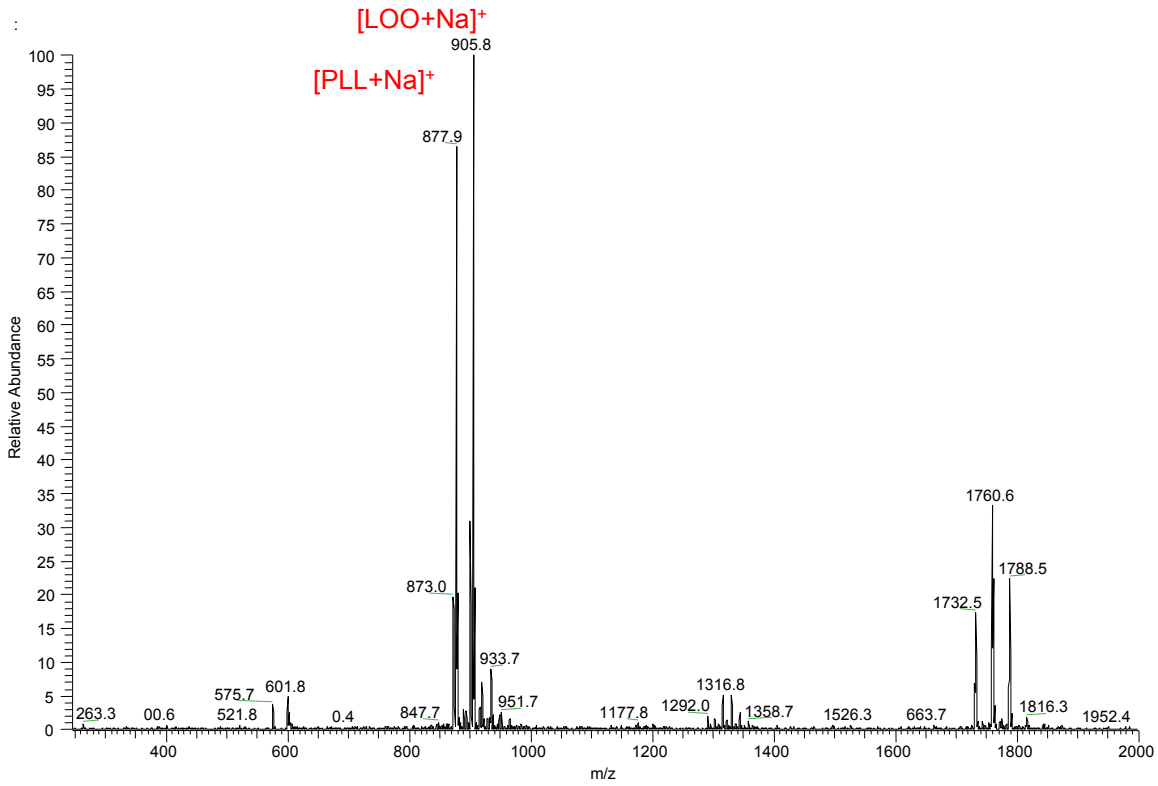


Fig 4.18. The ESI mass spectrum of F3.

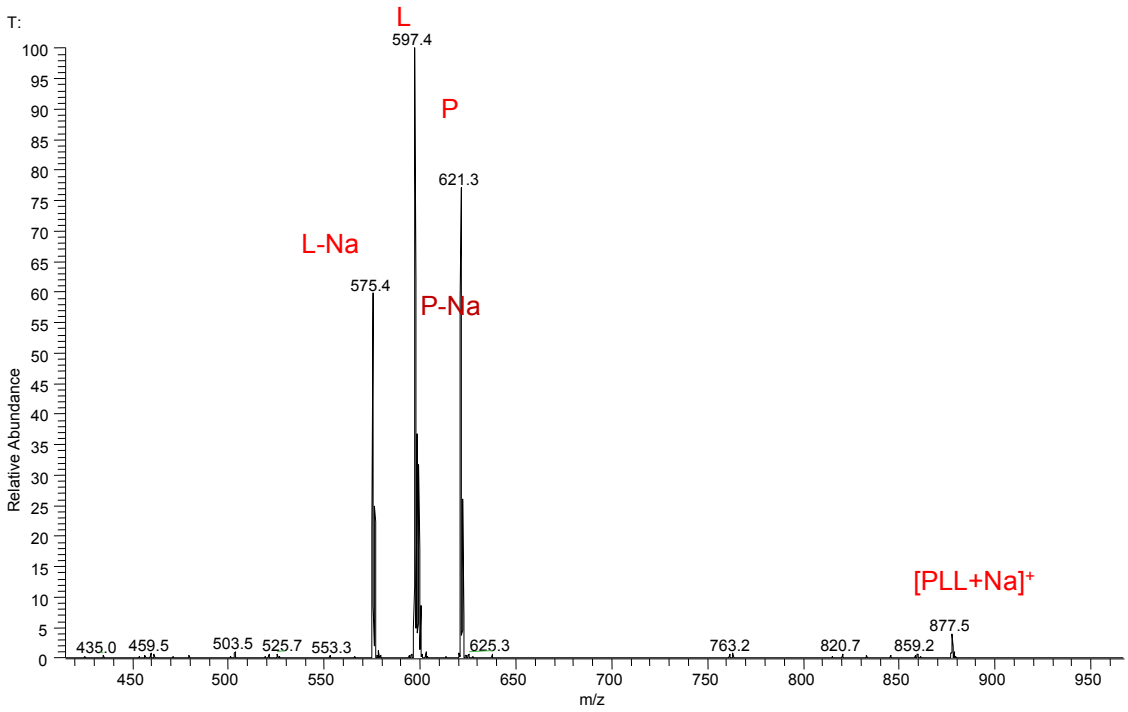


Fig 4.19. The CID spectrum of $[PLL+Na]^+$ at *m/z* 877.9.

Similarly MS/MS of *m/z* 905.8 gave fragment ions at *m/z* 625.5 (603.5), 623.5 (601.4), and 621.4 (599.5) corresponding to losses of the fatty acids L, O and S, and their sodium salts (Fig 4.20A, B), consistent with the formation of the $[M+Na]^+$ of OLO or SLL at *m/z* 905.8 (Fig. 4.18). With these similar properties, the two TAGs were not resolved in a one-dimensional run. Thus F3 (Fig. 4.17) was primarily a mixture of OLO and SLL with the same number DB (4) and PN (46). A similar MS/MS approach was used by Holčapek *et al*., [2003] for the analysis of a standard TAG mixture. It should be noted that ions at *m/z* 624, 626 602, and 604 were also evident in expanded CID separation of *m/z* 905.8 (Fig 4.20B). These were attributed to the capture of the isotopic ion at *m/z* 906.6 in the CID experiment.

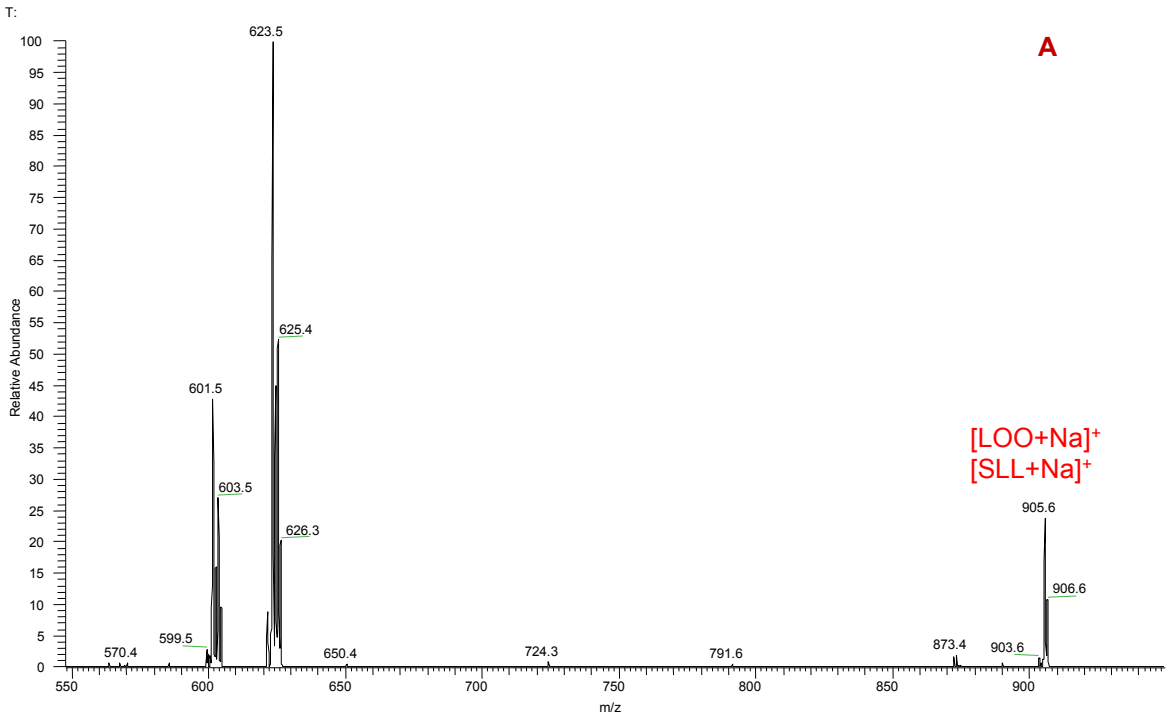


Fig 4.20A The CID spectrum of $[LOO+Na]^+$/ $[SLL+Na]^+$ at *m/z* 905.6.

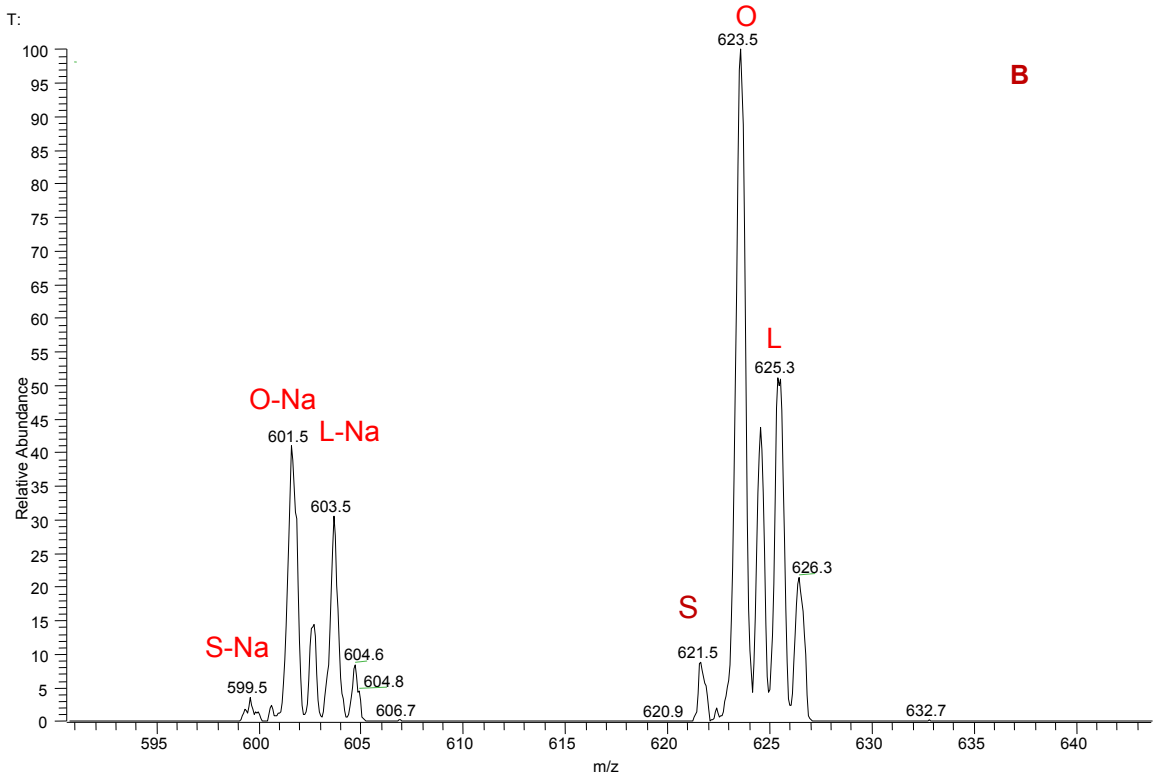


Fig 4.20B The expanded CID spectrum at *m/z* 580-650.

The comparison of the results obtained by the two methods, namely 2D off-line Ag-HPLC/ESI(+)/MS and 3D off-line Ag-HPLC/ ESI(+)/MS/GC/MS, is given below. In F3 (Fig 4.17), 2D off-line Ag-HPLC/ESI(+)/MS) gave two sodiated ions $[M+Na]^+$ at *m/z* 877.9 and 905.8 (Fig 4.18); however, in the 3D off-line Ag-HPLC/ ESI(+)/MS/GC/MS method F5 (Fig 4.14) gave two silver adduct $[M+ {}^{107/109}Ag]^+$ ions at *m/z* 961.7(963.6) and 989.7(991.6) (Fig 4.15A, B) and their FAMEs were identified as P, L, O, and S. And also their corresponded TAGs were identified as PLL and SLL/LOO. It is evident that the results obtained in F3 (Fig 4.17) by 2D off-line Ag-HPLC/ESI(+)/MS method are in good agreement with F5 (Fig 4.14) by 3D off-line Ag-HPLC/ESI(+)/MS/GC/MS method and both contained the same TAGs components (PLL and LOO/SLL).

The mass spectrum of F5 gave a ion at *m/z* 901.8 corresponding to $[OLnL+Na]^+$ (Fig 4.21). MS/MS spectrum of *m/z* 901.8 ion (Fig 4.22A, B) gave fragment ions at m/z 623.3 (601.6), 621.4 (599.5), and 619.5 (597.5) corresponding to losses of Ln, L, and O and their sodium salts.

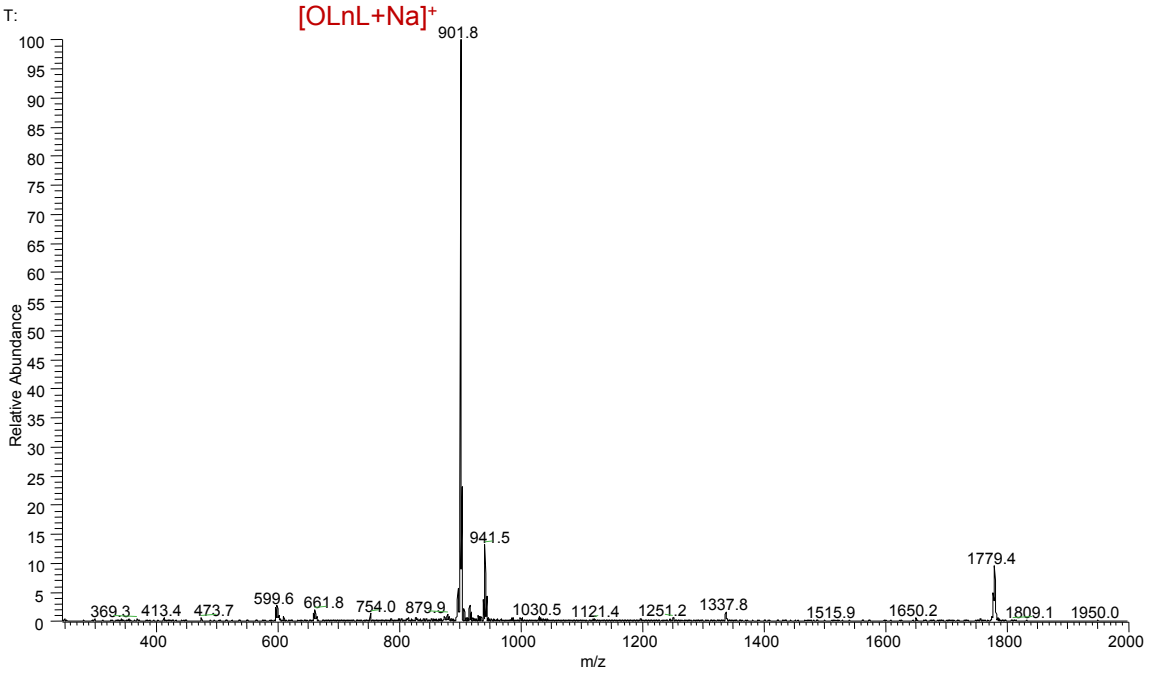


Fig 4.21. The ESI mass spectrum of F5 of $[OLnL+Na]^+$ ion at *m/z* 901.8.

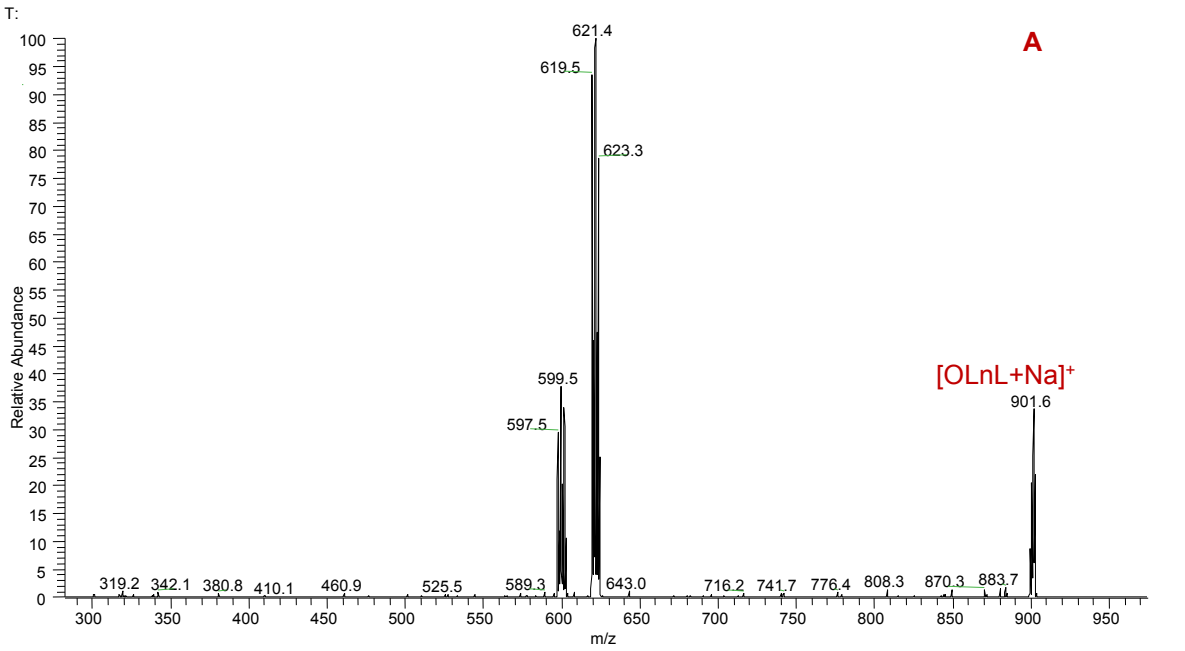


Fig 4.22A. The CID spectrum of F5 of $[OLnL+Na]^+$ ion at *m/z* 901.8.

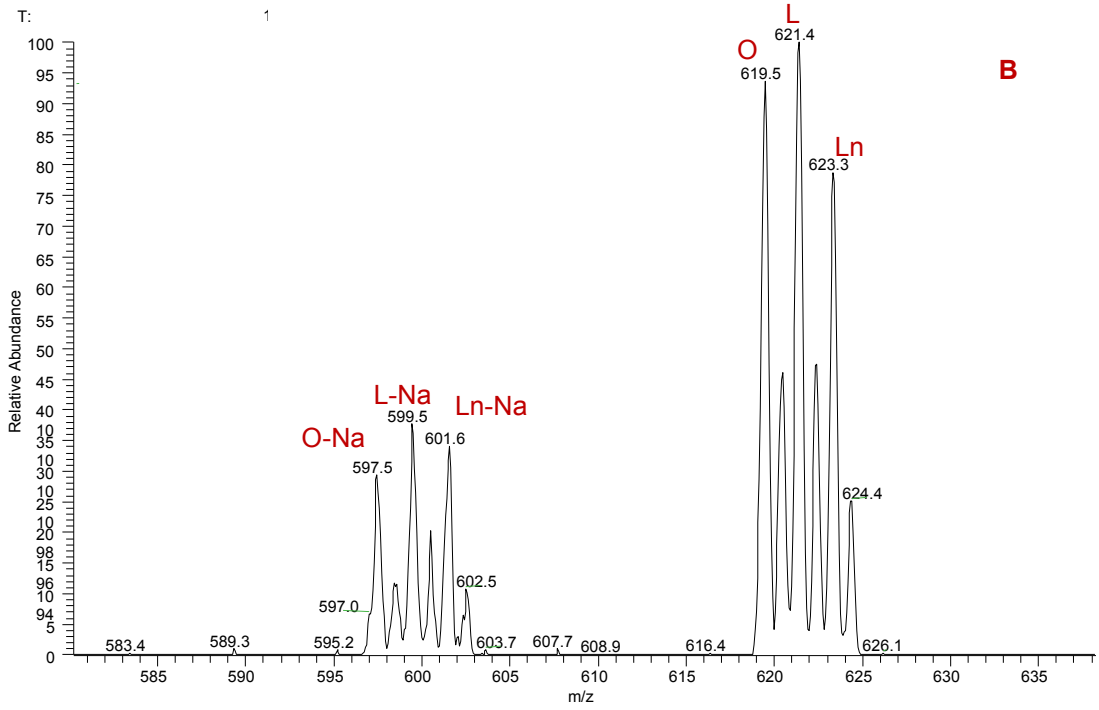


Fig 4.22B. The expanded CID spectrum at *m/z* 585-635

Also, F5 gave an ion at *m/z* 901.9 corresponding to [LLL+ Na]$^+$ (Fig 4.23). MS/MS spectrum at *m/z* 901.8 ion (Fig 4.24A, B) gave fragment ions at *m/z* 621.4 (599.5) corresponding to loss of L and its sodium ion. Thus, F5 (Fig. 4.18) was identified as [LLL+Na]$^+$ and [OLLn +Na]$^+$. Again the presence of other product ion in the expanded CID (Fig 4.22B) and (Fig 4.24B) spectrum indicated the capture and fragmentation of an isotopic ion during CID.

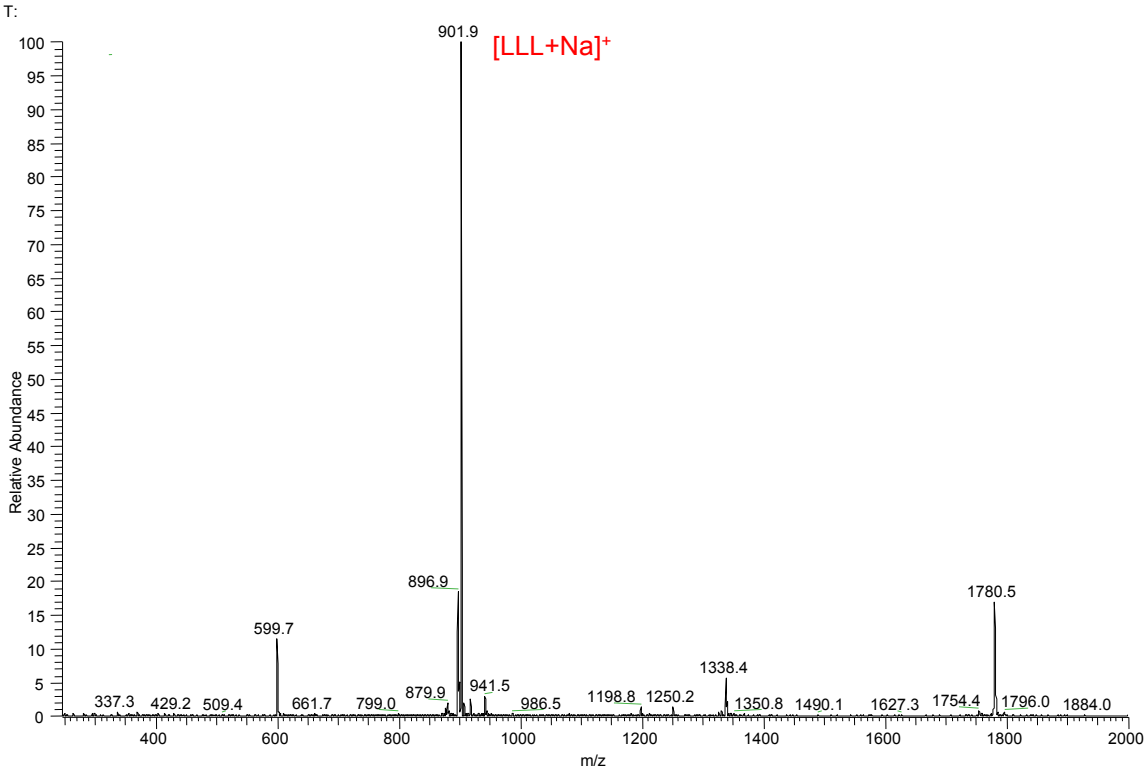


Fig 4.23. The ESI mass spectrum of F5 of [LLL+Na]$^+$ ion at *m/z* 901.9

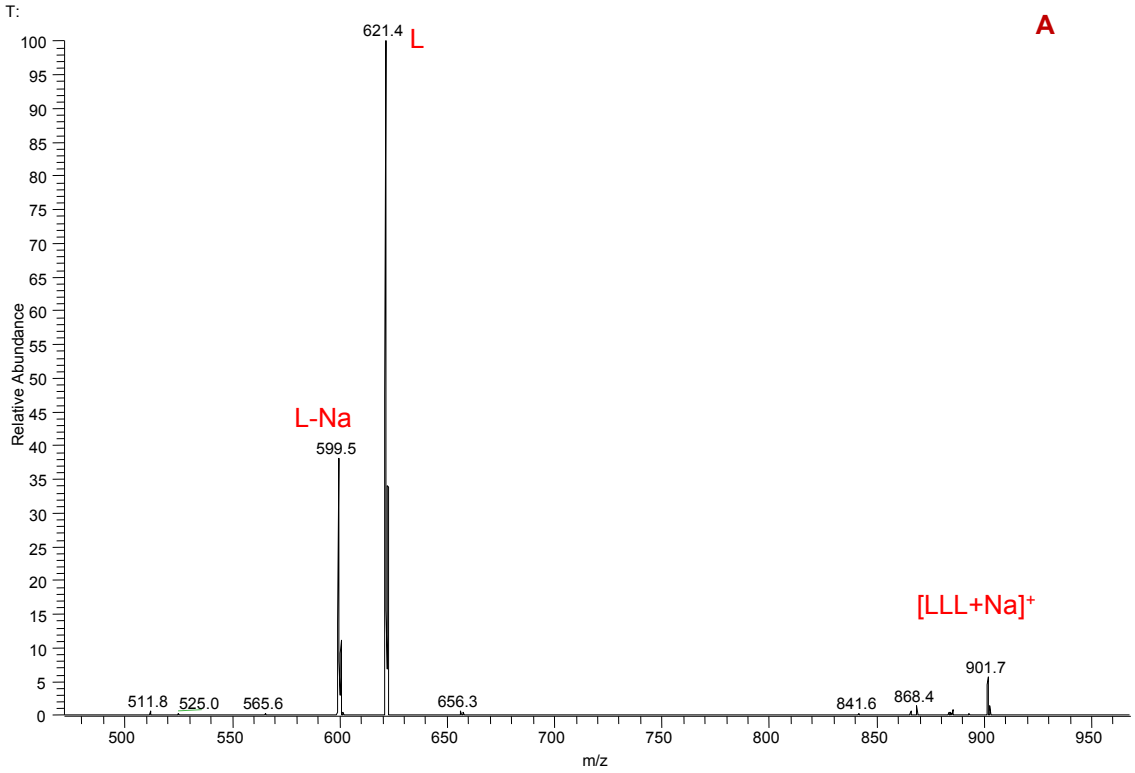


Fig 4.24A. The CID spectrum of F5 of $[LLL+Na]^+$ ion at *m/z* 901.9.

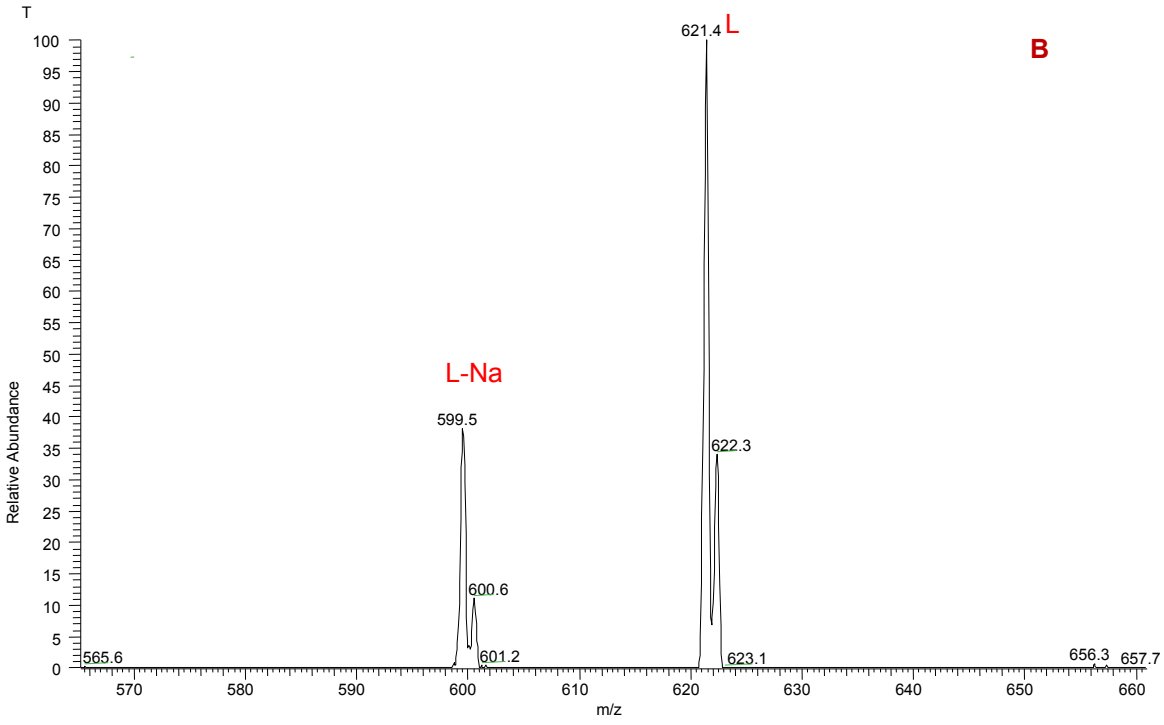


Fig 4.24B. The expanded CID spectrum at *m/z* 575-660.

In summary, 5 cases of co-elution of TAGs were detected by MS/MS (Table 4.4), namely OOO and SLO, LOO and SLL, LLO and OLnO, LLL and OLLn, LLLn and LnOLn.

Comparisons of the 2D results of F5 (both on-line and off-line of Ag-HPLC/RP-HPLC methods) were carried out. The F5 fraction gave one TAG by both on-line and off-line Ag-HPLC/RP-HPLC methods (Fig 4.10B and Fig 4.13); and this TAG was identified by Ag-HPLC/ESI(+)/MS as LLL and OLLn with identical DB (6), PN (42) and *m/z* 901.9 and 901.8 (Fig 4.21 and Fig 4.23). It is evident that the results obtained by all three methods are in good agreement.

Also, the results of F2 (on-line and off-line of Ag-HPLC/RP-HPLC methods, Ag-HPLC/ESI(+)/MS/GC/MS, and Ag-HPLC/ESI(+)/MS) were compared. The peak 2 or F2 was separated to 4 TAGs using both on-line (Fig 4.8-4,10A) and off-line (Fig 4.12A-C) Ag-HPLC/RP-HPLC methods. These TAGs were identified by Ag-HPLC/ESI(+)/MS (Fig 4.17, Table 4.4). The ESI mass spectrum of F2 (Fig 4.25) showed three sodium $[M+Na]^+$ ions at *m/z* 879.9, 907.8, and 935.5. These ions are well resolved and correspond to $[PLO+ Na]^+$ (PN: 46), $[SOL+Na]^+$ and $[OOO+Na]^+$ (PN: 48), and ALO (PN: 50), respectively with the same DB of 3. In addition, the results of 3D Ag-HPLC/ESI(+)/MS/GC/MS (Fig 4.14) method of F2 (Fig 4.26, Table 4.3) gave two major sliver $[M+ {}^{107/109}Ag]^+$ ions at *m/z* 963.7(965.6) and 991.7(993.6) corresponding to $[PLO+{}^{107/109}Ag]^+$ and $[OOO+{}^{107/109}Ag]^+$/$[SOL+{}^{107/109}Ag]^+$, respectively. And their FAMEs were found to be P, L, O, and S using GC/MS. However, F2 gave a lower intensity at *m/z* 1019.7 corresponding to $[ALO+{}^{107/109}Ag]^+$, but the FAME of A (C20:0) was not detected by GC/MS. It can therefore be concluded that the identification of F2 by the 4 methods agreed well.

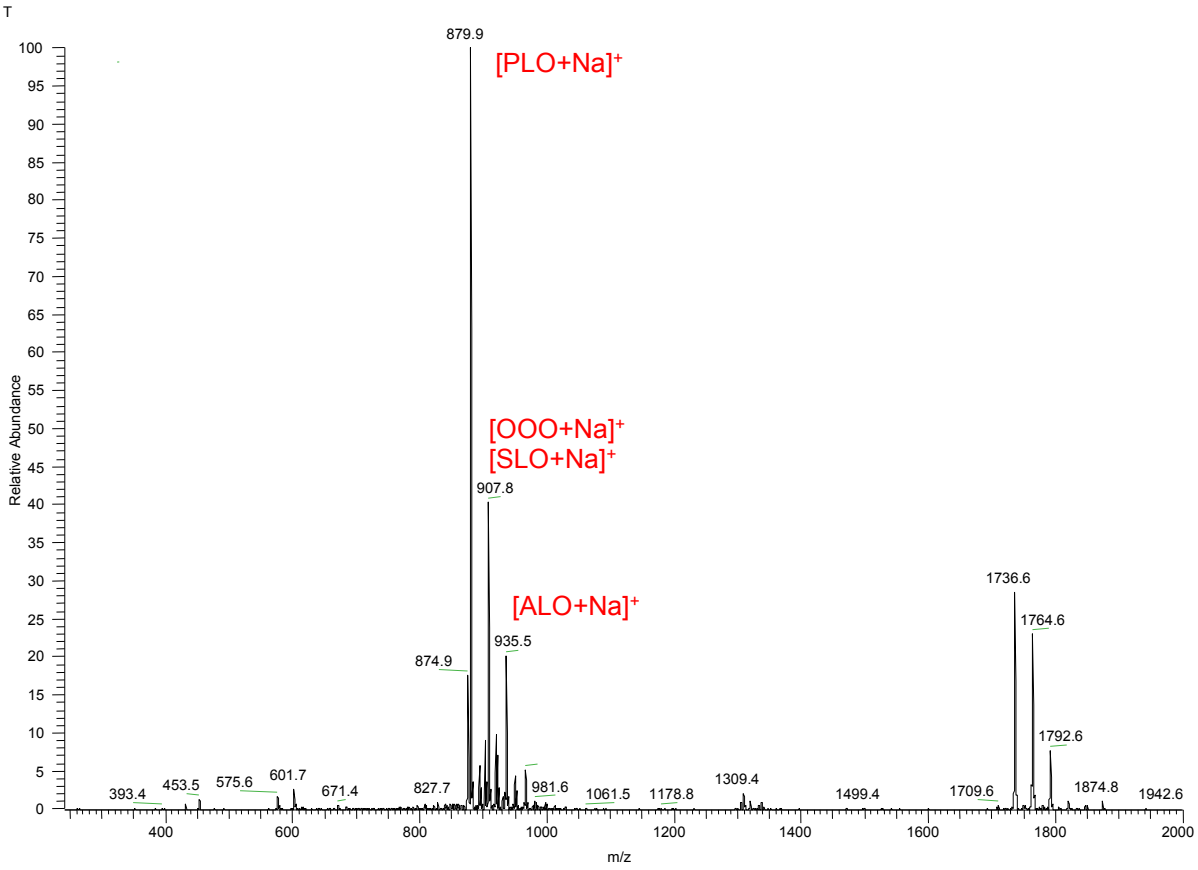


Fig 4.25. The ESI mass spectrum of F2 by the 2D off-line Ag-HPLC/ESI/MS method.

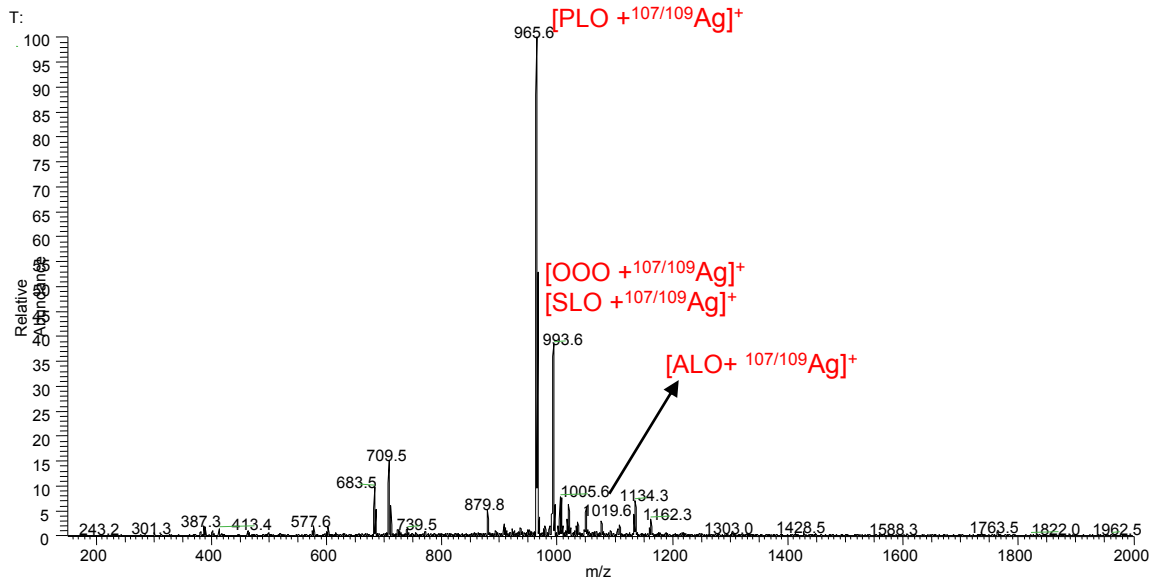


Fig 4.26. The ESI mass spectrum of F2 by 3D off-line Ag-HPLC/ESI/MS/GC/MS method.

In conclusion, our results show that the off-line Ag-HPLC/ESI(+)/MS method alone can be used for the detection of various TAG classes in corn oil while a combination of different methods is reported in the literature to achieve it. The summary of work done in this section on the identification of TAGs in corn oil is graphically presented in Fig 4.27. These data are in good agreement with those reported in the literature [van der Klift *et al*., 2008] shown in Fig 4.28.

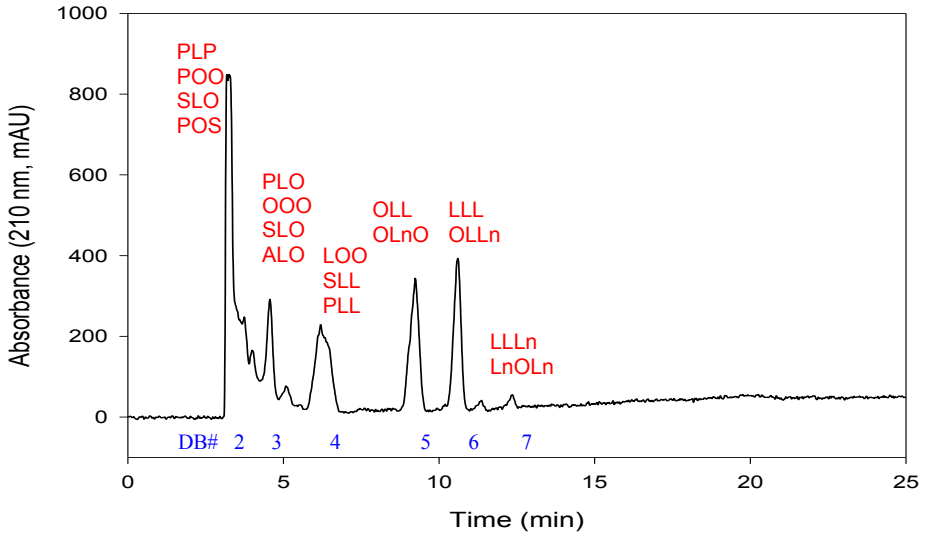


Fig 4.27. Identification of the TAGs in corn oil on a Ag(I)-coated ion exchanger column (250 mm X 4.6 mm) with gradient of MeOH and MeOH with 20% acetonitrile (100:50) at UV 210 nm.

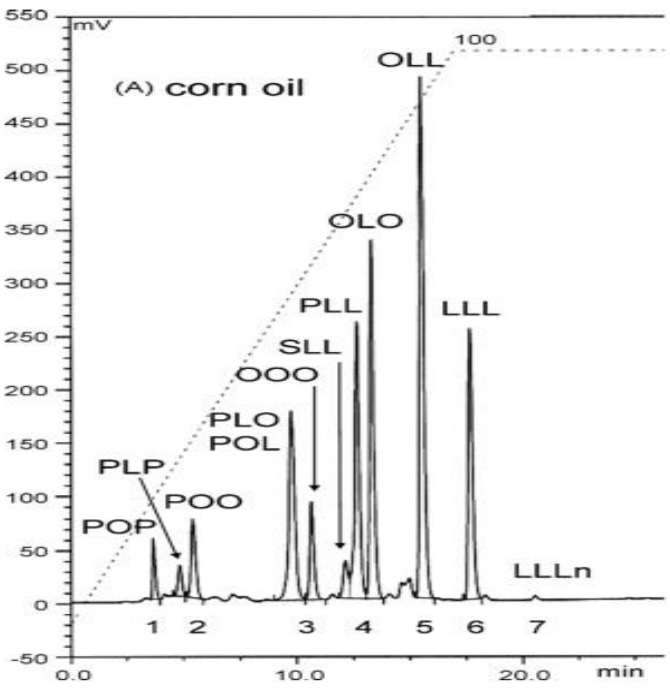


Fig 4.28. HPLC profile of corn oil TAGs on a Ag(I)-coated ion exchange (250 mm × 2.1 mm; gradient from 100%MeOH to MeOH–MeCN (94:6) in 17 min, flow 0.2 mL/min; detection: ELSD; numbers 1–7 below the peaks in the corn oil chromatogram refer to the number of double bonds. Adopted from [van der Klift *et al*., 2008].

4.4.4.2. Off-line 2D RP-HPLC/ESI(+)/MS for the identification of TAGs in corn oil

Seventeen fractions collected from the corn oil run using RP-HPLC with rapid gradient (Fig 4.29) were followed by ESI(+)/MS. The results are summarized in Table 4.5 which agrees with our results using Ag-HPLC/ESI(+)/MS. It should be noted that the order of TAGs was reversed in RP-HPLC compared to that in Ag-HPLC because the former separation mechanism is based on PN rather than DB.

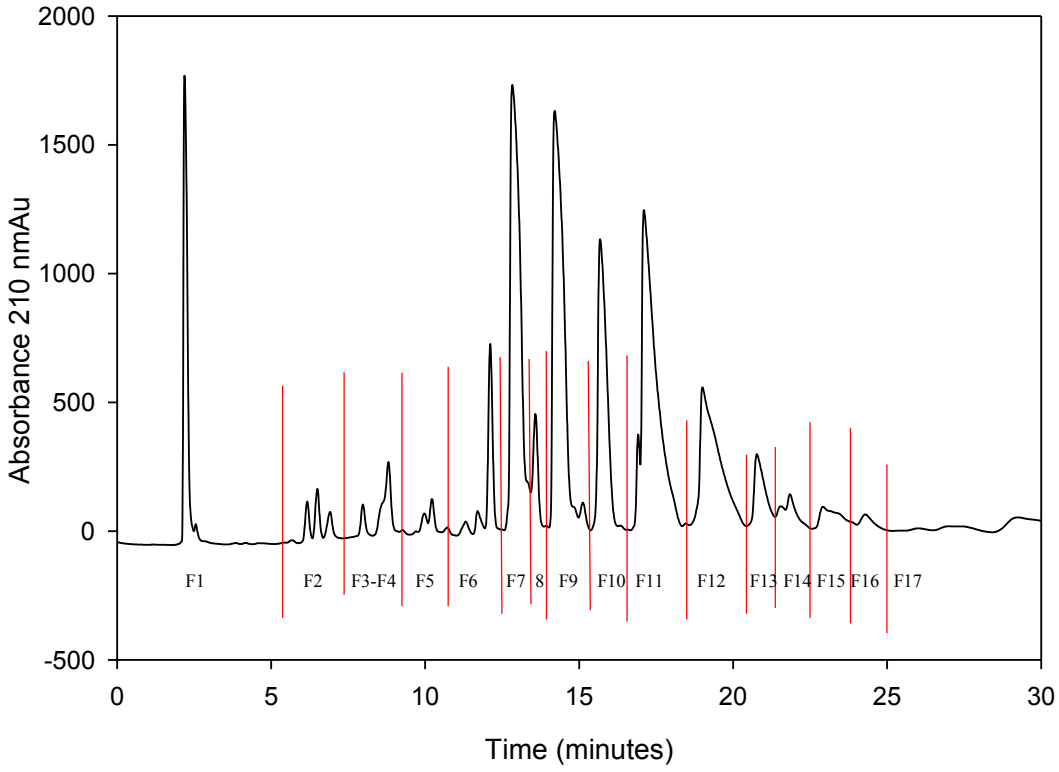


Fig 4.29. Seventeen fractions of corn oil collected form RP-HPLC.

Table 4.5. Peak identification of corn oil TAGs using PR-HPLC/ESI(+)/MS

F#	TAG	M	$[M+Na]^+$	$[M+Na-RCOOH]^+$ $[M+Na-RCOO-Na]^+$	Loss	DB	PN
F1-F6	Oxidized TAG	-	-	-	-	-	-
F7	LLL	878.7	901.7	621.2/ 599.3	L L-Na	6	42
F8	OLLn	878.8	901.7 909.6	623.2 621.3 619.4 601.3 599.4 597.3	Ln L O Ln-Na L-Na O-Na	6	42
F9	LLO	880.7	903.3	623.3 621.5 601.5 599.4	L O L-Na O-Na	5 5	44 44
F10	LOO LLS	882.7 882.7	905.7	625.4 623.5 622.3 603.6 601.4 600.1	L O S L-Na O-Na S-Na	4 4	46 46
F11	PLL	854.7	877.7	621.3 597.4 599.4 575.4	P L P-Na L-Na	4	
F12	PLO POL	856.7	879.7	623.4 599.4 597.5 601.4 577.5 575.5	P L O P-Na L-Na O-Na	3	46
F13	LLS	883	905.8	625.3 621.2 603.2 599.3	L S L-Na S-Na	4	46
F14	POO	858.7	881.9	625.5 599.5 603.6 577.4	P O P-Na O-Na	2	48

F15	OOO SOL SLO	884.7 884.7	907.9	627.5 625.5 623.5 605.6 603.6 601.6	L O S L-Na O-Na S-Na	3 3	48 48
	POP	832.5	855.9	599.3 573.3 577.1 551.1	P O P-Na O-Na	1	48
F16	POP	832.5	855.8	599.3 573.3 577.3 551.3	P O P-Na O-Na	1	49
	ALP SLS	886.5	909.8	653.3 629.2 625.3 598.9 631.1 607.2 603.3 577.2	P L S A P-Na L-Na S-Na A-Na	2	52
	ALL	910.3	933.5	653.3 621.3 631.3 599.2	L A L-Na A-Na	4	52
F17	SOO	886.6	909.8	627.3 625.3 605.4 603.4	O S O-Na S-Na	2	52
	ALO	912.6	935.7	655.5 653.2 623.3 633.5 631.3 601.2	L O A L-Na O-Na A-Na	3	53

P (C16:0); S (C18:0); O (C18:1); L (C18:2); Ln (C18:3); A (C20:0)

The RP-HPLC/ESI(+)/MS method allowed identification of TAG with long chain fatty acid, namely arachidic acid (C20:0, A). An examination of the ions in MS/MS revealed that the peaks belonged to $[ALP+Na]^+$, $[ALL+Na]^+$,and $[ALO+Na]^+$. For example F16 gave a ion at *m/z* 933.5 corresponding to $[ALL+Na]^+$, the MS/MS spectrum at m/z 933.5 ion (Fig. 4.30) gave fragment ions at m/z 653.3 (631.3) and 621.3 (599.5) corresponding to losses of L and A, and their sodium ions.

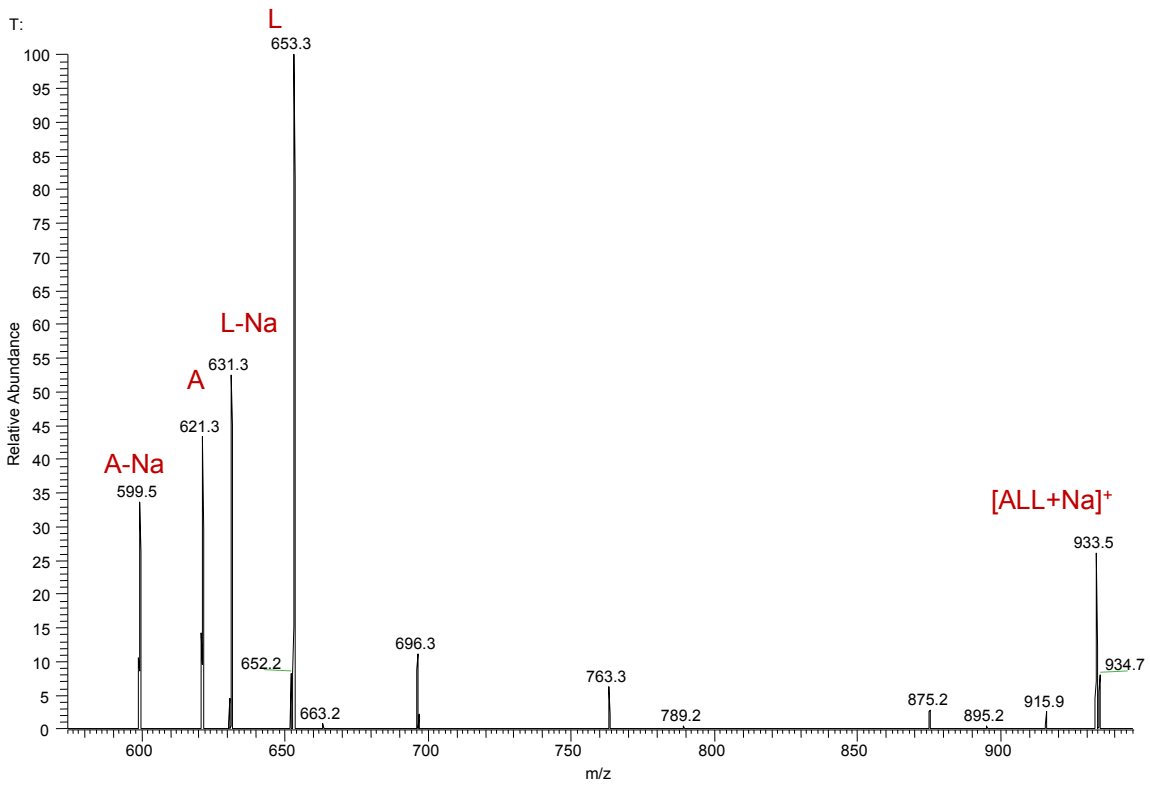


Fig 4.30. The CID spectrum of F16 of $[ALL+Na]^+$ ion at *m/z* 933.5.

The ESI(+) mass spectrum of F17 showed the sodium $[M+Na]^+$ ion at *m/z* 935.7 corresponding to $[ALO+Na]^+$. The MS/MS of the ion at *m/z* 935.7 (Fig 4.31) gave major product ions at *m/z* 655.4 (633.5), 653.3 (631.3) and 623.2 (601.2). These fractionations correspond to the losses of L, O and A as fatty acids and their sodium salts.

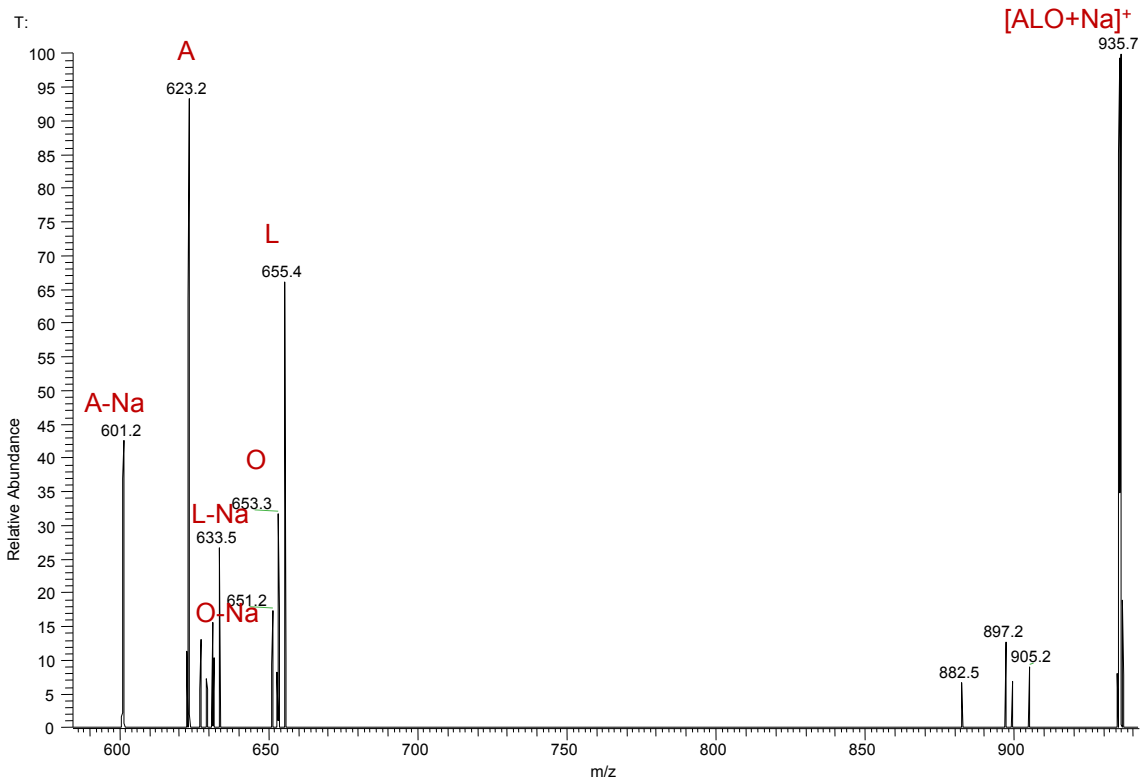


Fig 4.31. The CID spectrum of F16 of $[ALO+Na]^+$ ion at *m/z* 935.7.

A comparison of RP-HPLC data for two fractions obtained using two different detection methods was carried out. The results of the fatty acids found in F3 (C18:2 and C18:1 with 2:1 ratio) and F4 (C18:2 and C18:1 with 1: 2 ratio) using off-line 2D RP-HPLC/GC/MS shown in Fig 4.5 (in section 4.4.1.2) are in good agreement with the TAGs found in F9 ($[LLO+Na]^+$, *m/z* 903.4, and Figs 4. 32 and 33) and F10 (LOO, *m/z* 905.7 and Fig 4.34A, B) by off-line 2D RP-HPLC/ESI(+)/MS (Fig. 4.29 and Table 4.5). It is important to note that because of the high concentration of the TAG components in F9 at *m/z* 903.4 the dimmer at *m/z* 1784.2 was recorded and also in F10 at *m/z* 905.7 with dimmer at *m/z* 1788.2. To avoid this problem a small in-source energy (25%) was applied.

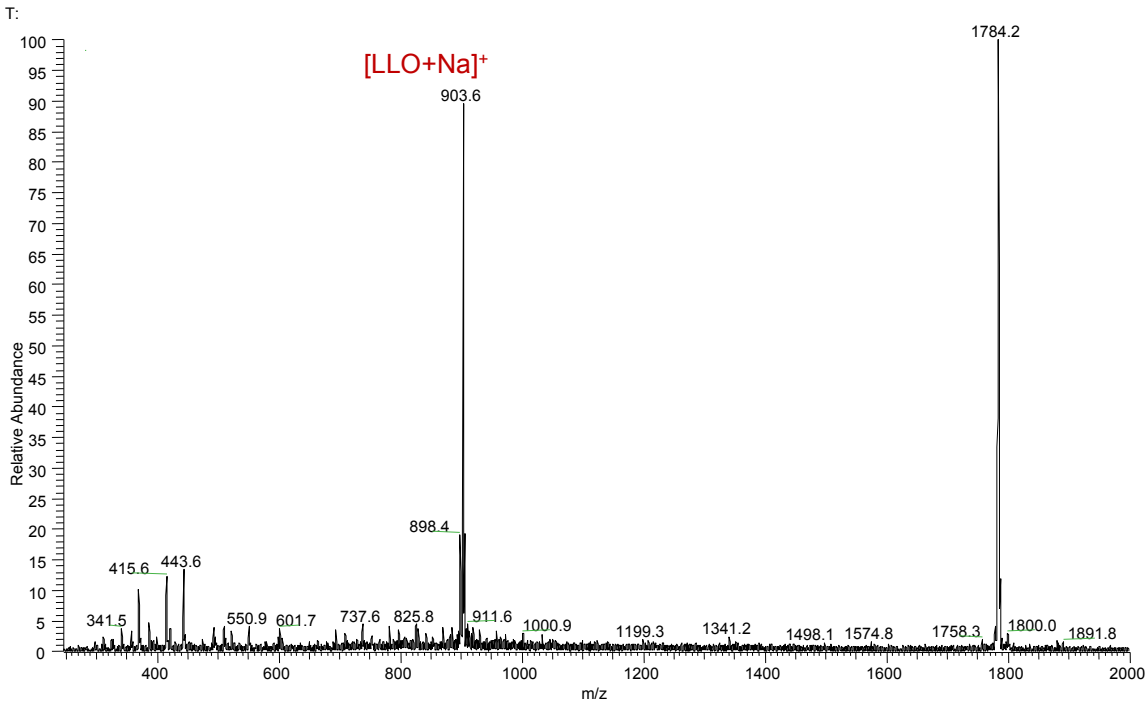


Fig 4.32. The ESI mass spectrum of F9 corn oil using RP-HPLC/ESI(+)/MS.

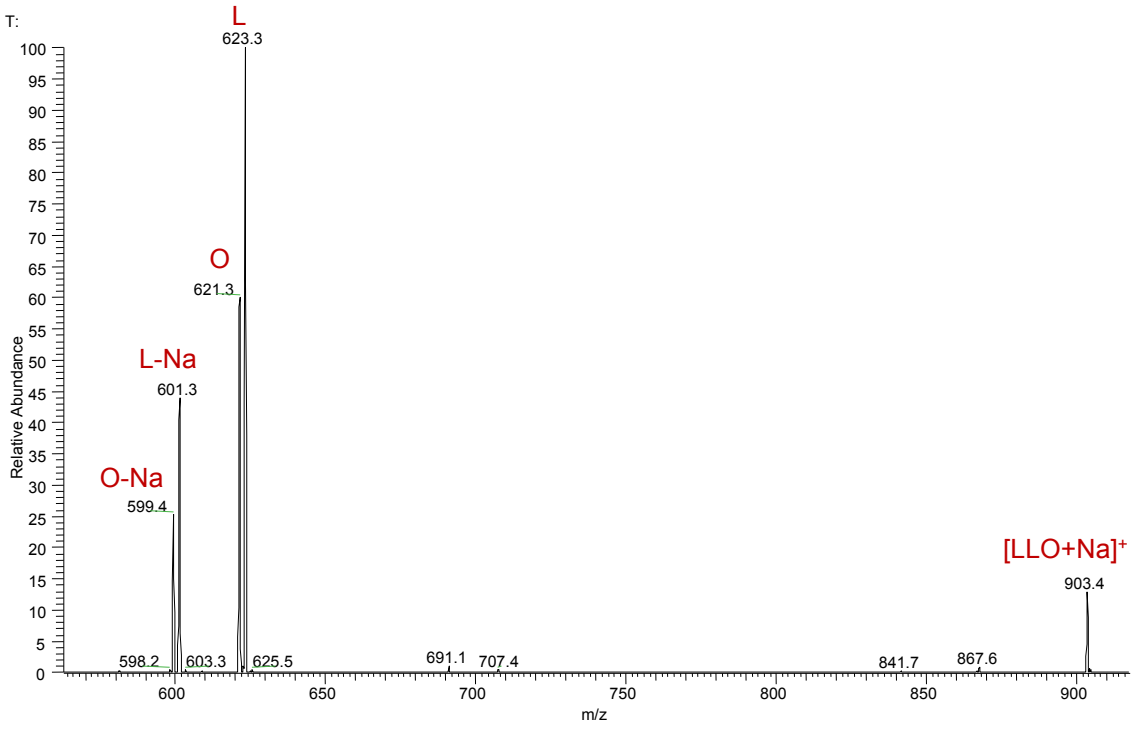


Fig 4.33. The CID spectrum of F9 $[Lorna]^+$ ion at *m/z* 903.4.

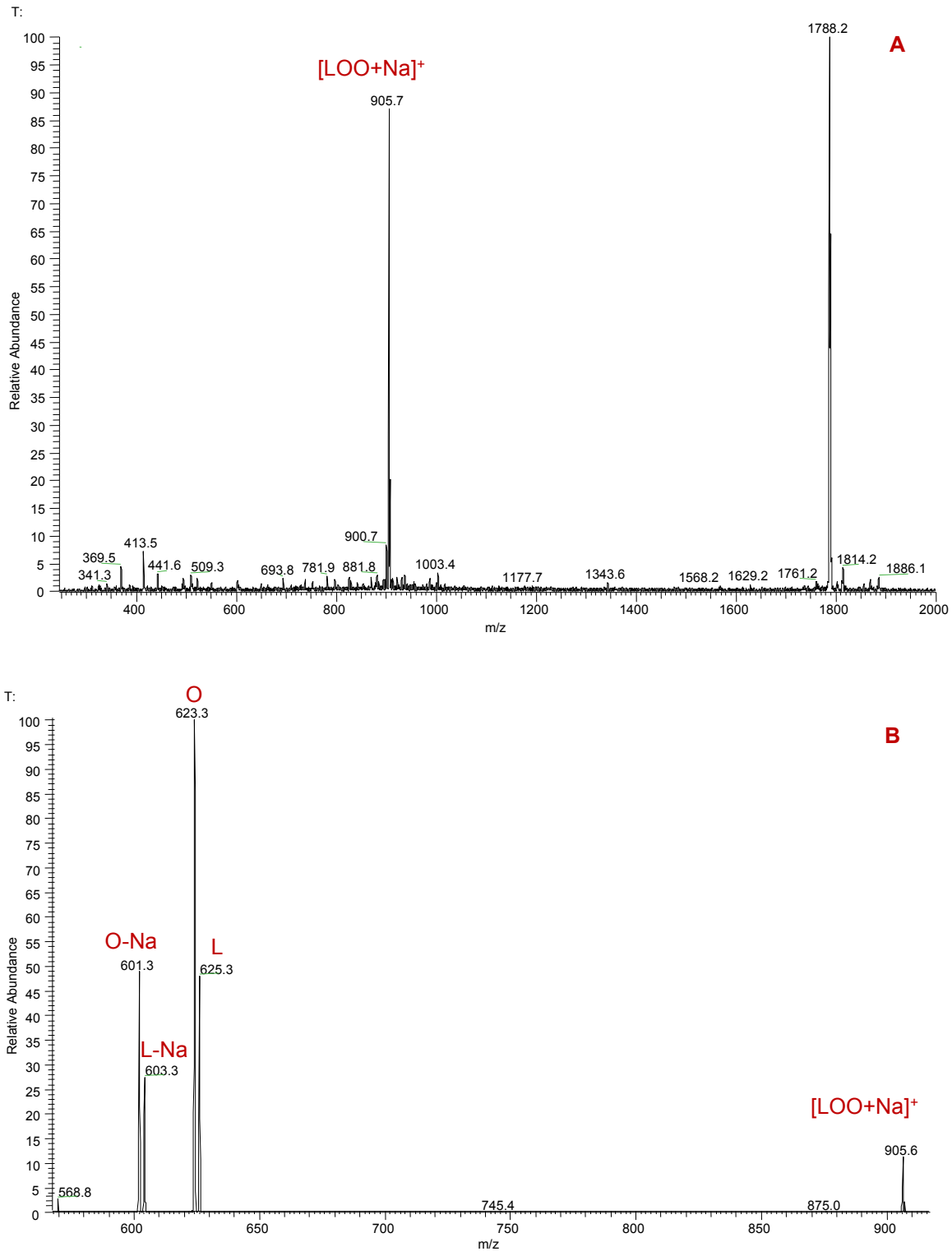


Fig 4.34. (A) The ESI mass spectrum of F10 of corn oil using PR-HPLC/ESI/MS; (B) the CID spectrum of F10 $[LOO+Na]^+$ ion at *m/z* 905.7.

The earlier studies [Zeb *et al.,* 2010] on corn oil identified 16 TAGs by RP-HPLC/ESI/MS (Fig 4.35), namely LLLn, LLL, OLL, OLO, SLL, POO, OOO, OSO. AOO, ALO, POS, SOL/SLO, ALL, PLO, PLP, and PLL. Our methods were able to identify a total of 20 TAGs which include the above 16 and additional 4 TAGS, namely OLLn, POP, ALP, and SLS in corn oil (Fig. 4.36).

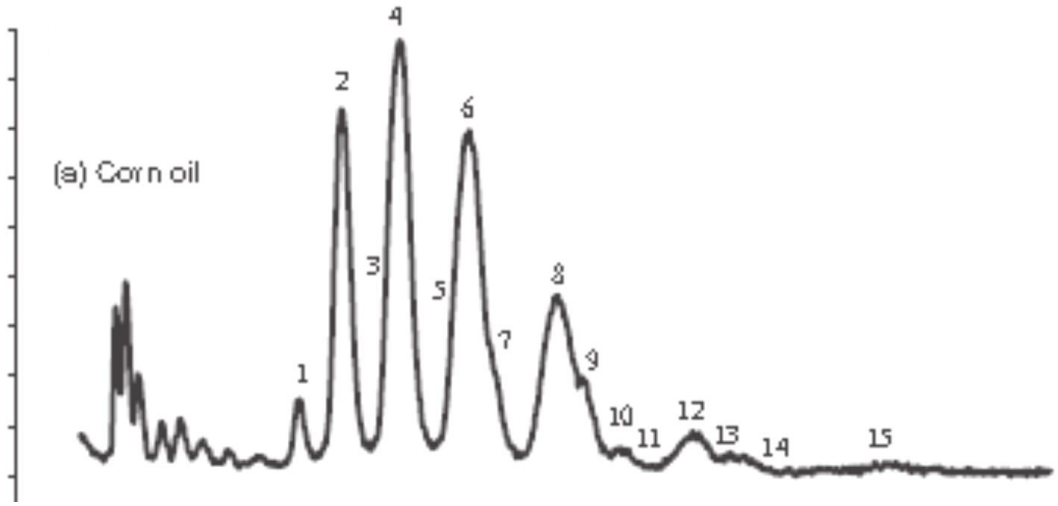


Fig 4.35. HPLC-ESI-MS chromatograms of edible oil. (A) Corn oil. The peak numbering represents identification TAGs. RP-HPLC was done using Phenomenex C18 (150 mm \times 3 mm) with an isocratic mode of 18% isopropanol in methanol. [Zeb *et al.,* 2010]

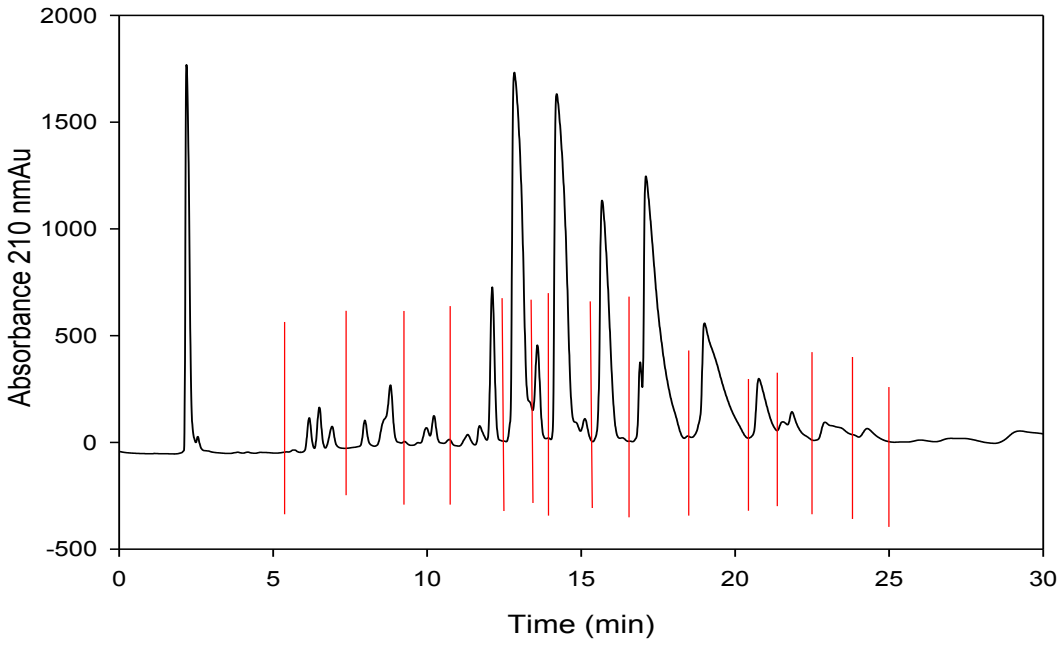


Fig 4.36. RP-HPLC/ESI(+)/MS using two Supelcosil LC-PAH (25 cm x 4.6 mm X 3 µ particles) with gradient 100% acetonitrile (solvent A) to 100% isopropanol (solvent B).

Further investigation was done on TAGs in F2, F3, F4, F5, and F6 fractions which eluted early in RP-HPLC (Fig 4.29) with retention times shorter than the corresponding TAGs in

the rest of the fractions (F7-F17). Their (F2-F6) sodiated molecular masses $[M+Na]^+$ could not be entirely interpreted. Furthermore, the MS/MS fragmentation results of these fractions (F2-F6) were difficult to interpret because all of them showed the loss of same linoleic (L) and linolenic (Ln) fatty acids. The unsaturated fatty acids are the key site of formation of oxidation TAG compounds. The oxidation chemistry of TAG is same as that of the oxidation of fatty acids. Several primary oxidation TAG compounds generated by the free radical included hydroxides, hydroperoxides, epidioxides, and hydroxyl epidioxides. Details of the oxidation chemistry and LC methods used for the analysis of oxidized TAG in different samples have been reviewed recently by Zeb [2015]. Oxidized TAG compounds in corn oil were reported in the literature [Lin and Chen 2010; Sjövall *et al*., 2001; Zeb *et al*., 2010, 2013, 2015]. The oxidized TAG compounds are formed during the refining and processing steps of edible oils as reported by Chen *et al.* [2011]. Several studies have focused on the negative impact of these oxidized TAG compounds and their decomposition on health related to heart diseases and aging [Lobo *et al*., 2010] and colon cancer [Turek *et al*., 2005].

The rate of oxidation usually is higher in pure TAGs than in edible oils due to the presence of natural antioxidants [Zeb, 2012]. This was observed in our lab when a sample of commercially available of trilinolein (TAG-C18:2) was found to be oxidzed and formed further oxidized TAG compounds. Thus oxidized TAG-C18:2 compounds were used as reference to determine the oxidized TAG compounds in F1-F6 of the corn oil. The ESI(+) mass spectrum of the oxidized TAG-C18:2 (Fig 4.37A, B) gave the sodium ion of TAG-C18:2 at *m/z* 901.8 and the oxidized TAG-C18:2 with two oxygen atoms at *m/z* 933.6, with 4 oxygen atoms at *m/z* 965.5, and with 6 oxygen atoms at *m/z* 997.5. Also, small peaks of oxidized TAG-C18:2 with one oxygen atom at *m/z* 917.7, three oxygen atoms at *m/z* 949, and 5 oxygen atoms at *m/z* 981.5 were detected. The peaks in the ESI mass spectrum most likely represent not only primary but also secondary oxidation of TAG-C18:2 compounds. According to Neff et al. [1998] the minor peaks of the primary oxidation TAG compounds elute before the main peaks of the secondary oxidation TAG compounds which agrees with the results obtained by us for oxidized TAG-C18:2 compounds.

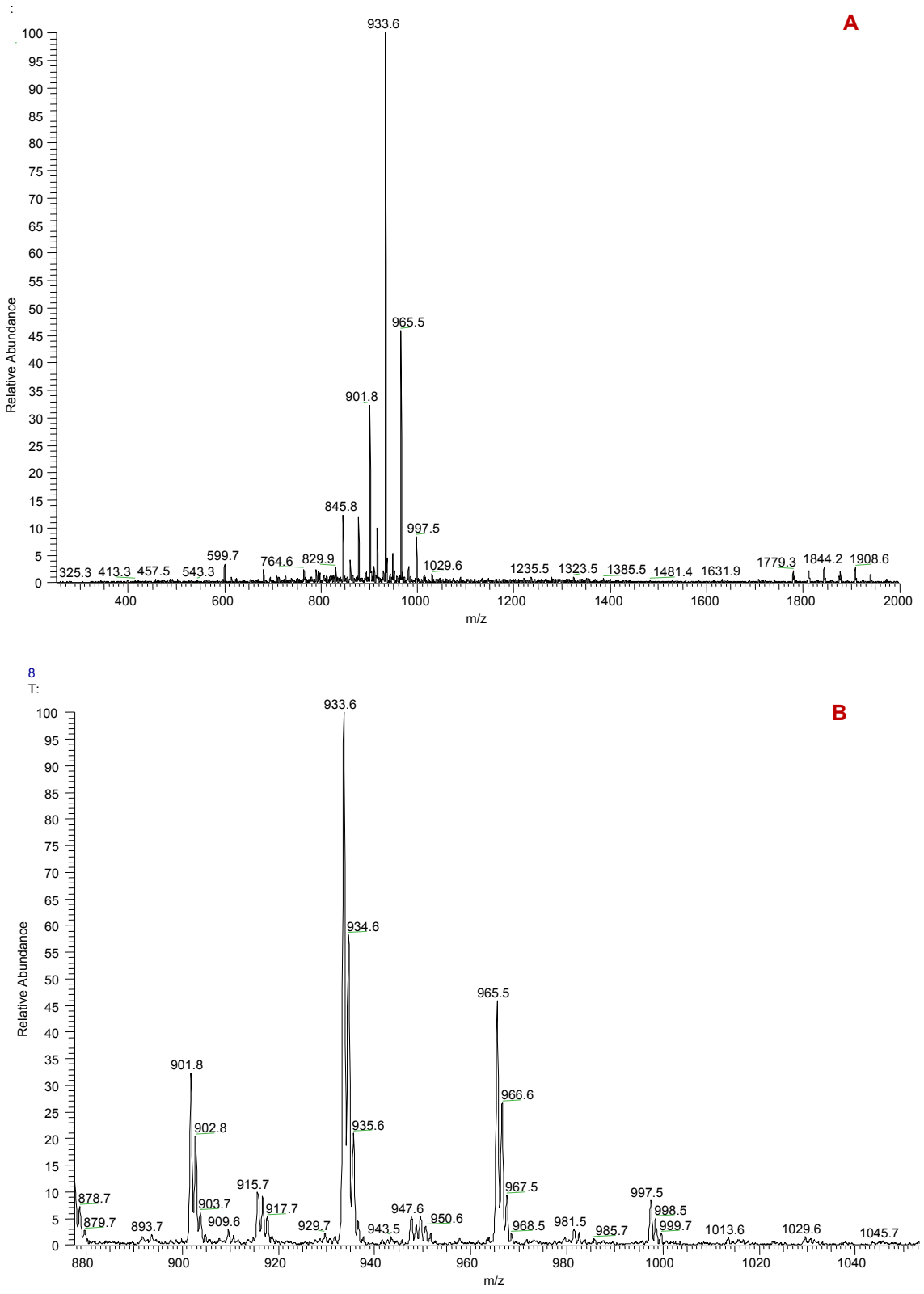


Fig 4.37. (A) The ESI mass spectrum of commercial TAG-C18:2 (*m/z* 901.8) showing possible oxidation at *m/z* 933.5 and 965.5; (B) expanded range at (880-1050).

As an example, CID of the oxidized commercial TAG-C18:2 sample gave a TAG ion at *m/z* 933.7 (Fig 4.38) showing the formation of a product ion at *m/z* 915.6 by loss of 18 u which is most likely H_2O. Similarly, a loss of 88 u product ion at *m/z* 845.5 corresponded to loss of $C_5H_{12}O$ or $C_4H_8O_2$. A loss of C18:2 detected as a major ion at *m/z* 653.5 and a loss of 296 u detected as a small peak at *m/z* 637.3 were consistent with the loss of an oxidized C18:2 fatty acid (C18:2+O).

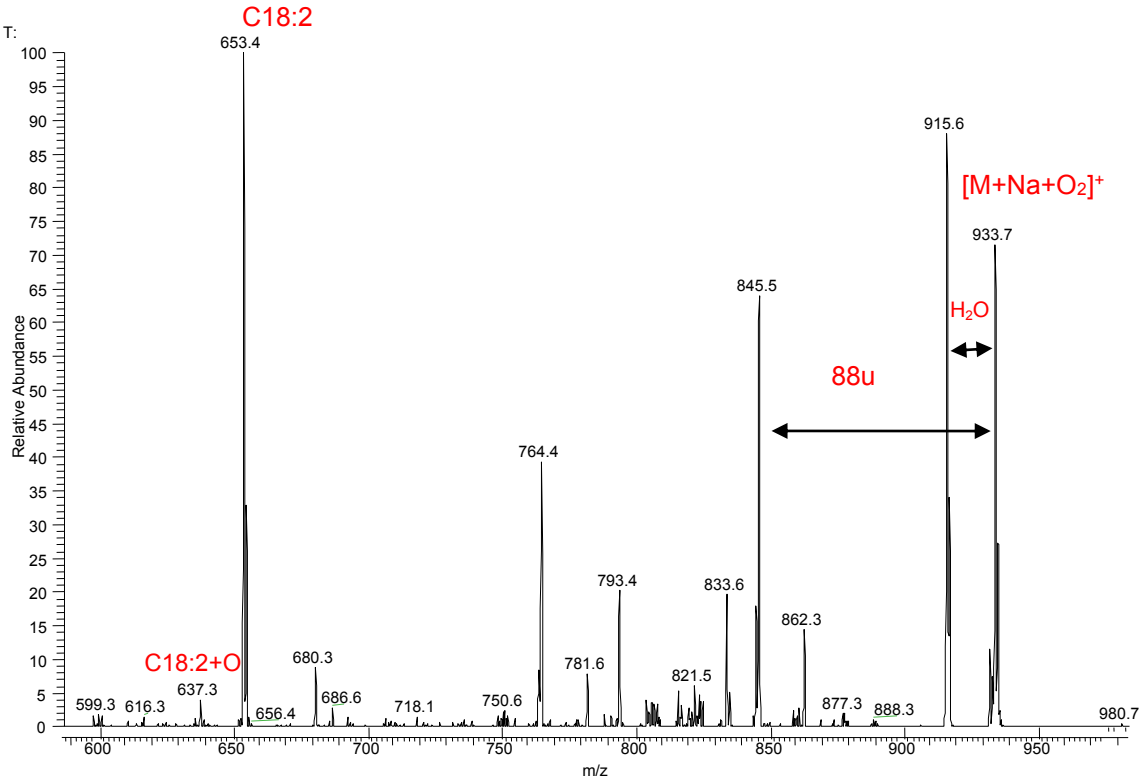


Fig 4.38. The CID spectrum of the oxidized standard TAG-C18:2 ion at *m/z* 933.7.

Thus ESI mass spectrometry was a valuable tool for the characterization of the oxidized TAG compounds in F1-F6 of the corn oil. For example, the ESI(+)/MS spectrum of F4 (Fig 4.39) of corn oil showed ions that were present in the mass spectrum of the oxidized commercial TAG-C18:2 sample. However, the sodiated ion $[M+Na]^+$ at *m/z* 901.7 corresponding to the non-oxidized TAG-C18:2 was not detected in the corn oil sample analyzed in this work (Fig. 4.28). The ions detected in F4 at *m/z* 915.9, 933.7, 949.5, and a small peak at 965.6 was also present in the mass spectrum of the oxidized standard (Fig 4.37). These indicated oxidation of TAG compounds with incorporation of one, two, three oxygen atoms. The oxidized TAG compounds with 4 oxygen atoms at

m/z 965.5 and 6 oxygen atoms at *m/z* 997.5 were are not detected in F4 of the corn oil due to the presence of natural antioxidants compound [Zeb, 2012].

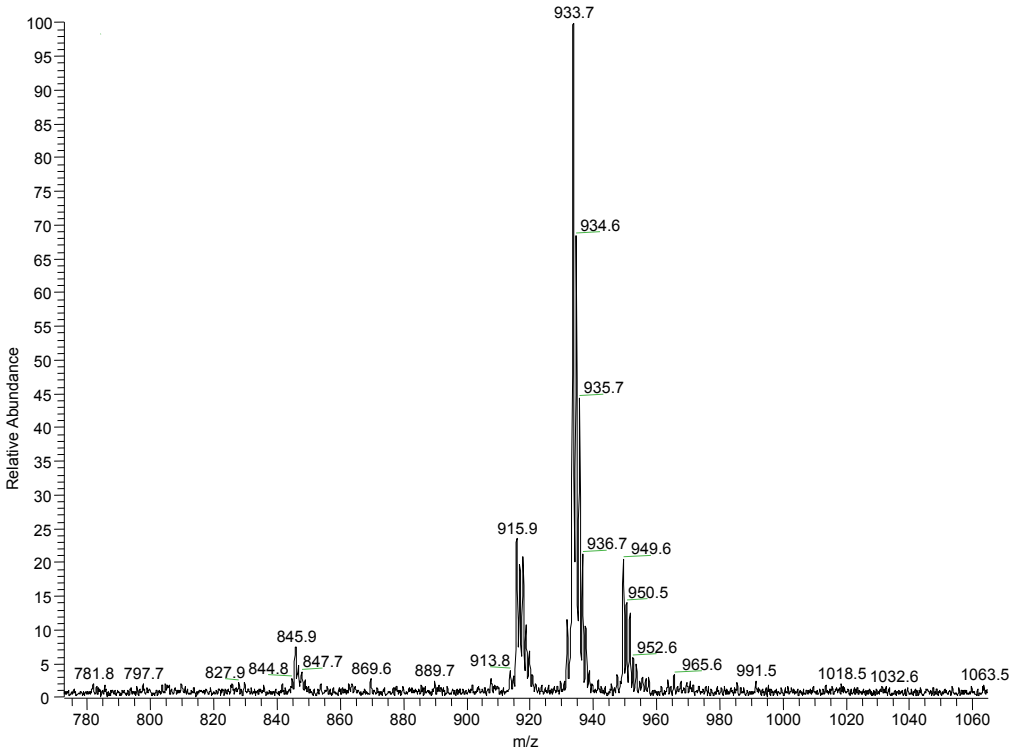


Fig.4.39. The ESI mass spectrum of the oxidized TAG compounds in F4 of corn oil (Fig 4.29).

The oxidized TAG compound at the ion m*/z* 933.7 in F4 of corn oil (Fig 4.40) fragmented upon CID. The observed loss of water (18 u) giving a production at *m/z* 915.6 and the loss of C18:2 detected at *m/z* 653.5 as major ion which indicated oxidation of a TAG-C18:2. The smaller peak at *m/z* 599.4 (334u) corresponded to the loss of the sodium salt of the oxidized C18:2 fatty acid ($[C18:2+Na+O_2]^+$).

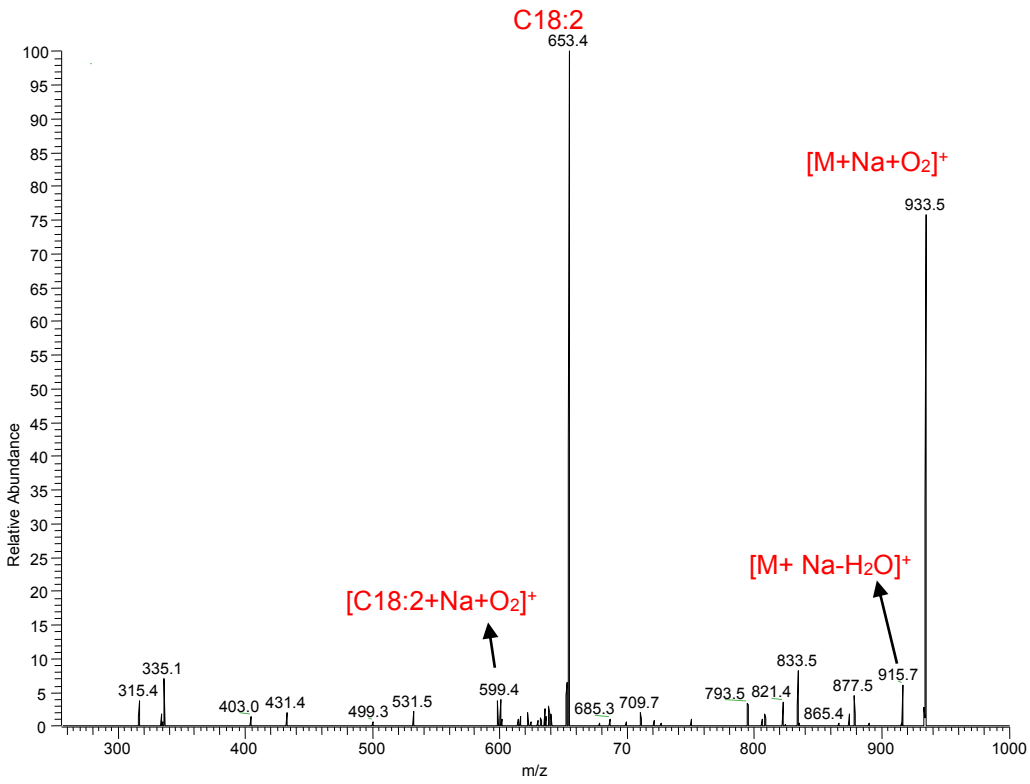


Fig 4.40. The CID spectrum of the oxidized TAG compounds in F4 of corn oil at *m/z* 933.5.

Similarly, other oxidized TAG compounds in corn oil were identified using RP-HPLC/ESI(+)/MS (Table 4.6). It is important to note that these oxidation products of TAGs were neither resolved nor detected by the off-line Ag-HPLC/ESI(+)//MS method (Fig. 4.17) but observed only using the off-line 2D RP-HPLC/ESI(+)/MS (Fig 4.29) method.

Table 4.6. Oxidation products of TAGs in corn oil using RP-HPLC/ESI(+)/MS

F #	M	$[M+Na]^+$	The oxidized TAG	MS/MS	The identified of oxidized TAG
F1	-	-	-	-	-
F2	-	-	-	-	-
F3	878.7	901.7	933.7	901.5/$[M+ Na]^+$ 653.4/C18:2	TAG3C18:2+O_2
F4	878.7	901.7	915.9	635.3/ C18:2 613.2/ C18:2-Na	TAG-3C18:2+O
			933.7	915.7/ H_2O 653.3/ C18:2 599.2/C18:2+O_2+Na	TAG-3C18:2+O_2
			949.6	931.6/ H_2O 917.2/ O_2 901.4/$[M+ Na]^+$ 669.4/ C18:2 653.2/ C18:2+O 651.0/ C18:2+H_2O 637.3/C18:2+O_2	TAG-3C18:2+O_3
F5	878.7	901.7	917.7	637.3/ C18:2 635.3/ C18:1	TAG-C18:2/C18:1/C18:3+O
			935.5	899.0/2 H_2O 655.5/ C18:2 653.1/C18:1	TAG-C18:2/C18:1/C18:3+O+ H_2O
			951.6	933.3/ H_2O 919.2/ O_2 673.9/ C18:3 669.3/ C18:1 653.4/ C18:1+O 639.4/C18:3+H_2O+O	TAG-C18:2/C18:1/C18:3+O_2+H_2O
F6	876.7	899.7	899.8	621.2/ C18:3 599.3/ C18:3-Na 619.3/ C18:2 597.2/ C18:2-Na	TAG-C18:2/C18:2/C18:3
		903.7	919.9	639.4/ C18:2 617.3/ C18:2-Na 637.3/ C18:1 615.4/ C18:1-Na 603.7/C18:1+H_2O+O	TAG-C18:2/C18:2/C18:1+O
		903.7	937.5	919.3/ H_2O 655.3/C18:1 657.4/C18:2 599.1/C18:1+H_2O +O+Na	TAG-C18:2/C18:2/C18:1+O+ H_2O

4.5. Conclusions

The optimization of separation methods for the identification of TAGs in the corn oil sample was done using 2D off-line RP-HPLC/GC/MS, RP-HPLC and Ag-HPLC (both on-line and off-line), 3D off-line Ag-HPLC/ESI(+)/MS/ GC/MS, 2D off-line Ag-HPLC/ESI(+)/MS and RP-HPLC/ESI(+)/MS allowing a good identification to 24 TAGs. All fatty acids in each TAG were identified successfully by the off-line 2D RP-HPL/GC/MS method as well as by the standard mixture. These results were very useful for the identification of the TAG peaks in 2D RP-HPLC/ESI(+)/MS. Some of the fatty acids detected by MS/MS in different fractions were not evident in GC/MS indicating that ESI(+)/MS was more sensitive than GC/MS. A summary of the results are presented in Table 4.5.

Table 4.7. A summary of the corn oil TAGs identified by RP-HPLC/GC/MS and RP-HPLC/ESI(+)/MS

Fraction #	TAG RP-HPLC/ESI(+)/MS	Fraction #	FAME RP-HPLC/GC/MS
F1-F6	-	F1	C16:0/C18:1
F7	LLL	F2	C18:2
F8	OLLn	F3	
F9	LLO		C18:1/C18:2
F10	LOO LLS	F4	C18:1/C18:2
F11	PLL OOO	F5	C16:0/C18:1/C18:2
F12	PLO POL	F6	C16:0/C18:1/C18:2
F13	LLS	F7	C16:0/C18:1/C18:2/ C18:0/C20:0
F14	POO		
F15	OOO SOL SLO POP		
F16	POP ALP SLS ALL		
F17	SOO ALO		

P (C16:0); S (C18:0); O (C18:1); L (C18:2); Ln (C18:3); A (C20:0)

The separation of six fractions using the 2D RP-HPLC and Ag-HPLC (both on-line and off-line) permitted a greater differentiation between several TAGs that contained the same number of double bonds but different partition numbers. The use of ESI-MS with MS/MS, as a second analytical dimension in both off-line RP-HPLC/ESI(+)/MS and Ag-HPLC/ESI(+)/MS methods, made the identification of several TAGs in the corn oil possible. The off-line RP-HPLC/ESI(+)/MS method gave significantly higher number of TAGs containing long chain fatty acid (C20:0; *e.g.* ALL, ALO, and ALP,) than the off-line Ag-HPLC/ESI(+)/MS method while more TAGs with polyunsaturated fatty acids were obtained by the off-line Ag-HPLC/ESI(+)/MS (*e.g.* OLnO, LLLn, and LnOLn). In addition, 9 oxidized species of TAGs were identified by the off-line RP-HPLC/ESI(+)/MS method while off-line Ag-HPLC/ESI(+)/MS could not detect them. A summary of these results are given in Table 4.8. These Methods should also be applicable to real salmon oil which is described in Chapter 6.

Table 4.8. A summary of TAGs identified in the corn oil by Ag-HPLC/ESI(+)/MS and RP-HPLC/ESI(+)/MS.

Fraction #	TAG Ag-HPLC/ESI/MS	Fraction #	TAG RP-HPLC/ESI/MS
F1	PLP POO	F7	LLL
F2	POL OOO SOL	F8	OLLn
F3	LOO SLL PLL	F9	LLO
F4	LLO OLnO	F10	LOO LLS
F5	LLL OLLn	F11	PLL
F6	LLLn LnOLn	F12	PLO POL
-	-	F13	LLS
-	-	F14	POO
-	-	F15	OOO SOL SLO POP
-	-	F16	POP ALP SLS ALL
-	-	F17	SOO GLO

P (C16:0); S (C18:0); O (C18:1); L (C18:2); Ln (C18:3); A (C20:0)

The next step was to spike the above corn oil sample with the Cl-TAG compounds. The Cl-TAG were not commercially available; so it was necessary to synthesize a number of them. Therefore, the work moved from analytical techniques to organic synthesis and chromatographic purification, followed by $^{1}H/^{13}C$ NMR and ESI/MS identification of Cl-TAGs which are described in detail in chapter 5

CHAPTER 5

Synthesis of chlorinated triacylglycerol compounds and their characterization in corn and salmon oils

5.1. Objectives

One of the main objectives of our work was to characterize triacylglycerol (TAG) and chlorinated TAG (Cl-TAG) compounds in salmon lipids. Since no certified Cl-TAG compounds were commercially available, our attention was first focussed on the synthesis of a series of Cl-TAG compounds using enzyme-catalyzed and carbodiimide-mediated coupling reactions. They were then characterized by ESI(+)/MS and NMR techniques. A mechanism describing sequential HCl losses and the formation of conjugated dienes from the Cl-TAG compounds was proposed for the loss of both vicinal chlorine atoms from an alkyl chain. Secondly, the model corn oil was spiked with these Cl-TAG compounds and characterized. Finally, known and unknown TAG compounds were characterized in real salmon skin and tissue oil samples.

This chapter consists of three parts. The first part of the chapter (Section 5.2) deals mostly with the synthesis of Cl-TAG compounds followed by their characterization using tandem mass spectrometry, as published by us [Lefsay *et al.*, 2013]. The paper entitled "Synthesis and tandem mass spectrometry of chlorinated triacylglycerols" coauthored by Abir M. Lefsay, Robert D. Guy, Amares Chatt, Robert L. White published in Chemistry and Physics of Lipids 174 (2013) 55-63, and is reproduced here with permission from the publisher Elsevier B.V. (Copyright 2013). The second part of this chapter (Section 5.3) contains details of spiking of the corn oil with Cl-TAG compounds and their characterization using a RP-HPLC/ESI(+)/MS method. The third part of the chapter (Section 5.4) consists of the application of the methods developed here to the skin and tissue oils extracted from a farmed Atlantic salmon (*Salmo salar*) and characterization of the TAG and Cl-TAG compounds in them.

5.2. Introduction

The occurrence and distribution of extractable, organically bound halogens in the marine environment [Dembitsky and Srebnik, 2002], particularly in the marine vertebrate [Björn *et al*., 1998b; Kiceniuk *et al*., 1997; Kiceniuk *et al*., 1998; King *et al.*, 2006; Lunde *et al*., 1976; Mu et al, 2004; Mu *et al*., 1996a; Mu *et al*., 1997b; Mu *et al*., 1996b; Tinsley and Lowry, 1980; Wan *et al*., 2010; Wesén *et al*., 1990; Wesén *et al*., 1992; Wesén *et al*.,

1995a; Wesén, 1995b; Zhuang *et al*., 2006] and shellfish [Bottaro *et al*., 1999; Lunde *et al*., 1976; Milley *et al*., 1997; Mu *et al*., 1997b; Tinsley and Lowry, 1980] constituents of the human food chain, is of ongoing ecotoxicological concern [Björn *et al*., 1998a; Ewald, 1999; Spickett, 2007]. However, well-known, environmentally persistent, halogenated anthropogenic substances, such as polychlorinated biphenyls (PCBs), hexachlorocyclohexanes (HCHs) and certain pesticides (*e.g*., DDT), accounted for only a minor portion of the total halogen [Björn, 1998a; Ewald, 1999; Håkansson *et al*., 1991; Lunde *et al*., 1976; Milley *et al*., 1997; Newsome *et al*., 1993; Wan *et al*., 2010; Zhuang *et al*., 2006]. Instead, most (up to 90%) of the bromine [Mu *et al*., 1997b; Tinsley and Lowry, 1980; Wan *et al*., 2010] and chlorine [Björn, 1998a; Ewald, 1999; Milley *et al*., 1997; Mu *et al*., 2004; Mu *et al*., 1997b; Mu *et al*., 1996a; Wesén *et al*., 1995b; Wesén *et al*., 1992; Zhuang *et al*., 2006] was associated with storage and membrane lipids, and subsequently, several physiological effects were attributed to halogenated lipids and fatty acids [Björn *et al*., 1998a; Ewald, 1999; Håkansson *et al*., 1991; Høstmark *et al*., 1999; Lystad *et al*., 2001].

Three dichlorinated fatty acids, 9,10-dichlorooctadecanoic acid (9,10-dichlorostearic acid; $HO_2C(CH_2)_7(CHCl)_2(CH_2)_7CH_3$), 7,8-dichlorohexadecanoic acid (7,8-dichloropalmitic acid; $HO_2C(CH_2)_5(CHCl)_2(CH_2)_7CH_3$) and 5,6-dichlorotetradecanoic acid (5,6-dichloromyristic acid; $HO_2C(CH_2)_3(CHCl)_2(CH_2)_7CH_3$), were identified in several organisms by analysis of fatty acid methyl esters [Björn *et al*., 1998b; King *et al*., 2006; Milley *et al*., 1997; Mu *et al*., 1996a; Mu *et al*., 1996b; Mu *et al*., 1997b; Wesén *et al*., 1992; Wesén *et al*., 1995a; Wesén, 1995b; Zhuang *et al*., 2003; Zhuang *et al*., 2006] and pentafluorobenzyl [Zhuang *et al*., 2004b] derived from extracted lipids. The position of chlorine in 5,6-dichlorotetradecanoic acid was confirmed by interpreting the electron ionization mass spectral fragmentation patterns of 4,4-dimethyloxazoline [Zhuang *et al*., 2004a] and picolinyl [Åkesson-Nilsson and Wesén, 2004] derivatives.

The metabolic relationship inferred by the common structural features of the three dichloroalkanoic acids was supported by the detection of shorter-chain, halogenated fatty acids in human cell cultures and rats administered 9,10-dichlorooctadecanoic acid or 9,10-dibromooctadecanoic acid [Åkesson-Nilsson and Wesén, 2004; Conacher *et al*., 1984; Gustafson-Svärd *et al*., 2001; Lawrence *et al*., 1984] and by the release of radioactive carbon dioxide when [1-^{14}C]-9,10-dichlorooctadecanoic acid was fed to goldfish [Björn *et al*., 2004]. Other experiments demonstrating the incorporation of 9,10-dichlorooctadecanoic acid into phospholipids and triacylglycerol [Björn *et al*., 1998a;

Björn *et al*., 2004; Ewald *et al*., 1996; Gustafson-Svärd *et al*., 2001] are consistent with the proposed uptake of 9,10-dichlorooctadecanoic acid from pulp mill effluents [Björn *et al*., 2004; Gustafson-Svärd *et al*., 2001; Zhuang *et al*., 2003; Zhuang *et al*., 2006] and transfer within food chains [Mu *et al*., 2004; Ewald, 1999]. Chlorinated fatty acids were not recognized as xenobiotic compounds by various organisms [Ewald, 1999; Björn *et al*., 1998a] and, when present in phospholipids, modified the properties of membranes [Ewald, 1999; Håkansson *et al*., 1991]. Also, growth inhibitory [Björn *et al*., 1998a; Høstmark *et al*., 1999] and apoptotic effects [Lystad *et al*., 2001] on human cell lines have been demonstrated for 9,10-dichlorooctadecanoic acid and 5,6-dichlorotetradecanoic acid.

In previous studies [Björn *et al*., 1998b; King *et al*., 2006; Milley *et al*., 1997; Mu *et al*., 1996a; Mu *et al*., 1997a; Mu *et al*., 1997b; Wesén *et al*., 1992; Wesén *et al*., 1995a; Wesén *et al*., 1995b; Zhuang *et al*., 2003; Zhuang *et al*., 2004a; Zhuang *et al*., 2006], the presence of halogen in complex lipids was detected only after transesterification to monoesters. Additional structural information, however, can be obtained using analytical methods that determine intact neutral glycerolipids and phospholipids. Herein, regioselective syntheses of chlorinated triacylglycerols by coupling 9,10-dichlorooctadecanoic acid to mono- and didodecanoylglycerols are described to provide reference standards of defined structure for the development and validation of liquid chromatography-tandem mass spectrometry (LC-MS/MS) [Herrera *et al*., 2010; Segall *et al*., 2004], or perhaps high-temperature gas chromatography (GC) [Ruiz-Samblás *et al*., 2012], methods for the direct determination of halogenated triacylglycerols in complex mixtures. The mass spectra of the synthetic standards showed a characteristic, predominant loss of two molecules of HCl from alkyl chains containing vicinal chlorine substituents.

5.2.1 Experimental

5.2.1.1 Chemicals

Recombinant lipases from *Candida antarctica* (produced in *Aspergilis niger* and immobilized on macroporous acrylic resin) and *Rhizomucor miehei* (produced in *Aspergillus oryzae*), vinyl laurate (dodecanoate), boron trifluoride-methanol solution, 4-dimethylaminopyridine (DMAP), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDCI), glyceryl trioleate (tri(*cis*-9-octadecenoyl)glycerol), DL-1,2-isopropylideneglycerol, sulfuryl chloride, silica gel 60 (230-400 mesh), and analytical

TLC plates were purchased from Sigma-Aldrich (Oakville, ON). Oleic acid (*cis*-9-octadecenoic acid), lauric acid (dodecanoic acid) and ion-exchange resin C-244 (strong acid, sulfonated polystyrene, 10% cross linked, 30-80 mesh) were obtained from Fisher Scientific (Ottawa, ON), Eastman Organic Chemicals (Rochester, NY) and the J.T. Baker Chemical Company (Phillipsburg, NJ), respectively.

5.2.1.2. Instruments and their operation conditions

5.2.1.2.1. Melting point measurements

Melting points (uncorrected) were obtained in open capillary tubes on a GallenKamp apparatus.

5.2.1.2.2. NMR

Proton (^{1}H, 500 MHz) and carbon (^{13}C, 125 MHz) nuclear magnetic resonance (NMR) spectra were recorded on a Bruker AVANCE 500 MHz spectrometer. Samples were dissolved in either $CDCl_3$ or CD_2Cl_2 (30 mg mL^{-1}). Chemical shifts (δ) in parts per million (ppm) were referenced to solvent signals ($CDCl_3$: δ_H 7.26, δ_C 77.16; CD_2Cl_2: δ_H 5.32, δ_C 54.00). Coupling constants (*J*) are given in Hertz (Hz); resonance multiplicities are described as: singlet (s), doublet (d), triplet (t), quartet (q), doublet of doublets (dd), broad singlet (br s), and multiplet (m). The number of carbon nuclei is indicated in parenthesis when one signal corresponds to two or more carbon atoms.

5.2.1.2.3. ESI(+)MS

Electrospray ionization mass spectrometry (ESI-MS) and ESI-MS/MS were done using a Thermo-Finnigan LCQ DUO ion trap mass spectrometer running under Xcalibur software and equipped with an ESI probe set at 3.7 kV. Nitrogen was used as the source gas and the capillary temperature was maintained at 200°C. Tandem mass spectra (MS/MS) were acquired using helium collision gas at collision-induced dissociation (CID) energies given in arbitrary units (%). Sample solutions (10 µL; 4 µM in methanol) were introduced into the ion source of the mass spectrometer by flow injection in methanol (syringe pump, 1.2 mL h^{-1}) via a Rheodyne 7725 injection valve. Accurate masses were determined using a Bruker microTOF mass spectrometer.

5.2.1.2.4 GC/MS

A portion (1 μL) of the sample was injected onto a poly(5% diphenyl/95% dimethylsiloxane) fused silica capillary (Supleco PTE-5; 30 m x 0.25 mm id, 0.25 μm film thickness; 100:1 split injection) in an HP6890 GC connected to an HP5937 mass selective detector and an HP6890 autosampler/injector. Helium carrier gas (1 mL min^{-1}) and a temperature gradient of 10°C min^{-1} from 100 to 320°C with a five-min hold were employed. The injection port and transfer line were at 260°C, while the ionization source and quadrupole were maintained at 230 and 150°C, respectively. Mass spectra (*m/z* 40–550) were acquired at 2.3 s^{-1}.

5.2.3. Synthesis procedures

As mentioned above, we needed to synthesize Cl-TAG compounds which are not commercially available. These compounds are: 1-(9,10-dichlorooctadecanoyl)-2,3-didodecanoylglycerol (compound 13), 1,2-Di-(9,10-dichlorooctadecanoyl)-3-dodecanoyl-glycerol (compound 14), and Tri-(9,10-dichlorooctadecanoyl)glycerol (compound 16). For the syntheses of compounds 13 and 14, it was necessary to first synthesize 9,10-dichlorooctadecanoic acid (compound 2), 1,3-didodecanoylglycerol (compound 5), 2-(9,10-dichloro-octadecanoyl)-1,3-didodecanoyl-glycerol (compound 6), 1,2-isopropyl -idene-3-(9,10-dichlorooctadecanoyl)glycerol (compound 9), *rac*-1,2-isopropylidene-3-dodecanoylglycerol (compound 10), 1-(9,10-dichlorooctadecanoyl)glycerol (compound 11), and *rac*-1-dodecanoyl-glycerol (compound 12). Briefly, compound 13 was obtained by coupling two equivalents of dodecanoic acid (compound 7) and compound 11**.** The compound 14 was obtained by coupling two equivalents of compound 2 with compound 12. Reaction of tri-(*cis*-9-octadecenoyl)glycerol (compound 15) with glyceryl trioleate and sulfuryl chloride gave hexachlorinated triacylglycerol (compound 16). The commercially available compounds were: *cis*-9-octadecenoic acid (also known as oleic acid, compound 1), vinyl dodecanoate (compound 3), glycerol (compound 4), DL-1,2-isopropylideneglycerol (compound 8), and compound 15. The syntheses of the rest of the compounds are described below in detail.

5.2.3.1. Synthesis of 9,10-Dichlorooctadecanoic acid (compound 2)

Sulfuryl chloride (3 mL, 37 mmol) was added to a chloroform solution of *cis*-9-octadecenoic acid (compound 1also known as oleic acid; 2.0 g, 7.1 mmol) in a round-bottomed flask covered with aluminum foil and immersed in an ice bath (Turnhofer *et al*.,

2008). After stirring for 3 h, water (5 mL) was added, and stirring was continued overnight at room temperature. The reaction mixture was washed with water (5 x 10 mL) until the aqueous wash was about pH 7. The organic layer was dried (anhydrous sodium sulfate) and rotary evaporated. Crude product was purified using column chromatography on silica gel (hexane-ethyl acetate, 2:1 v/v), yielding compound 2 as a yellow oil (2.3 g, 92%). ^{1}H and ^{13}C NMR spectra were consistent with published data (Denton et al., 2010); ESI(+)MS (relative intensity): *m*/*z* 375.3 (100, $[M+Na]^+$); MS/MS (33% CID) of *m*/*z* 375.3: *m/z* 339.0 (12), 303.1 (100).

For GC analysis, a mixture of 9,10-dichlorooctadecanoic acid (0.10 g, 0.3 mmol; in 1 mL CH_2Cl_2) and BF_3-MeOH (14%, 1 mL, 2 mmol) was heated at 80°C for 5 min. Saturated aqueous sodium chloride solution (2 mL) was added and the mixture was extracted with hexane (2 mL). The organic layer was dried (anhydrous sodium sulfate) and a portion (1 μL) was injected onto GC/MS.

5.2.3.2. Synthesis of 2-(9,10-dichlorooctadecanoyl)-1,3-didodecanoyl-glycerol (compound 6)

In order to synthesize 2-(9,10-dichlorooctadecanoyl)-1,3-didodecanoyl-glycerol (compound 6), it was first necessary to synthesize another compound, namely 1,3-didodecanoylglycerol (compound 5), as given below.

5.2.3.2.1. Synthesis of 1,3-didodecanoylglycerol (compound 5)

Immobilized *Candida antarctica* lipase (1.5 g) was added to a mixture of vinyl dodecanoate (compound 3; 5.0 g, 22 mmol) and glycerol (compound 4; 0.51 g, 5.5 mmol) in dichloromethane (2 mL) [Halldorsson *et al*., 2003]. The suspension was allowed to stand at 4°C for 18 h. The immobilized lipase was separated by filtration and the filtrate was rotary evaporated to afford a white crystalline product (2.4 g, 96%). The mp was 44-46°C [Halldorsson *et al*., 2003, mp 54.3-54.7°C]. The ^{1}H and ^{13}C NMR spectra were consistent with published data [Halldorsson *et al*., 2003]: ESI(+)/MS (relative intensity): *m/z* 935.2 (82, $[2M+Na]^+$) and 479.5 (100, $[M+Na]^+$); and MS/MS (35% CID) of *m/z* 479.5: *m/z* 279.1 (44), 257.1 (100).

5.2.3.2.2 Synthesis of compound 6

A solution of 1,3-didodecanoylglycerol (compound 5; 1.8 g, 3.9 mmol), 9,10-dichlorooctadecanoic acid (compound 2; 1.43 g, 4.1 mmol), DMAP (0.164 g, 1.3 mmol) and EDCI (0.76 g, 4.0 mmol) in dichloromethane (20 mL) was stirred at room

temperature for 24 h. The solvent was removed by rotary evaporation, and the crude product was purified by flash chromatography on silica gel (ethyl acetate-hexane, 1:4 v/v). The separated product was a colorless oil (2.6 g, 83%). ^{1}H NMR (CD_2Cl_2) δ: 5.25–5.21 (1H, m), 4.26 (2H, dd, *J* = 11.9 and 4.3), 4.13 (2H, dd, *J* = 11.9 and 6.1), 4.08–4.04 (2H, m), 2.30 (2H, t, *J* = 7.5), 2.29 (4H, t, *J* = 7.6), 1.93–1.85 (2H, m), 1.84–1.76 (2H, m), 1.63–1.56 (8H, m), 1.33–1.26 (50H, m), 0.89 (3H, t, *J* = 7), 0.88 (6H, t, *J* = 7); ^{13}C NMR (CD_2Cl_2) δ: 173.6 (2C), 173.3, 69.3, 66.4, 66.3, 62.6 (2C), 35.4, 35.3, 34.7, 34.5 (2C), 32.5 (2C), 32.4, 31.1, 30.2 (4C), 30.0 (2C), 29.9 (2C), 29.84 (2C), 29.76, 29.64 (2C), 29.61 (2C), 29.5, 29.4, 27.22, 27.17, 25.4 (2C), 25.3, 23.25 (2C), 23.21, 14.4 (3C); ESI(+)MS (relative intensity): *m*/*z* 813.6 (100, $[M+Na]^+$); MS/MS (30% CID) of *m*/*z* 813.6: *m*/*z* 777.4 (15), 741.4 (100), 613.2 (5), 595.2 (5); MS/MS (40% CID) of *m*/*z* 741.4: *m*/*z* 541.1 (100), 519.3 (50), 461.1 (20), 439.3 (14); HRMS: 813.5509 (813.5537 calculated for $C_{45}H_{84}Cl_2O_6Na$).

5.2.3.3. Syntheses of chlorinated unsymmetrical triacylglycerols (compounds 13 and 14)

5.2.3.3.1. Synthesis of monoacylisopropylidenes

A solution of 1,2-isopropylideneglycerol (compound 8), fatty acid (1 equiv. of 2 or 7), EDCI (1 equiv.) and DMAP (0.5 equiv.) in dichloromethane (20 mL) was stirred at room temperature for 24 h. The solvent was removed by rotary evaporation and crude product was purified using flash chromatography on silica gel eluted using ethyl acetate-hexane (2:1 v/v).

5.2.3.3.1.1. Synthesis of 1,2-isopropylidene-3-(9,10-dichlorooctadecanoyl) glycerol (compound 9)

Coupling of 9,10-dichlorooctadecanoic acid (compound 2**;** 2.6 g, 7.4 mmol) and 1,2-isopropylideneglycerol (compound 8; 0.97 g, 7.3 mmol) yielded 1,2-isopropylidene-3-(9,10-dichlorooctadecanoyl)glycerol (compound 9) as an oil (2.9 g 85%). ^{1}H NMR (CD_2Cl_2) δ: 4.30–4.25 (1H, m), 4.14–4.02 (5H, m), 3.72 (1H, dd, *J* = 8.3 and 6.1), 2.35–2.30 (2H, m), 1.98–1.90 (2H, m), 1.85–1.75 (2H, m), 1.64–1.52 (4H, m), 1.40 (3H, s), 1.34 (3H, s), 1.34–1.28 (18H, m), 0.90 (3H, t, *J* = 7); ^{13}C NMR (CD_2Cl_2) δ: 173.9, 110.3, 74.4, 67.1, 66.49, 66.45, 65.1, 35.52, 35.49, 34.7, 32.5, 30.1, 29.9, 29.7 (2C), 29.6, 29.5, 27.35, 27.30, 27.2, 25.9, 25.5, 23.3, 14.5; ESI(+)MS (relative intensity): *m/z* 489.3 (100,

[M + Na]$^+$): MS/MS (35% CID) of 489: *m/z* 452.9 (16), 416.9 (100); HRMS: 489.2498 (489.2509 calculated for $C_{24}H_{44}Cl_2O_4Na$).

5.2.3.3.1.2. Synthesis of *rac*-1,2-Isopropylidene-3-dodecanoylglycerol (compound 10)

Coupling of dodecanoic acid (2.8 g, 14 mmol) and 1,2-isopropylideneglycerol (compound 8; 1.9 g, 14 mmol) yielded 1,2-Isopropylidene-3-dodecanoylglycerol (compound 10) as a white crystalline solid (3.8 g, 86%). The mp was < 20°C [Batovska *et al*., 2004; 32.5°C)]. The ^{1}H and ^{13}C NMR spectra were consistent with published data [Batovska *et al*., 2004]; ESI(+)MS: *m/z* 337.3 ([M+Na]$^+$); MS/MS (35% CID) of 337.3: *m/z* 279.1.

5.2.3.3.2. Syntheses of monoacylglycerols

Purified 1,2-isopropylidene-3-acylglycerol (compound 9) was mixed with ion-exchange resin C-244 (2 g, H^+ form) in MeOH (20 mL). The mixture was stirred at room temperature and monitored using TLC. When the starting material was consumed (4 h) the resin was filtered. The filtrate was dried (anhydrous sodium sulfate) and removed by rotary evaporation. The same procedure was followed for *rac*-1,2-Isopropylidene-3-dodecanoylglycerol (compound 10).

5.2.3.3.2.1. Synthesis of 1-(9,10-Dichlorooctadecanoyl)glycerol (compound 11)

Hydrolysis of 1,2-isopropylidene-3-(9,10-dichlorooctadecanoyl)glycerol (1.62 g, 3.5 mmol) yielded 11 as a colorless oil (1.35 g, 92%).^{1}H NMR (CD_2Cl_2) δ: 4.18–4.02 (4H, m), 3.91–3.87 (1H, m), 3.68–3.55 (2H, m), 2.34 (2H, t, *J* = 7.4), 1.99–1.88 (2H, m), 1.85–1.75 (2H, m), 1.65–1.51 (4H, m), 1.45–1.28 (18H, m), 0.90 (3H, m); ^{13}C NMR (CD_2Cl_2) δ: 174.6, 71.0, 67.1, 66.5, 65.9, 64.1, 35.5, 35.3, 34.7, 32.5, 30.3, 30.0, 29.8, 29.7, 29.6, 29.5, 27.3, 26.9, 25.5, 23.3, 14.5; ESI(+)MS (relative intensity): *m/z* 449.2 (100, [M+ Na]$^+$); MS/MS (25% CID) of 449.2: *m/z* 412.9 (20), 376.9 (100); HRMS: 449.2206 (449.2196 calculated for $C_{21}H_{40}Cl_2O_4Na$).

5.2.3.3.2.2. Synthesis of *rac*-1-Dodecanoylglycerol (compound 12)

Hydrolysis of 1,2-isopropylidene-3-dodecanoylglycerol (1.5 g, 4.8 mmol) yielded 12 as white crystals (1.25 g, 93%). mp 60-61°C (lit [Rytczak et al., 2010] mp 62–63°C); ^{1}H and ^{13}C NMR spectra were consistent with published data [Batovska et al., 2004;

Rytczak et al., 2010]; ESI(+)MS: *m/z* 297.4 ([M+Na]$^+$); MS/MS (28% CID) of 297.4: *m*/*z* 115.1.

5.2.3.3.3. Coupling of monoacyl glycerol with two equivalent fatty acids

The coupling procedure described in Section 5.2.3.3.1 and 33-h reaction times were used.

5.2.3.3.3.1. Synthesis of 1-(9,10-Dichlorooctadecanoyl)-2,3-didodecanoyl glycerol (compound 13)

Coupling of 1-(9,10-dichlorooctadecanoyl)glycerol (11; 1.5 g, 3.5 mmol) and dodecanoic acid (**7**; 1.4 g, 7.0 mmol) yielded 13 as a waxy solid (2.4 g, 84%). mp 30–33°C; ^{1}H NMR ($CDCl_3$) δ: 5.28–5.24 (1H, m), 4.29 (2H, dd, *J* = 11.9 and 4.3), 4.14 (2H, dd, *J* = 11.9 and 6.0), 4.04–4.01 (2H, m), 2.31 (6H, td, *J* = 7.5 and 3.2), 1.93–1.86 (2H, m), 1.81–1.73 (2H, m), 1.63–1.56 (8H, m), 1.32–1.24 (50H, m), 0.88 (9H, t, *J* = 7); ^{13}C NMR ($CDCl_3$) δ: 173.4, 173.3, 173.0, 69.0, 65.7, 65.6, 62.2 (2C), 34.53, 34.49, 34.3, 34.2, 34.1, 32.05 (2C), 31.96, 29.84, 29.75 (4C), 29.63, 29.61, 29.52, 29.48 (2C), 29.42, 29.41, 29.34, 29.25, 29.21, 29.17, 29.09, 29.0, 26.9, 26.8, 25.04, 24.99, 24.9, 22.82 (2C), 22.79, 14.3 (3C); ESI(+)MS (relative intensity): *m*/*z* 813.5 (100, [M+Na]$^+$); MS/MS (36% CID) of *m*/*z* 813.5: *m*/*z* 777.5 (20), 741.5 (100), 613.2 (5); MS/MS (33% CID) of *m*/*z* 741.5: *m*/*z* 541.3 (100), 519.3 (50), 461.2 (40), 439.1 (25); HRMS: 813.5560 (813.5537 calculated for $C_{45}H_{84}Cl_2O_6Na$).

5.2.3.3.3.2. Synthesis of 1,2-Di-(9,10-dichlorooctadecanoyl)-3-dodecanoyl glycerol (compound 14)

Coupling of 1-dodecanoylglycerol (12; 1.0 g, 3.6 mmol) with 9,10-dichlorooctadecanoic acid (2.5 g, 7.2 mmol) yielded 14 as an oil (2.5 g, 73%). ^{1}H NMR ($CDCl_3$) δ: 5.26–5.21 (1H, m), 4.29–4.25 (2H, m), 4.11 (2H, dd, *J* = 11.9 and 6.0), 4.02–3.98 (4H, m), 2.30–2.25 (6H, m), 1.92–1.84 (4H, m), 1.79–1.70 (4H, m), 1.61–1.49 (10H, m), 1.31–1.22 (52H, m), 0.86 (3H, t, *J* = 7), 0.85 (6H, t, *J* = 7); ^{13}C NMR ($CDCl_3$) δ: 173.3, 173.2, 172.8, 69.0, 65.7 (2C), 65.6 (2C), 62.17, 62.14, 34.5 (4C), 34.2, 34.1, 34.0, 32.0, 31.9 (2C), 29.8, 29.75, 29.71 (2C), 29.6, 29.5, 29.43, 29.36, 29.30, 29.2, 29.1 (4C), 29.04, 29.01, 28.93, 28.91, 26.81 (2C), 26.76 (2C), 24.94, 24.88, 24.85, 22.8, 22.7 (2C), 14.2 (3C); ESI(+)MS (relative intensity): *m/z* 965. 7 (71, [M+Na]$^+$), 966.8 (44), 967.7 (100); MS/MS (30% CID) of *m*/*z* 967.7: *m*/*z* 931.4 (15), 895.5 (100), 893.5 (76), 821.6 (3); MS/MS (30% CID) of *m*/*z* 893.5: *m*/*z* 857.5 (26), 821.5 (100); MS/MS (36% CID) of

m/*z* 821.5: *m*/*z* 621.3 (64), 599.2 (21), 541.1 (100), 519.3 (35); HRMS: 965.5726 (965.5697 calculated for $C_{51}H_{94}Cl_4O_6Na$).

5.2.3.4. Hexahalogenated triacylglycerols

5.2.3.4.1. Tri-(9,10-dichlorooctadecanoyl)glycerol (compound 16)

Reaction of tri-(*cis*-9-octadecenoyl)glycerol (15, glyceryl trioleate; 0.52 g, 0.59 mmol) with sulfuryl chloride (3 mL, 37 mmol) as described in Section 2.2 yielded hexachlorinated triacylglycerol (16) as a colorless oil (0.54 g, 84%). ^{1}H NMR (CD_2Cl_2) δ: 5.25–5.21 (m, 1H), 4.26 (2H, dd, *J* = 12.0 and 4.3), 4.13 (2H, dd, *J* = 11.8 and 6.0), 4.08–4.05 (6H, m), 2.30 (6H, t, *J* = 7.5), 1.92–1.86 (6H, m), 1.84–1.76 (6H, m), 1.63–1.50 (12H, m), 1.42–1.26 (54H, m), 0.88 (9H, t, *J* = 7.0); ^{13}C NMR (CD_2Cl_2) δ: 173.6 (2C), 173.2, 69.4, 66.41 (3C), 66.37 (3C), 62.6 (2C), 35.4 (6C), 34.7, 34.5 (2C), 32.4 (3C), 30.0 (3C), 29.8 (3C), 29.6 (6C), 29.5 (3C), 29.4 (3C), 27.23 (3C), 27.19 (3C), 25.3 (3C), 23.2 (3C), 14.4 (3C); ESI(+)MS (relative intensity): *m*/*z* 1117.5 (60, $[M+Na]^+$); MS/MS (35% CID) of *m*/*z* 1119.5: *m*/*z* 1083.3 (24), 1047.4 (100), 1045.6 (40), 1009.6 (23), 973.5 (42); MS/MS (33% CID) of *m*/*z* 1047.5: *m*/*z* 1011.5 (28), 975.5 (100), 973.7 (69), 901.6 (25); MS/MS (33% CID) of *m*/*z* 973.5: *m*/*z* 937.3 (40), 901.5 (100); MS/MS (38% CID) of *m*/*z* 901.6: *m*/*z* 621.6 (100), 599.5 (38); HRMS: 1117.5812 (1117.5856 calculated for $C_{57}H_{104}Cl_6O_6Na$).

5.2.3.4.2. Tri-(9,10-dibromooctadecanoyl)glycerol (compound17)

Bromine (1.5 mL, 29 mmol) was added over 20 min to a stirred solution of tri-(*cis*-9-octadecenoyl)glycerol (15; 0.20 g, 0.23 mmol) in dichloromethane (5 mL) at –10°C. After 1 h and an additional 3 h at room temperature, solvent was removed by rotary evaporation, and purification of the residue by flash chromatography on silica gel (dichloromethane) yielded hexabrominated triacylglycerol (17) as a yellow oil (0.16 g, 50%). ^{1}H NMR (CD_2Cl_2) δ: 5.25–5.21 (1H, m), 4.27 (2H, dd, *J* = 11.9 and 4.4), 4.25–4.21 (6H, m), 4.13 (2H, dd, *J* = 11.8 and 6.0), 2.31 (6H, t, *J* = 7.5), 2.06–1.89 (6H, m), 1.90–1.82 (6H, m), 1.64–1.53 (12H, m), 1.43–1.25 (54H, m), 0.88 (9H, t, *J* = 7.0); ^{13}C NMR (CD_2Cl_2) δ: 173.6 (2C), 173.2, 69.4, 62.6 (2C), 60.7 (3C), 60.6 (3C), 36.0 (6C), 34.6, 34.5 (2C), 32.4 (3C), 29.9 (3C), 29.8 (3C), 29.6 (3C), 29.5 (3C), 29.4 (3C), 29.2 (3C), 28.32 (3C), 28.27 (3C), 25.3 (3C), 23.2 (3C), 14.4 (3C); ESI(+)MS (relative intensity): *m*/*z* 1391.6 (17), 1390.7 (35), 1389.7 (42), 1388.7 (75), 1387.7 (59), 1386.7 (100), 1385.7 (48), 1384.7 (73), 1383.8 (23), 1382.7 (34), 1381.9 (12), 1380.7 (10, $[M+Na]^+$), 1230.0 (8), 1228.9 (16), 1227.9 (21), 1226.9 (43); 1225.9 (28), 1225.1 (50), 1224.1 (22),

1222.9 (39), 1221.9 (9), 1221.1 (14, $[M + Na - 2HBr]^+$), 1067.2 (10), 1066.2 (17), 1065.2 (37), 1064.2 (23), 1063.1 (40), 1062.3 (14), 1061.2 (19, $[M + Na - 4HBr]^+$); MS/MS (24% CID) of *m*/*z* 1386.7: *m*/*z* 1226.9 (41), 1224.9 (100), 1222.9 (31); MS/MS (36% CID) of *m*/*z* 901.5: *m*/*z* 621.3 (100), 599.1 (20); HRMS: 1381.2780 (1381.2825 calculated for $C_{45}H_{84}Cl_2O_6Na$).

5.2.4 Results and Discussion

5.2.4.1 Chlorination of oleic acid

The analytical studies of halogenated lipids in marine organisms [Björn *et al*., 1998a; Dembitsky and Srebnik, 2002; King *et al*., 2006; Milley *et al*, 1997; Mu *et al*., 1997b; Wesén *et al*., 1992; Wesén *et al*., 1995a; Zhuang *et al*., 2003; Zhuang *et al*., 2004a; Zhuang *et al*., 2006] most commonly detected dichlorinated fatty acids. Accordingly, 9,10-dichlorooctadecanoic acid (compound 2) was prepared from cis-9-octadecenoic acid (compound 1) and sulfuryl chloride [Bouquet and Paquot, 1946; Thurnhofer *et al*., 2008] (Fig. 5.1). By comparison of ^1H and ^{13}C NMR data with published values [Denton *et al*., 2010], the relative stereochemistry of the predominant isomer of the reaction product was assigned as *threo* (*syn*). Given the relative ^1H NMR chemical shifts of methyl *threo*- and erythro-9,10-dichlorooctadecanoate [Wesén *et al*., 1995a], a distinct resonance (δ 3.98-3.95) was assigned to *erythro*-9,10-dichlorooctadecanoic acid and a 7:1 threo:erythro ratio was calculated. Two closely eluting peaks (retention times of 22.4 and 22.5 min; ratio 1:5) were observed upon GC of the methyl esters in accord with the *erythro-threo* order noted previously [Björn *et al*., 1998b; Mu *et al*., 1996a; Wesén *et al*., 1995a; Zhuang *et al*., 2003]. Consequently, the *threo* product of the sulfuryl chloride reaction [Bouquet and Paquot, 1946; Thurnhofer *et al*., 2008] is formed by anti-addition of chlorine to the cis double bond in 9-octadecenoic acid (compound 1).

5.2.4.2. Discussion synthesis of 2-(9,10-dichlorooctadecanoyl)-1,3-didodecanoylglycerol (compound 6)

An enzymatic approach was adopted to achieve regioselective acylation of the terminal hydroxyl groups in glycerol [Magnusson and Haraldsson, 2010; Andrews *et al*., 2008; Fraser *et al*., 2007; Halldorsson *et al*., 2003; Irimescu *et al*., 2001]. Initially, vinyl dodecanoate (compound 3) and glycerol (compound 4) (3:1 ratio) were incubated with the 1,3-specific lipase from *Rhizomucor miehei* produced in *Aspergillus oryzae* [Halldorsson *et al*., 2003; Irimescu *et al*., 2001]. ESI(+)MS analysis detected the sodium adduct ions of didodecanoylglycerol (compound 5, *m*/*z* 479.6) and tridodecanoylglycerol

(*m*/*z* 661.7), as well as higher mass ions (*m*/*z* 935.4, 1117.5 and 1299.6) corresponding to the sodium adduct ions of the three possible dimers of di- and tridodecanoylglycerol. The similar abundances of the five ions indicated that the *Rhizomucor miehei* lipase-catalyzed reaction yielded compound 5 and tridodecanoylglycerol as major products, as noted previously [Halldorsson *et al*., 2003].

On the other hand, incubation of glycerol (compound 4) and excess vinyl dodecanoate (compound 3) with immobilized *Candida antarctica* lipase at 4°C [Magnusson and Haraldsson, 2010; Andrews *et al*., 2008; Halldorsson *et al*., 2003] gave the desired 1,3-didodecanoylglycerol (compound 5) in excellent yield and purity (Fig. 5.1). Low-intensity resonances at δ 5.25, 5.08, 4.33-4.19 and 3.72 in the ^{1}H NMR spectrum of the product indicated trace amounts of 1,2-dodecanoylglycerol and tridodecanoylglycerol side products [Haraldsson *et al*., 1995].

Carbodiimide-mediated coupling [Magnusson and Haraldsson, 2010; Andrews *et al*., 2008; Fraser *et al*., 2007; Halldorsson *et al*., 2003] of dihalogenated fatty acid (compound 2) with 1,3-didodecanoylglycerol (compound 5) using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDCI) and 4-dimethylaminopyridine (DMAP) in dichloromethane at room temperature gave (after chromatography) 2-(9,10-dichlorooctadecanoyl)-1,3-didodecanoylglycerol (compound 6). The observation of two ^{13}C NMR resonances and an $(AB)_2X$ ^{1}H NMR splitting pattern for nuclei in the glycerol backbone of compound 6 demonstrated the symmetry of this triacylglycerol product, in accord with the reported absence of acyl-migration side products under these coupling conditions [Magnusson and Haraldsson, 2010; Andrews *et al*., 2008; Halldorsson *et al*., 2003]. Also, no mono- or diacylglycerols were detected by ESI(+)MS.

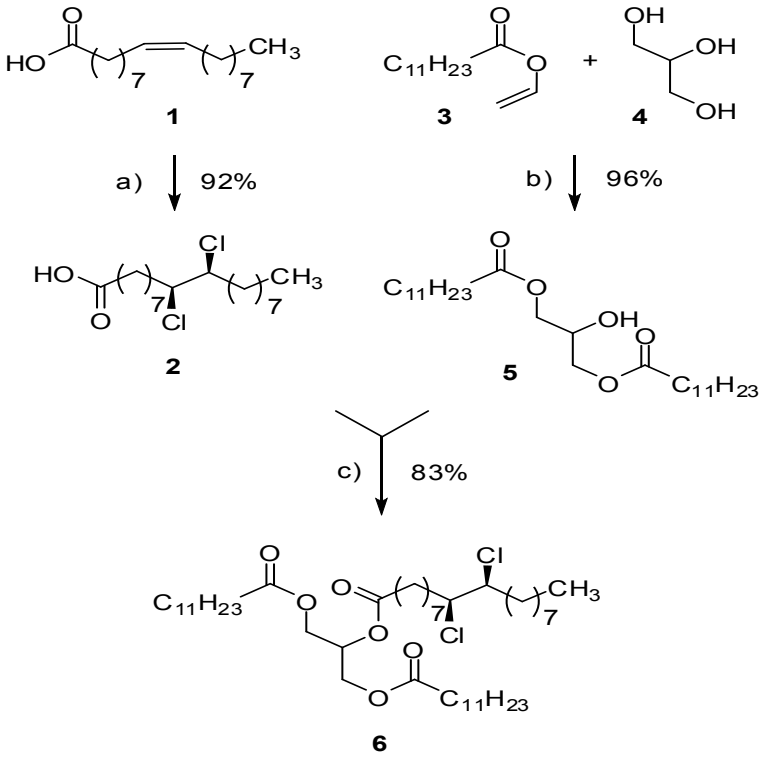


Fig 5.1. Synthesis of the symmetrical triacylglycerol (compound 6). Reagents and conditions: (a) SO2Cl2, CHCl3, 3 h; (b) Immobilized C. *antarctica* lipase, 4°C, overnight; (c) EDCI, DMAP, CH2Cl2, 24 h.

5.2.4.3. Discussion synthesis of two unsymmetrical chlorinated triacylglycerols (compound 13 and compound 14)

The preparation of unsymmetrical triacylglycerols containing one dichlorinated chain in a terminal position or two dichlorinated chains at adjacent positions (Fig. 5.2) started with a protected glycerol [Andrews *et al*., 2008; Fraser *et al*., 2007]. Separate carbodiimide-mediated couplings of 9,10-dichlorooctadecanoic acid (compound 2) and dodecanoic acid (compound 7) with rac-1,2-isopropylideneglycerol (compound 8) added an acyl group specifically at a terminal position. Hydrolysis of the acylated isopropylidenes compound 9 and compound 10 was achieved in high yield under the acidic conditions provided by a sulfonated polystyrene ion-exchange resin. The racemic monoacylglycerol products compound 11 and compound 12 contained about 6–7% of their regioisomer by ^{1}H NMR analysis [Haraldsson *et al*., 1995], indicating only small amounts of acyl group migration. Carbodiimide coupling of dodecanoic acid compound (7) and 9,10-dichlorooctadecanoic acid (compound 2) to the monoacylglycerols compound 11 and

compound 12, respectively, yielded chlorinated unsymmetrical triacylglycerols (compound 13 and compound 14) with complementary substitution patterns.

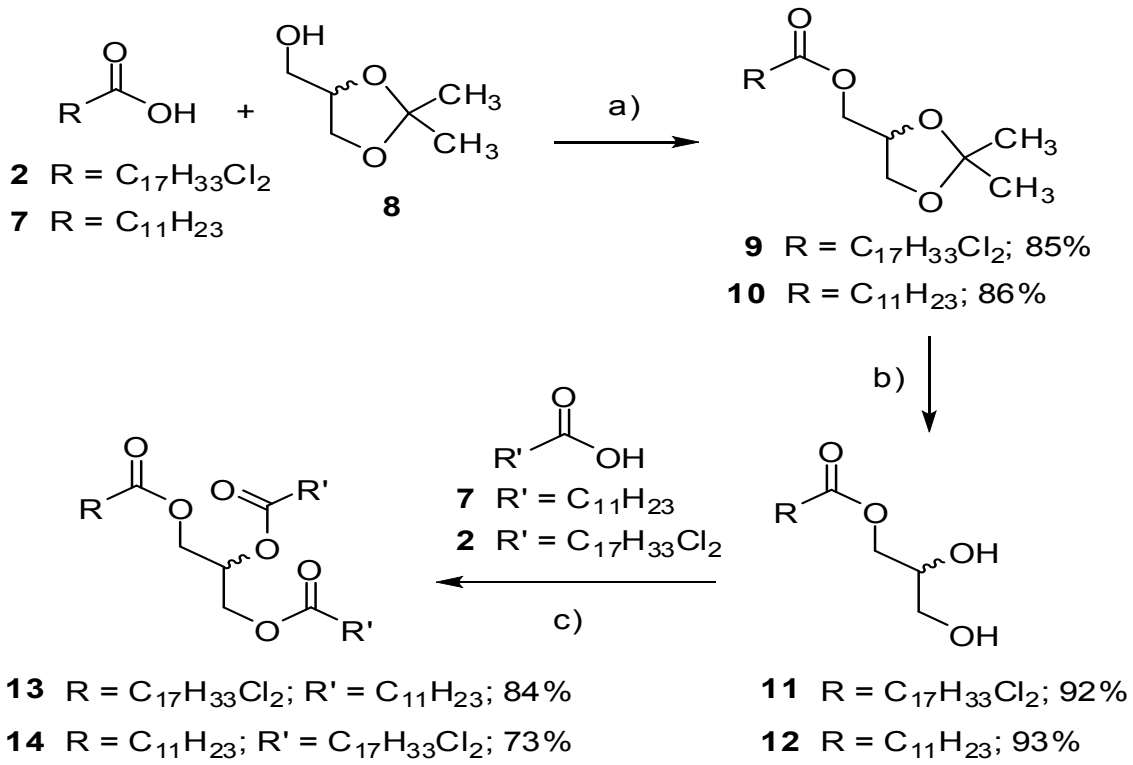


Fig 5.2. Synthesis of the unsymmetrical triacylglycerols **13** and **14**. Reagents and conditions: (A) EDCI, DMAP, CH_2Cl_2, 24 h; (B) cation-exchange resin, MeOH, 4 h; (C) EDCI, DMAP, CH_2Cl_2, 33 h.

5.2.4.4. Discussion synthesis of hexahalogenated triacylglycerols

Tri-(9,10-dichlorooctadecanoyl)glycerol (compound 16) was obtained in high yield (90%) by treating commercially available tri-(*cis*-9-octadecenoyl)glycerol (compound 15) with excess sulfuryl chloride. Similarly, tri-(9,10-dibromooctadecanoyl)glycerol (compound 17) was prepared by adding excess bromine to tri-(*cis*-9-octadecenoyl)glycerol (compound 15). The glyceryl resonances in the ^{1}H NMR spectrum of each product appeared as the typical pattern of a triacylglycerol [Haraldsson *et al*., 1995], and no olefinic resonances were detected in the ^{1}H and ^{13}C NMR spectra, indicating complete conversion of unsaturated triacylglycerol to the hexahalogenated product.

5.2.4.5. Mass spectrometry

When subjected to electrospray ionization mass spectrometry (ESI(+)/MS), 9,10-dichlorooctadecanoic acid (compound 2) and all mono-, di-, and triacylglycerols (*i.e.*, 5, 6, 9–14, 16, 17) were detected as sodium adduct ions. The $[M+Na]^+$ ions of the chlorinated compounds showed distinctive isotope clusters (e.g., Fig. 5.3A inset and Table 5.1) in which the relative intensities of the individual peaks closely corresponded to the values calculated [Ramaley and Herrera, 2008] using the expected elemental compositions (including two, four or six chlorine atoms) and natural isotopic abundances.

Table 5.1: Isotope clusters observed upon ESI(+)MS of the chlorinated compounds.

The program Isotope Pattern Generator was used to provide the calculated relative intensities [Ramaley and Herrera, 2008].

Comp'd	$[M + Na]^+$ Formula	*m*/*z* (Observed Relative Intensity, Calculated Relative Intensity)						
		A	A + 1	A + 2	A + 3	A + 4	A + 5	A + 6
2	$C_{18}H_{34}Cl_2O_2Na$	375.3 (100,100)	376.3 (24,20)	377.3 (68,66)	378.3 (15,13)	379.2 (11,12)	-	-
6	$C_{45}H_{84}Cl_2O_6Na$	813.6 (100,100)	814.6 (49,50)	815.5 (73,77)	816.5 (31,34)	817.5 (20,19)	818.4 (9,7)	-
9	$C_{24}H_{44}Cl_2O_4Na$	489.3 (100,100)	490.1 (36,27)	491.3 (75,68)	492.1 (22,17)	493.1 (15,13)	-	-
11	$C_{21}H_{40}Cl_2O_4Na$	449.2 (100,100)	450.1 (28,23)	451.1 (68,67)	452.1 (16,15)	453.1 (22,12)	-	-
13	$C_{45}H_{84}Cl_2O_6Na$	813.5 (100,100)	814.5 (50,50)	815.5 (72,77)	816.5 (32,34)	817.4 (19,19)	818.5 (6,7)	-
14	$C_{51}H_{94}Cl_4O_6Na$	965.7 (71,69)	966.8 (44,39)	967.7 (100,100)	968.7 (52,52)	969.7 (58,57)	970.7 (30,27)	971.7 (20,17)
16	$C_{57}H_{104}Cl_6O_6Na$	1117.5 (60,47)	1118.6 (35,30)	1119.5 (100,100)	1120.5 (56,59)	1121.5 (95,91)	1122.5 (50,50)	1123.6 (48,47)

The sodiated ions of the chlorinated compounds (*i.e.*, 2, 6, 9, 11, 13, 14) showed no fragmentation upon electrospray ionization. By contrast, the ammonium adduct ions of methyl 9,10-dichlorooctadecanoate [Mu *et al*., 1996a; Wesén *et al*., 1995a] and methyl dichlorooctadecenoate [Mu *et al*., 1996b] fragmented under chemical ionization conditions, losing Cl_2, as well as one or two molecules of HCl from methyl

dichlorooctadecenoate [Mu *et al*., 1996b]. When subjected to plasma spray ionization mass spectrometry [Sundin *et al*., 1992], chlorinated triacylglycerols readily lost a fatty acid residue from the $[M + H]^+$ ions. Upon plasma spray ionization of tri-(9,10-dichlorooctadecanoyl)glycerol, loss of the fatty acid was accompanied by up to four molecules of HCl, but, unlike the CID fragmentations described below, there was no preference for losses of an even number of HCl molecules. For triacylglycerols containing one chlorine per acyl group, no loss of HCl was observed upon plasma spray ionization.

Loss of a fatty acid and its sodium salt are typically observed upon CID of the sodiated ions of di- and triacylglycerols [Herrera *et al*., 2010; Hsu and Turk, 2010; Segall *et al*., 2004]; however, the ESI(+)MS/MS spectra showed predominantly two losses of HCl (*e.g.*, Fig.5.3A and B) from the sodium adduct ions of 9,10-dichlorooctadecanoic acid (compound 2) and all chlorinated mono-, di-, and triacylglycerols (i.e., 6, 9, 11, 13 and 14) containing 9,10-dichlorooctadecanoyl groups in both terminal and central positions. The lithium adduct ion of compound 13 showed a similar pattern of HCl losses . The isotopic composition of the product ions reflected that of the ion selected within the isotopic cluster. For example, losses of two $H^{35}Cl$ and $H^{35}Cl + H^{37}Cl$ from the $^{35}Cl_3{}^{37}Cl$ isotopologue (*m*/*z* 967.7) of sodiated compound 14 (Fig. 5.3A) yielded product ions at *m*/*z* 895.5 and 893.5 with relative abundances matching the calculated ratio (i.e., 76:100 vs. 79:100 [Ramaley and Herrera, 2008]. Double losses of HCl also were observed as in-source fragmentations. Subsequent CID of the $[M + Na – 2HCl]^+$ ion of compound 14 (Fig.5.3B) and the $[M + Na – 2HCl]^+$ and $[M + Na – 4HCl]^+$ ions of compound 16 also led to minor single and major double losses of HCl.

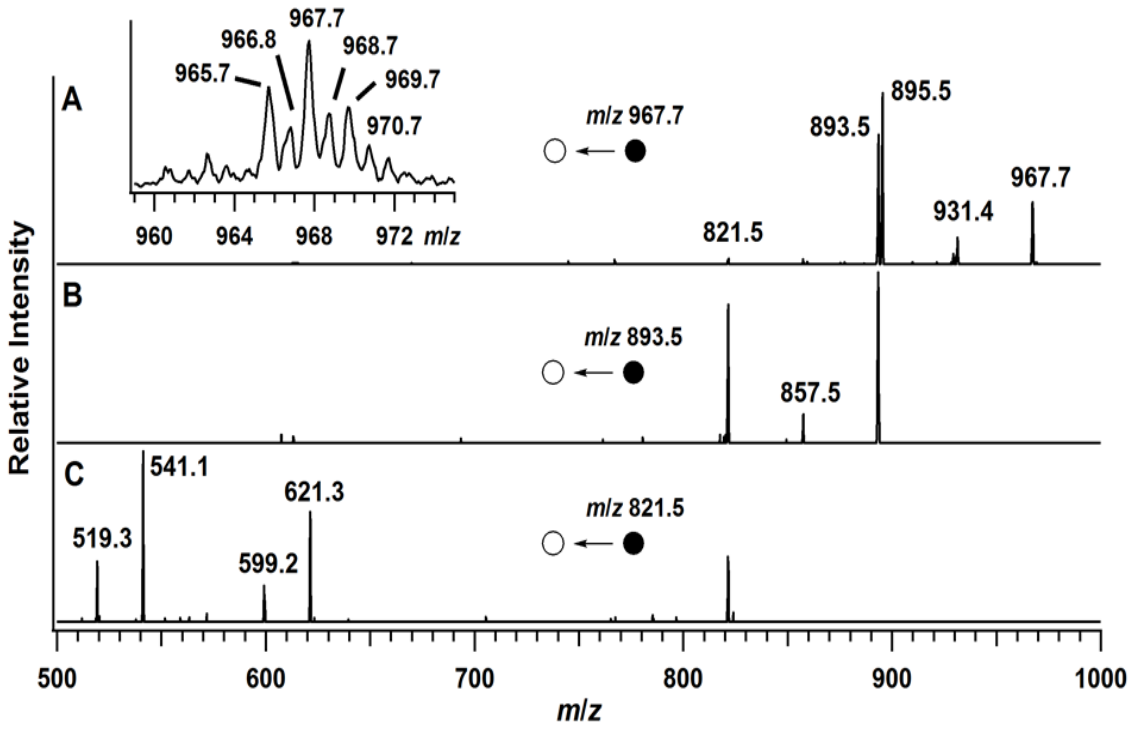


Fig 5.3. Mass spectra of 1,2-di-(9,10-dichlorooctadecanoyl)-3-decanoylglycerol (compound 14). A: CID (30%) spectrum of $[M + Na]^+$. B: CID (30%) spectrum of in-source generated $[M+Na – 2HCl]^+$. C: CID (36%) spectrum of in-source generated $[M+Na – 4HCl]^+$.

Similar minor single and major double losses of HBr were observed upon CID of sodiated tri-(9,10-dibromooctadecanoyl)glycerol (compound 17). Losses of HBr occurred at lower CID energies and isotopic clusters corresponding to $[M+Na]^+$, $[M+Na–2HBr]^+$ and $[M+Na – 4HBr]^+$ were prominent in the electrospray mass spectrum. The relative peak intensities within each isotope cluster were consistent with calculated values [Ramaley and Herrera, 2008], and product ions with the expected isotopic compositions were obtained upon CID of the individual isotopologues of $[M+Na]^+$.

When all halogen atoms were lost by in-source fragmentation, then the expected neutral losses of acyl groups as fatty acids and their sodium salts [Herrera *et al*., 2010; Hsu and Turk, 2010; Segall *et al*., 2004] were observed (e.g., Fig.5.3C and 5.4 (inset spectra)). The losses corresponded to dodecanoic acid and a doubly unsaturated C18 acid, the expected product formed upon elimination of two HCl. Preferred cleavage of acyl groups

from the terminal position of sodiated triacylglycerol ions [Herrera *et al*., 2010; Hsu and Turk, 2010; Segall *et al*., 2004] allowed the isomeric triacylglycerols compounds 6 and 13 to be distinguished by mass spectrometry (Fig.5.4 inset spectra). For the symmetrical triacylglycerol compound 6, the loss of a dodecanoyl group from either position 1 or 3 of was about four times more likely than the loss of the 9,10-dichlorooctadecanoyl group from the central position (Fig. 5.4B inset). On the other hand, the loss of a dodecanoyl group from position 1 or 2 of the unsymmetrical triacylglycerol compound 13 was favored by about 2:1 over the loss of the 9,10-dichlorooctadecanoyl group from position 3 (Fig.5.4A inset). For comparison, the loss of a doubly unsaturated group from position 1 or 2 of the unsymmetrical triacylglycerol compound 14 with the complementary substitution pattern was also about twice as frequent as the loss of the dodecanoyl group from position 3 (Fig.5.3C).

By contrast, HCl losses occurred readily from 9,10-dichlorooctadecanoyl substituents at both the central and terminal positions of sodiated triacylglycerols (Fig.5.4) and from ions having only one fatty acid chain (*i.e*., the sodium adduct ions of compounds 2, 9 and 11). The latter results identify the dichlorinated alkyl chain, one oxygen-containing functional group and a cation as the minimum common structural requirements and suggest that each double loss of HCl observed for the sodiated tetra- and hexachlorinated triacylglycerols compound 14 (Fig. 5.3) and compound 16 occur from a single 9,10-dichlorooctadecanoyl group. Also, the conjugated diene formed by two HCl losses from the same dichloroalkyl group provides another favorable binding site for the sodium ion and more favorable energetics than the formation of two separate double bonds.

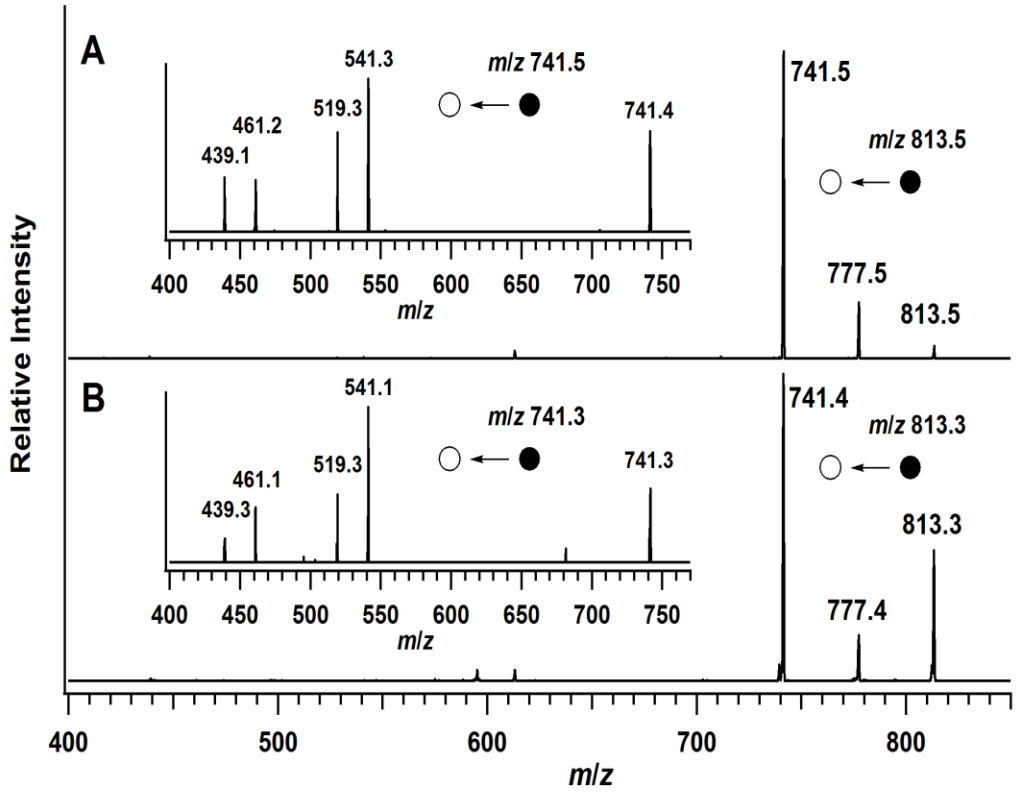


Fig 5.4. CID spectra of A: 1-(9,10-dichlorooctadecanoyl)-2,3-didodecanoylglycerol (compound 13, 33% CID) and B: 2-(9,10-dichlorooctadecanoyl)-1,3-didodecanoylglycerol (compound 6, 36% CID). The CID (35%) spectra of the corresponding in-source generated $[M+Na– 2HCl]^+$ ions are shown as insets.

A mechanistic hypothesis incorporating the minimum required structural features is presented in Fig 5.5. Sodium ions bind to a wide range of functional groups [Hoyau *et al*., 1999; Rodgers and Armentrout, 2000], including chlorine in HCl, and some of the ions formed by ESI will be stabilized by interaction of the sodium ion with both a carbonyl oxygen and a chlorine at C9, or C10, in the same acyl group. The intervening alkyl chain in **18** is able to adopt a favorable chair-chair conformation, and the relative stereochemistry of the two chlorine atoms is fixed by the initial *anti* addition to a *cis* double bond (Section **5.2.4.1**). Upon CID of **18**, C–Cl bond cleavage is assisted by sodium ion coordination and possible anchimeric assistance. With participation by the vicinal chlorine [Denmark *et al*., 2010], an intermediate chloronium ion (**19**) is formed, and abstraction of an acidic hydrogen from C8 and/or C11 by the proximate chloride ion facilitates double bond formation. Only the *cis* and *trans* product ions (**20** and **22**,

respectively) resulting from loss of hydrogen at C11 are shown in Fig 5.5. In the *cis* isomer **20**, interaction with the sodium ion weakens the C–Cl bond and increases the acidity of the allylic hydrogen at C12, which, in turn, promote HCl loss and conjugated diene (**21**) formation via an electrocyclic rearrangement. Alternatively, a conjugated diene product ion (e.g., **24**) could arise by allylic cation formation (e.g., **23**) and hydrogen abstraction from C12. The reactions presented in Fig 5.5 are a subset of the possible routes arising from two initial binding sites for sodium ion with chlorine and the alternative sites for proton abstraction. A mixture of product ions differing in the position and stereochemistry of conjugated double bonds likely results from the observed HCl losses from 9,10-dichlorooctadecanoyl substituents (e.g., Fig.5.1A,1B and 5.2).

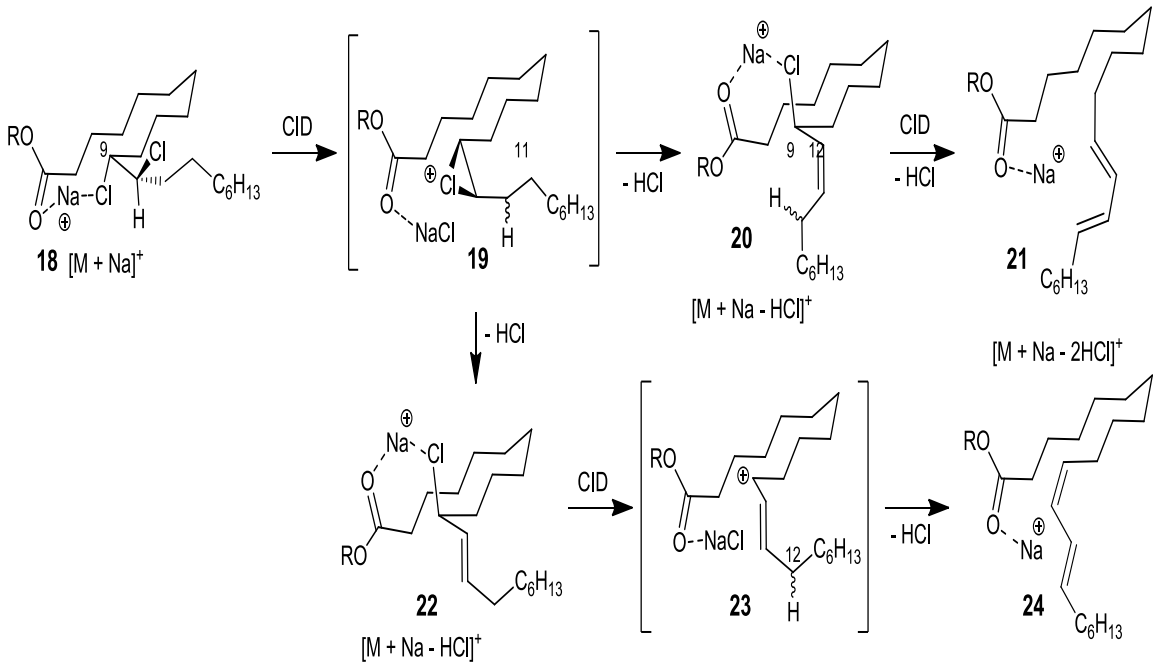


Fig 5.5. Mechanistic hypothesis for CID-initiated, sequential HCl losses from a 9,10-dichlorooctadecanoyl group, the common structural subunit in the sodiated ions formed from compounds 2, 6, 9, 11, 13, 14 and 16. The two possible proton abstractions from C11 in **19** are shown. Analogous routes follow from C8 proton abstractions in **19** and from initial binding of the sodium ion to the chlorine at C10, rather than C9 as shown in **18**.

5.3. Spiking corn oil with Cl-TAG compounds

Before spiking corn oil it was necessary to determine the sensitivity of the ESI(+)/MS technique used for Cl-TAG compounds. A quantitative study was done with 1 nm-6 μM concentrations of the commercially available TAG of tri-(*cis*-9-octadecenoyl) glycerol (TAG-C18:1). The calibration curves of the peak area and peak height are shown in Figs 5.6, 5.7, and 5.8. Similar calibration curves of TAGC12-C18:Cl2-C12 for the concentration range 1 nM-1 μM are given in Figs 5.9, 5.10 and 5.11. On visual examination it may appear that the point at 0.2 μM of the peak height graph (Fig. 5.10) is an outlier from the rest but a Q-Test showed that it is not a significant outlier (P > 0.05); so the data were not rejected.

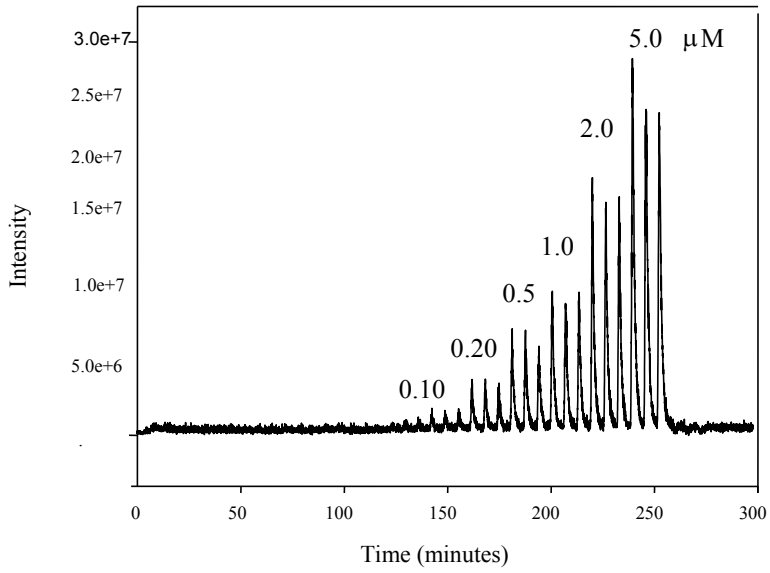


Fig 5.6. TIC of TAGC18:1 (1 nM- 6 μM) range *m/z* 907.7 -908.7.

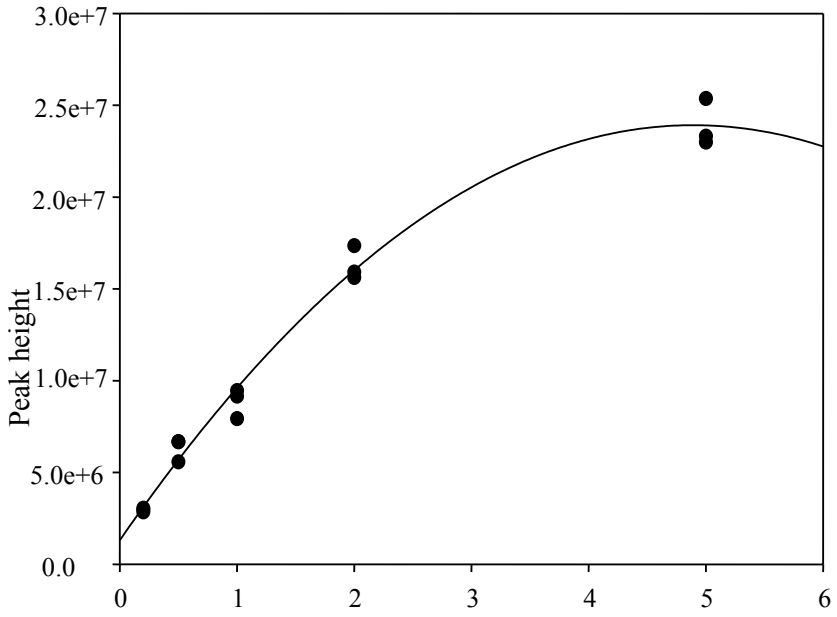


Fig 5.7. The calibration curve (1nM- 6 µM) of the peak height study of TAGC18:1.

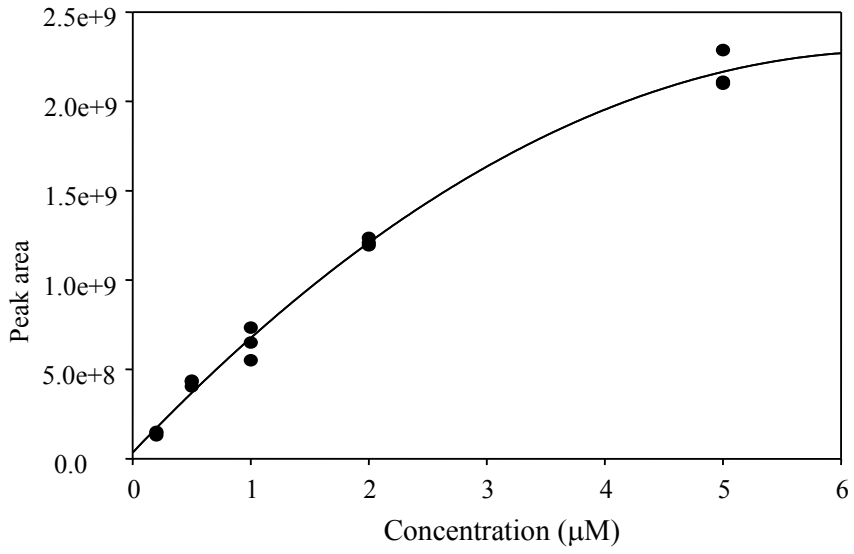


Fig 5.8. The calibration curve (1nM- 6 µM) of the peak area study of TAGC18:1.

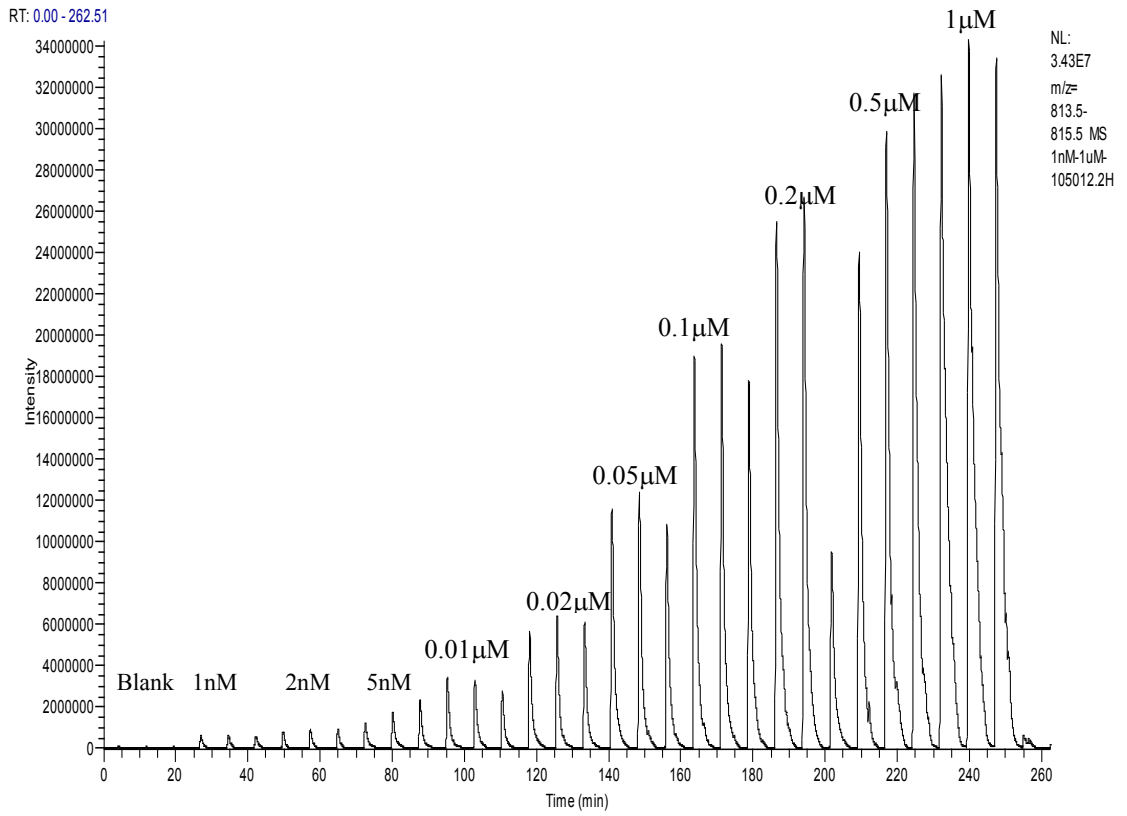


Fig 5.9. TIC of TAGC12-C18Cl$_2$-C12 (1 nM- 1 µM) range *m/z* 813.5 -815.5.

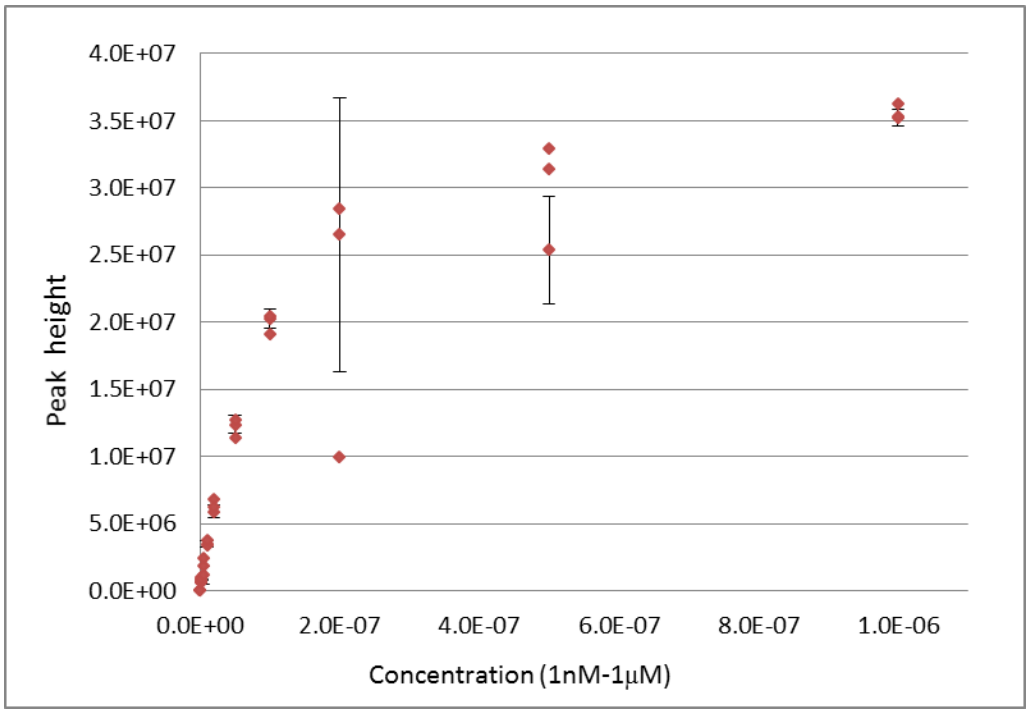


Fig 5.10. Calibration curve (1nM- 1 µM) of the peak height of TAGC12-C18Cl_2-C12.

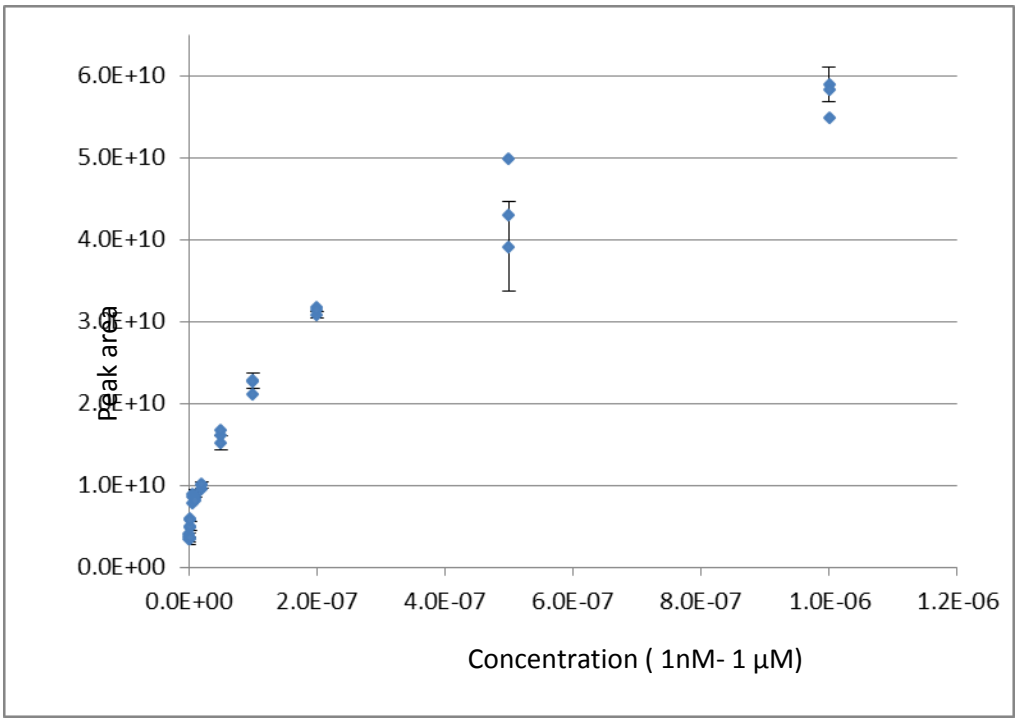


Fig 5.11. Calibration curve (1 nM- 1 µM) of the peak area of TAGC12-C18Cl_2-C12.

An interesting observation of the above results was that the ESI/MS was more sensitive to Cl-TAG (Fig. 5.9) than non-Cl-TAG (Fig. 5.6). Its sensitivity was estimated to

be 0.03 μM. From a theoretical point of view, Cl is supposed to replace the double bond in a TAG therby reducing the sensitivity of ESI/MS which is the opposite of our observation. The reason for this improved sensitivity for Cl-TAG is not very clear at this stage. Perhaps the mechanism given Fig. 5.5 could explain it somewhat.

In order to estimate the lowest detection limit of a Cl-TAG in corn oil a series of experiments were carried out. The Cl-TAG chosen was the one with the lowest number of Cl atoms studied in this thesis, which was TAG-C12:0/C18Cl_2/C12:0 with only two Cl atoms. The corn oil samples were spiked with different concentrations of this Cl-TAG as shown in Table 5.2. Seventeen fractions were collected from RP-HPLC column (Fig.5.12) and the first 6 fractions were analyzed by ESI(+)/MS.

Table 5.2. The limit of detection of TAG-C12:0/C18Cl_2/C12:0 by ESI(+)/MS

Concentration #	ESI(+)/MS observation
1 nM	Not detected
2 nM	Not detected
5 nM	Not detected
0.01 μM	Not detected
0.02 μM	Not detected
0.05 μM	Not detected
0.1 μM	Not detected
0.2 μM	Detected
0.5 μM	Detected
1 μM	Detected

It is evident from the data in Table 5.2 that the concentration of TAG-C12:0/C18Cl_2/C12:0 below 0.2 μM cannot be detected by the ESI(+)/MS instrument used in this thesis. It was also observed that the signal had high intensity and was highly reproducible at above the concentrion of 1 μM of the Cl-TAG. It was then decided to spike corn oil with 1 μM of all Cl-TAGs.

Consequently, 1 μM of TAG-C12:0/C18Cl_2/C12:0 (compound 13), TAG-C12:0/C18Cl_2/C18Cl_2 (compound 14) and TAG-C18Cl_6 (compound 16) were added to

corn oil. Seventeen fractions collected from the corn oil using RP-HPLC with rapid gradient (Fig.5.12) were followed by ESI(+)/MS.

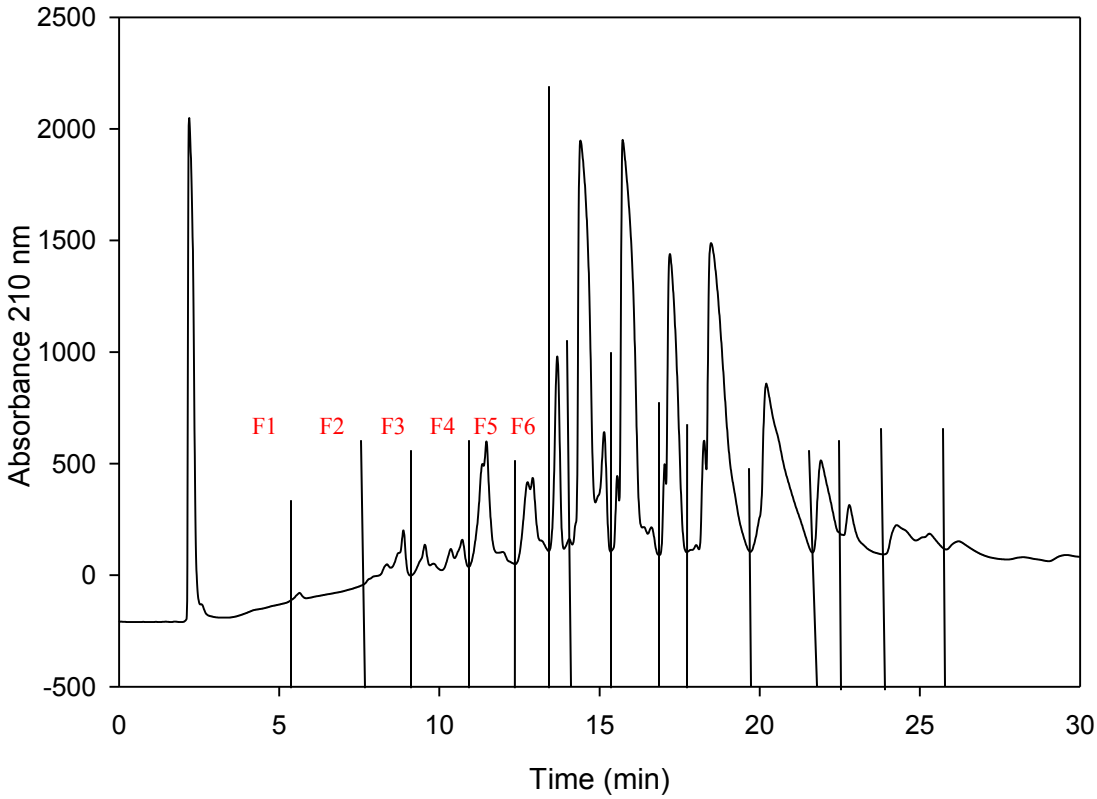


Fig 5.12. Seventeen fractions collected from the corn oil run RP-HPLC after spiked with Cl compounds (13, 14, and16).

The Cl-TAG compounds were eluted in the RP-HPLC chromatogram of the corn oil before its TAG peak. The ESI(+)/MS method gave the following results: TAG-C12:0-C18Cl_2-C12:0 (compound 13) with 2 Cl atoms eluted at 2 min (F1, Fig 5.13A,B); the TAG-C12:0-C18Cl_2-C18Cl_2 (compound 14) with 4Cl atoms eluted at 8 min (F3, Fig 5.14A,B); finally the TAG-C18Cl_6 (compound 16) with 6 Cl atoms eluted at 12 min (F6, Fig 5.15A,B). The rest of the fractions were identified to be the same TAGs of the corn oil which were previously identified (Chapter 4) and listed in Table 4.4. No Cl-TAG compounds were detected.

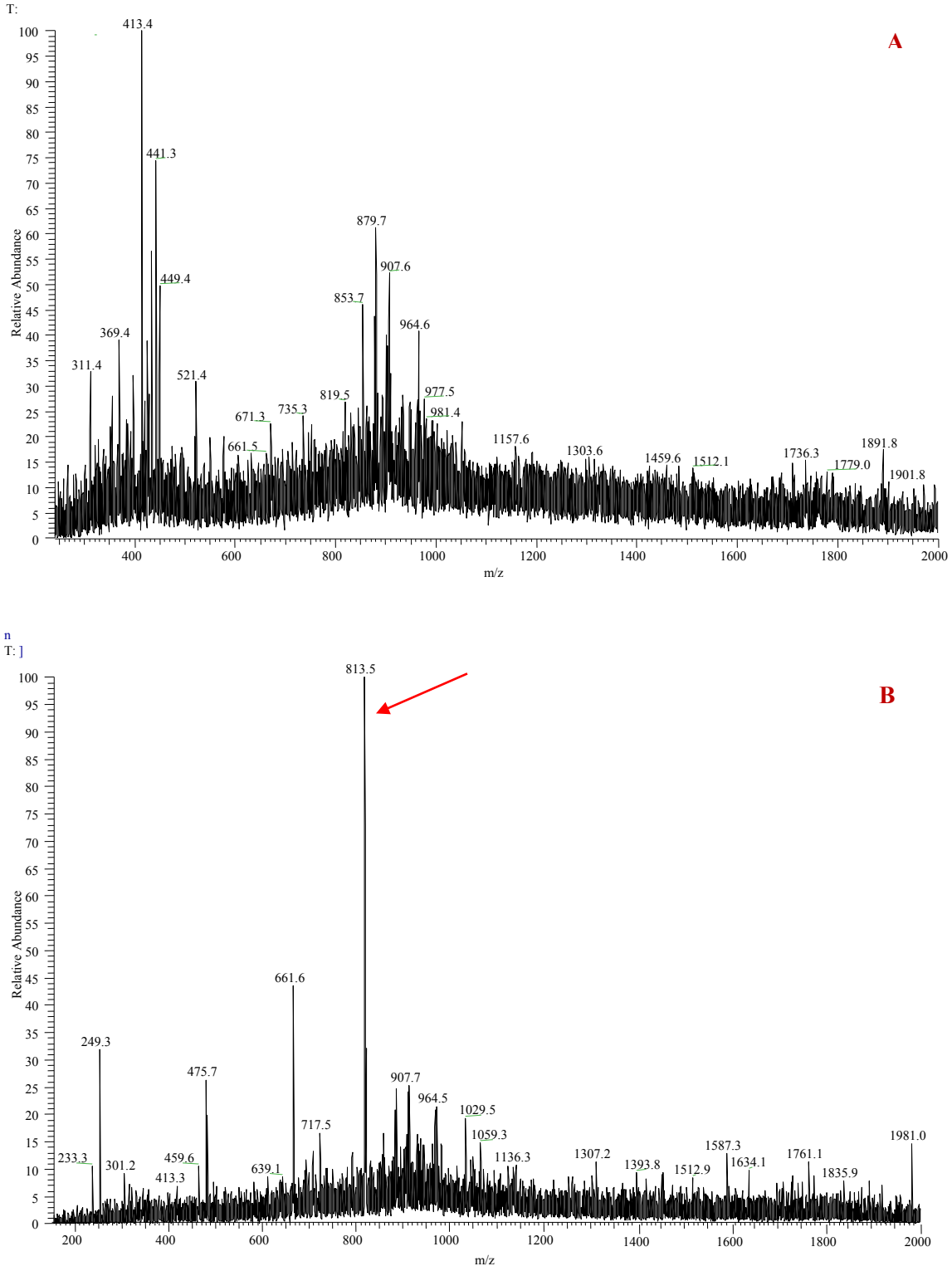


Fig 5.13. (A) Fraction 1 of the corn oil by RP-HPLC/ESI(+)/MS before spiking; (B) detected TAG-C12:0-C18Cl_2-C12:0 in F1 after spiking.

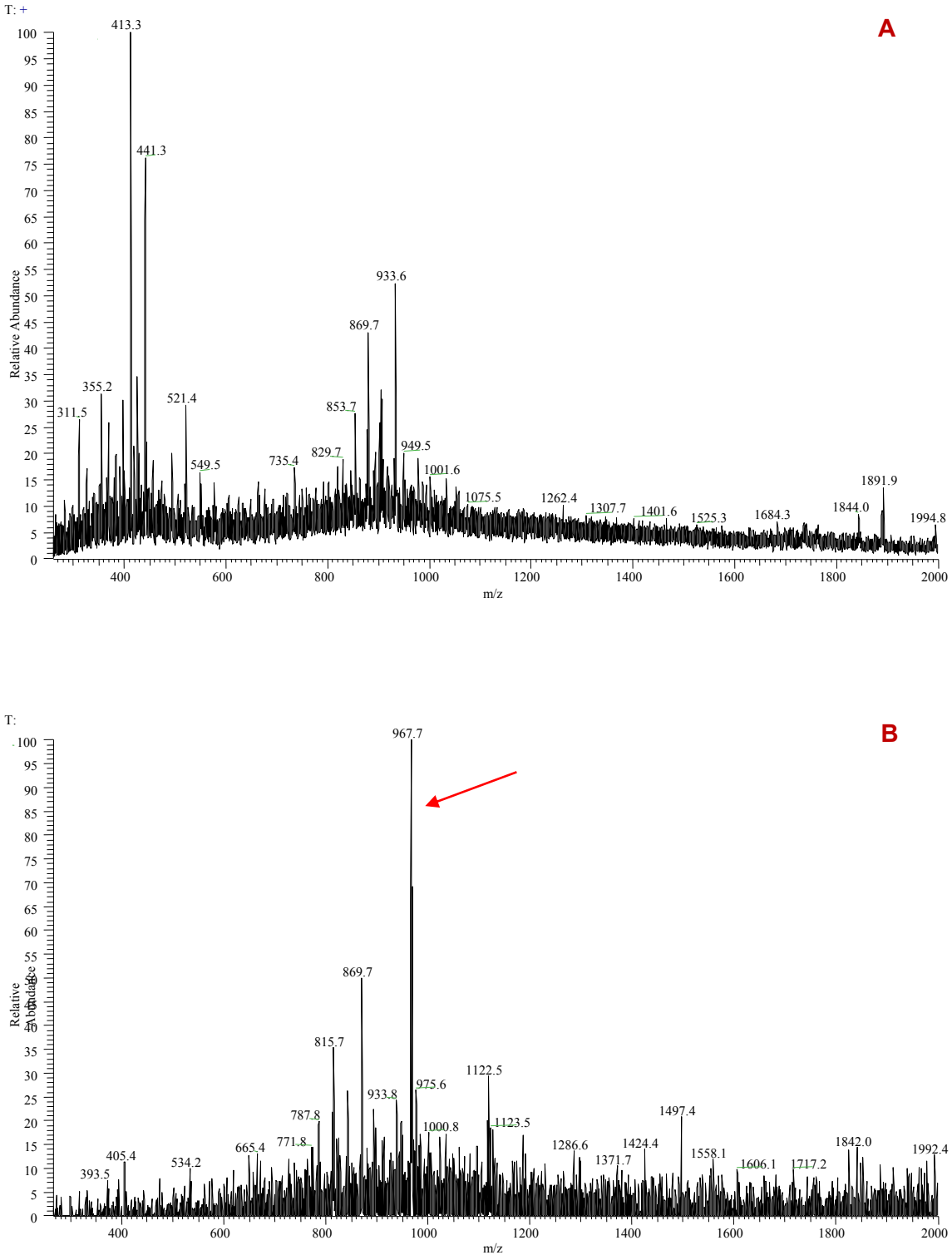


Fig 5.14. (A) Fraction 3 of the corn oil by RP-HPLC/ESI(+)/MS before spiking; (B) detected the TAG-C18Cl$_2$-C18Cl$_2$-C12:0 in F3 after spiking.

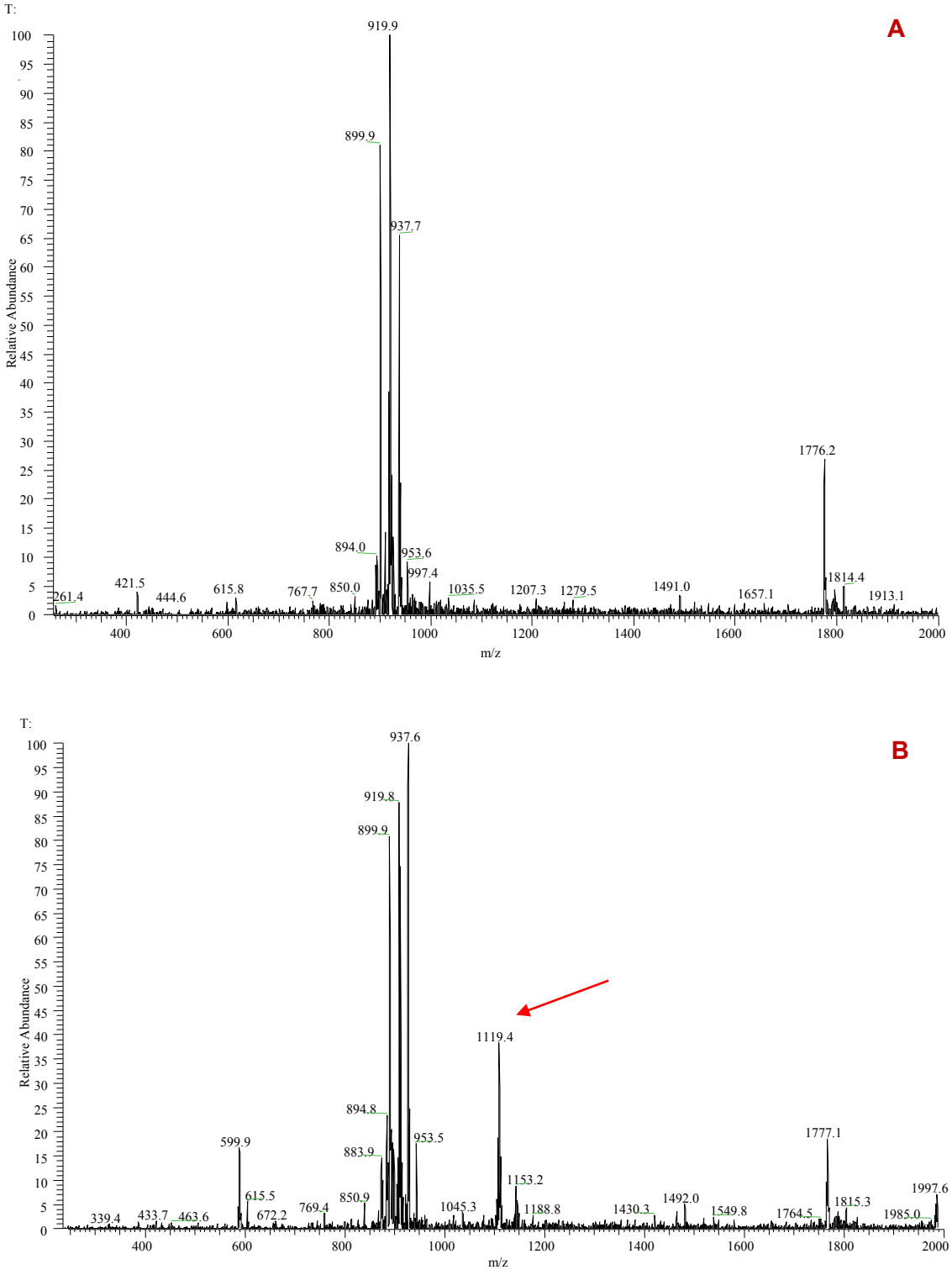


Fig 5.15. (A) Fraction 6 of the corn oil by RP-HPLC/ESI(+)/MS before spiking; (B) detected the TAG-C18Cl_6 in F6 after spiking.

In conclusion, the retention time of the Cl-TAG compounds increased by increasing the number of the chlorine atoms in them. Also, the number of the carbon atoms may have influenced the retention time since The TAG-C12-C18Cl_2-C12 (compound 13) with low number of carbon ($C_{45}H_{84}Cl_2O_6Na$) eluted early followed by the TAG-C12-C18Cl_2-C18Cl_2 (compound 14) containing more carbon ($C_{51}H_{94}Cl_4O_6Na$). Finally, the TAG-C18Cl_6 (compound 16) with highest carbon number $C_{57}H_{104}Cl_6O_6Na$ eluted last.

5.4. Studies of Cl-TAG compounds in farmed Atlantic salmon oil

5.4.1. Objectives

As mentioned previously, the overall objective of my thesis was to investigate the chlorinated compounds in Atlantic salmon (*Salmo salar*) obtained from a local retailer in Halifax, Nova Scotia.

The qualitative and quantitative methods developed in this thesis work and described above were applied to identify Cl-TAG compounds in salmon oil. Separate salmon skin and tissue samples were first homogenized and then the oil was extracted from them using a hexane-acetone sonication method. The analytical techniques used for the characterization of the TAG and Cl-TAG compounds in the salmon skin and tissue oils, namely the off-line 2D RP-HPLC/ESI(+)/MS and NMR, and of the fatty acid composition by GC/MS, were developed in this work and described above. The rest of the methods are given below.

5.4.2. Introduction

Recent research on salmon has largely focused on its importance as a source of omega-3 and omega-6 polyunsaturated fatty acids (PUFAs), mainly eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) which have beneficial effects on human health [Swanson et al., 2012]. These fatty acids play a preventive role in cardiovascular diseases and other health problems [Yagi *et al.*, 2015; Nishizaki *et al.*, 2014; Hara *et al.*, 2013; von Schacky, 2009; and Lavie *et al.*, 2009]. Several studies have reported that diet is one of the most important entry points for persistent organic pollutants into humans [Kiviranta *et al.* 2004; Darnerud *et al.* 2006]. The relatively high levels of persistent organic pollutants in farmed Atlantic salmon and the increased global consumption of farmed salmon have raised public health concerns [Hites *et al.*, 2004; Shaw *et al.*, 2008]. The rapid growth of aquaculture has increased the pressure on fish stocks market and led to the development of salmon feeds with less marine ingredients [Torstensen *et al.*, 2008]. The replacement of marine ingredients with vegetable

ingredients has been shown to reduce the levels of these traditional persistent organic pollutants in salmon feeds, but as a consequence introduced new plant-oil derived contaminants, such as polycyclic aromatic hydrocarbons (PAHs), pesticides, and other nonpolar contaminants that have not previously been associated with farming [Berntssen *et al.*, 2010; Søfteland *et al.*, 2014; Montory and Barra, 2006; Montory *et al.*, 2011].

In order to assess the effects of various pollutants on lipid composition and fatty acid profiles many laboratory experiments were performed [Kakela and Hyvarinen, 1999; Kudo *et al*. 2011]. The persistent organic pollutants are characterized by high lipophilicity and have the potential to bioaccumulate in aquatic organisms. Their presence in membrane lipids (Endo *et al.*, 2011) caused severe effects on the cell homeostasis such as fluidity [Zepik *et al.*, 2008; Tekpli *et al.*, 2011]. The higher membrane fluidity led to an increase in saturated fatty acids (SFA) and decrease in polyunsaturated fatty acid (PUFA) in the membrane lipids. This restructure of the membrane to maintain the fluidity was called a mechanism of homeoviscous adaptation [Sinensky, 1974; Henderson and Tocher, 1987; Tocher, 1995; Rilfors and Lindblom, 2002]. The reduction in the amount of n-3/n-6 PUFA has been reported in wild fish such as the Atlantic Cod (*Gadus morhua*) and haddock (*Melanogrammus aeglefinus*) collected near oil installation area [Meier *et al.*, 2007; Grøsvik *et al.*, 2009; Balk *et al.*, 2011; Bratberg *et al.*, 2013].

Larger salmon fed diets including plant oils, which are consequently rich in alpha linolenic acid (C18:3n-3, ALA) but low in EPA and DHA, cause significant reduction in total ω3, in particular DHA and EPA (Polvi and Ackman, 1992; Bell *et al*., 2003a; Bransden *et al*., 2003; Bell *et al*., 2004). Pan (2013) in his study found that the n-3 PUFA in rapeseed oil, linseed oil and soy oil are mostly build up by C18 PUFA like the two fatty acids α-linolenic (C18:3) acid and linoleic (C18:2) acid. By replacing fish oil with plant oil in fish feed, the result will be a reduction of the EPA and DHA content in the fish as well as a lowering of the nutritional quality of the fillet [Pan (2013) Asche *et al*., 2011]. These statements could explain the lower proportion of EPA and DHA in farmed Atlantic salmon compared to wild pink salmon [Megdal *et al.*, 2009]. Herein the characterization of TAGs in Atlantic salmon (*Salmo salar*) obtained from a local store was carried out using RP-HPLC/ESI(+)/MS and NMR; their fatty acids were investigated using GC-MS.

Although the RP-HPLC/Ag-HPLC (offline and online) and Ag-HPLC/ESI(+)/MS (off-line) methods were developed and applied successfully to detect TAGs in corn oil (Chapter 4), It could not be applied to the salmon oil samples because of the non-availability of the Ag-HPLC instrument.

5.4.3. Experimental

5.4.3.1. Sample Preparation

A whole farmed Atlantic salmon weighing about 5.74 kg (*Salmo salar*) was purchased from Sobeys (a local retailer in Halifax, NS). The bones were removed first followed by the skin from the muscle (tissue). Chunks of salmon tissue and skin samples were homogenized separately in a blender with dry ice resulting in fine granular powders. The powders were divided into small portions, transferred into pre-cleaned polyethylene bags, and placed in a freezer at -25°C to ensure that any liquid oil were frozen. Next day the frozen salmon tissue and skin samples were weighed and stored (-25°C) in clean glass vials ready for use in individual extraction experiments (Fig 5.16).

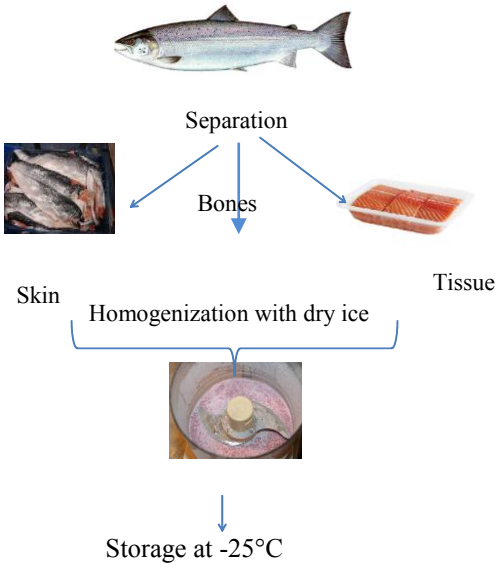


Fig 5.16. Prepartion of salmon skin and tissue samples.

5.4.3.2. Extraction method for salmon skin and tissue oils

The extraction of oils from salmon skin and tissue was carried out using an acetone:hexane (1:1) mixture which was found to be most suitable for such purposes among various solvent mixtures evaluated in our laboratory over the years [Bahroun, 2007]. Approximately 15 g of salmon tissue were placed in a square glass extraction container with the solvent mixture fitted with a Brinkman Polytron and sonically agitated for 5-7 min. The liquid mixture was transferred to a 500-mL Teflon separator funnel and

the organic layer (top layer) was pipetted out leaving the residue behind. These steps were repeated three times and the organic layers were combined, centrifuged for 5 min, transferred to a fresh separatory funnel, washed three times with DW to remove any inorganic halides, and dried using anhydrous Na_2SO_4.The excess solvent was evaporated under N_2 in a water bath to minimize any aerial oxidation that may occur (Fig 5.17).

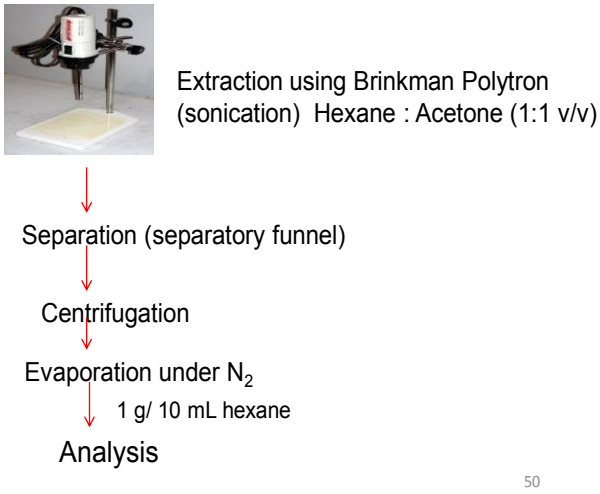


Fig 5.17. The extraction steps of the salmon skin and tissue samples.

5.4.3.3. Instruments

The extraction was done using (i) a Brinkman Polytron (sonication), Brinkman instruments Division, Sybran Canada Limited; (ii) a clinical centrifuge, International Equipments Co; (iii) a Vortex, VWR Analog Vortex Mixer; and (iv) a food processer,
The RP-HPLC and ESI(+)/MS apparatus were the same as described in the Experimental section (5.2.3).

As mentioned early in this thesis (section 3.3.3), two GC-MS instruments were used. A PerkinElmer® GC equipped with a PerkinElmer TurboMass™ Mass Spectrometer and a PerkinElmer® autosampler/injector was used for study the Cl-TAG compounds of the salmon skin and tissue oils. The separation of FAME of the salmon oils was done using a 5% diphenyl/95% dimethylsilane stationary phase (SPB®-5 Capillary GC Column, 30 m Lx0.25 mm I.D. and 0.25 µm film thickness). The chromatographic conditions were spilt injection (1.0 µL, 100:1), He carrier gas at 1 mL min^{-1} and a temperature gradient starting at 100°C (no delay), a ramp rate of 10°C min^{-1}

to 320 °C and a hold of 5 min. The injection port was at 260°C, the transfer line at 260°C, the ionization source at 230°C and the quadrupole at 150°C Mass spectral conditions were from 45 to 300 *m/z* at a rate of 1 scans per s^{-1}.

5.4.4. Results and discussion

This section contains details of the characterization of TAGs by RP-HPLC/ESI(+)/MS in salmon skin (Section 5.4.3.1) and tissue oils (Section 5.4.3.1.1), and unknown TAG compounds in skin and tissue oils (Section 5.4.3.1.2). The results of TAGs in salmon skin and tissue oils by NMR are described (Section 5.4.3.2). Finally, the identification fatty acids in salmon skin and tissue oils by GC-MS are also discussed (Section 5.4.3.3).

5.4.4.1. RP-HPLC/ESI(+)/MS

5.4.4.1.1. Identification of TAGs in salmon skin and tissue oils using an off-line 2D RP-HPLC/ ESI(+)/MS method

The 18 fractions collected from the salmon skin oil were analyzed using RP-HPLC with rapid gradient (Fig 5.18) and detected by ESI(+)/MS. A total of 87 TAGs were identified in salmon skin oil by the off-line RP-HPLC/ESI(+)/MS method developed in this thesis and the characterization results of every fraction are presented in Table App-1-Table-1.

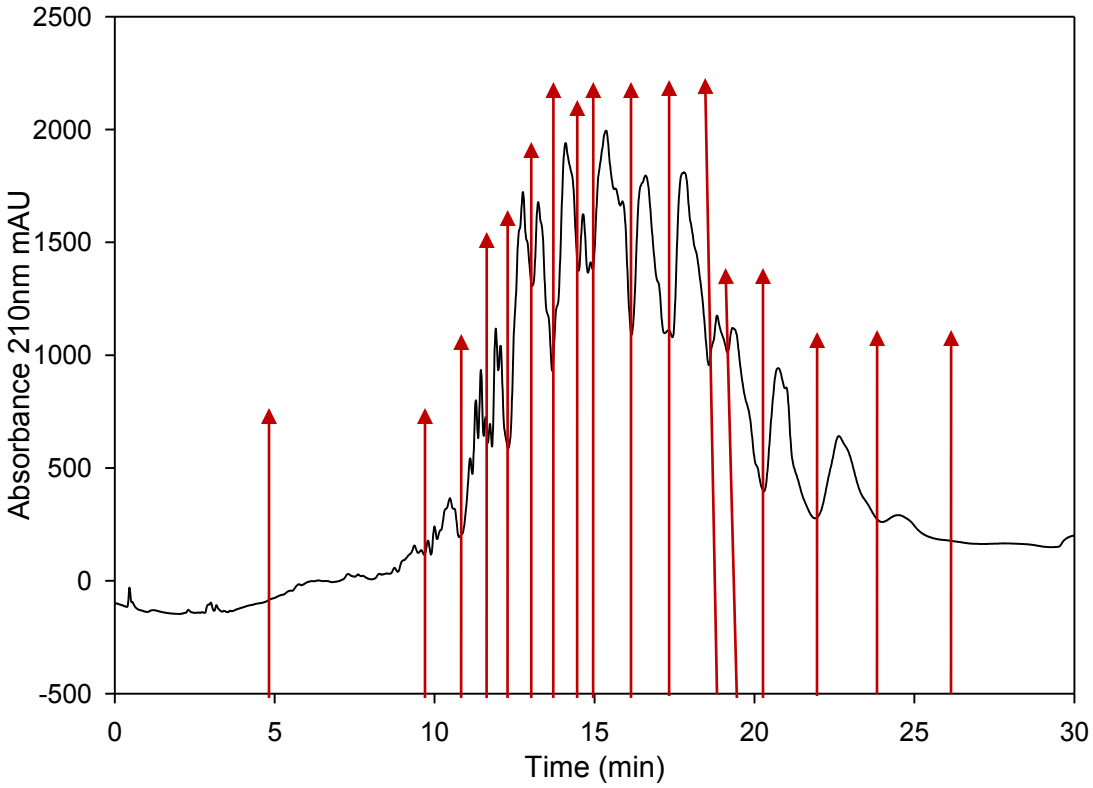


Fig 5.18. Eighteen fractions of salmon skin oil using RP-HPLC/ESI(+)/MS.

Sixteen fractions collected from the salmon tissue oil were analyzed using RP-HPLC with rapid gradient (Fig 5.19) and ESI(+)/MS. The tissue oil shows very complicated chromatograms containing large numbers of peak and up to 5 TAGs in each peak similar to that found in skin oil. The ESI(+)/MS and MS/MS were capable to detect about 80 TAGs in tissue oil their characterization results are presented in Table App-1-Table-2.

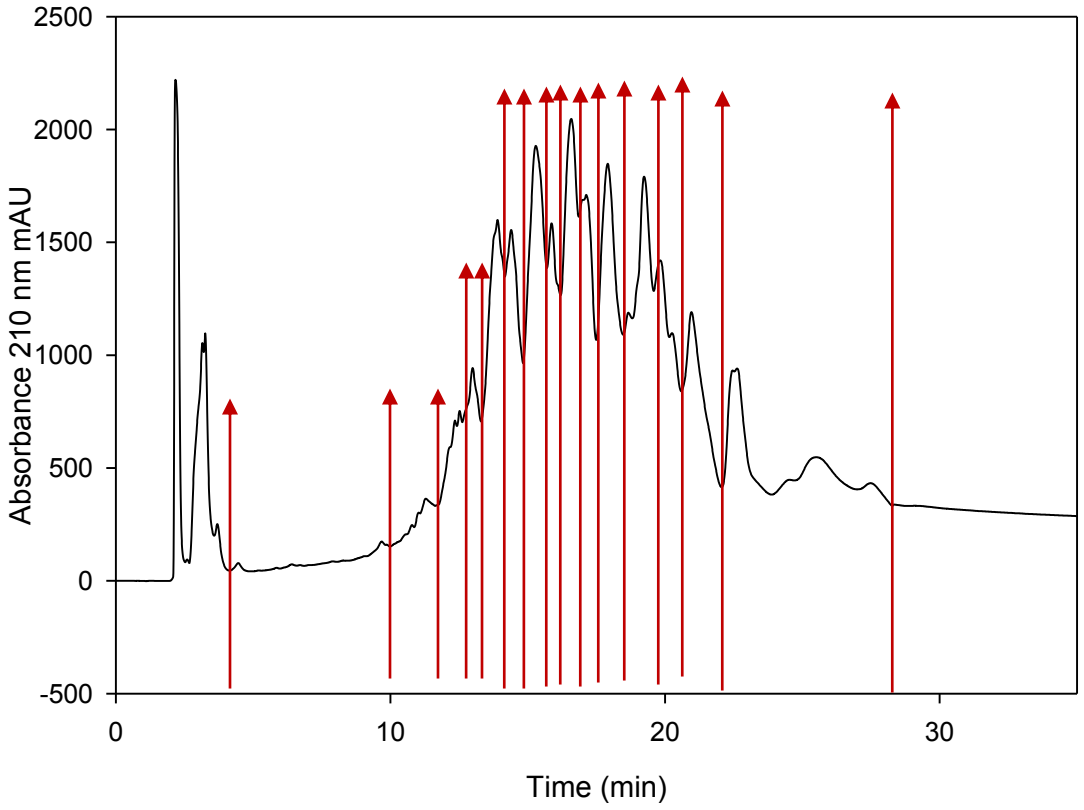


Fig 5.19. Sixteen fractions of salmon tissue oil using RP-HPLC/ESI(+)/MS.

Each collected peak in the chromatograms of the skin and tissue oils shows a large number of TAGs in the ESI(+)/MS spectrum compared to that in corn oil. For example, fraction 9 of salmon skin oil shows sodiated ions for 5 different TAGs (Fig.5.20A); however, a typical fraction 9 of the corn shows predominate one TAG (Fig.5.20B). The interpretation of ESI(+)/MS spectra can be complicated by the presence of up to 5 TAGs in one fraction. However, the $[M+Na]^+$ ions are more stable and hence the fragmentation in MS/MS and the relative abundances of the $[M+Na-RCOOH]^+$ ions are sufficient for the identification of the individual acyls with their sodiated fragment ions.

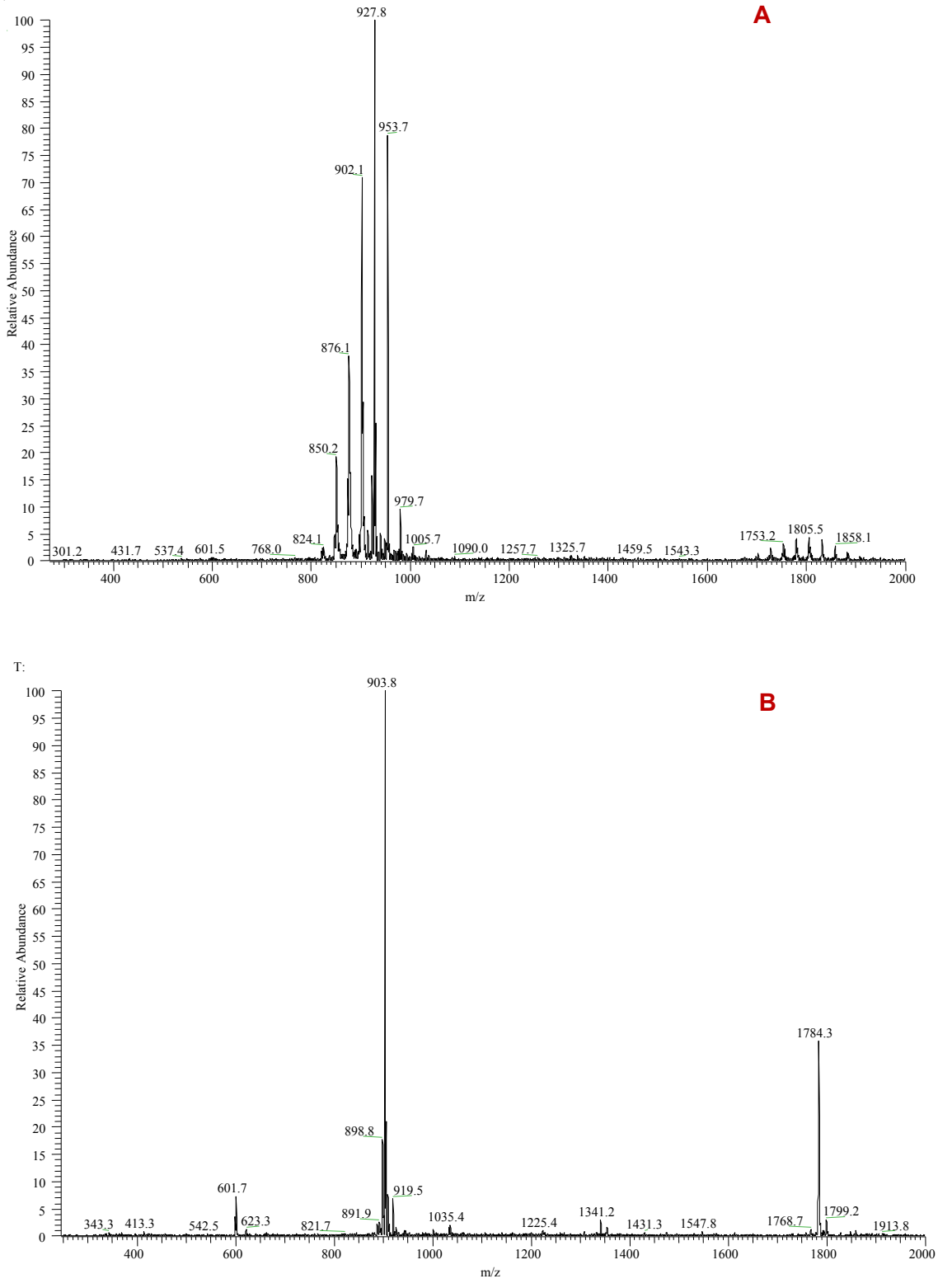


Fig 5.20. Comparison of the ESI(+) mass spectra of F9 (A)of salmon skin oil with F9(B) of corn oil.

Only one study on TAGs and their fatty acids in salmon (skin + tissue) oil has so far been reported in the literature. Tarvainen *et al*. [2015] investigated the effect of plant extracts produced by supercritical CO_2 technology and used UHPLC/ESI(+)/MS method to detect 180 TAGs and their fatty acids in wild Atlantic salmon (*salar*). In that study many odd-chain fatty acids (C19:0 and C17:0) were found. The authors claimed their source from algae, as by. These odd-chain fatty acids have not been detected by any other investigator including us in any fish species.

Most of the fractions obtained from the salmon skin and tissue oils contained TAG species as shown in Table 1 and 2 in Appendix 1. More than 5 sodiated ion $[M+Na]^+$ of TAGs were detected in one single fraction and up to two TAGs were identified in the same sodiated ions value. In many cases when different fraction numbers were found, TAG contained the same value of $[M+Na]^+$ by the ESI(+)/MS. However, upon CID fragmentations gave different fatty acid compositions. In other cases, known and unknown TAGs fragmented with consistent number of 148 u which include fatty acids and their sodiated ions. The examples of all of the above cases are given below in more detail.

For example, the ESI(+) mass spectrum of fraction 10 (Fig 5.21) showed 4 major ions TAGs at *m/z* 878.2, 904.1, 927.9, and 953.7 and TAG ions of lower abandance at *m/z* 848.1, 901.7, 947.5, and 981.7.

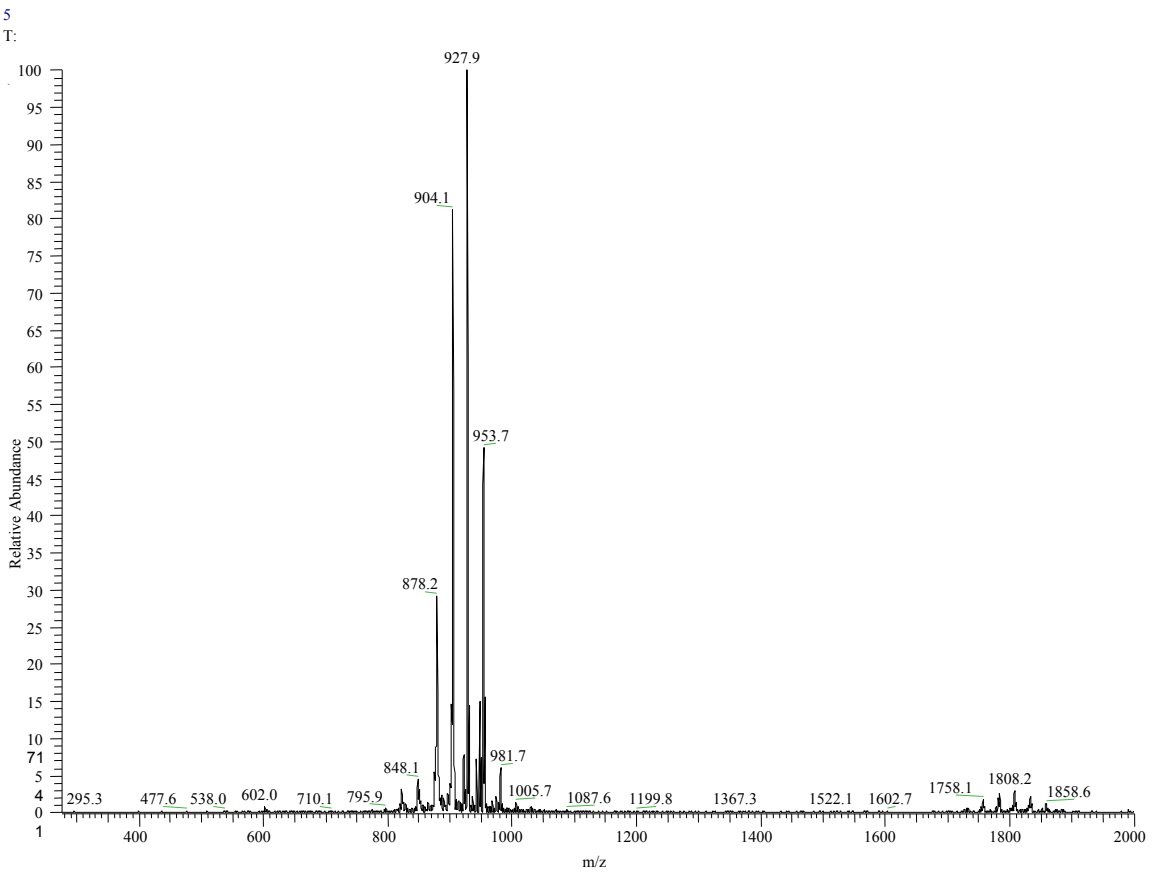


Fig 5.21. The ESI(+) mass spectrum of F10 of salmon tissue oil.

The CID spectra of the ions found in fraction 10 of salmon tissue oil showed prominent neutral losses of fatty acids accompanied by minor losses of the corresponding sodium salts. This fragmentation behavior is characteristic of sodiated TAGs (Herrera *et al*., 2010; Hsu and Turk, 2010; Segall *et al*., 2004). The assignments of the acyl groups in the TAG were based on the mass different between the precursor $[M+Na]^+$ ion and the product ions $[M+Na\text{-}RCOOH]^+$ and $[M+Na\text{-}RCOO\text{-}Na]^+$. For example, CID of the $[M+Na]^+$ ion at *m/z* 927.9 (Fig 5.22) gave the product $[M+Na\text{- }RCOOH]^+$ and $[M+Na\text{-}RCOO\text{-}Na]^+$ ions at *m/z* 645.3 (623.3) and 625.4 (603.5) ; the mass different of 282 and 302 corresponding to C18:1 and C20:5. The great intensity of the product ion at *m/z* 645.3 indicated two C18:1 acyl chains and the sum of two and one C20:5 acyl groups, a glycerol back bone, and sodium ion equalled 927, the mass of the sodiated TAG subjected to CID. Thus, the TAG was identified as C18:1/C20:5/C18:1. The C20:5 acyl group likely corresponded to the well known omega-3 fatty acid, EPA.

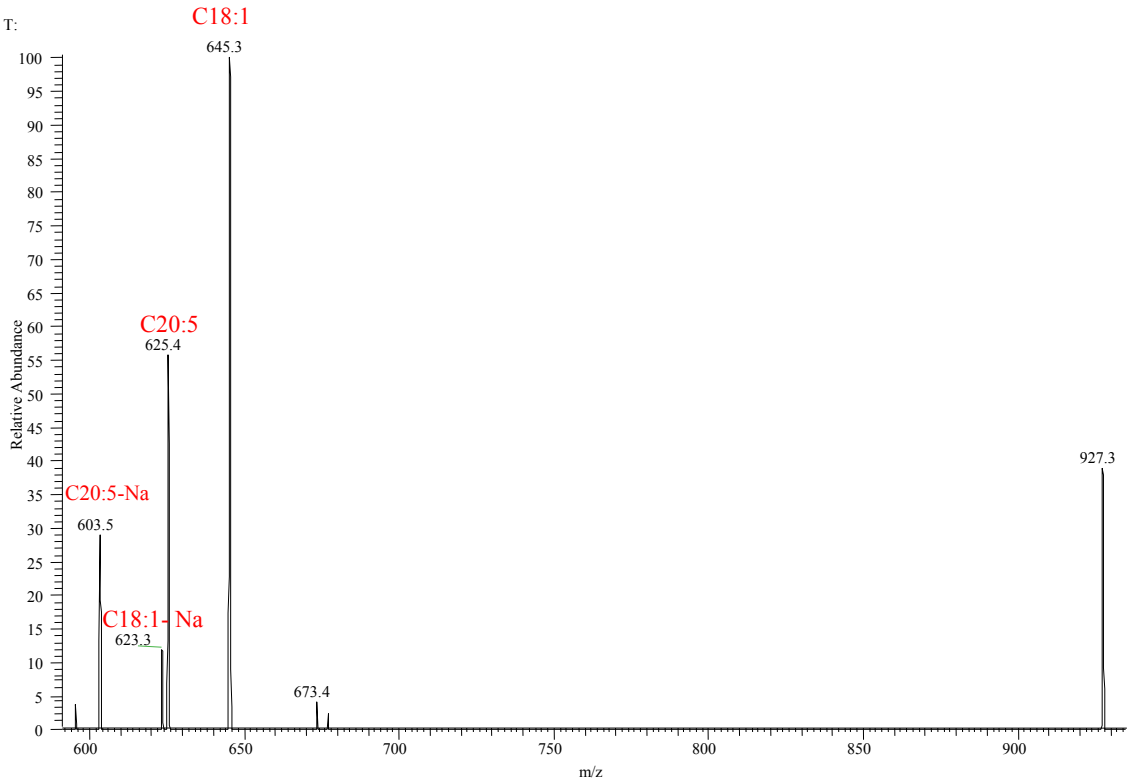


Fig 5.22. The CID spectrum of F10 of salmon tissue oil at *m/z* 927.9.

Another omega-3 fatty acid C22:6 (which is well known as DHA) was found in fraction 10 of salmon tissue oil at *m/z* 953.7 and the MS/MS spectrum (Fig 5.23) gave the fatty acid fragment ions $[M+Na-RCOOH]^+$ (and their minor ions $[M+Na-RCOONa]^+$) as follows: C18:1 at *m/z* 671.3 (649.5), C22:6 at *m/z* 625.3 (603.4) and the TAG is identified as TAG-C18:1/C22:6/C18:1.

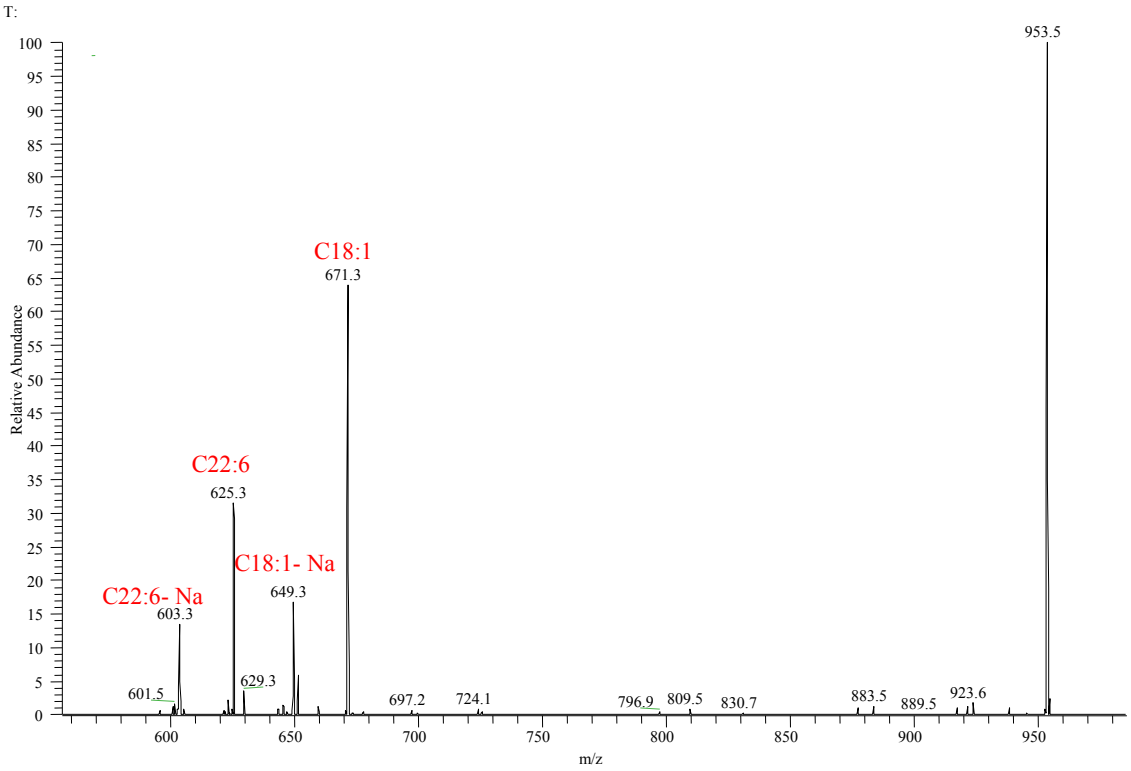


Fig 5.23. The CID spectrum of F10 of salmon tissue oil at *m/z* 953.5.

Both omega-3 fatty acids (EPA and DHA) were found in one TAG-C16:0/C22:6/C20:5 in F10 of salmon tissue oil at small at *m/z* 947.5 and the MS/MS spectrum (Fig 5.24) gave the following fatty acid fragment ions (and their sodiated ions): C16:0 at *m/z* 691.1 (699.5), C20:5 at *m/z* 645.4 (623.2), and C22:6 at *m/z* 619.2 (597.3).

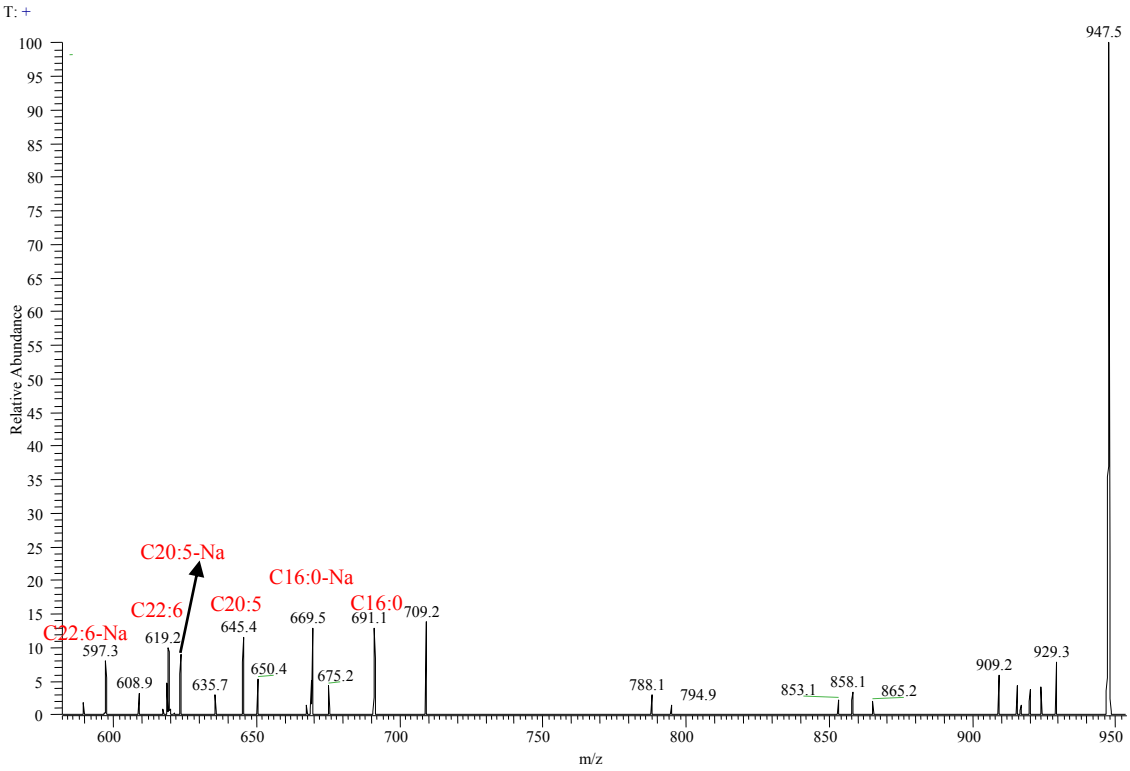
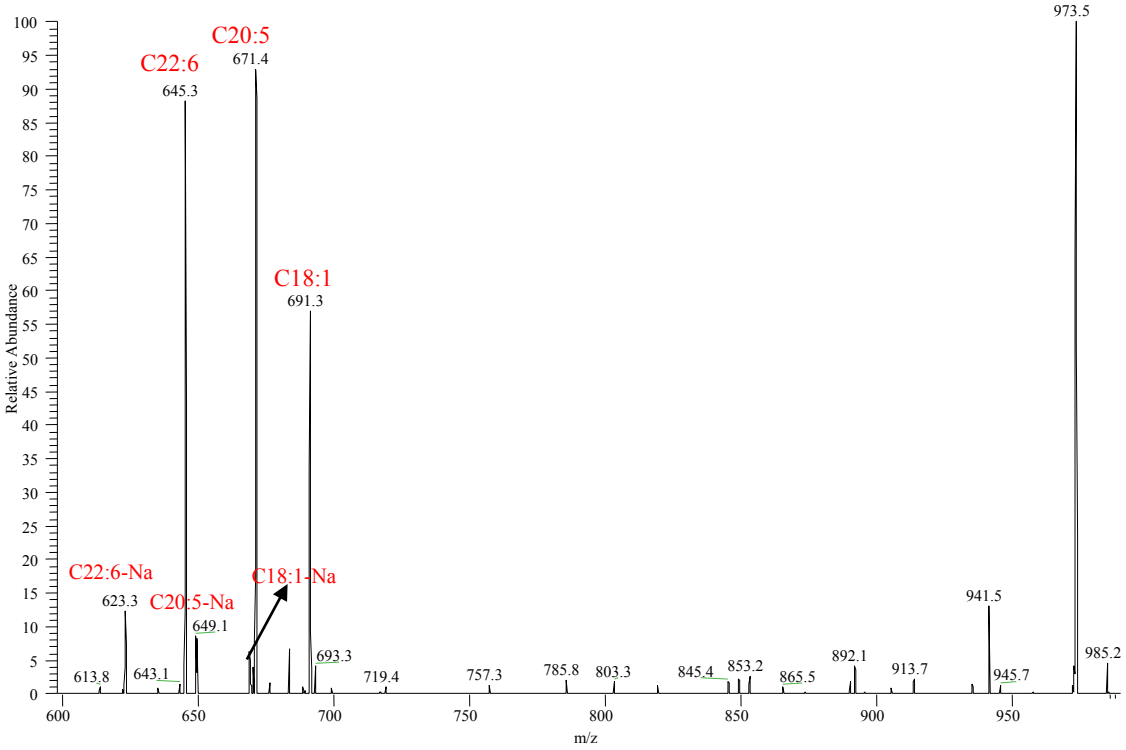


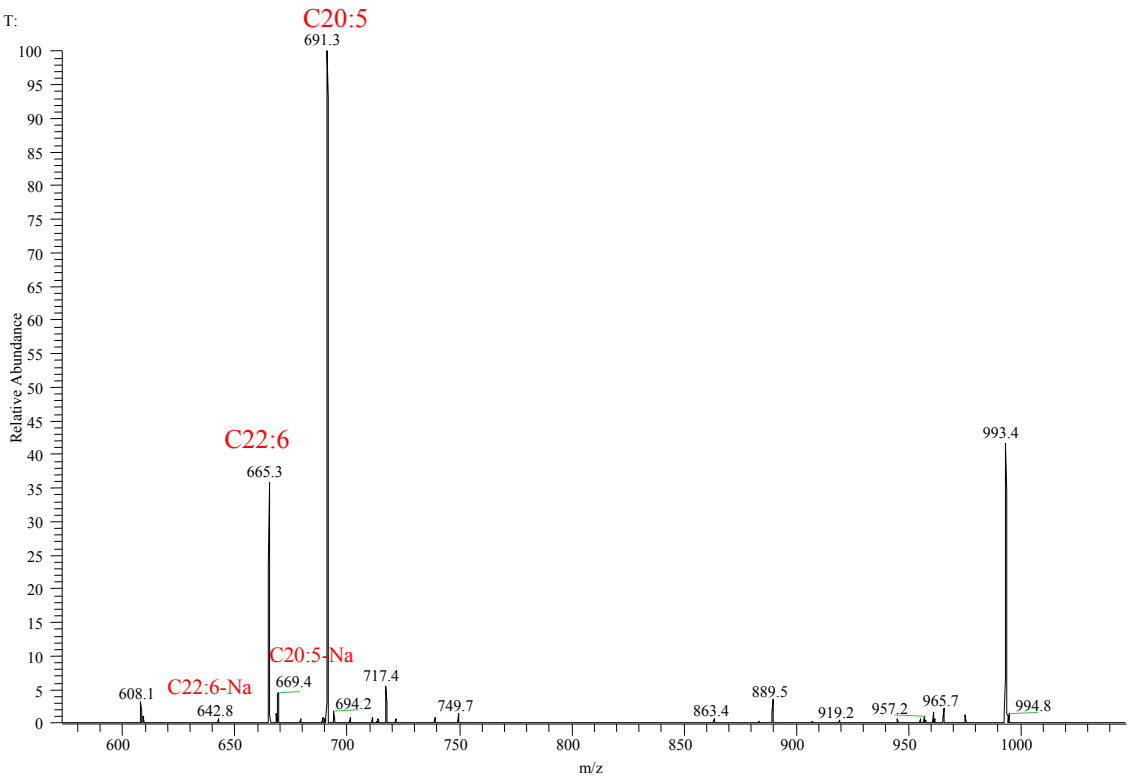
Fig 5.24. The CID spectrum of F10 of salmon tissue oil at *m/z* 947.5.

In all of the identified TAGs of salmon skin and tissue oils, omega-3 (EPA and DHA) fatty acids were found in the same TAG components. In F3 of salmon skin and tissue oils at *m/z* 943.7 was identified as TAGC20:5/C20:5/C18:3. In F4 of salmon skin and tissue oils at *m/z* 971.7 was identified as TAG-C18:2/C22:6/C20:5 and at *m/z* 997.7 as TAG-C18:2/C22:6/C22:6. Also, in F6 of salmon skin and tissue oils at *m/z* 999.6 (TAG-C22:6/C22:6/C18:1), at *m/z* 949.7 (TAG-C22:6/C22:6/C18:2), and at *m/z* 973.7 (Fig 5.25) were identified as TAG-C18:1/C22:6/C20:5. The MS/MS fragmentations are shown in App-1-Table-1 and 2.



5.25. The CID spectrum of F6 of salmon skin oil at *m/z* 973.5.

Among all the fractions collected from the RP-HPLC chromatograms of skin and tissue oils and subjected to ESI(+)/MS, only two TAGs containing just the omega-3 fatty acids were detected in F3 of salmon tissue oil at *m/z* 967.7 and 993.8. MS/MS of the ion at *m/z* 967.7 led to the loss of a fatty acid (and its sodiated ion) at *m/z* 667.3 (645.4) which correspond to C20:5 and the TAG identified as TAG-3C20:5. The MS/MS spectrum of *m/z* 993.8 (Fig 5.26) gave the loss of both omega-3 fatty acids with their sodiated ions as follows: at *m/z* 691.4 (669.0) of C20:5 and 665.3 (642.8) of C22:6. The TAG at *m/z* 993.8 was found to be TAG-C20:5/C22:6/C20:5. Overall the TAGs containing up to two omega-3 fatty acids were detected in the early eluted fractions (F3-F6) of salmon skin and tissue oils which agrees with the RP-HPLC separation mechanism of TAG according to their partition number (PN= CN-2DB). The higher the number of the double bonds the lower the partition number. The PNs of all of the TAGs of salmon skin and tissue oils are listed in Appendix-1-Table-1 and 2.



5.26. The CID spectrum of F3 of salmon tissue oil at *m/z* 993.8.

Several cases of TAGs yielding sodiated ions $[M+Na]^+$ with the same *m/z* values were found in different fractions. When MS/MS was applied to the ions from different fractions, different fatty acid components were obtained. In other words, they were completely different TAGs. For example in F14 of salmon tissue oil, an ion at *m/z* 853.8 gave the loss of the following fatty acids (sodium salt): loss of C14:0 at *m/z* 625.3 (603.3) and loss of C18:1 at *m/z* 591.2 (569.3) and the TAG is TAG-2C18:1/C14:0. On the other hand, the sodiated ion $[M+Na]^+$ at *m/z* 853.9 in F15 gave the loss of C16:1 at *m/z* 599.4 (577.3); loss of C16:0 at *m/z* 597.3 (575.3); and loss of C8:1 at *m/z* 571.4 (549.5). The identified TAG is TAG-C16:0/C18:1/C16:1. More of these examples are presented in Appendix -1-Table-1 and 2.

In other cases, when the sodiated ion $[M+Na]^+$ gave up to three fatty acid compositions upon CID fragmentations, most likely two TAGs with the same PN coeluted. For example, F13 of the salmon tissue oil gave 6 TAGs and two of them showed the combination of more than one TAGs and their MS/MS spectra gave up to three fatty acids. The TAG ion of F13 at *m/z* 851.8 showed a loss of 5 different fatty

acids (C14:0, C16:1, C16:0, C18:2, and C18:1) which were identified as TAG-C18:1/C18:2/C14:0 and TAG-C16:0/C16:1/C18:2 with the same CN and DB (CN:DB 53:3) and PN of 50, respectively. Also, in the same F13 the sodiated ion $[M+Na]^+$ at *m/z* 933.7 fragmented by CID gave 6 fatty acids as follows: C16:1; C18:2; C18:1; C20:1; C20:2; and C22:1, and the TAGs were identification as TAG-2C18:2/C20:2, TAG-C18:1/C20:1/C18:2, TAG-C18:2/C16:1/C22:1, and TAG-C16:1/C20:1/C20:2 with the same(CN:DB 59:4) and PN of 55, respectively. More such examples are presented in App-1-Table 1 and 2.

However, in the 88 identified TAGs in salmon skin oil and 81 TAGs in salmon tissue oil some TAGs (and unknown TAGs, Table 5.3) showed losses of 74 u and 148 u upon CID. These TAGs were detected in fractions F7 at *m/z* 871.7 (Fig 5.27), F8 at *m/z* 873.5 (Fig 5.29), and F9 at *m/z* 875.5 (App-1-Fig 18A, B) of salmon skin oil. For example, in F7 (Fig 5.27) of salmon skin oil, an ion at *m/z* 871.7 (Fig 5.28) gave an unidentified loss of 74 u upon CID yielding an ion at *m/z* 797.0 as major peak (which probably presented the loss of $C_3H_6O_2$ or $C_4H_{10}O$), and a loss of 32 u at *m/z* 839.1 as small peak which is probably the loss of two oxygen atoms. The fatty acid fragment ion $[M+Na-RCOOH]^+$ and sodium salt ion $[M+Na-RCOO-Na]^+$ were observed upon CID and the MS/MS spectrum of *m/z* 871.7 gave the loss corresponded to several doubly unsaturated C16 fatty acid (and their sodium salts) as follows: C16:0 at *m/z* 615.4 (953.2), C16:1 at *m/z* 617.3 (595.3), and C16:4 *m/z* at 623.2 (601.3). Also, the loss of C20:5 was detected at *m/z* 569.4 (547.2). In addition, small peaks were observed at *m/z* 606.2 and 517.4, which could be the oxidation of C16:4+OH and C20:5+2H_2O+O. The possible identification of the TAG and/or the oxidized TAG compound of F7 at *m/z* 871.7 was TAG-C16:1/C16:4/C16:1+3H_2O and TAG-C16:1/C16:1/C20:5.

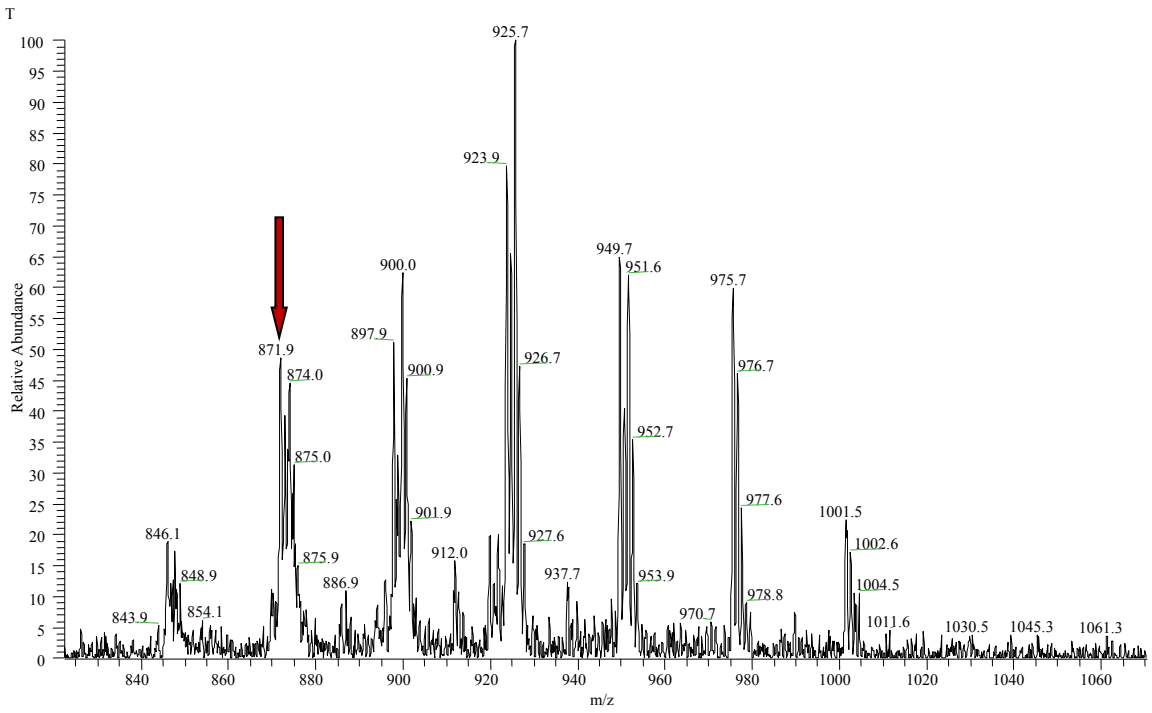


Fig 5.27. ESI(+) mass spectrum of F7 of salmon skin.

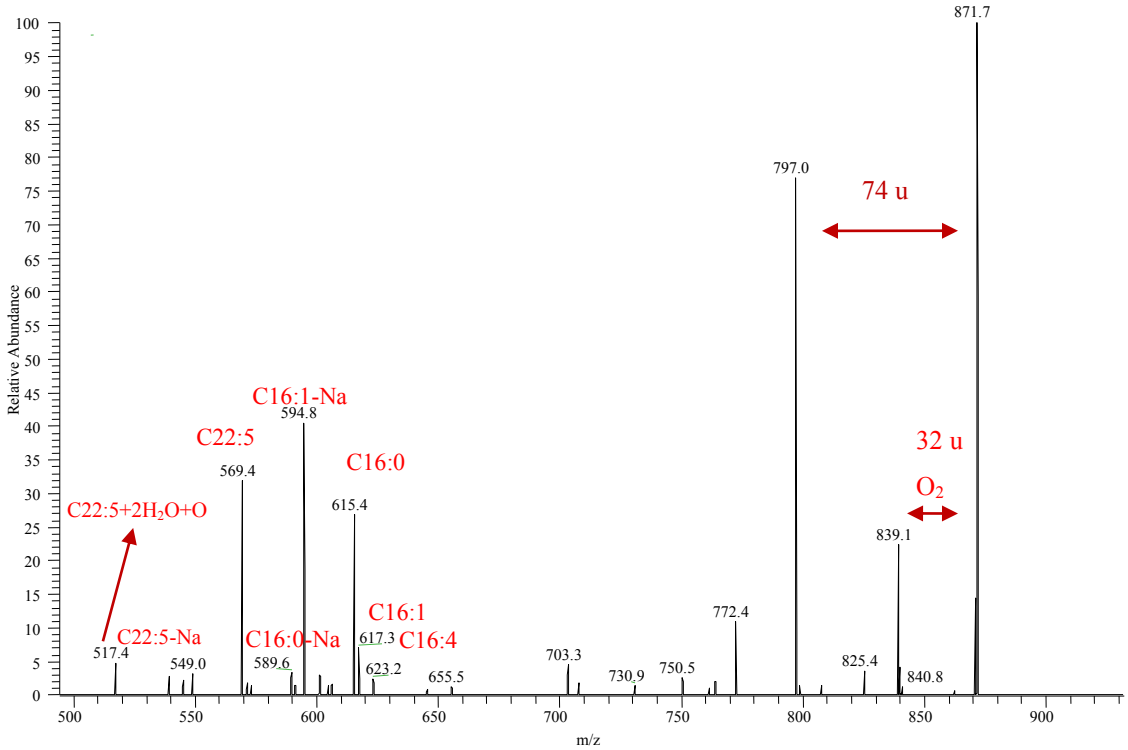


Fig 5.28. The CID spectrum of F7 of salmon skin oil at *m/z* 871.

Another example, F8 (Fig 5.29) gave a TAG ion at *m/z* 873.5 that showed a loss of 148 u upon CID yielding an ion at *m/z* 725.5 ion, and a loss of 18 u at *m/z* 855.5 as a small peak which is most likely H_2O and 32 u at *m/z* 841.5 which is probably the loss of two oxygen atoms, other unidentified loss at *m/z* 799.3 of 74 u (Fig 5.30A). The expected neutral losses of acyl group as fatty acids and their sodium salts showed up early in the spectrum followed by the oxidized species (Fig 5.30B). The MS/MS spectrum at *m/z* 873.5 gave the fatty acid fragment ion $[M+Na-RCOOH]^+$ and sodium salt ion $[M+Na-RCOO-Na]^+$. This loss corresponded to C14:0 at *m/z* 645.4 (622.3). An interesting observation was that the loss corresponded to several doubly unsaturated C18 fatty acid (and their sodium salts) as follows: C18:1 at *m/z* 591.3 (569.3), C18:2 at *m/z* 593.3 (571.0) and C18:3 at *m/z* 595.9 (573.4). Also the losses of several doubly unsaturated C16 were noted, namely C16:1 at *m/z* 619.3 (597.3), C16:3 at *m/z* 623.4 (601.3), and C16:4 at *m/z* 625.4 (603.3). In addition, small peaks were observed at *m/z* 581.5 which probably corresponded to C16:0+2H_2O and at *m/z* 563.0 and 561.3 were consistent with the loss of C18:3+O_2 and C18:2+O_2. These oxidation fragments of the fatty acids indicated oxidation of TAG compound with the incorporation of two oxygen atoms. Therefore, the possible composition of this unknown TAG compound at *m/z* 873.5 is TAG-C14/C18:2/C16:1+O_2+H_2O or TAG-C18:1/C16:1/C14:0+O_3.

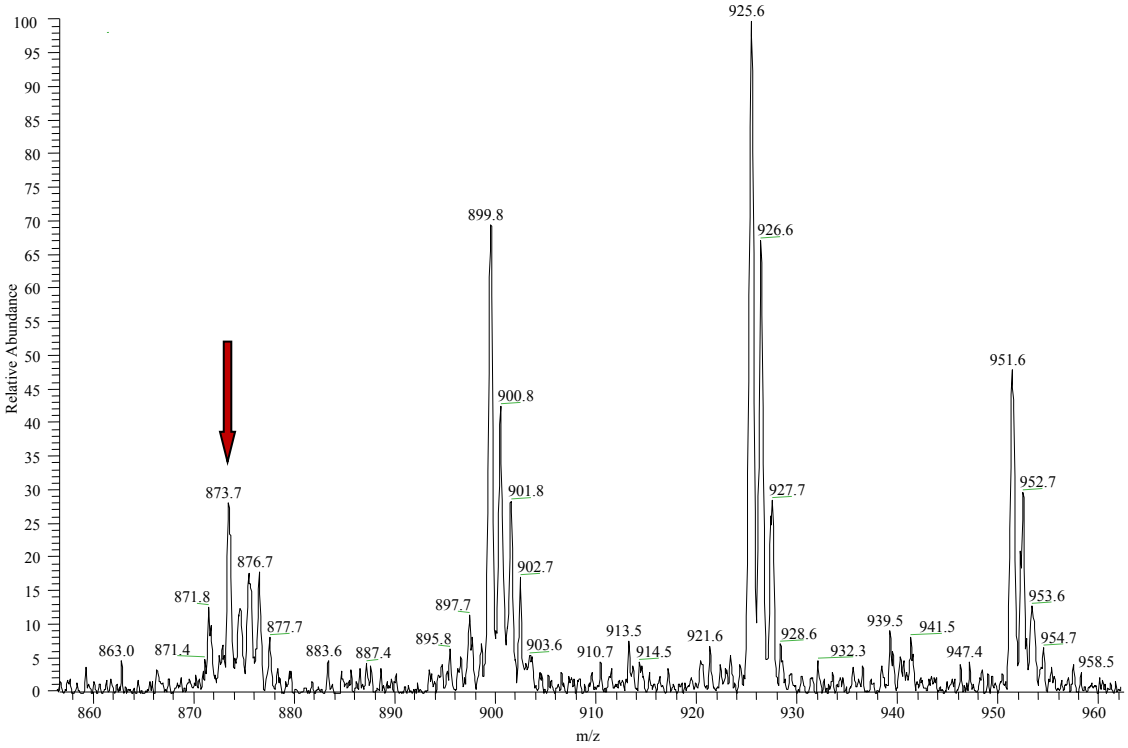


Fig 5.29. ESI(+) mass spectrum of F8 of salmon skin.

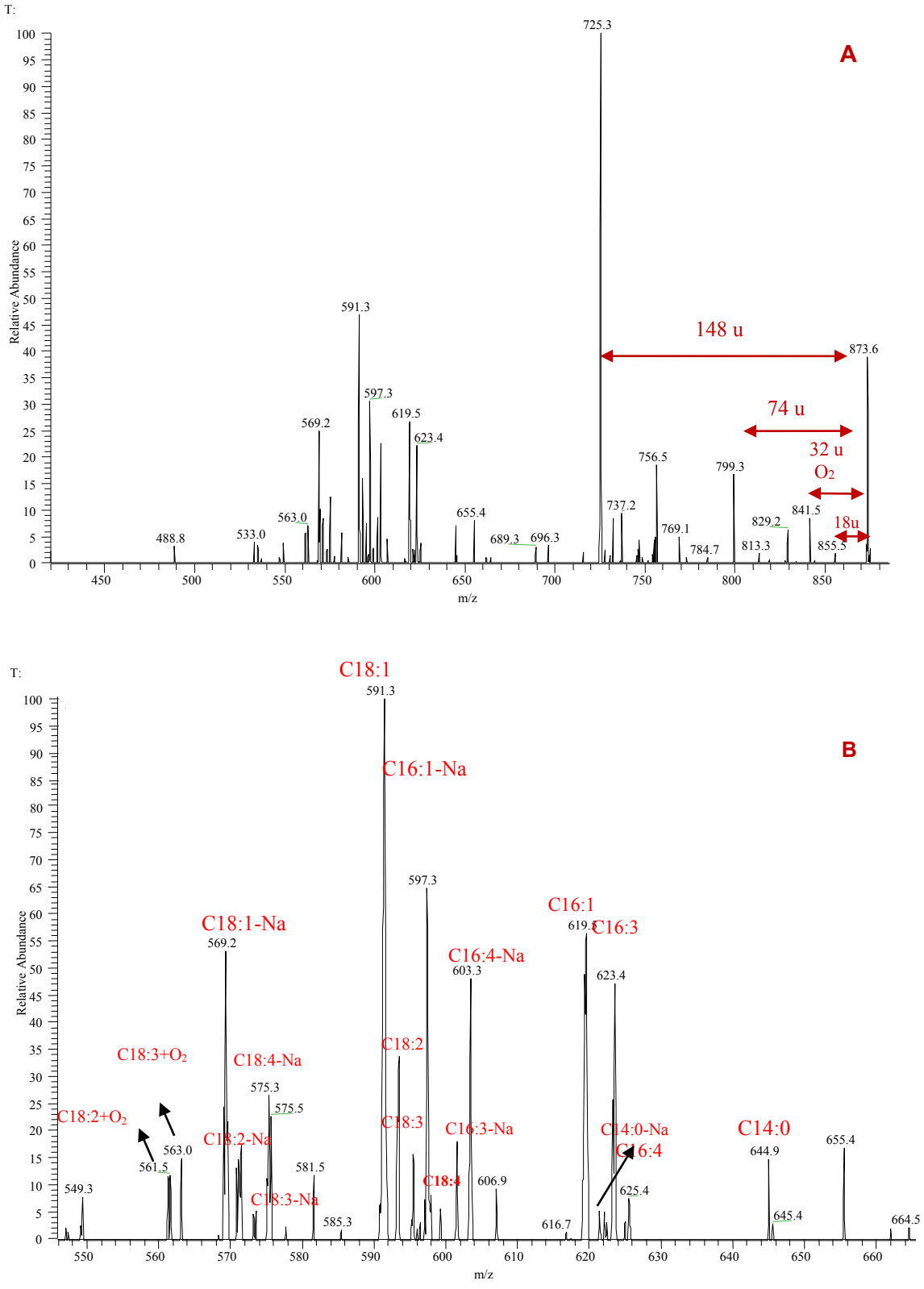


Fig 5.30. (A)The CID spectrum of F8 of of salmon skin at *m/z* 873.5; (B) expanded range (*m/z* 560-665).

Table 5.3. Peak identification of the possible oxidized TAG compounds in salmon skin oil.

F#	$[M+Na]^+$	MS/MS $[M+Na-RCOOH]^+$ $[M+Na-RCOO-Na]^+$	Identification ClTAG
7 Skin	871.7	835.3/ O_2 797.0/74 u ($2H_2O+O+Na$) 623.2/C16:4 601.3/C16:4-Na 617.3/C16:1 594.8/C16:1-Na 615.4/C16:0 593.2/C16:0-Na 606.2/C16:4+HO 569.4/C20:5 547.2/C20:5-Na 517.0/C20:5+$2H_2O$+O	TAG- C16:1/C16:4/C16:1+$3H_2O$ TAG-C16:1/C16:1/C20:5
F8 Skin	873.5	799.3/ 74 u ($2H_2O+O+Na$) 725.5/ 148 u 644.9/ C14:0 622.2/ C14-Na 625.4/ C16:4 603.3/ C16:4-Na 623.4/ C16:3 601.2/ C16:3-Na 619.3/ C16:1 597.3/ C16:1-Na 595.2/ C18:3 573.1/ C18:3-Na 593.2/ C18:2 571.1/ C18:2-Na 591.3/ C18:1 569.5/ C18:1-Na 563.0/ C18:3+O_2 561.3/ C18:2+O_2	TAG-C14:0/C16:1/ C18:2+O_2+H_2O TAG-C18:1/C14:0/C16:1+ O_3 TAG- C16:0/C16:0/C16:3+H_2O+ O_2 TAG- C18:2/C18:3/C16:1
9 Skin	875.5	801.3/74 u 727.3/148 u 647.5/C14:0 625.3/C14:0-Na 623.5/C16:2 601.2/C16:2-Na 621.5/C16:1 599.2/C16:1-Na 597.5/C18:3 575.3/C18:3-Na 595.3/C18:2 573.0/C18:2-Na 593.3/C18:1 570.9/C18:1-Na 562.4/C16:1+$2H_2O$+Na 550.8/C16:2+$2H_2O$+O+Na 532.9/C16:1+$2H_2O$+O_2+Na 521.9/C18:2+$4H_2O$ 505.3/C18:2+$2H_2O$+O_2+Na	TAG- C14:0/C16:2/C18:2+$3H_2O$ TAG-C18:1/C16:1/C14:0+ +H_2O+O_2 TAG- C18:1/C18:3/C16:1

In summary, 88 TAG species were identified in salmon skin and 81 TAGs in tissue oils. The TAGs in tissue oil contained more unsaturated fatty acids than the TAGs of skin oil. The elution order of the TAGs in both types of salmon oil was according to their PNs (TAGs with low PNs eluted early) which agreed with the RP-HPLC mechanism. The overlapping of up to 5 TAGs in the same fraction was difficult to identify, especially when they have the same sodiated $[M+Na]^+$ ions. Whether the oxidized TAG compounds are present in salmon oil or not is still unconfirmed. Despite all of the Cl-TAG standards that were synthesized and characterized by ESI(+)/MS in this thesis work, the ESI(+)/MS has not yet been applied to detection of Cl-TAG compounds in a real sample. To the best of our knowledge no Cl-TAG compounds in salmon oil have been reported in the literature.

5.4.4.2. NMR

Even though ^{13}C NMR is a less sensitive technique compared to GC, the analysis of fish oil by NMR techniques is less labor intensive [Sacchi *et al*., 1993a; Gunstone and Seth, 1994; Aursand *et al.,* 1995; Vlahov *et al*., 2006] and does not require chemical or enzymatic esterification/transesterification. However, small and broad NMR signals could arise due to oxidation of some lipids that overlapped with the signals used for the determination of EPA and DHA [Tyl *et al*., 2008; Guillen *et al*., 2008]. NMR can be advantageously used to analyze intact lipids non-destructively and also to provide information about the type of lipid [Siddiquie *et al*., 2003; Falch *et al*., 2005]. ^{13}C NMR can give information on the class of fatty acid (saturated and unsaturated) and regiospecific distribution in TAGs which are important characteristics for distinguishing oils of different fish species. Research by Gunstone (1990, 1991) and Aursand and Grasdalen (1992) first established the relationship between omega-3 fatty acid structure and chemical shifts.

The ^1H NMR spectra of the salmon skin oil (Fig. 5.31) and tissue oil (Fig 5.32) did not show any evidence of phospholipids, and the spectra were dominated by what appeared to be signals from triacylglycerols. The ^1H spectra clearly showed signals associated with the carbons of the glycerol which could be found downfield at 5.27-5.37, ppm corresponding to the proton on the C-2 and a pattern of eight lines at 4.06, 4.25 associated with the protons on the C-1 and C-3. The lack of resolution in some signals

such as the one at 1.23 ppm indicated that the sample was a mixture of long chain fatty acids. An additional triplet caused by a terminal methyl on chain could be seen at 0.83 ppm. From the ^{1}H NMR spectra it was possible to identify the omag-3 fatty acids C22:6 (known as DHA) and C20:5 (known as EPA) by the unique signals [Igarashia *el al*., 2000] with chemical shifts of 2.362-32 and 0.95-98 ppm (triplet), respectively, as shown in Figs 5.33 and 5.34. These signals are well separated from the signals of methylene protons of non-DHA fatty acids. The DHA signals in ^{1}H NMR spectrum of the salmon skin oil were smaller than those in tissue oil which agreed with the GC-MS results.

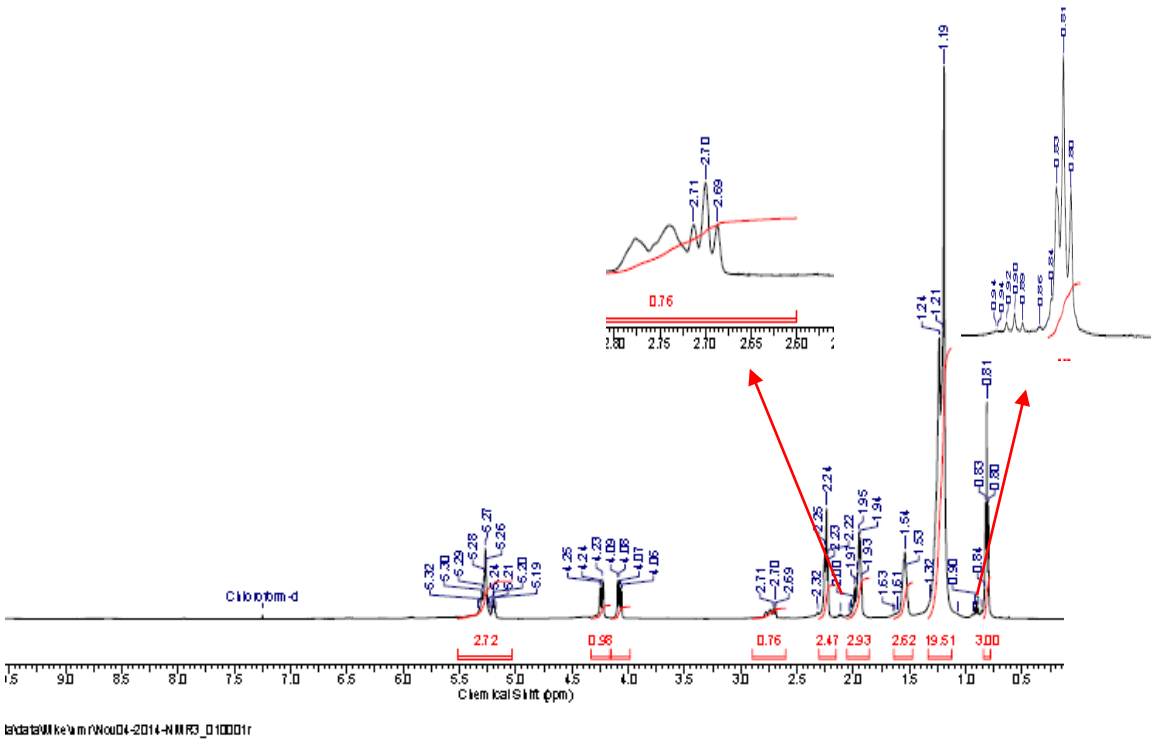


Fig 5.31. ^{1}H NMR spectrum of salmon skin oil

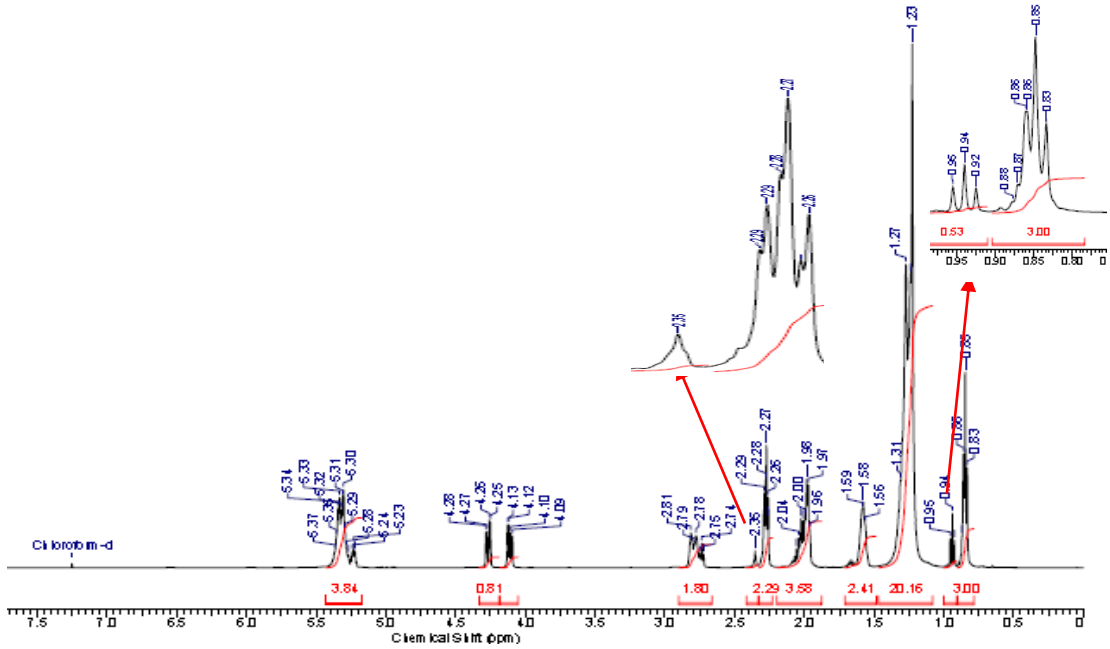


Fig 5. 32. ^{1}H NMR spectrum of salmon tissue oil

The ^{13}C spectra of both salmon oils (Figs 5.33 and Fig 5.34) gave signals at 130.0 and 129.7 ppm corresponding to olefinic carbons with slightly different environments. In addition the omag-3 fatty acid signal at 126 ppm was in accordance with data reported in the literature [Aursand *et al.,* 1995; Faucnnot *et al.,* 2006; Ruiz-Lopez *et al.,*2015]; this signal was smaller in salmon skin oil than in the tissue oil (Fig 5.35 A,B).

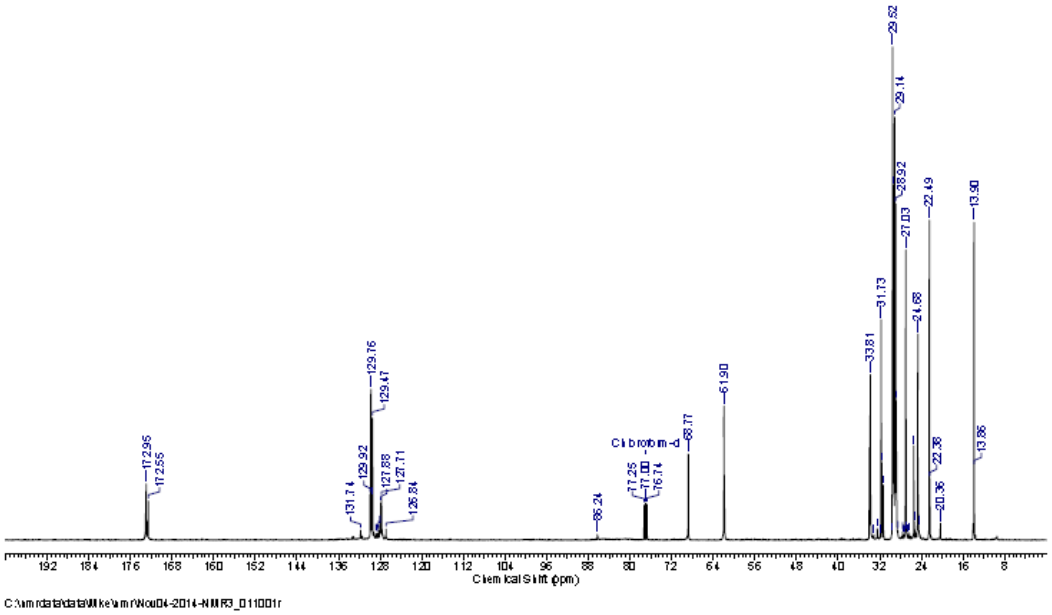


Fig 5.33. ^{13}C NMR spectrum of salmon skin oil

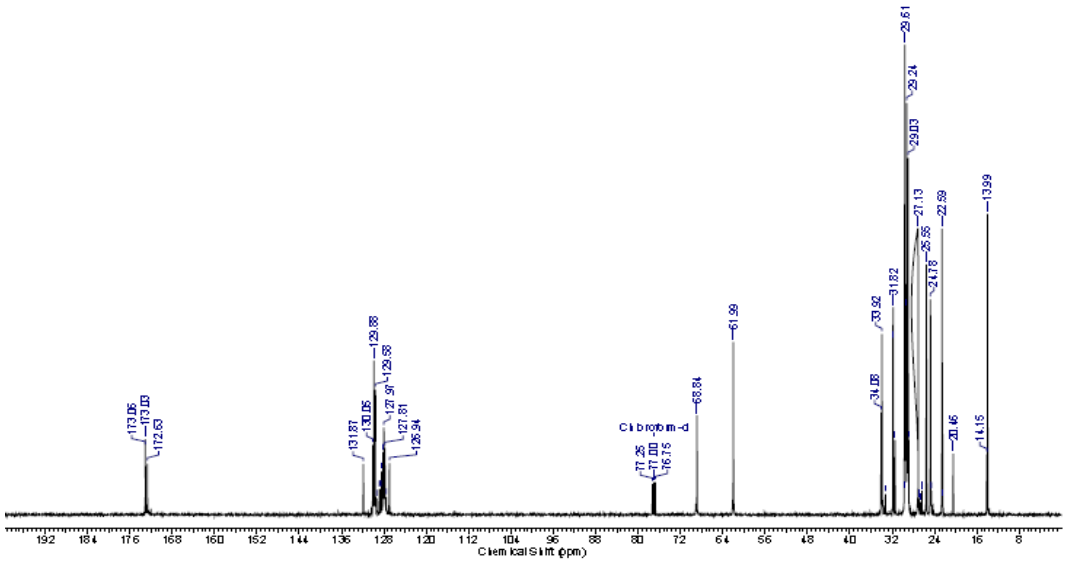


Fig 5.34. 13C NMR spectrum of salmon Tissue

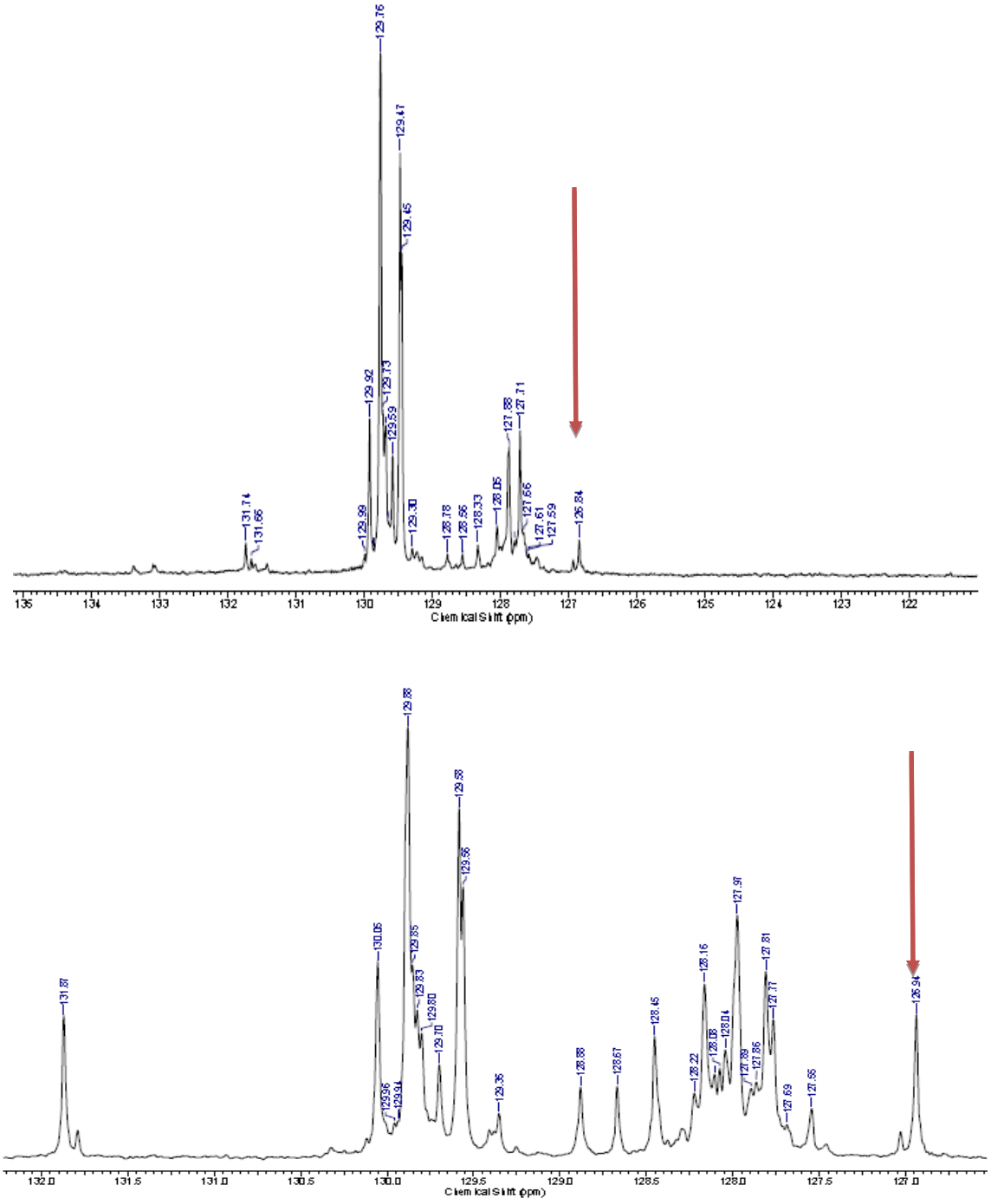


Fig 5. 35. (A) ^{13}C NMR spectrum of salmon skin expanded (122-136 ppm); (B) tissue oils (126-133 ppm)

5.4.4.3. GC/MS

The GC/MS study was carried out to examine the fatty acid composition of the farmed Atlantic salmon. The salmon skin and tissue oils were converted to their methyl ester derivatives using the BF_3-MeOH procedure individually then analyzed by GC/MS. The methyl ester chromatograms for the salmon skin and tissue oils are shown in Figs. 5.36 and 5.37, respectively, and the fatty acid methyl ester profiles are given in Tables 5.4 and 5.5, respectively. The fatty acid composition of each of the oils was obtained from relative areas of the peaks as determined by the TurboMass program. The results showed that the predominant fatty acids present are C18:1, C18:2 and C16:0 with small amounts of C18:0, C20:5 and C22:6. The concentrations of C16:0 and C18:1 were significantly higher in the salmon tissue oil and their characteristic fatty acids from fish fed with diet rich in vegetable oils. Also, a significant increase of linoleic acid (C18:2) and their elongation products, namely eicosatrienoic acid (C20:3) and eicosatetraenoic acid (C20:4), were detected. This is in accordance with recent studies in Atlantic salmon fed diets including vegetable oils [Pan, 2013; Asche *et al.,* 2011; Ruiz-López *et al*., 2015]. It is well known that the fatty acid composition of farmed salmon greatly varies depending upon their diet [Bell *et al*., 2001] and since the commercial feed is generally very different from natural diet, the fat content and lipid composition of fish muscle have been used as criteria to differentiate farmed salmon from the wild ones [Aursand *et al*., 2000; Busetto *et al*., 2008]. In this study, the composition of the diets for the farmed salmon is unknown. It was felt necessary to identify the existence of omag-3 fatty acids in salmon oil, and ^{1}H and ^{13}C NMR were used for this purpose. The NMR results were in agreement with the GC/MS and showed that the salmon skin oil contained smaller signals of omag-3 fatty acids than the tissue oil.

Because of the limited access to GC/MS for this part of the thesis project, no preconcentration steps were applied to the transesterified skin and tissue oil samples. Consequently, no chlorinated fatty acids were detected. Also, no further investigation using this off-line 2D RP-HPLC/GC/MS was done to the salmon oil.

Table 5.4 . FAME in salmon tissue oil.

Retention time	Fatty Acid	% of Total
14.70	C14:0	4.02
17.48	C16:1	5.01
17.79	C16:0	25.86
21.69	C18:2	9.93
21.86	C18:1	37.57
21.99	C18:1	6.24
22.48	C18:0	3.92
27.06	C20:5	2.17
27.71	C20:4	2.17
28.2	C20:3	0.12
29.0	C20:2	0.5
29.3	C20:1	0.89
30.4	C20:0	0.21
38.2	C22:6	0.71
39.3	C22:1	0.27
42.05	C22:0	0.2
		Σ = 97.62

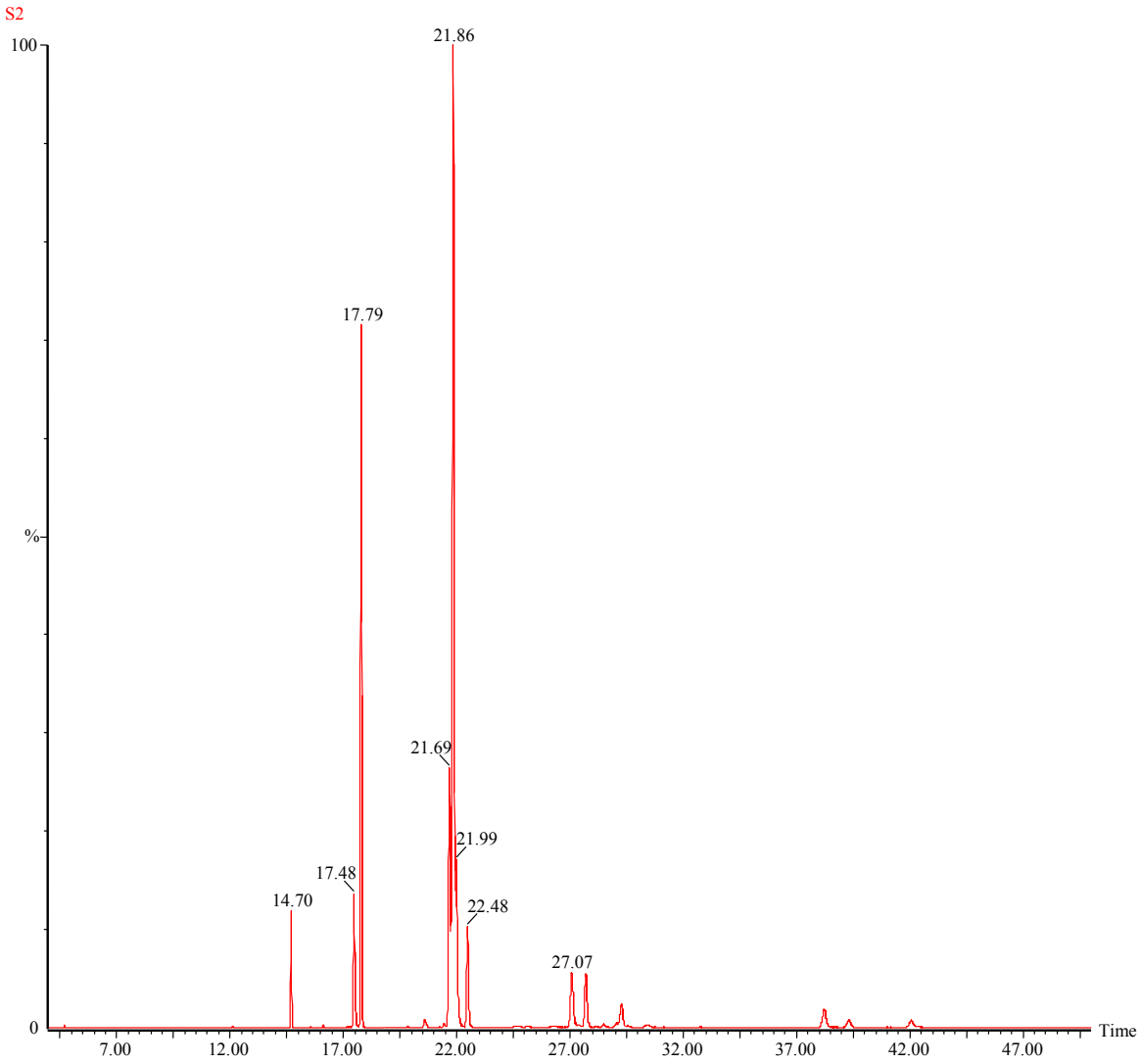


Fig 5.36. GC/MS of the fatty acid methyl esters chromatogram for the salmon tissue.

Table 5.5. FAME in salmon skin oil

Retention time	Fatty Acid	% of Total
14.70	C14:0	2.88
17.47	C16:1	3.30
17.79	C16:0	23.51
21.67	C18:2	7.39
21.83	C18:1	37.19
21.97	C18:1	10.13
22.49	C18:0	3.24
27.06	C20:5	5.24
27.71	C20:4	1.72
28.2	C20:3	0.10
29.26	C20:2	1.00
30.35	C20:0	0.41
38.19	C22:6	0.73
39.27	C22:1	0.27
42.05	C22:0	0.36
		Σ = 96.28

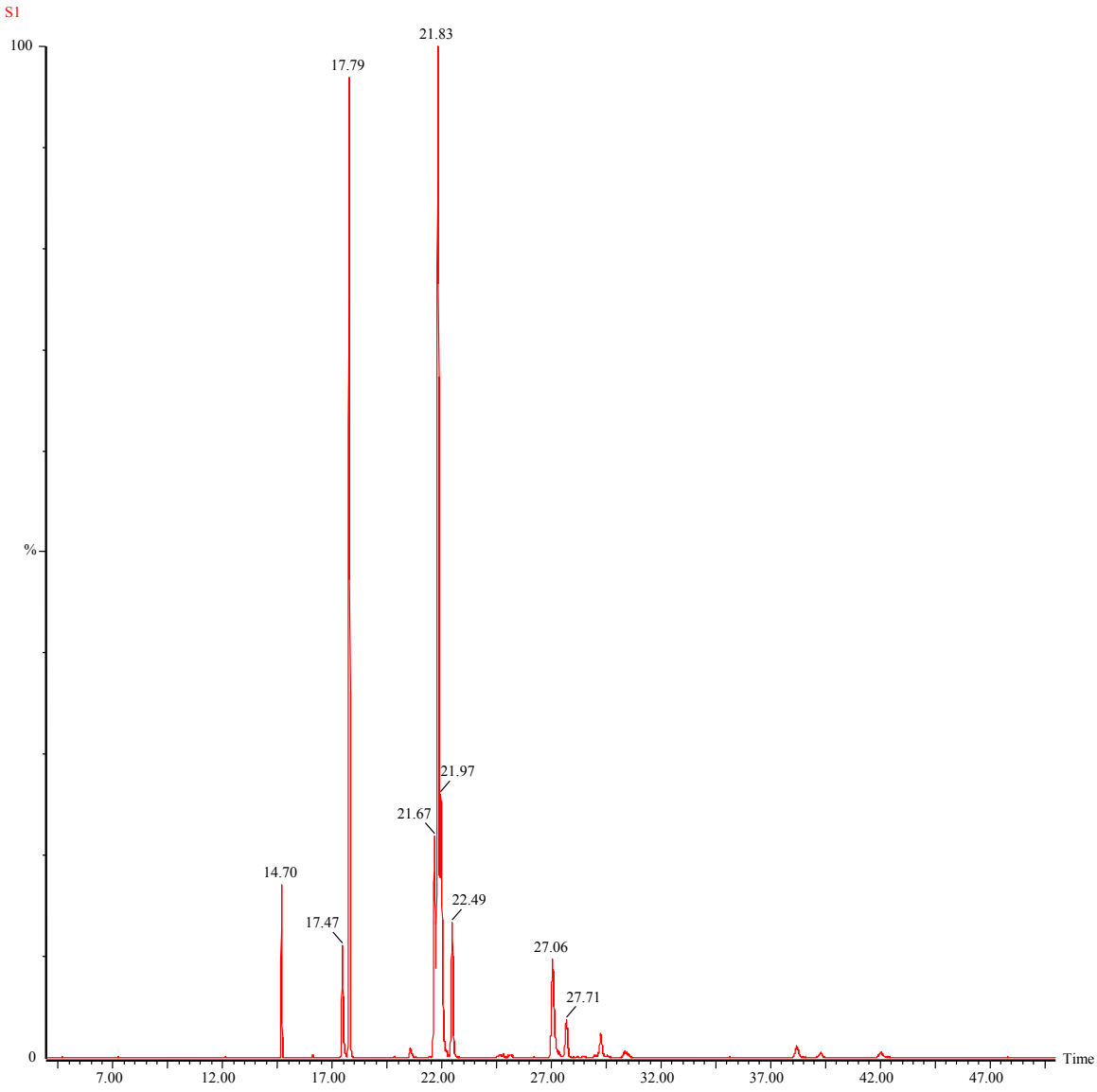


Fig 5.37. GC/MS of the fatty acid methyl esters chromatogram for the salmon skin oil.

5.4.5. Conclusions

In summary, the Cl-TAG model standards were synthesized in this thesis with very good yield (73-98%). The full characterization of each Cl-TAG compound using NMR and ESI(+)/MS techniques was achieved. The $[M+Na]^+$ ions of the chlorinated compounds showed distinctive isotope clusters in which the relative intensities of the individual peaks closely corresponded to the values calculated (Ramaley and Herrera, 2008) using the expected elemental compositions (including two, four or six chlorine

atoms) and natural isotopic abundances. Mechanistic hypothesis for CID-initiated, sequential HCl losses from a 9,10-dichlorooctadecanoyl group was predicated and evaluated. The retention time of the Cl-TAG compounds in the spiked corn oil was observed to increase with increasing number of chlorine and carbon atoms. It can therefore be concluded that the development analytical methods for qualitative as well as quantitative study of the Cl-TAG compounds in the corn oil sample using RP-HPLC/ESI(+)/MS were successful.

A comparison of the fatty acids results in salmon skin and tissue oils by two detection methods was carried out. In the 2D RP-HPLC/ESI(+)/MS method the fatty acids in each TAG were identified successfully upon CID. The assignments of the fatty acids in the TAG were based on the mass difference between the precursor $[M+Na]^+$ ion and the product ions $[M+Na-RCOOH]^+$ and $[M+Na-RCOO-Na]^+$. However, in the GC/MS method the fatty acids in each TAG were identified as fatty acid methyl esters (FAMEs) using the BF_3-MeOH method. These results were very useful for the identification of the TAG peaks in salmon oils. Some of the doubly unsaturated C18 and C16 fatty acids detected by ESI(+)/MS upon CID in different fractions were not evident in GC/MS indicating that ESI(+)/MS was more sensitive than GC/MS. In addition, the doubly unsaturated C18 and C16 fatty acids could contain polar groups which could be removed with a nonpolar solvent like hexane during the extraction step after transesterification. A summary of the results is presented in Table 5.6.

Table 5.6. A summary of the fatty acids in the salmon (skin and tissue) oils identified by GC/MS as FAME and RP-HPLC/ESI(+)/MS upon CID.

FAMEs GC/MS	FA upon CID RP-HPLC/ESI(+)/MS
C14:0	C14:0
-	C16:4
-	C16:3
-	C16:2
C16:1	C16:1
C16:0	C16:0
-	C18:4
-	C18:3
C18:2	C18:2
C18:1	C18:1
C18:1	C18:1
C18:0	C18:0
C20:5	C20:5
C20:4	C20:4
C20:3	C20:3
C20:2	C20:2
C20:1	C20:1
C20:0	C20:0
C22:6	C22:6
-	C22:5
-	C22:4
C22:1	C22:1
C22:0	C22:0

The possible oxidized TAG compounds were detected in salmon skin and their possible structural formulae were proposed. The RP-HPLC/ESI(+)/MS turned out to be a more reliable method for the detection of The oxidized TAG compounds compared to NMR and GC/MS. The ^{13}C and ^{1}H NMR spectra revealed that the Omega-3 fatty acids in salmon skin oil had lower levels than those in the tissue oil.

The amount of the fatty acid C22:6 (DHA) by GC/MS in both salmon oils was not as high as that reported in literature [Chow, 2008]. It appears from our work that farmed salmon fed with diets containing vegetable oils have increased level of C18:1 and decreased level of Omega-3, as also reported in the literature. We detected Omega-3 in the commercially available salmon oil tablet, as reported in Section 3.4.4. It is possible that Omega-3 was added externally to the commercial sample.

The use of the GC/MS and NMR spectroscopic techniques for the determination of fatty acid composition and TAG in salmon oils was successful; however, these techniques revealed that the Omega-3 fatty acids in salmon skin oil had lower levels than those in the tissue oil.

CHAPTER 6

Conclusions and recommendations

It has been mentioned before that the overall objective of this thesis was to characterize extractable organochlorine compounds (EOCls) in farmed salmon sample. A series of methods were developed and a number of steps were followed for meeting these objectives. These are summarized below.

The BF_3-MeOH method was applied to esterify fatty acids and transesterify TAGs in several natural oil samples, namely corn, grape seed, canola, and salmon oils. In general, 5 min was used by other researchers to convert both free FAs and TAGs to their FAMEs. In this thesis we carried out a study on the time required for the complete conversion of free FAs and TAGs using GC/MS. Although 5 min was found to be sufficient for the conversion of free FAs to their FAMEs, we observed that at least 15 min was required for the 100% conversion of TAGs to their FAMEs. Consequently, we used 15 min henceforth for TAG conversion.

It has been noted in the literature survey that corn oil has been most extensively studied among all the natural oils. A maximum number of 15 TAGs in corn oil were identified by other researchers using on-line Ag-HPLC followed by RP-HPLC then detection by either GC/MS, ESI(+)/MS or APCI(-)/MS. In this thesis, a greater differentiation between several corn oil TAGs, with the same number of double bonds but different partition numbers, was achieved by developing a combination of 2D off-line and on-line Ag-HPLC and RP-HPLC methods followed by ESI(+)/MS. It should be noted here that the Ag-HPLC column we prepared in our laboratory was superior to the ones used by others. The off-line RP-HPLC/ESI(+)/MS method gave significantly higher number of TAGs containing long chain fatty acids (C20:0; *e.g.* ALL, ALO, and ALP) than the off-line Ag-HPLC/ESI(+)/MS method. On the other hand, more TAGs with polyunsaturated fatty acids were obtained by the off-line Ag-HPLC/ESI(+)/MS (*e.g.* OLnO, LLLn, and LnOLn). We identified a total of 25 TAGs in corn oil using both the off-line RP-HPLC/ESI(+)/MS and Ag-HPLC/ESI(+)/MS methods developed here. We then developed a 3-D Ag-HPLC/ESI(+)/MS/GC/MS and a 2D RP-HPLC/GC/MS both off-line methods and combined them to determine the composition of fatty acids in each TAG in the corn oil. To the best of my knowledge no paper on the 3-D Ag-HPLC/ESI(+)/MS/GC/MS has yet been published.

In addition to 25 natural TAGs mentioned above, 10 oxidized TAGs were identified in the corn oil sample by an off-line RP-HPLC/ESI(+)/MS method developed here. The fatty acid components of the oxidized TAGs were detected by CID. However, all of them could not definitively be identified as mono- and di-hydroperoxides, mono- and bis-epoxides, epoxy epidioxides, and hydroxyl epidioxides because of the lack of appropriate standards. It should be pointed here out that no paper on the identification of oxidized TAGs in un-treated commercial corn oil sample has yet been published in the literature. However, one paper was found on oxidized TAGs in fried corn oil [Yoon *et al*., 2007] and another in corn oil where oxidation was purposely induced for the study [Zeb *et al*., 2013]. A comparison of their mass results with ours reveal the possible existence of 7 common oxidized TAGs in our corn oil sample. Further studies on the oxidized TAGs in commercially available oils and their bioavailability, especially in human, are needed to assess their adverse health effects.

It has been mentioned that although the main objective of this thesis was to characterize Cl-TAGs, no standard was commercially available for their identification by comparison. So a lot of effort was spent in this thesis to synthesize 4 Cl-TAGs, namely TAG-C12:0/C18:Cl_2/C12:0, TAG-C12:0/C12:0/C18:Cl_2, TAG-C12:0/C18:Cl_2/C18:Cl_2, and TAG-C18:Cl_2/C18:Cl_2/C18:Cl_2 and one brominated TAG (TAG-C18:Br_2/C18:Br_2/ C18:Br_2 all with very good yields (73-98%). Each of these TAGs was characterized using NMR and ESI(+)/MS techniques. The mechanism for the loss of HCl from Cl-TAGs was hypothesised and evaluated by CID using a 9,10-dichlorooctadecanoyl group as a model. The Cl-TAGs were found to contain Cl-FAs in their symmetrical and asymmetrical structures but so far not reported in the literature. As mentioned before, corn oil was selected as a representative oil in this thesis; it was spiked with the 4 Cl-TAGs synthesized here. Both qualitative and quantitative studies were done using the off-line RP-HPLC/ESI(+)/MS method. It was observed that a minimum of 1 μM solution of the Cl-TAG was necessary for detection purposes. The retention times of the Cl-TAGs spiked on the corn oil were observed to increase with increasing number of chlorine and carbon atoms.

The methods developed above and the knowledge acquired so far were applied to identify TAG components in the farmed salmon skin and tissue oils using the off-line RP-HPLC/ESI(+)/MS method. This method was able to identify 88 TAGs in the salmon skin oil and 81 TAGs in the salmon tissue oil. The CID spectra provided significant information on the FA composition of each TAG in salmon (skin and tissue) oils. Some

CID spectra were too complicated to interpret because of the high probability of two structurally different TAGs having the same sodiated $[M+Na]^+$ ions. To the best our knowledge this the first time TAGs in raw farmed salmon skin and tissue oils were directly identified using an off-line RP-HPLC/ESI(+)/MS method. Tarvainen *et al*. [2015] published a paper on TAGs and oxidized TAGs in cooked wild salmon portions after marinating them with several types of herb and storing in a refrigerator. They analyzed the cooked/treated samples by a UHPLC/ESI(+)/MS method after 0, 7, 14 and 26 days of storage. They put forward possible compositions of the TAGs and oxidized TAGs from their *m/z* without any further proof. We went a step forward and used tandem mass spectrometry (MS^n) to deduce more reliable FA content of the TAGs in raw farmed salmon skin and tissue oils.

Two additional observations were made during our framed salmon oil study. Firstly, we found 3 unknown TAGs which lost either 74 u or 148 u, as evident from their CID spectra. We hypothesized them as oxidized TAGs but no proof can be provided at this stage. Secondly, doubly unsaturated FAs of C16 and C18 (*e.g.,* 3 and 4 DB) were detected in the CID spectra but not detected by GC/MS. These FAs were perhaps somehow got removed during the conversion steps of the TAGs to their FAMEs. No information on 3 or 4 DB in C16 and C18 is available in the literature.

The NMR technique was used to confirm that we were dealing with TAGs and not phospholipids in salmon skin and tissue oils. Consequently, experiments were carried out with a ^{31}P probe which indicated that there were no phospholipids in either tissue or skin oils. In addition, the ^{1}H NMR and ^{13}C NMR spectra were dominated by the signals from TAGs which agreed with ESI(+)/MS results. It was then concluded that only TAGs in salmon oils were being investigated.

It should be pointed out here that 8 methods were originally developed and applied in this thesis for the analysis of corn oils. These methods are: GC/MS, both off-line and on-line 2D-Ag-HPLC and RP-HPLC, off-line 2D-RP-HPLC/GC/MS, off-line 2D-Ag-HPLC/ESI(+)/MS, off-line 2D-RP-HPLC/ESI(+)/MS, and off-line 3-D Ag-HPLC/ESI(+)/MS/GC/MS. However, only GC/MS and the off-line RP-HPLC/ESI(+)/MS were applied to the analysis of salmon oils. Other 6 methods involving GC/MS and Ag^+-column in both 2D and 3D modes could not be applied to salmon oils because of the inaccessibility of the instrument during the remaining time of this thesis work.

Although series of methods were developed and a number of new Cl-TAGs were synthesized and characterize, no Cl-TAG could be detected in the raw farmed salmon skin and tissue oils. There could be several reasons for it. (i) One of the reasons could be the location where the salmon was caught. To the best of our knowledge, this salmon was obtained from a hatchery in New Brunswick. It is quite possible that this hatchery is fairly clean and not contaminated with OCls. (ii) It is quite reasonable to assume that the feed in this hatchery did not contain significant amounts of OCls. Since the exact location the hatchery is not known, it is not possible to investigate the food source. (iii) The third reason could be the instruments used were not sensitive enough for the detection of very low levels of OCls in TAGs. (iv) It is also possible that relatively large amounts of non-halogenated TAGs interfered with the determination of low levels of Cl-TAGs in the methods used here. (v) Another reason could be the solvents used in this work. The OCs were extracted from the farmed salmon using hexane:acetone (1:1) mixture which was successfully used in our laboratory for many years. However for the purification of the Cl-TAGs synthesized in this thesis, acetone could not be used in column chromatography due to its high volatility instead a mixture of hexane:ethyl acetate (2:1, 4:1, and 8:1 ratio) was used. In view of the above observations, several recommendations can be made. Firstly, it is recommended to evaluate the efficacy of the hexane:ethyl acetate and other solvent mixtures which are also suitable for the purification of Cl-TAGs be investigated with respect to the extraction OCls from salmon.

Since the levels of Cl-TAGs could be very low in presence of large amounts of non-halogenated ones, pre-concentration methods can be developed. These methods could include the conversion of TAGs to FAMEs first, then removal of polyunsaturated FAs by complexation with silver ions, followed by the removal of saturated fatty acids by urea, and finally detection by GC/MS, as recommended by Brown and Järlskog [2015].

The sensitivity of a method can also be improved by using better instrumentation. The ESI/MS has been used here for the first time to identify Cl-TAGs. It has not been used by others for Cl-TAGs for various reasons. Natural sample is a very complex mixture and finding low levels of Cl-TAGs is rather difficult. Finding a ‘fingerprint’ of chlorine from a chlorine-containing compound in a mixture either by ESI/MS or ESI/MS/MS is not easy compared to GC/MS. Since chlorine has two natural isotopes, ^{35}Cl and ^{37}Cl with an abundance ratio of 3:1, compounds containing different numbers of Cl should give different chlorine patterns by MS. However, if the concentration of this compound is very low, or the chlorine pattern of this compound is partly overlapped with those of other

compounds in a mixture, then it would be difficult to spot these patterns and recognize the presence of chlorine. In these instances, ESI/MS/MS may be helpful. It is obvious from this thesis that Ag-column was very efficient in separating TAGs containing unsaturated FAs. It is recommended to develop 3D methods of RP-HPLC/ESI/MS/GC/MS and Ag-HPLC/ESI/GC/MS for the characterization of low levels of Cl-TAGs in salmon oils. Finally, the methods can be evaluated by analyzing contaminated natural salmon oils or experimental salmon raised in the laboratory with contaminated feed.

CHAPTER 7

REFERENCES

Ackman, R. G., 1998, Remarks on official methods employing boron trifluoride in the preparation of methyl esters of the fatty acids of fish oils: Journal of the American Oil Chemists Society, v. 75, p. 541-545.

Adlof, R. O., 1997, Normal-phase separation effects with lipids on a silver ion high-performance liquid chromatography column: Journal of Chromatography A, v. 764, p. 337-340.

Akesson-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, v. 996, p. 173-180.

Akesson-Nilsson, G., and C. Wesen, 2004, Structural characterization of 5,6-dichlorotetradecanoic acid, an isolated metabolite of 9,10-dichlorooctadecanoic acid, by studying picolinyl esters, pyrrolidides and methyl esters with electron ionization mass spectrometry: Journal of Mass Spectrometry, v. 39, p. 1313-1320.

Alaee, M., P. Arias, A. Sjodin, and A. Bergman, 2003, An overview of commercially used brominated flame retardants, their applications, their use patterns in different countries/regions and possible modes of release: Environment International, v. 29, p. 683-689.

Aronson, K. J., A. B. Miller, C. G. Woolcott, E. E. Sterns, D. R. McCready, L. A. Lickley, E. B. Fish, G. Y. Hiraki, C. Holloway, T. Ross, W. M. Hanna, S. K. SenGupta, and J. P. Weber, 2000, Breast adipose tissue concentrations of polychlorinated biphenyls and other organochlorines and breast cancer risk: Cancer Epidemiology Biomarkers & Prevention, v. 9, p. 55-63.

Aursand, M., L. Jorgensen, and H. Grasdalen, 1995, Positional distribution of omega-3-fatty-acids in marine lipid triacylglycerols by high-resolution C-13 nuclear-magnetic-resonance spectroscopy: Journal of the American Oil Chemists Society, v. 72, p. 293-297.

Aursand, M., J. R. Rainuzzo, and H. Grasdalen, 1993, Quantitative high-resolution C-13 and H-1 nuclear-magnetic-resonance of omega-3-fatty-acids from white muscle of Atlantic salmon (*Salmo salar*): Journal of the American Oil Chemists Society, v. 70, p. 971-981.

Aursand, M., I. B. Standal, A. Prael, L. McEvoy, J. Irvine, and D. E. Axelson, 2009, C-13 NMR pattern recognition techniques for the classification of atlantic salmon (Salmo salar L.) according to their wild, farmed, and geographical origin: Journal of Agricultural and Food Chemistry, v. 57, p. 3444-3451.

Andrews, P.C., Fraser, B.H., Junk, P.C., Massi, M., Perlmutter, P., Thienthong, N., Wijesundera, C, 2008, Large-scale synthesis of both symmetrical and unsymmetrical triacylglycerols containing docosahexaenoic acid: Tetrahedron, v .64, p. 9197–9202.

Bahroun, 2007, Studies of Extractable Organohalogens in Salmon by Neutron Activation Analysis and Other Techniques: Dalhousie University, Canada.

Ballschmiter, K., 2003, Pattern and sources of naturally produced organohalogens in the marine environment: biogenic formation of organohalogens: Chemosphere, v. 52, p. 313-324.

Batterman, S., J. Y. Chin, C. Jia, C. Godwin, E. Parker, T. Robins, P. Max, and T. Lewis, 2012, Sources, concentrations, and risks of naphthalene in indoor and outdoor air: Indoor Air, v. 22, p. 266-278.

Beceiro-Gonzalez, E., M. J. Gonzalez-Castro, S. Muniategui-Lorenzo, P. Lopez-Mahia, and D. Prada-Rodriguez, 2012, Analytical methodology for the determination of organochlorine pesticides in vegetation: Journal of Aoac International, v. 95, p. 1291-1310.

Beermann, C., N. Winterling, A. Green, M. Moebius, J. J. Schmitt, and G. Boehm, 2007, Comparison of the structures of triacylglycerols from native and transgenic medium-chain fatty acid-enriched rape seed oil by liquid chromatography-atmospheric pressure chemical ionization ion-trap mass spectrometry (LC-APCI-ITMS): Lipids, v. 42, p. 383-394.

Bell, J. G., J. McEvoy, D. R. Tocher, F. McGhee, P. J. Campbell, and J. R. Sargent, 2001, Replacement of fish oil with rapeseed oil in diets of Atlantic salmon (Salmo salar) affects tissue lipid compositions and hepatocyte fatty acid metabolism: Journal of Nutrition, v. 131, p. 1535-1543.

Bergkvist, C., M. Oberg, M. Appelgren, W. Becker, M. Aune, E. H. Ankarberg, M. Berglund, and H. Hakansson, 2008, Exposure to dioxin-like pollutants via different food commodities in Swedish children and young adults: Food and Chemical Toxicology, v. 46, p. 3360-3367.

Berntssen, M. H. G., K. Julshamn, and A.-K. Lundebye, 2010a, Chemical contaminants in aquafeeds and Atlantic salmon (Salmo salar) following the use of traditional- versus alternative feed ingredients: Chemosphere, v. 78, p. 637-646.

Berntssen, M. H. G., A. Maage, K. Julshamn, B. E. Oeye, and A. K. Lundebye, 2011, Carry-over of dietary organochlorine pesticides, PCDD/Fs, PCBs, and brominated flame retardants to Atlantic salmon (Salmo salar L.) fillets: Chemosphere, v. 83, p. 95-103.

Berntssen, M. H. G., P. A. Olsvik, B. E. Torstensen, K. Julshamn, T. Midtun, A. Goksoyr, J. Johansen, T. Sigholt, N. Joerum, J. V. Jakobsen, A. K. Lundebye, and E. J. Lock, 2010b, Reducing persistent organic pollutants while maintaining long chain omega-3 fatty acid in farmed Atlantic salmon using decontaminated fish oils for an entire production cycle: Chemosphere, v. 81, p. 242-252.

Beyer, A., and M. Biziuk, 2008, Applications of sample preparation techniques in the analysis of pesticides and PCBs in food: Food Chemistry, v. 108, p. 669-680.

Bjermo, H., P. O. Darnerud, S. Lignell, M. Pearson, P. Rantakokko, C. Nalsen, H. E. Barbieri, H. Kiviranta, A. K. Lindroos, and A. Glynn, 2013, Fish intake and breastfeeding time are associated with serum concentrations of organochlorines in a Swedish population: Environment International, v. 51, p. 88-96.

Bjorn, H., G. Ewald, P. Sundin, C. Wesen, J. Skramstad, and P. Froyen, 2004, Metabolic fate of (14)C-labelled chlorinated and non-chlorinated fatty acids in goldfish (Carassius auratus): Fish Physiology and Biochemistry, v. 30, p. 13-20.

Bjorn, H., P. Sundin, C. Wesen, H. L. Mu, K. Martinsen, A. L. Kvernheim, and G. Odham, 1998, Chlorinated fatty acids in membrane lipids of fish: Naturwissenschaften, v. 85, p. 229-232.

Batovska, D.I., Tsubota, S., Kato, Y., Asano, Y., Ubukata, M, 2004, Lipase-mediated desymmetrization of glycerol with aromatic and aliphatic anhydrides: Tetrahedron Asymmetry, v. 15, p .3551–3559.

Bjorn, H., Sundin, P., Wesen, C, 1998a, On chlorinated fatty acids as environmental pollutants. In: Rose, J. (Ed.), Environmental Toxicology: Current Developments. Gordon and Breach Science Publishers, pp. 159–170.

Bratberg, M, 2012, Effects of oil compounds and persistent organic pollutants (POPs) on phospholipid composition in liver and brain of Atlantic cod (*Gadus morhua*): Dissertation for the degree philosophiae doctor (PhD) at the University of Bergen

Bottaro, C.S., Kiceniuk, J.W., Chatt, A, 1999, Spatial dstribution of extractable organohalogens in northern pink shrimp in the North Atlantic: Biological Trace Element Research, v. 71–72, p. 149–166.

Bouquet, F., Paquot, C, 1946, Action du chlorure de sulfuryle sur les acides oleique et stearique : Comptes Rendus Hebdomadaires Des Seances De L’ Academie DesSciences, v. 223, p. 481–483.

Bonora, S., M. Di Foggia, and M. Iafisco, 2008, DSC and Raman study on the interaction of DDT 1,1,1-trichloro-2,2-bis(p-chlorophenyl)-ethane with liposomal phospholipids: Pesticide Biochemistry and Physiology, v. 92, p. 144-149.

Bouchard, M. F., Y. Oulhote, S. K. Sagiv, D. Saint-Amour, and J. Weuve, 2014, Polychlorinated Biphenyl exposures and cognition in older US adults: NHANES (1999-2002): Environmental Health Perspectives, v. 122, p. 73-78.

Boyd, R.K, 1993, “Extractable organically bound chlorine (EOCl) in fish and sediments; the unidentified 85%”: IMB Technical Report No. 69, Halifax, and National Research Council.

Brauner, E. V., O. Raaschou-Nielsen, E. Gaudreau, A. Leblanc, A. Tjonneland, K. Overvad, and M. Sorensen, 2011, Predictors of Polychlorinated Biphenyl concentrations in adipose tissue in a general danish population: Environmental Science & Technology, v. 45, p. 679-685.

Brondz, I., 2002, Development of fatty acid analysis by high-performance liquid chromatography, gas chromatography, and related techniques: Analytica Chimica Acta, v. 465, p. 1-37.

Buchgraber, M., F. Ulberth, and E. Anklam, 2004, Interlaboratory evaluation of injection techniques for triglyceride analysis of cocoa butter by capillary gas chromatography: Journal of Chromatography A, v. 1036, p. 197-203.

Bustnes, J. O., E. Lie, D. Herzke, T. Dempster, P. A. Bjorn, T. Nygard, and I. Uglem, 2010a, Salmon farms as a source of organohalogenated contaminants in wild fish: Environmental Science & Technology, v. 44, p. 8736-8743.

Bustnes, J. O., E. Lie, D. Herzke, T. Dempster, P. A. Bjorn, T. Nygard, and I. Uglem, 2010b, Salmon Farms as a Source of Organohalogenated Contaminants in Wild Fish: Environmental Science & Technology, v. 44, p. 8736-8743.

Byrdwell, W. C., W. E. Neff, and G. R. List, 2001, Triacylglycerol analysis of potential margarine base stocks by high-performance liquid chromatography with atmospheric pressure chemical ionization mass spectrometry and flame ionization detection: Journal of Agricultural and Food Chemistry, v. 49, p. 446-457.

Byrdwell, W.C., Emken. A.E, 1995, Analysis of triglycerides using atmospheric pressure chemical ionization mass spectrometry: Lipids, v. 30, p. 173–175.

Cai, H., Y. Chen, X. Cui, S. Cai, and Z. Chen, 2014, High-resolution H-1 NMR spectroscopy of fish muscle, eggs and small whole fish via hadamard-Encoded Intermolecular Multiple-Quantum Coherence: Plos One, v. 9.

Campbell, A. S., Y. Yu, S. Granick, and A. A. Gewirth, 2009, PCB association with model phospholipid bilayers: Abstracts of Papers of the American Chemical Society, v. 237.

Calvosa, F., Lagalante, A, 2010, Supercritical fluid extraction of polybrominated diphenyl ethers (PBDEs) from house dust with supercritical 1,1,1,2-tetrafluoroethane: Talanta, v. 80, p. 1116–1120.

Cao, Y., W. Wang, Y. Xu, B. Yang, and Y. Wang, 2013, Enzymatic synthesis of extremely pure triacylglycerols enriched in conjugated linoleic acids: Molecules, v. 18, p. 9704-9716.

Carvalho, M. S., M. A. Mendonca, D. M. M. Pinho, I. S. Resck, and P. A. Z. Suarez, 2012, Chromatographic analyses of fatty acid methyl esters by HPLC-UV and GC-FID: Journal of the Brazilian Chemical Society, v. 23, p. 763-769.

Canadian Environmental Protection Act, 1991, Priority Substances List Assessment Report No. 2 Effluents from Pulp Mills Using Bleaching.

Clark, K, 2003, Chlorinated fatty acids in muscle lipids and blubber of harbour porpoise and harbour seals:Dept. Environmental Assessment, Uppsala, Sweden

Chang J-H, S., C. R. Vogt, G. Y. Sun, and A. Y. Sun, 1981, Effects of acute administration of chlorinated water on liver lipids: Lipids, v. 16, p. 336-340.

Charlier, C. J., A. I. Albert, L. Y. Zhang, N. G. Dubois, and G. J. Plomteux, 2004, Polychlorinated biphenyls contamination in women with breast cancer: Clinica Chimica Acta, v. 347, p. 177-181.

Christie, W and Han, X, 2010, Lipid Analysis - Isolation, Separation, Identification and Lipidomic Analysis (4th edition): Oily Press, Bridgwater, U.K.

Christie, W.W,1997, Structural analysis of fatty acids: Advances in Lipid Methodology, v. 4, p. 119-169.

Christie, W.W, 1998, Gas chromatography mass spectrometry methods for structural analysis of fatty acids: Lipids,v. 33, p.343-353.

Cheek, A. O., K. Kow, J. Chen, and J. A. McLachlan, 1999, Potential mechanisms of thyroid disruption in humans: Interaction of organochlorine compounds with thyroid receptor, transthyretin, and thyroid-binding globulin: Environmental Health Perspectives, v. 107, p. 273-278.

Cherr, G. N., J. M. Shenker, C. Lundmark, and K. O. Turner, 1987, Toxic effects of selected bleached kraft mill effluent constituents on the sea-urchin sperm cell: Environmental Toxicology and Chemistry, v. 6, p. 561-569.

Chow, C, 2008, Fatty acids in food and their health implication, CRC press.

Cohen, M., 2007, Environmental toxins and health - The health impact of pesticides: Australian Family Physician, v. 36, p. 1002-1004.

Cohn, B. A., 2011, Developmental and environmental origins of breast cancer: DDT as a case study: Reproductive Toxicology, v. 31, p. 302-311.

Cole, D. W., R. Cole, S. J. Gaydos, J. Gray, G. Hyland, M. L. Jacques, N. Powell-Dunford, C. Sawhney, and W. W. Au, 2009, Aquaculture: Environmental, toxicological, and health issues: International Journal of Hygiene and Environmental Health, v. 212, p. 369-377.

Cole, P., D. Trichopoulos, H. Pastides, T. Starr, and J. S. Mandel, 2003, Dioxin and cancer: a critical review: Regulatory Toxicology and Pharmacology, v. 38, p. 378-388.

Conacher, H.B.S., Chadha, R.K., Lawrence, J.F., Charbonneau, S.M., Bryce, F, 1984, Mammary transfer and metabolism in the rat of halogenated fatty acids of halogenated olive oil: Lipids, v. 19, p. 637‑642.

Costa, M., J. Costa-Rodrigues, M. H. Fernandes, P. Barros, V. Vasconcelos, and R. Martins, 2012, Marine cyanobacteria compounds with anticancer properties: a review on the implication of apoptosis: Marine Drugs, v. 10, p. 2181-2207.

Crinnion, W. J., 2011a, Polychlorinated Biphenyls: persistent pollutants with immunological, neurological, and endocrinological consequences: Alternative Medicine Review, v. 16, p. 5-13.

Crinnion, W. J., 2011b, Polychlorinated Biphenyls: persistent pollutants with immunological, neurological, and endocrinological consequences: Alternative Medicine Review, v. 16, p. 5-13.

Crinnion, W. J., 2011c, The role of persistent organic pollutants in the worldwide epidemic of type 2 diabetes mellitus and the possible connection to farmed Atlantic salmon (Salmo salar): Alternative Medicine Review, v. 16, p. 301-313.

Cozzolino, R and De Giulio, B, 2011, Application of ESI and MALDI-TOF MS for triacylglycerols analysis in edible oils: Eur. J. Lipid Sci. Technol, v. 113, p. 160–167

Cunha, S. C., and M. Oliveira, 2006, Discrimination of vegetable oils by triacylglycerols evaluation of profile using HPLC/ELSD: Food Chemistry, v. 95, p. 518-524.

DeAngelo, A. B., F. B. Daniel, B. M. Most, and G. R. Olson, 1996, The carcinogenicity of dichloroacetic acid in the male fischer 344 rat: Toxicology, v. 114, p. 207-221.

Deshmukh, A. P., S. W. Kim, A. G. Marshall, and S. C. B. Myneni, 2005, Organohalogens in soils: Anthropogenic or natural?: Abstracts of Papers of the American Chemical Society, v. 230, p. U1506-U1506.

Dembitsky, V.M., Srebnik, M, 2002, Natural halogenated fatty acids: their analogues and derivatives: Progress in Lipid Research , v. 41, p.315‑367.

Denmark, S.E., Burk, M.T., Hoover, A.J, 2010, On the absolute configurational stability of bromonium and chloronium ions: Journal of the American Chemical Society , v. 132, p.1232‑1233.

Denton, R.M., Tang, X., Przeslak, A, 2010, Catalysis of phosphorus (V)-mediated transformations: dichlorination reactions of epoxides under appel conditions: Organic Letters, v. 12, p.4678‑4681.

Duffin, K., Henion, J., Shieh, J, 1991, Electrospray and tandem mass spectrometric characterization of acylglycerol mixtures that are dissolved in nonpolar solvents: Anaytical Chemistry, v. 63, p. 1781–1788

Deutch, B., H. S. Pedersen, and J. C. Hansen, 2004, Dietary composition in Greenland 2000, plasma fatty acids and persistent organic pollutants: Science of the Total Environment, v. 331, p. 177-188.

Dewailly, E., P. Ayotte, M. Lucas, and C. Blanchet, 2007, Risk and benefits from consuming salmon and trout: A Canadian perspective: Food and Chemical Toxicology, v. 45, p. 1343-1348.

Dewan, P., V. Jain, P. Gupta, and B. D. Banerjee, 2013a, Organochlorine pesticide residues in maternal blood, cord blood, placenta, and breastmilk and their relation to birth size: Chemosphere, v. 90, p. 1704-1710.

Dewan, P., V. Jain, P. Gupta, and B. D. Banerjee, 2013b, Organochlorine pesticide residues in maternal blood, cord blood, placenta, and breastmilk and their relation to birth size: Chemosphere, v. 90, p. 1704-1710.

Dingemans, M. M. L., A. de Groot, R. G. D. M. van Kleef, A. Bergman, M. van den Berg, H. P. M. Vijverberg, and R. H. S. Westerink, 2008a, Hydroxylation increases the neurotoxic potential of BDE-47 to affect exocytosis and calcium homeostasis in PC12 cells: Environmental Health Perspectives, v. 116, p. 637-643.

Dingemans, M. M. L., A. de Groot, R. G. D. M. van Kleef, A. Bergman, M. van den Berg, H. P. M. Vijverberg, and R. H. S. Westerink, 2008b, Hydroxylation increases the neurotoxic potential of BDE-47 to affect exocytosis and calcium homeostasis in PC12 cells: Environmental Health Perspectives, v. 116, p. 637-643.

Dobson, G., W. W. Christie, and B. Nikolovadamyanova, 1995, Silver ion chromatography of lipids and fatty-acids: Journal of Chromatography B-Biomedical Applications, v. 671, p. 197-222.

Dolowy, M., and A. Pyka, 2015, Chromatographic Methods in the Separation of Long-Chain Mono- and Polyunsaturated Fatty Acids: Journal of Chemistry.

Donato, P., F. Cacciola, P. Q. Tranchida, P. Dugo, and L. Mondello, 2012, Mass spectrometry detection in comprehensive liquid chromatography: Basic concepts, instrumental aspects, applications and trends: Mass Spectrometry Reviews, v. 31, p. 523-559.

Donia, M., and M. T. Hamann, 2003, Marine natural products and their potential applications as anti-infective agents: Lancet Infectious Diseases, v. 3, p. 338-348.

Dugo, P., F. Cacciola, T. Kumm, G. Dugo, and L. Mondello, 2008, Comprehensive multidimensional liquid chromatography: Theory and applications: Journal of Chromatography A, v. 1184, p. 353-368.

Dugo, P., O. Favoino, P. Q. Tranchida, G. Dugo, and L. Mondello, 2004, Off-line coupling of non-aqueous reversed-phase and silver ion high-performance liquid chromatography-mass spectrometry for the characterization of rice oil triacylglycerol positional isomers: Journal of Chromatography A, v. 1041, p. 135-142.

Dugo, P., T. Kumm, M. L. Crupi, A. Cotroneo, and L. Mondello, 2006, Comprehensive two-dimensional liquid chromatography combined with mass spectrometric detection in the analyses of triacylglycerols in natural lipidic matrixes: Journal of Chromatography A, v. 1112, p. 269-275.

Eckenrode, B, 2001, Environmental and forensic applications of field-portable GC-MS: an overview: J Am Soc Mass Spectrom 2001, v. 12, p. 683–693.

Eguchi, A., K. Nomiyama, G. Devanathan, A. Subramanian, K. A. Bulbule, P. Parthasarathy, S. Takahashi, and S. Tanabe, 2012, Different profiles of anthropogenic and naturally produced organohalogen compounds in serum from residents living near a coastal area and e-waste recycling workers in India: Environment International, v. 47, p. 8-16.

Endo, S., B. I. Escher, and K. Goss, 2011a, Capacities of membrane lipids to accumulate neutral organic chemicals: Environmental Science & Technology, v. 45, p. 5912-5921.

Endo, S., B. I. Escher, and K. Goss, 2011b, Capacities of membrane lipids to accumulate neutral organic chemicals: Environmental Science & Technology, v. 45, p. 5912-5921.

Engelke, M., H. Tahti, and L. Vaalavirta, 1996, Perturbation of artificial and biological membranes by organic compounds of aliphatic, alicyclic and aromatic structure: Toxicology in Vitro, v. 10, p. 111-115.

Ewald, G., 1998, Chlorinated fatty acids - Environmental pollutants with intriguing properties: Chemosphere, v. 37, p. 2833-2837.

Ewald, G., and P. Sundin, 1993, ATP leakage from eld cells after exposure to stearic, monochlorostearic, dichlorostearic, and oleic acids: Pharmacology & Toxicology, v. 73, p. 159-162.

Ewald, G., P. Sundin, J. Skramstad, and P. Froyen, 1996, Distribution of C-14 from ingested, radiolabelled dichlorostearic, stearic and oleic acids in body and in lipids of perch, Perca fluviatilis: Aquatic Toxicology, v. 36, p. 129-139.

Falandysz, J., 2003, Chloronaphthalenes as food-chain contaminants: a review: Food additives and contaminants, v. 20, p. 995-1014.

Faroon, O., D. Jones, and C. De Rosa, 2000, Effects of polychlorinated biphenyls on the nervous system: Toxicology and Industrial Health, v. 16, p. 305-333.

Fraser, B.H., Perlmutter, P., Wijesundera, C, 2007, Practical syntheses of triacylglycerol regioisomers containing long-chain polyunsaturated fatty acids: Journal of the American Oil Chemists' Society, v. 84, p. 11–21.

Fiedler, H., 1996, Sources of PCDD/PCDF and impact on the environment: Chemosphere, v. 32, p. 55-64.

Foran, J. A., D. O. Carpenter, M. C. Hamilton, B. A. Knuth, and S. J. Schwager, 2005, Dioxin-contaminated farmed salmon: Environmental Health Perspectives, v. 113, p. A656-A657.

Fowden, L, 1968, The occurrence and metabolism of carbon-halogen compounds. Proc. Roy. Soc. B. Soc. B, v. 171, p. 5.

Gabrielsen, K. M., J. S. Krokstad, G. D. Villanger, D. A. D. Blair, M. Obregon, C. Sonne, R. Dietz, R. J. Letcher, and B. M. Jenssen, 2015, Thyroid hormones and deiodinase activity in plasma and tissues in relation to high levels of organohalogen contaminants in East Greenland polar bears (Ursus maritimus): Environmental Research, v. 136, p. 413-423.

Gasull, M., M. Bosch de Basea, E. Puigdomenech, J. Pumarega, and M. Porta, 2011, Empirical analyses of the influence of diet on human concentrations of persistent

organic pollutants: A systematic review of all studies conducted in Spain: Environment International, v. 37, p. 1226-1235.

Gibson, T., Haley, J, 1986, Chlorinated fatty acids during water treatment studies of disinfectionReativity and product identifaction: Environ, Technol Letters, v. 7, p. 365-372

Gilles, K. A., C. A. Anker, D. H. Wheeler, and J. S. Andrews, 1958, Some observations on the constitution of wheat flour lipids isolated from unbleached and chlorine dioxide-treated flours: Cereal Chem, v. 35, p. 374-379.

Gjoen, T., E. J. Kleveland, C. Moya-Falcn, M. K. Froystad, A. Vegusdal, E. Hvatturn, R. K. Berge, and B. Ruyter, 2007, Effects of dietary thia fatty acids on lipid composition, morphology and macrophage function of Atlantic salmon (Salmo salar L.) kidney: Comparative Biochemistry and Physiology B-Biochemistry & Molecular Biology, v. 148, p. 103-111.

Glynn, A., A. Thuvander, M. Aune, A. Johannisson, P. O. Darnerud, G. Ronquist, and S. Cnattingius, 2008, Immune cell counts and risks of respiratory infections among infants exposed pre- and postnatally to organochlorine compounds: a prospective study: Environmental Health, v. 7.

Gribble, G. W., 1992, Naturally-occurring organohalogen compounds - a survey: Journal of Natural Products, v. 55, p. 1353-1395.

Gribble, G. W., 2012a, Occurrence of halogenated alkaloids: The Alkaloids. Chemistry and biology, v. 71, p. 1-165.

Gribble, G. W., 2012b, Recently discovered naturally occurring heterocyclic organohalogen compounds: Heterocycles, v. 84, p. 157-207.

Gribble, G. W., 2015, A recent survey of naturally occurring organohalogen compounds: Environmental Chemistry, v. 12, p. 396-405.

Gutleb, A. C., P. Cenijn, M. van Velzen, E. Lie, E. Ropstad, J. U. Skaare, T. Malmberg, A. Bergman, G. W. Gabrielsen, and J. Legler, 2010, In vitro assay shows that pcb metabolites completely saturate thyroid hormone transport capacity in blood of wild polar bears (*Ursus maritimus*): Environmental Science & Technology, v. 44, p. 3149-3154.

Haglund, P., M. Harju, C. Danielsson, and P. Marriott, 2002, Effects of temperature and flow regulated carbon dioxide cooling in longitudinally modulated cryogenic systems for comprehensive two-dimensional gas chromatography: Journal of Chromatography A, v. 962, p. 127-134.

Haines, D. A., and J. Murray, 2012, Human biomonitoring of environmental chemicals-Early results of the 2007-2009 Canadian health measures survey for males and females: International Journal of Hygiene and Environmental Health, v. 215, p. 133-137.

Hakansson, H., P. Sundin, T. Andersson, B. Brunstrom, L. Dencker, M. Engwall, G. Ewald, M. Gilek, G. Holm, S. Honkasalo, J. Idestamalmquist, P. Jonsson, N. Kautsky, G. Lundberg, A. Lundkvernheim, K. Martinsen, L. Norrgren, M. Personen, M. Rundgren, M. Stalberg, M. Tarkpea, and C. Wesen, 1991,Invivo and invitro toxicity of fractionated fish lipids, with particular regard to their content of chlorinated organic-compounds: Pharmacology & Toxicology, v. 69, p. 459-471.

Hamilton, M. C., R. A. Hites, S. J. Schwager, J. A. Foran, B. A. Knuth, and D. O. Carpenter, 2005, Lipid composition and contaminants in farmed and wild salmon: Environmental Science & Technology, v. 39, p. 8622-8629.

Hara, M., Y. Sakata, D. Nakatani, S. Suna, M. Usami, S. Matsumoto, T. Hamasaki, Y. Doi, M. Nishino, H. Sato, T. Kitamura, S. Nanto, M. Hori, I. Komuro, and I. Osaka Acute Coronary, 2013, Low Levels of Serum n-3 Polyunsaturated Fatty Acids Are Associated With Worse Heart Failure-Free Survival in Patients After Acute Myocardial Infarction: Circulation Journal, v. 77, p. 153-162.

Harrison, E. Z., S. R. Oakes, M. Hysell, and A. Hay, 2006, Organic chemicals in sewage sludges: Science of the Total Environment, v. 367, p. 481-497.

Heikes, D. L., 1992, Mass-spectral identification and gas-chromatographic determination of chlorinated bleaching adducts in flour-containing food items: Journal of Agricultural and Food Chemistry, v. 40, p. 489-491.

Heikes, D. L., 1993, Procedure for supercritical-fluid extraction and gas-chromatographic determination of chlorinated fatty-acid bleaching adducts in flour and flour-containing food items utilizing acid hydrolysis-methylation and florisil column cleanup techniques: Journal of Agricultural and Food Chemistry, v. 41, p. 2034-2037.

Halldorsson, A., Magnusson, C.D., Haraldsson, G.G, 2003, Chemoenzymatic synthesis of structured triacylglycerols by highly regioselective acylation: Tetrahedron, v. 59, p.9101‑9109.

Haraldsson, G.G., Gudmundsson, B.O., Almarsson, O, 1995, The synthesis of homogeneous triglycerides of eicosapentaenoic acid and docosahexaenoic acid by lipase: Tetrahedron, v. 51, p.941‑952.

Harrad, S, 1996, Sources and fates of polychlorinated dibenzo-*p*-dioxins, dibenzofurans and biphenyls: the budget and source inventory approach: The royal Society of Chemistry Retrieved from www.knovel.com.

Herrera, L.C., Potvin, M.A., Melanson, J.E, 2010, Quantitative analysis of positional isomers of triacylglycerols *via* electrospray ionization tandem mass spectrometry of sodiated adducts: Rapid Communications in Mass Spectrometry , v. 24, p. 2745‐2752.

Hostmark, A.T., Lystad, E., Jebens, E., Skramstad, J., Froyen, P, 1999, Growth modulatingeffects of dichloromyristic and dichlorostearic acid in cell cultures: Pharmacology & Toxicology, v. 85, p.162‐168.

Hoyau, S., Norrman, K., McMahon, T.B., Ohanessian, G, 1999, A quantitative basis for a scale of Na^+ affinities of organic and small biological molecules in the gas phase: Journal of the American Chemical Society , v. 121, p. 8864‐8875.

Hsu, F., Turk, J, 2010, Electrospray ionization multiple-stage linear ion-trap mass spectrometry for structural elucidation of triacylglycerols: assignment of fatty acyl groups on the glycerol backbone and location of double bonds: Journal of the American Society for Mass Spectrometry, v. 21, p. 657‐669.

Helmfrid, I., M. Berglund, O. Lofman, and G. Wingren, 2012, Health effects and exposure to polychlorinated biphenyls (PCBs) and metals in a contaminated community: Environment International, v. 44, p. 53-58.

Herrera, L. C., L. Ramaley, M. A. Potvin, and J. E. Melanson, 2013, A method for determining regioisomer abundances of polyunsaturated triacylglycerols in omega-3 enriched fish oils using reversed-phase liquid chromatography and triple-stage mass spectrometry: Food Chemistry, v. 139, p. 655-662.

Hird, S. J., B. P. Y. Lau, R. Schuhmacher, and R. Krska, 2014, Liquid chromatography-mass spectrometry for the determination of chemical contaminants in food: Trac-Trends in Analytical Chemistry, v. 59, p. 59-72.

Hites, R. A., J. A. Foran, D. O. Carpenter, M. C. Hamilton, B. A. Knuth, and S. J. Schwager, 2004, Global assessment of organic contaminants in farmed salmon: Science, v. 303, p. 226-229.

Holcapek, M., P. Jandera, P. Zderadicka, and L. Hruba, 2003, Characterization of triacylglycerol and diacylglycerol composition of plant oils using high-performance liquid chromatography-atmospheric pressure chemical ionization mass spectrometry: Journal of Chromatography A, v. 1010, p. 195-215.

Holcapek, M., M. Lisa, P. Jandera, and N. Kabatova, 2005, Quantitation of triacylglycerols in plant oils using HPLC with APCI-MS, evaporative light-scattering, and UV detection: Journal of Separation Science, v. 28, p. 1315-1333.

Holcapek, M., M. Ovcacikova, M. Lisa, E. Cifkova, and T. Hajek, 2015, Continuous comprehensive two-dimensional liquid chromatography-electrospray ionization mass spectrometry of complex lipidomic samples: Analytical and Bioanalytical Chemistry, v. 407, p. 5033-5043.

Hori, S., 2010, Organohalogen compounds in foodstuffs and human breast milk: Shokuhin eiseigaku zasshi. Journal of the Food Hygienic Society of Japan, v. 51, p. 373-82.

Horvath, K., J. N. Fairchild, and G. Guiochon, 2009, Detection issues in two-dimensional on-line chromatography: Journal of Chromatography A, v. 1216, p. 7785-7792.

Hostmark, A. T., E. Lystad, E. Jebens, J. Skramstad, and P. Froyen, 1998, Growth modulating effects of chlorinated oleic acid in cell cultures: Pharmacology & Toxicology, v. 83, p. 29-35.

HSDB. 2003. Polychlorinated Biphenyls. Compiled by the National Library of Medicine. Hazardous Substance Data Bank Web page: http://csi.micromedex.com/ Data/ HS/HS3945H.htm.

Hutchings, J. A., and M. E. B. Jones, 1998, Life history variation and growth rate thresholds for maturity in Atlantic salmon (*Salmo sal*ar): Canadian Journal of Fisheries and Aquatic Sciences, v. 55, p. 22-47.

Hutzinger, O., and H. Fiedler, 1993, From source to exposure - some open questions: Chemosphere, v. 27, p. 121-129.

Igarashi, M., T. Tsuzuki, T. Kambe, and T. Miyazawa, 2004, Recommended methods of fatty acid methylester preparation for conjugated dienes and trienes in food and biological samples: Journal of Nutritional Science and Vitaminology, v. 50, p. 121-128.

Igarashi, T., M. Aursand, Y. Hirata, I. S. Gribbestad, S. Wada, and M. Nonaka, 2000, Nondestructive quantitative determination of docosahexaenoic acid and n-3 fatty acids in fish oils by high-resolution H-1 nuclear magnetic resonance spectroscopy: Journal of the American Oil Chemists Society, v. 77, p. 737-748.

Igbinosa, E. O., E. E. Odjadjare, V. N. Chigor, I. H. Igbinosa, A. O. Emoghene, F. O. Ekhaise, N. O. Igiehon, and O. G. Idemudia, 2013, Toxicological profile of chlorophenols and their derivatives in the environment: The Public Health Perspective: Scientific World Journal.

Irimescu, R., Furihata, K., Hata, K., Iwasaki, Y., Yamane, T, 2001, Utilization of reaction medium-dependent regiospecificity of *Candida antarctia* lipase (Novozym 435) for the synthesis of 1,3-dicaproyl-2-docosahexaenoyl (or eicosapentaenoyl)

glycerol: Journal of the American Oil Chemists' Society, v. 78, p. 285–290.

Iwata, H., E.-Y. Kim, M. Yamauchi, S. Inoue, T. Agusa, and S. Tanabe, 2007, Chemical contamination in aquatic ecosystems: Yakugaku Zasshi-Journal of the Pharmaceutical Society of Japan, v. 127, p. 417-428.

Jacobson, J. L., and S. W. Jacobson, 1996, Intellectual impairment in children exposed to polychlorinated biphenyls in utero: New England Journal of Medicine, v. 335, p. 783-789.

Johnston, P., Stringer, R., Santillo, D., Stephenson, A., Labounskaia, I.,McCartney,H, 1996, Towards zero-effluent pulp and paper production: the pivotal role of totally chlorine free bleaching :Greenpeace Research Laboratories, Earth Resources Centre, University of Exeter,Exeter, UK

Janssen, H. G., W. Boers, H. Steenbergen, R. Horsten, and E. Floter, 2003, Comprehensive two-dimensional liquid chromatographyXgas chromatography: evaluation of the applicability for the analysis of edible oils and fats: Journal of Chromatography A, v. 1000, p. 385-400.

Jolley, A, 1975, Chlorine-containing organic costituents in chlorinated effeluents: WPCF,V. 47, P. 601-618

Johnson, K. J., B. J. Prazen, R. K. Olund, and R. E. Synovec, 2002, GC x GC temperature programming requirements to produce bilinear data for chemometric analysis: Journal of Separation Science, v. 25, p. 297-303.

Jordan, K. D., and R. K. Upmacis, 2013, Mass spectrometric analysis of oxidized eicosapentaenoic Acid sodium salt: Lipid insights, v. 6, p. 21-35.

Josef Olli, J., H. Breivik, T. Morkore, B. Ruyter, J. Johansen, P. Reynolds, O. Thorstad, and G. Berge, 2010, Removal of persistent organic pollutants from Atlantic salmon (Salmo solar L.) diets: Influence on growth, feed utilization efficiency and product quality: Aquaculture, v. 310, p. 145-155.

Keyzers, R. A., and M. T. Davies-Coleman, 2005, Anti-inflammatory metabolites from marine sponges: Chemical Society Reviews, v. 34, p. 355-365.

Kim, S., J. Park, H.-J. Kim, J. J. Lee, G. Choi, S. Choi, S. Kim, S. Y. Kim, H.-B. Moon, S. Kim, and K. Choi, 2015, Association between Several Persistent Organic Pollutants and Thyroid Hormone Levels in Cord Blood Serum and Bloodspot of the Newborn Infants of Korea: Plos One, v. 10.

King, M. D., L. D. Rea, and J. M. Kennish, 2006, Chlorinated fatty acids in lipid class fractions from cardiac and skeletal muscle of Chinook salmon: Lipids, v. 41, p. 1133-1140.

Kiceniuk, J.W., Holzbecher, J., Chatt, A, 1997, Extractable organohalogens in tissues of beluga whales from the Canadian arctic and the St. Lawrence estuary: Environmental Pollution, v. 97, p. 205–211.

Kiceniuk, J.W., Zwicker, B., Chatt, A, 1998, Determination of extractable organic bromine and chlorine in biological compartments of Atlantic cod (Gadus morhua) by neutron activation analysis: Journal of Radioanalytical and Nuclear Chemistry , v. 235, p. 291–294.

Kjaer, M. A., A. Vegusdal, T. Gjoen, A. C. Rustan, M. Todorevic, and B. Ruyter, 2008, Effect of rapeseed oil and dietary n-3 fatty acids on triacylglycerol synthesis and secretion in Atlantic salmon hepatocytes: Biochimica Et Biophysica Acta-Molecular and Cell Biology of Lipids, v. 1781, p. 112-122.

Kleijn, R., Elshkaki, A., Koning, A., Tukker,A, 2001, Literature study on unknown organochlorine compounds in the environment from anthropogenic sources: Project within chlorine chain follow-up research programme on chlorinated microcontaminants (ovoc), Centre of Environmental Science (CML), Leiden University

Knothe, G., M. O. Bagby, and D. Weisleder, 1996, Evaluation of the olefinic proton signals in the H-1-NMR spectra of allylic hydroxy groups in long-chain compounds: Chemistry and Physics of Lipids, v. 82, p. 33-37.

Knothe, G., M. Jie, C. C. Lam, and M. O. Bagby, 1995, Evaluation of the c-13-nmr signals of the unsaturated carbons of triacylglycerols: Chemistry and Physics of Lipids, v. 77, p. 187-191.

Kodavanti, P. R. S., and M. C. Curras-Collazo, 2010, Neuroendocrine actions of organohalogens: thyroid hormones, arginine vasopressin, and neuroplasticity: Frontiers in neuroendocrinology, v. 31, p. 479-96.

Komosuwelack, C., L. Acker, and E. Schulte, 1983a, on the chemistry and analysis of flour bleaching .3. Investigation of flours and baked products of domestic and foreign origin as to whether bleaching with chlorine and chlorine dioxide has occurred: Zeitschrift Fur Lebensmittel-Untersuchung Und-Forschung, v. 176, p. 285-288.

Komosuwelack, C., E. Schulte, and L. Acker, 1983b, On the chemistry and analysis of flour bleaching .1.The treatment of flours with chlorine and its analytical detection: Zeitschrift Fur Lebensmittel-Untersuchung Und-Forschung, v. 176, p. 169-172.

Komosuwelack, C., E. Schulte, and L. Acker, 1983c, On the chemistry and analysis of flour bleaching .2. The treatment of flours with chlorine dioxide and its detection: Zeitschrift Fur Lebensmittel-Untersuchung Und-Forschung, v. 176, p. 173-175.

Korchowiec, B., Y. Corvis, T. Viitala, C. Feidt, Y. Guiavarch, C. Corbier, and E. Rogalska, 2008, Interfacial approach to polyaromatic hydrocarbon toxicity: phosphoglyceride and cholesterol monolayer response to phenantrene, anthracene, pyrene, chrysene, and benzo a pyrene: Journal of Physical Chemistry B, v. 112, p. 13518-13531.

Kosikowska, M., and M. Biziuk, 2009, Methods of determination of pesticides residues in atmosphere - a review: Ecological Chemistry and Engineering S-Chemia I Inzynieria Ekologiczna S, v. 16, p. 207-220.

Kosikowska, M., and M. Biziuk, 2010, Review of the determination of pesticide residues in ambient air: Trac-Trends in Analytical Chemistry, v. 29, p. 1064-1072.

Kudo, N., T. Yamazaki, T. Sakamoto, K. Sunaga, T. Tsuda, A. Mitsumoto, and Y. Kawashima, 2011, Effects of perfluorinated fatty acids with different carbon chain length on fatty acid profiles of hepatic lipids in mice: Biological & Pharmaceutical Bulletin, v. 34, p. 856-864.

Kujawski, M. W., A. Barganska, K. Marciniak, E. Miedzianowska, J. K. Kujawski, M. Slebioda, and J. Namiesnik, 2014, Determining pesticide contamination in honey by LC-ESI-MS/MS - Comparison of pesticide recoveries of two liquid-liquid extractions based approaches: Lwt-Food Science and Technology, v. 56, p. 517-523.

Laakso P., Voutilainen. P, 1996, Analysis of triacylglycerols by silver-ion high-performance liquid chromatography–atmospheric pressure chemical ionization mass spectrometry: Lipids, v. 31, p. 1311–1322.

Labrada, E. G., 2003, Studies of Organohalogens in Cod Brain Tissues by Neutron Activation Analysis. PhD thesis, Dalhousie University.

Lall, S. P., 2010, The health benefits of farmed salmon: fish oil decontamination processing removes persistent organic pollutants: British Journal of Nutrition, v. 103, p. 1391-1392.

Lambropoulou, D. A., 2010, An overview of modern extraction techniques for the determination of organic pollutants in environmental matrices: A Review: Current Organic Chemistry, v. 14, p. 2247-2267.

Langer, P., 2010, The impacts of organochlorines and other persistent pollutants on thyroid and metabolic health: Frontiers in Neuroendocrinology, v. 31, p. 497-518.

LeBel, G. L., F. M. Benoit, and D. T. Williams, 1997, A one-year survey of halogenated disinfection by-products in the distribution system of treatment plants using three different disinfection processes: Chemosphere, v. 34, p. 2301-2317.

Lefsay, A. M., R. D. Guy, A. Chatt, and R. L. White, 2013, Synthesis and tandem mass spectrometry of chlorinated triacylglycerols: Chemistry and Physics of Lipids, v. 174, p. 55-63.

Leri, A. C., J. A. Hakala, M. A. Marcus, A. Lanzirotti, C. M. Reddy, and S. C. B. Myneni, 2010, Natural organobromine in marine sediments: New evidence of biogeochemical Br cycling: Global Biogeochemical Cycles, v. 24.

Leri, A. C., and S. C. B. Myneni, 2012, Natural organobromine in terrestrial ecosystems: Geochimica Et Cosmochimica Acta, v. 77, p. 1-10.

Letcher, R. J., J. O. Bustnes, R. Dietz, B. M. Jenssen, E. H. Jorgensen, C. Sonne, J. Verreault, M. M. Vijayan, and G. W. Gabrielsen, 2010, Exposure and effects assessment of persistent organohalogen contaminants in arctic wildlife and fish: The Science of the total environment, v. 408, p. 2995-3043.

Lewis, T., P. D. Nichols, and T. A. McMeekin, 2000, Evaluation of extraction methods for recovery of fatty acids from lipid-producing microheterotrophs: Journal of Microbiological Methods, v. 43, p. 107-116.

Lie Ken Jie, M. S. F., and C. C. Lam, 1995a, 13C-NMR studies of polyunsaturated triacylglycerols of type AAA and mixed triacylglycerols containing saturated, acetylenic and ethylenic acyl groups: Chemistry and Physics of Lipids, v. 78, p. 1-13.

Lie Ken Jie, M. S. F., and C. C. Lam, 1995b, 13C-nuclear magnetic resonance spectroscopic studies of triacylglycerols of type AAA containing (Z)- and (E)-monoethylenic acyl groups: Chemistry and Physics of Lipids, v. 78, p. 15-27.

Lin, J.-T., and G. Q. Chen, 2010, Acylglycerols Containing Trihydroxy Fatty Acids in Castor Oil and the Regiospecific Quantification of Triacylglycerols: Journal of the American Oil Chemists Society, v. 87, p. 1371-1379.

Lin, J.-T., McKeon, T, 2005, HPLC of Acyl Lipids. (2nd): HNB Publishing, New York, NY,

Lind, P. M., B. Zethelius, and L. Lind, 2012, Circulating Levels of Phthalate Metabolites Are Associated with Prevalent Diabetes in the Elderly: Diabetes Care, v. 35, p. 1519-1524.

Lundebye, A. K., M. H. G. Berntssen, O. Lie, G. Ritchie, P. Isosaari, H. Kiviranta, and T. Vartiainen, 2004, Dietary uptake of dioxins (PCDD/PCDFs) and dioxin-like PCBs in Atlantic salmon (Salmo salar): Aquaculture Nutrition, v. 10, p. 199-207.

Lawrence, J.F., Chadha, R.K., Iverson, F., McGuire, P., Conacher, H.B.S, 1984. Brominated fatty acid distribution in tissues and fluids of rats fed brominated vegetable oils: Lipids, v. 19, p. 704‑707.

Lunde, G., Gether, J., Steinnes, E, 1976, Determination of volatility and chemical persistence of lipid-soluble halogenated organic substances in marine organisms: AMBIO, v. 5, p. 180‑182.

Lunde, G. and E. Steinnes, 1975, Presence of lipid-soluble chlorinated hydrocarbons in marine oils: Environ. Sci.Technol, v. 9, p. 155-157

Lyn Patrick, 2008, Iodine: deficiency and therapeutic considerations: Alternative Medicine Review,v. 13,p. 116-127.

Lystad, E., Hostmark, A.T., Jebens, E, 2001, Apoptotic effects of dichloro stearic and dichloro myristic acid in human hepatoma cells (HepG2): Pharmacology & Toxicology, v.89, p.85‑91.

Macrae, A. R., and R. C. Hammond, 1985, Present and future applications of lipases: Biotechnology & Genetic Engineering Reviews, v. 3, p. 193-217.

Maervoet, J., G. Vermeir, A. Covaci, N. Van Larebeke, G. Koppen, G. Schoeters, V. Nelen, W. Baeyens, P. Schepens, and M. K. Viaene, 2007, Association of thyroid hormone concentrations with levels of organochlorine compounds in cord blood of neonates: Environmental Health Perspectives, v. 115, p. 1780-1786.

Marangoni, A. G., and D. Rousseau, 1995, Engineering triacylglycerols - the role of interesterification: Trends in Food Science & Technology, v. 6, p. 329-335.

Martı´nez-Moral, M., Tena, M, 2013, Focused ultrasound solid–liquid extraction of perfluorinated compounds from sewage sludge: Talanta, v. 109, p. 197–202.

Mayer, A. M. S., and M. T. Hamann, 2005, Marine pharmacology in 2001-2002: Marine compounds with anthelmintic, antibacterial, anticoagulant, antidiabetic, antifungal, anti-inflammatory, antimalarial, antiplatelet, antiprotozoal, antituberculosis, and antiviral activities; affecting the cardiovascular, immune and nervous systems and other miscellaneous mechanisms of action: Comparative Biochemistry and Physiology C-Toxicology & Pharmacology, v. 140, p. 265-286.

Magnusson, C.D., Haraldsson, G.G, 2010, Chemoenzymatic synthesis of symmetrically structured triacylglycerols possessing short-chain fatty acids: Tetrahedron , v. 66, p. 2728‑2731.

Mbatia, B., D. Adlercreutz, P. Adlercreutz, A. Mahadhy, F. Mulaa, and B. Mattiasson, 2010, Enzymatic oil extraction and positional analysis of omega-3 fatty acids in Nile perch and salmon heads: Process Biochemistry, v. 45, p. 815-819.

McKague, A. B., and D. W. Reeve, 1991, CHLORINATION OF LINOLEIC-ACID: Chemistry and Physics of Lipids, v. 58, p. 213-218.

McMaster, Marvin, 2008, GC/MS: a practical user's guide. 2nd.ed Published by John Wiley & Sons, Inc., Hoboken, New Jersey.

Meeker, J. D., A. Maity, S. A. Missmer, P. L. Williams, S. Mahalingaiah, S. Ehrlich, K. F. Berry, L. Altshul, M. J. Perry, D. W. Cramer, and R. Hauser, 2011, Serum concentrations of polychlorinated biphenyls in relation to in vitro fertilization outcomes: Environmental Health Perspectives, v. 119, p. 1010-1016.

Meerts, I., Y. Assink, P. H. Cenijn, J. H. J. van den Berg, B. M. Weijers, A. Bergman, J. H. Koeman, and A. Brouwer, 2002, Placental transfer of a hydroxylated polychlorinated biphenyl and effects on fetal and maternal thyroid hormone homeostasis in the rat: Toxicological Sciences, v. 68, p. 361-371.

Megdal, P. A., N. A. Craft, and G. J. Handelman, 2009a, A Simplified Method to Distinguish Farmed (Salmo salar) from Wild Salmon: Fatty Acid Ratios Versus Astaxanthin Chiral Isomers: Lipids, v. 44, p. 569-576.

Megdal, P. A., N. A. Craft, and G. J. Handelman, 2009b, A Simplified Method to Distinguish Farmed (Salmo salar) from Wild Salmon: Fatty Acid Ratios Versus Astaxanthin Chiral Isomers: Lipids, v. 44, p. 569-576.

Menditto, A., and L. Turrio-Baldassarri, 1999, Environmental and biological monitoring of endocrine disrupting chemicals: Chemosphere, v. 39, p. 1301-7.

Metcalfe, L. D., A. A. Schmitz, and J. R. Pelka, 1966, RAPID PREPARATION OF FATTY ACID ESTERS FROM LIPIDS FOR GAS CHROMATOGRAPHIC ANALYSIS: Analytical Chemistry, v. 38, p. 514-&.

Miller, M. R., P. D. Nichols, and C. G. Carter, 2007, Replacement of dietary fish oil for Atlantic salmon parr (Salmo salar L.) with a stearidonic acid containing oil has no effect on omega-3 long-chain polyunsaturated fatty acid concentrations: Comparative Biochemistry and Physiology B-Biochemistry & Molecular Biology, v. 146, p. 197-206.

Milley, J. E., R. K. Boyd, J. M. Curtis, C. Musial, and J. F. Uthe, 1997, Dichloromyristic acid, a major component of organochlorine load in lobster digestive gland: Environmental Science & Technology, v. 31, p. 535-541.

Mizukawa, H., K. Nomiyama, S. Nakatsu, H. Iwata, J. Yoo, A. Kubota, M. Yamamoto, M. Ishizuka, Y. Ikenaka, S. M. M. Nakayama, T. Kunisue, and S. Tanabe, 2016, Organohalogen compounds in pet dog and cat: do pets biotransform natural brominated products in food to harmful hydroxlated substances?: Environmental Science & Technology, v. 50, p. 444-452.

Mottran H., Evershed R, 1996, Structure analysis of triacylglycerol positional isomers using atmospheric pressure chemical ionization mass spectrometry: Tetrahedron Letters, v. 37, p. 8593–8596.

Momchilova, S. M., and B. M. Nikolova-Damyanova, 2012, Advances in Silver Ion Chromatography for the Analysis of Fatty Acids and Triacylglycerols-2001 to 2011: Analytical Sciences, v. 28, p. 837-844.

Monde, K., H. Satoh, M. Nakamura, M. Tamura, and M. Takasugi, 1998, Organochlorine compounds from a terrestrial higher plant: Structures and origin of chlorinated orcinol derivatives from diseased bulbs of Lilium maximowiczii: Journal of Natural Products, v. 61, p. 913-921.

Mondello, L., P. Q. Tranchida, P. Dugo, and G. Dugo, 2006, Rapid, micro-scale preparation and very fast gas chromatographic separation of cod liver oil fatty acid methyl esters: Journal of Pharmaceutical and Biomedical Analysis, v. 41, p. 1566-1570.

Mondello, L., P. Q. Tranchida, V. Stanek, P. Jandera, G. Dugo, and P. Dugo, 2005, Silver-ion reversed-phase comprehensive two-dimensional liquid chromatography combined with mass spectrometric detection in lipidic food analysis: Journal of Chromatography A, v. 1086, p. 91-98.

Montory, M., and R. Barra, 2006, Preliminary data on polybrominated diphenyl ethers (PBDEs) in farmed fish tissues (Salmo salar) and fish feed in Southern Chile: Chemosphere, v. 63, p. 1252-1260.

Montory, M., E. Habit, P. Bahamonde, P. Fernandez, J. O. Grimalt, K. Saez, I. Rudolph, and R. Barra, 2011, Polychlorinated biphenyls in farmed and wild Onchorhynchus kisutch and Onchorhynchus mykiss from the Chilean Patagonia: Environmental Science and Pollution Research, v. 18, p. 629-637.

Morris, L. J., 1966, Separations of lipids by silver ion chromatography: Journal of Lipid Research, v. 7, p. 717-&.

Mu, H. L., G. Ewald, E. Nilsson, P. Sundin, and C. Wesen, 2004, Fate of chlorinated fatty acids in migrating sockeye salmon and their transfer to arctic grayling: Environmental Science & Technology, v. 38, p. 5548-5554.

Mu, H. L., P. Sundin, and C. Wesen, 1997a, Halogenated fatty acids .2. Methods of determination in lipids: Trac-Trends in Analytical Chemistry, v. 16, p. 274-286.

Mu, H. L., C. Wesen, T. Novak, P. Sundin, J. Skramstad, and G. Odham, 1996a, Enrichment of chlorinated fatty acids in fish lipids prior to analysis by capillary gas chromatography with electrolytic conductivity detection and mass spectrometry: Journal of Chromatography A, v. 731, p. 225-236.

Mu, H. L., C. Wesen, I. Odenbrand, and K. G. Wahlund, 1999, Response factors of organochlorine compounds in the electrolytic conductivity detector: Journal of Chromatography A, v. 849, p. 285-292.

Mu, H. L., C. Wesen, and P. Sundin, 1997b, Halogenated fatty acids .1. Formation and occurrence in lipids: Trac-Trends in Analytical Chemistry, v. 16, p. 266-274.

Mu, H. L., C. Wesen, P. Sundin, and E. Nilsson, 1996b, Gas chromatographic and mass spectrometric identification of tetrachloroalkanoic and dichloroalkenoic acids in eel lipids: Journal of Mass Spectrometry, v. 31, p. 517-526.

Munro, M. H. G., J. W. Blunt, E. J. Dumdei, S. J. H. Hickford, R. E. Lill, S. X. Li, C. N. Battershill, and A. R. Duckworth, 1999, The discovery and development of marine compounds with pharmaceutical potential: Journal of Biotechnology, v. 70, p. 15-25.

Murata, T., 1977, Analysis of triglycerides by gas chromatography chemical ionization mass-spectrometry: Analytical Chemistry, v. 49, p. 2209-2213.

Murata, T., and Takahash.S, 1973, Analysis of triglyceride mixtures by gas-chromatography - mass-spectrometry: Analytical Chemistry, v. 45, p. 1816-1823.

Neff, W. E., and W. C. Byrdwell, 1998, Characterization of model triacylglycerol (triolein, trilinolein and trilinolenin) autoxidation products via high-performance liquid chromatography coupled with atmospheric pressure chemical ionization mass spectrometry: Journal of Chromatography A, v. 818, p. 169-186.

Neff, W. E., G. R. List, and W. C. Byrdwell, 1999, Quantitative composition of high palmitic and stearic acid soybean oil triacylglycerols by reversed phase high performance liquid chromatography: Utilization of evaporative light scattering and flame ionization detectors: Journal of Liquid Chromatography & Related Technologies, v. 22, p. 1649-1662.

Newman, D. J., and G. M. Cragg, 2004, Marine natural products and related compounds in clinical and advanced preclinical trials: Journal of Natural Products, v. 67, p. 1216-1238.

Newsome, W.H., Andrews, P., Conacher, H.B.S., Rao, R.R., Chatt, A, 1993, Total

organochlorine content of fish from the great lakes: Journal of the Association of Analytical Communities International , v. 76, p. 703‐706.

Nilsson, G. A., O. Nilsson, I. Odenbrand, and C. Wesen, 2001, New halogen-specific detector applied to the analysis of chlorinated fatty acids: Journal of Chromatography A, v. 912, p. 99-106.

Nishizaki, Y., K. Shimada, S. Tani, T. Ogawa, J. Ando, M. Takahashi, M. Yamamoto, T. Shinozaki, K. Miyauchi, K. Nagao, A. Hirayama, M. Yoshimura, I. Komuro, R. Nagai, and H. Daida, 2014, Significance of Imbalance in the Ratio of Serum n-3 to n-6 Polyunsaturated Fatty Acids in Patients With Acute Coronary Syndrome: American Journal of Cardiology, v. 113, p. 441-445.

Nostbakken, O. J., H. T. Hove, A. Duinker, A.-K. Lundebye, M. H. G. Berntssen, R. Hannisdal, B. T. Lunestad, A. Maage, L. Madsen, B. E. Torstensen, and K. Julshamn, 2015, Contaminant levels in Norwegian farmed Atlantic salmon (Salmo salar) in the 13-year period from 1999 to 2011: Environment International, v. 74, p. 274-280.

Nostbakken, O. J., and A. Maage, 2015, Reply to the comments to our paper "Contaminant levels in Norwegian farmed Atlantic salmon (Salmo salar) in the 13-year period from 1999 to 2011": Environment International, v. 80, p. 100-101.

Ofstad, E. B., G. Lunde, and H. Drangsholt, 1979, Chlorinated organic-compounds in the fatty surface-film on water: International Journal of Environmental Analytical Chemistry, v. 6, p. 119-131.

Oros, G., Cserháti, T., Szőgyi, M, 2012, Chromatography of xenobiotics in biological and environmental matrices: Eur. Chem. Bull*,* v. 1, p. 81-93.

Gustafson-Svard, C., Akesson-Nilsson, G., Mattsson, M., Sundin, P., Wesen, C, 2001, Removal of xenobiotic dichlorostearic acid from phospholipids and neutral lipids in cultured human cell lines by s-oxidation and secretion of Dichloromyristic acid: Pharmacology & Toxicology, v. 89,p. 56‐64.

Pal, D., J. B. Weber, and M. R. Overcash, 1980, Fate of polychlorinated biphenyls (PCBs) in soil-plant systems: Residue reviews, v. 74, p. 45-98.

Park, H.-Y., I. Hertz-Picciotto, E. Sovcikova, A. Kocan, B. Drobna, and T. Trnovec, 2010, Neurodevelopmental toxicity of prenatal polychlorinated biphenyls (PCBs) by chemical structure and activity: a birth cohort study: Environmental Health, v. 9.

Park, Y., K. J. Albright, Z. Y. Cai, and M. W. Pariza, 2001, Comparison of methylation procedures for conjugated linoleic acid and artifact formation by commercial

(trimethylsilyl) diazomethane: Journal of Agricultural and Food Chemistry, v. 49, p. 1158-1164.

Pereira Fontenele, E. G., M. R. Alves Martins, A. R. Pinto Quidute, and R. M. Montenegro Junior, 2010, Environmental contaminants and endocrine disruptors: Arquivos Brasileiros De Endocrinologia E Metabologia, v. 54, p. 6-16.

Pernilla, C, 2013, Selective processes for bioaccumulative up-take of persistent organic pollutants (POPs) in Arctic food webs: Faculty of Medicine, Department Of Pharmacy, and University of Tromsø

Pflieger-Bruss, S., and W. B. Schill, 2000, Effects of chlorinated hydrocarbons on sperm function in vitro: Andrologia, v. 32, p. 311-5.

Porta, M., M. Gasull, E. Puigdomenech, M. Gari, M. Bosch de Basea, M. Guillen, T. Lopez, E. Bigas, J. Pumarega, X. Llebaria, J. O. Grimalt, and R. Tresserras, 2010, Distribution of blood concentrations of persistent organic pollutants in a representative sample of the population of Catalonia: Environment International, v. 36, p. 655-664.

Poster, D. L., J. R. Kucklick, M. M. Schantz, B. J. Porter, S. D. Leigh, and S. A. Wise, 2003, Determination of polychlorinated biphenyl congeners and chlorinated pesticides in a fish tissue standard reference material: Analytical and Bioanalytical Chemistry, v. 375, p. 223-241.

Provencio, M., A. Sanchez, J. Gasent, P. Gomez, and R. Rosell, 2009, Cancer Treatments: Can We Find Treasures at the Bottom of the Sea?: Clinical Lung Cancer, v. 10, p. 295-300.

Ramaley, L., Herrera, L.C., 2008. Software for the calculation of isotope patterns in tandem mass spectrometry: Rapid Communications in Mass Spectrometry , v. 22, p. 2707‐2714.

Rahman, F., K. H. Langford, M. D. Scrimshaw, and J. N. Lester, 2001, Polybrominated diphenyl ether (PBDE) flame retardants: Science of the Total Environment, v. 275, p. 1-17.

Reddy, C. M., L. Xu, T. I. Eglinton, J. P. Boon, and D. J. Faulkner, 2002, Radiocarbon content of synthetic and natural semi-volatile halogenated organic compounds: Environmental Pollution, v. 120, p. 163-168.

Reddy, C. M., L. Xu, G. W. O'Neil, R. K. Nelson, T. I. Eglinton, D. J. Faulkner, R. Norstrom, P. S. Ross, and S. A. Tittlemier, 2004, Radiocarbon evidence for a naturally produced, bioaccumulating halogenated organic compound: Environmental Science & Technology, v. 38, p. 1992-1997.

Reiner, E. J., A. R. Boden, T. Chen, K. A. MacPherson, and A. M. Muscalu, 2010, Advances in the Analysis of Persistent Halogenated Organic Compounds: Lc Gc Europe, v. 23, p. 60.

Rezaei, F., Bidari, A., Birjandi, A., Hosseini,M., Assadi, Y, 2008, Development of a dispersive liquid–liquid microextraction method for the determination of polychlorinated biphenyls in water: Journal of Hazardous Materials, v. 158, p. 621–627.

Richard, F., Spande, T., Garraffo, M., Yeh, C., John, W, 2010, Phantasmidine: an epibatidine congener from the Ecuadorian poison frog *epipedobates anthonyl*: J Nat Prod, v. 73, p. 331–337.

Rodgers, M.T., Armentrout, P.B, 2000, Noncovalent metal‐ligand bond energies as studied by threshold collision-induced dissociation: Mass Spectrometry Reviews, v. 19, p. 215‐247.

Ruiz-Samblas, C., Cuadros-Rodriguez, L., Gonzalez-Casadoa, A., Rodriguez-Garcia, F.P, 2012, A straightforward quantification of triacylglycerols (and fatty acids) in monovarietal extra virgin olive oils by high-temperature GC: Analytical Methods, v.4, p. 753‐758.

Ruiz-Lopez, N., I. Stubhaug, I. Ipharraguerre, G. Rimbach, and D. Menoyo, 2015, Positional Distribution of Fatty Acids in Triacylglycerols and Phospholipids from Fillets of Atlantic salmon (Salmo Salar) Fed Vegetable and Fish Oil Blends: Marine Drugs, v. 13, p. 4255-4269.

Ruiz-Rodriguez, A., G. Reglero, and E. Ibanez, 2010, Recent trends in the advanced analysis of bioactive fatty acids: Journal of Pharmaceutical and Biomedical Analysis, v. 51, p. 305-326.

Ruiz-Samblas, C., F. Marini, L. Cuadros-Rodriguez, and A. Gonzalez-Casado, 2012, Quantification of blending of olive oils and edible vegetable oils by triacylglycerol fingerprint gas chromatography and chemometric tools: Journal of Chromatography B-Analytical Technologies in the Biomedical and Life Sciences, v. 910, p. 71-77.

Ruxton, C. H. S., S. C. Reed, M. J. A. Simpson, and K. J. Millington, 2004, The health benefits of omega-3 polyunsaturated fatty acids: a review of the evidence: Journal of Human Nutrition and Dietetics, v. 17, p. 449-459.

Ruzzin, J., C. Bethune, A. Goksoyr, K. Hylland, D. H. Lee, D. R. Jacobs, Jr., and D. O. Carpenter, 2015, Comment on "Contaminant levels in Norwegian farmed Atlantic salmon (Salmo salar) in the 13-year period from 1999 to 2011" by Nostbakken et al: Environment International, v. 80, p. 98-99.

Ruzzin, J., R. Petersen, E. Meugnier, L. Madsen, E.-J. Lock, H. Lillefosse, T. Ma, S. Pesenti, S. B. Sonne, T. T. Marstrand, M. K. Malde, Z.-Y. Du, C. Chavey, L. Fajas, A.-K. Lundebye, C. L. Brand, H. Vidal, K. Kristiansen, and L. Froyland, 2010, Persistent Organic Pollutant Exposure Leads to Insulin Resistance Syndrome: Environmental Health Perspectives, v. 118, p. 465-471.

Rylander, C., T. M. Sandanger, and M. Brustad, 2009, Associations between marine food consumption and plasma concentrations of POPs in a Norwegian coastal population: Journal of Environmental Monitoring, v. 11, p. 370-376.

Rytczak, P., Koziołkiewicz, M., Okruszek, A, 2010, The chemical synthesis of phosphorothioate and phosphorodithioate analogues of lysophosphatidic acid (LPA) and cyclic phosphatidic acid (CPA): New Journal of Chemistry , v. 34, p. 1008–1017

Sahena, F., I. S. M. Zaidul, S. Jinap, N. Saari, H. A. Jahurul, K. A. Abbas, and N. A. Norulaini, 2009, PUFAs in Fish: Extraction, Fractionation, Importance in Health: Comprehensive Reviews in Food Science and Food Safety, v. 8, p. 59-74.

Salem, N., T. Moriguchi, R. S. Greiner, K. McBride, A. Ahmad, J. N. Catalan, and B. Slotnick, 2001, Alterations in brain function after loss of docosahexaenoate due to dietary restriction of n-3 fatty acids: Journal of Molecular Neuroscience, v. 16, p. 299-307.

Salihovic, S., H. Nilsson, J. Hagberg, and G. Lindstrom, 2013, Trends in the analysis of persistent organic pollutants (POPs) in human blood: Trac-Trends in Analytical Chemistry, v. 46, p. 129-138.

Salvadó, J., Sobek, A., Carrizo, D., Gustafsson, O, 2016, Observation-based assessment of pbde loads in arctic oceanwaters: Environ. Sci. Technol,v. 50, p. 2236−2245.

Sandanger, T. M., M. Brustad, C. D. Sandau, and E. Lund, 2006, Levels of persistent organic pollutants (POPs) in a coastal northern Norwegian population with high fish-liver intake: Journal of Environmental Monitoring, v. 8, p. 552-557.

Santos, F. J., and M. T. Galceran, 2002, The application of gas chromatography to environmental analysis: Trac-Trends in Analytical Chemistry, v. 21, p. 672-685.

Segall, S.D., Artz, W.E., Raslan, D.S., Ferraz, V.P., Takahashi, J.A, 2004, Ouricuri (Syagrus coronata) triacylglycerol analysis using HPLC and positive ion electrospray tandem MS: Journal of the American Oil Chemists' Society, v. 81, p. 143–149.

Seppanen-Laakso, T., I. Laakso, and R. Hiltunen, 2002, Analysis of fatty acids by gas chromatography, and its relevance to research on health and nutrition: Analytica Chimica Acta, v. 465, p. 39-62.

Sharma, D., A. Nagpal, Y. B. Pakade, and J. K. Katnoria, 2010, Analytical methods for estimation of organophosphorus pesticide residues in fruits and vegetables: A review: Talanta, v. 82, p. 1077-1089.

Shaw, S. D., M. L. Berger, D. Brenner, D. O. Carpenter, L. Tao, C.-S. Hong, and K. Kannan, 2008, Polybrominated diphenyl ethers (PBDEs) in farmed and wild salmon marketed in the Northeastern United States: Chemosphere, v. 71, p. 1422-1431.

Shaw, S. D., D. Brenner, M. L. Berger, D. O. Carpenter, C.-S. Hong, and K. Kannan, 2007, PCBs, PCDD/Fs, and organochlorine pesticides in Farmed Atlantic Salmon from Maine, Eastern Canada, and Norway, and Wild Salmon from Alaska (vol 40, pg 5347, 2006): Environmental Science & Technology, v. 41, p. 4180-4180.

Sipkema, D., M. C. R. Franssen, R. Osinga, J. Tramper, and R. H. Wijffels, 2005, Marine sponges as pharmacy: Marine Biotechnology, v. 7, p. 142-162.

Sinkkonen, S, 1997, PCDTs in the environment: *Chemosphere,* v. 34, p. 2585-2594,

Smith, A. J., 2004, Medicinal and pharmaceutical uses of seaweed natural products: A review: Journal of Applied Phycology, v. 16, p. 245-262.

Siuda, J., DeBernardis F, 1973, Naturally occurring halogenated organic compounds: Llojdia, v.36, p. 107.

Softeland, L., J. A. Kirwan, T. S. F. Hori, T. R. Storseth, U. Sommer, M. H. G. Berntssen, M. R. Viant, M. L. Rise, R. Waagbo, B. E. Torstensen, M. Booman, and P. A. Olsvik, 2014, Toxicological effect of single contaminants and contaminant mixtures associated with plant ingredients in novel salmon feeds: Food and Chemical Toxicology, v. 73, p. 157-174.

Sonne, C., Letcher, R., Bechshøft, T., Rigét, F, 2011, Two decades of biomonitoring polar bear health in Greenland: a review: From Environmental contaminants and animal health. The 26th Symposium of the Nordic Committee for Veterinary Scientific Cooperation (NKVet) Helsinki, Finland. 6-7 October 2011

Stuart j. Harrad Sources and Fates of Polychlorinated Dibenzo-*p*-dioxins, Dibenzofurans and Biphenyls: The Budget and Source Inventory Approach

Spickett, C.M, 2007, Chlorinated lipids and fatty acids: an emerging role in pathology. Pharmacology & Therapeutics, v. 115, p. 400‐409.

Sundin, P., Larsson, P., Wesen, C., Odham, G, 1992, Chlorinated triacylglycerols in fish lipids? Chromatographic and mass spectrometric studies of model compounds: Biological Mass Spectrometry, v. 21, p. 633‐641.

Sonne, C., 2010, Health effects from long-range transported contaminants in Arctic top predators: An integrated review based on studies of polar bears and relevant model species: Environment international, v. 36, p. 461-91.

Soumanou, M. M., M. Perignon, and P. Villeneuve, 2013, Lipase-catalyzed interesterification reactions for human milk fat substitutes production: A review: European Journal of Lipid Science and Technology, v. 115, p. 270-285.

Sprague, M., E. A. Bendiksen, J. R. Dick, F. Strachan, J. Pratoomyot, M. H. G. Berntssen, D. R. Tocher, and J. G. Bell, 2010, Effects of decontaminated fish oil or a fish and vegetable oil blend on persistent organic pollutant and fatty acid compositions in diet and flesh of Atlantic salmon (Salmo solar): British Journal of Nutrition, v. 103, p. 1442-1451.

Standal, I. B., D. E. Axelson, and M. Aursand, 2009, Differentiation of Fish Oils According to Species by C-13-NMR Regiospecific Analyses of Triacyglycerols: Journal of the American Oil Chemists Society, v. 86, p. 401-407.

Steenland, K., P. Bertazzi, A. Baccarelli, and M. Kogevinas, 2004, Dioxin revisited: Developments since the 1997 IARC classification of dioxin as a human carcinogen: Environmental Health Perspectives, v. 112, p. 1265-1268.

Stern, N., M. Oberg, H. Casabona, C. Trossvik, E. Manzoor, N. Johansson, M. Lind, J. Orberg, R. Feinstein, A. Johansson, I. Chu, R. Poon, A. Yagminas, A. Brouwer, B. Jones, and H. Hakansson, 2002, Subchronic toxicity of Baltic herring oil and its fractions in the rat II: Clinical observations and toxicological parameters: Pharmacology & Toxicology, v. 91, p. 232-244.

Suarez, E. R., P. F. Mugford, A. J. Rolle, I. W. Burton, J. A. Walter, and J. A. Kralovec, 2010, C-13-NMR Regioisomeric Analysis of EPA and DHA in Fish Oil Derived Triacylglycerol Concentrates: Journal of the American Oil Chemists Society, v. 87, p. 1425-1433.

Sundin, P., P. Larsson, C. Wesen, and G. Odham, 1992, Chlorinated triacylglycerols in fish lipids - chromatographic and mass-spectrometric studies of model compounds: Biological Mass Spectrometry, v. 21, p. 633-641.

Suwalsky, M., C. Rodriguez, F. Villena, F. Aguilar, and C. P. Sotomayor, 1998, The organochlorine pesticide lindane interacts with the human erythrocyte membrane: Pesticide Biochemistry and Physiology, v. 62, p. 87-95.

Svendsen, T. C., K. Vorkamp, B. Ronsholdt, and J.-O. Frier, 2008a, Retrospective determination of primary feeding areas of Atlantic salmon (Salmo salar) using fingerprinting of chlorinated organic contaminants: Ices Journal of Marine Science, v. 65, p. 921-929.

Svendsen, T. C., K. Vorkamp, B. Ronsholdt, and J.-O. Frier, 2008b, Retrospective determination of primary feeding areas of Atlantic salmon (Salmo salar) using fingerprinting of chlorinated organic contaminants: Ices Journal of Marine Science, v. 65, p. 921-929.

Tadeo, J. L., R. Ana Perez, B. Albero, A. I. Garcia-Valcarcel, and C. Sanchez-Brunete, 2012, Review of Sample Preparation Techniques for the Analysis of Pesticide Residues in Soil: Journal of Aoac International, v. 95, p. 1258-1271.

Tan, Y. S., C. H. Chen, D. Lawrence, and D. O. Carpenter, 2004, Ortho-substituted PCBs kill cells by altering membrane structure: Toxicological Sciences, v. 80, p. 54-59.

Tang, H. P.-o., 2013, Recent development in analysis of persistent organic pollutants under the Stockholm Convention: Trac-Trends in Analytical Chemistry, v. 45, p. 48-66.

Tankiewicz, M., J. Fenik, and M. Biziuk, 2010, Determination of organophosphorus and organonitrogen pesticides in water samples: Trac-Trends in Analytical Chemistry, v. 29, p. 1050-1063.

Tarvainen, M., A. Nuora, K.-W. Quirin, H. Kallio, and B. Yang, 2015, Effects of CO2 plant extracts on triacylglycerol oxidation in Atlantic salmon during cooking and storage: Food Chemistry, v. 173, p. 1011-1021.

Taylor, K. W., R. F. Novak, H. A. Anderson, L. S. Birnbaum, C. Blystone, M. DeVito, D. Jacobs, J. Koehrle, D.-H. Lee, L. Rylander, A. Rignell-Hydbom, R. Tornero-Velez, M. E. Turyk, A. L. Boyles, K. A. Thayer, and L. Lind, 2013, Evaluation of the Association between Persistent Organic Pollutants (POPs) and Diabetes in Epidemiological Studies: A National Toxicology Program Workshop Review: Environmental Health Perspectives, v. 121, p. 774-783.

Teuten, E. L., G. M. King, and C. M. Reddy, 2006, Natural C-14 in Saccoglossus bromophenolosus compared to C-14 in surrounding sediments: Marine Ecology Progress Series, v. 324, p. 167-172.

Tinsley, I.J., Lowry, R.R, 1980, Bromine content of lipids of marine organisms: Journal of the American Oil Chemists' Society , v. 57, p. 31–33.

Thurnhofer, S., Lehnert, K., Vetter, W, 2008, Exclusive quantification of methylbranched fatty acids and minor 18:1-isomers in foodstuffs by GC/MS in the SIM mode using 10,11-dichloroundecanoic acid and fatty acid ethyl esters as internal standards: European Food Research and Technology , v. 226, p. 975–983.

Toledo-Neira, C., Enríquezb,P., Richte. P, 2013, Integrated pressurized solvent extraction-cleanup for the rapid determination of polychlorinated biphenyls in meat samples: J. Braz. Chem. Soc, v. 24, p .743-748.

Torstensen, B. E., M. Espe, M. Sanden, I. Stubhaug, R. Waagbo, G. I. Hemre, R. Fontanillas, U. Nordgarden, E. M. Hevroy, P. Olsvik, and M. H. G. Berntssen, 2008, Novel production of Atlantic salmon (Salmo salar) protein based on combined replacement of fish meal and fish oil with plant meal and vegetable oil blends: Aquaculture, v. 285, p. 193-200.

Tyl, C. E., L. Brecker, and K.-H. Wagner, 2008, H-1 NMR spectroscopy as tool to follow changes in the fatty acids of fish oils: European Journal of Lipid Science and Technology, v. 110, p. 141-148.

Ucan-Marin, F., A. Arukwe, A. S. Mortensen, G. W. Gabrielsen, and R. J. Letcher, 2010, Recombinant Albumin and Transthyretin Transport Proteins from Two Gull Species and Human: Chlorinated and Brominated Contaminant Binding and Thyroid Hormones: Environmental Science & Technology, v. 44, p. 497-504.

van der Klift, E. J. C., G. Vivo-Truyols, F. W. Claassen, F. L. van Holthoon, and T. A. van Beek, 2008, Comprehensive two-dimensional liquid chromatography with ultraviolet, evaporative light scattering and mass spectrometric detection of triacylglycerols in corn oil: Journal of Chromatography A, v. 1178, p. 43-55.

van Leeuwen, S. P. J., and J. de Boer, 2008, Advances in the gas chromatographic determination of persistent organic pollutants in the aquatic environment: Journal of Chromatography A, v. 1186, p. 161-182.

Vereskuns, G., C. Wesen, K. Skog, and M. Jagerstad, 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-Genetic Toxicology and Environmental Mutagenesis, v. 416, p. 149-157.

Vetter, W., and G. W. Gribble, 2007, Anthropogenic persistent organic pollutants-lessons to learn from halogenated natural products: Environmental Toxicology and Chemistry, v. 26, p. 2249-2252.

Vetter, W., and D. Janussen, 2005, Halogenated natural products in five species of antarctic sponges: Compounds with POP-like properties?: Environmental Science & Technology, v. 39, p. 3889-3895.

Vetter, W., E. Stoll, M. J. Garson, S. J. Fahey, C. Gaus, and J. F. Muller, 2002, Sponge halogenated natural products found at parts-per-million levels in marine mammals: Environmental Toxicology and Chemistry, v. 21, p. 2014-2019.

von Schacky, C., 2009, Cardiovascular disease prevention and treatment: Prostaglandins Leukotrienes and Essential Fatty Acids, v. 81, p. 193-198.

Vonderheide, A. P., 2009, A review of the challenges in the chemical analysis of the polybrominated diphenyl ethers: Microchemical Journal, v. 92, p. 49-57.

Wagner, L., S. Trattner, J. Pickova, P. Gomez-Requeni, and A. A. Moazzami, 2014, H-1 NMR-based metabolomics studies on the effect of sesamin in Atlantic salmon (Salmo salar): Food Chemistry, v. 147, p. 98-105.

Warner, M., B. Eskenazi, P. Mocarelli, P. Gerthoux, S. Samuels, L. Needham, D. Patterson, and P. Brambilla, 2002, Serum dioxin concentrations and breast cancer risk in the Seveso Women's Health Study: American Journal of Epidemiology, v. 155, p. s102-s102.

Warner, M., P. Mocarelli, S. Samuels, L. Needham, P. Brambilla, and B. Eskenazi, 2011, Dioxin Exposure and Cancer Risk in the Seveso Women's Health Study: Environmental Health Perspectives, v. 119, p. 1700-1705.

Wan, Y., Jones, P.D., Wiseman, S., Chang, H., Chorney, D., Kannan, K., Zhang, K., Hu, J.-Y., Khim, J.S., Tanabe, S., Lam, M.H.W., Giesy, J.P, 2010,Contribution of synthetic and naturally occurring organobromine compounds to bromine mass in marine organisms: Environmental Science & Technology , v. 44, p. 6068‑6073.

Wesen, C., Carlberg, G.E., Martinsen, K, 1990, On the identity of chlorinated organic substances in aquatic organisms and sediments: AMBIO , v. 19, p. 36‑38.

Wei, F., N. Hu, X. Lv, X.-Y. Dong, and H. Chen, 2015, Quantitation of triacylglycerols in edible oils by off-line comprehensive two-dimensional liquid chromatography-atmospheric pressure chemical ionization mass spectrometry using a single column: Journal of Chromatography A, v. 1404, p. 60-71.

Wesen, C., H. L. Mu, A. L. Kvernheim, and P. Larsson, 1992, identification of chlorinated fatty-acids in fish lipids by partitioning studies and by gas-chromatography with hall electrolytic conductivity detection: Journal of Chromatography, v. 625, p. 257-269.

Wesen, C., H. L. Mu, P. Sundin, P. Froyen, J. Skramstad, and G. Odham, 1995, Gas-chromatographic mass-spectrometric identification of chlorinated octadecanoic acids in eel lipids: Journal of Mass Spectrometry, v. 30, p. 959-968.

Wright, A. D., H. Q. Wang, M. Gurrath, G. M. Konig, G. Kocak, G. Neumann, P. Loria, M. Foley, and L. Tilley, 2001, Inhibition of heme detoxification processes underlies the antimalarial activity of terpene isonitrile compounds from marine sponges: Journal of Medicinal Chemistry, v. 44, p. 873-885.

Xia, Y., and R. C. Larock, 2010, Vegetable oil-based polymeric materials: synthesis, properties, and applications: Green Chemistry, v. 12, p. 1893-1909.

Xu, X. B., 2000, Production of specific-structured triacylglycerols by lipase-catalyzed reactions: a review: European Journal of Lipid Science and Technology, v. 102, p. 287-303.

Yagi, S., K.-i. Aihara, D. Fukuda, A. Takashima, M. Bando, T. Hara, S. Nishimoto, T. Ise, K. Kusunose, K. Yamaguchi, T. Tobiume, T. Iwase, H. Yamada, T. Soeki, T. Wakatsuki, M. Shimabukuro, M. Akaike, and M. Sata, 2015, Reduced ratio of eicosapentaenoic acid and docosahexaenoic acid to arachidonic acid is associated with early onset of acute coronary syndrom: Nutrition Journal, v. 14, P. 111.

Yunker, M. B., M. G. Ikonomou, P. J. Sather, E. N. Friesen, D. A. Higgs, and C. Dubetz, 2011, Development and Validation of Protocols To Differentiate PCB Patterns between Farmed and Wild Salmon: Environmental Science & Technology, v. 45, p. 2107-2115.

Zeliger, H, 2011, Human Toxicology of Chemical Mixtures Toxic Consequences Beyond the Impact of One-Component Product and Environmental Exposures, Second Edition

Zeb, A., 2015, Chemistry and liquid chromatography methods for the analyses of primary oxidation products of triacylglycerols: Free Radical Research, v. 49, p. 549-564.

Zeb, A., and M. Murkovic, 2010, Analysis of triacylglycerols in refined edible oils by isocratic HPLC-ESI-MS: European Journal of Lipid Science and Technology, v. 112, p. 844-851.

Zeb, A., and M. Murkovic, 2013, Determination of thermal oxidation and oxidation products of beta-carotene in corn oil triacylglycerols: Food Research International, v. 50, p. 534-544.

Zeng, S., Cao, Yu,. Sang,W., Li, T., Gan,T.,Zheng, L, 2012, Enrichment of polychlorinated biphenyls from aqueous solutions using fe_3o_4 grafted multiwalled carbon nanotubes with poly dimethyl diallyl ammonium chloride: Int. J. Mol. Sci, v. 13, p. 6382-63

Zeng, Y., P. Araujo, B. Grung, and L. Zhang, 2011, Evaluation of different fingerprinting strategies for differentiating marine oils by liquid chromatography ion-trap mass spectrometry and chemometrics: Analyst, v. 136, p. 1507-1514.

Zhang, X. R., R. A. Minear, Y. B. Guo, C. J. Hwang, S. E. Barrett, K. Ikeda, Y. Shimizu, and S. Matsui, 2004, An electrospray ionization-tandem mass spectrometry method for identifying chlorinated drinking water disinfection byproducts: Water Research, v. 38, p. 3920-3930.

Zhao, J., Yin. P., Zhao, L., Yu, Q., Lu, G, 2015, Spatial and Seasonal Variations of Total Petroleum Hydrocarbon in Surface Water and Sediment in Pearl River Delta: Bull Environ Contam Toxicol, v. 95, p. 298–303:

Zhao,Z., Li,H., Wang,Y., Li, G, 2013, Source and migration of short-chain chlorinated paraffins in the coastal east china sea using multiproxies of marine organic geochemistry: Environ. Sci. Technol, v. 47,p. 5013−5022

Zhuang, W., A. B. McKague, D. W. Reeve, and J. Carey, 2006, Characterization and sources of extractable organochlorine in white sucker downstream from bleached kraft pulp mills: International Journal of Environmental Analytical Chemistry, v. 86, p. 867-887.

Zhuang, W. S., A. B. McKague, D. W. Reeve, and J. H. Carey, 2004a, Identification of chlorinated fatty acids in fish by gas chromatography/mass spectrometry with negative ion chemical ionization of pentafluorobenzyl esters: Journal of Mass Spectrometry, v. 39, p. 51-60.

Zhuang, W. S., A. B. McKague, D. W. Reeve, and J. H. Carey, 2005, Evaluation of halogen-specific detector (XSD (TM)) for trace analysis of chlorinated fatty acids in fish: Instrumentation Science & Technology, v. 33, p. 481-507.

Zhuang, W. S., B. McKague, J. Carey, and D. Reeve, 2003a, Enrichment of trace chlorinated species in a complex matrix of fatty acids using HPLC in conjunction with gas chromatography-halogen specific detection: Journal of Liquid Chromatography & Related Technologies, v. 26, p. 1809-1826.

Zhuang, W. S., B. McKague, and D. Reeve, 2004b, Mass spectrometric elucidation of chlorine location in dichloro fatty acids following 4,4-dimethyloxazoline derivatization, and its application to chlorinated fatty acids in fish: International Journal of Mass Spectrometry, v. 232, p. 127-137.

Zhuang, W. S., B. McKague, D. Reeve, and J. Carey, 2003b, Identification and confirmation of traces of chlorinated fatty acids in fish downstream of bleached kraft pulp mills by gas chromatography with halogen-specific detection: Journal of Chromatography A, v. 994, p. 137-157.

Zhuang, W. S., B. McKague, D. Reeve, and J. Carey, 2004c, A comparative evaluation of accelerated solvent extraction and polytron extraction for quantification of lipids and extractable organochlorine in fish: Chemosphere, v. 54, p. 467-480.

Appendix 1

Table 1. Peak identification of salmon tissue oil TAGs using 2D off-line RP-HPLC/ESI(+)/MS

Fraction #	[M+Na]	MS/MS $[M+Na-RCOOH]^+$ $[M+Na-RCOO-Na]^+$	Identification TAG	PN	DB
1	-	-	-		
2	-	-	-		
3	841.8	593.3/C16:4 571.3/C16:4-Na 591.5/C16:3 569.3/C16:3-Na 589.4/C16:2 567.3/C16:2-Na 563.5/C18:3 541.3/C18:3-Na 561.2/C18:2 539.3/C18:2-Na	TAG-C18:2/C16:4/C16:3 TAG-C16:2/C18:3/C16:3	32 34	9 9
	855.9	605.7/C16:3 583.2/C16:3-Na 599.3/C16:0 577.3/C16:0-Na 573.2/C18:2 550.3/C18:2-Na	TAG-C16:0/C16:0/C18:2	2	48
	865.9	636.5/C14:0 617.3/C16:4 595.6/C16:4-Na 611.2/C16:1 589.3/C16:1-Na 609.6/C16:0 563.4/C20:5 541.3/C20:5-Na	TAG-C16:4/C16:4/C22:5	28	13
	917.8	669.5/C16:4 647.2/C16:4-Na 643.5/C18:4 621.3/C18:4-Na 637.5/C18:2 615.5/C18:2-Na 635.2/C18:1 614.8/C18:1-Na 589.4/C22:6 567.3/C22:6-Na	TAG-C22:6-C18:4-C16:4	28	14
	943.7	665.3/C18:3 643.9/C18:3-Na 641.3/ C20:5 619.6/C20:5-Na	TAG-2C20:5/C18:3	32	13

Fraction #	[M+Na]	MS/MS $[M+Na-RCOOH]^+$ $[M+Na-RCOO-Na]^+$	Identification TAG	PN	DB
	967.7	665.3/C20:5 643.4/C20:5-Na	TAG-3C20:5	30	15
	993.8	717.3/C18:4 694.8/C18:4-Na 691.4/C20:5 669.0/C20:5-Na 665.2/C22:6 643.2/C22:6-Na	TAG-2C20:5/C22:6	30	16
4	997.7	717.3/C18:2 695.4/C18:2-Na 669.3/C22:6 647.5/C22:6-Na	TAG-2C22:6/C18:2	34	14
	893.7	645.6/C16:4 623.0/C16:4-Na 643.4/C16:2 621.3/C16:2-Na 639.2/C16:1 617.5/C16:1-Na 611.2/C18:1 589.3/C18:1-Na 591.2/C20:5 569.4/C20:5-Na	TAG- C20:5/C18:1/C16:4	34	10
	869.4				
	971.7	691.4/C18:2 669.1/C18:2-Na 669.4/C20:5 647.4/C20:5-Na 643.3/C22:6 621.3/C22:6-Na	TAG- C18:2/C20:5/C22:6	34	13
	919.6	665.4/C16:1 643.3/C16:1-Na 617.4/C20:5 595.6/C20:5-Na	TAG-2C20:5/C16:1	34	11
5					
	843.4	595.5/C16:4 573.4/C16:4-Na 589.3/C16:2 567.4/C16:2-Na 563.3/C20:5 541.1/C20:5-Na			
	921.8		TAG- C22:6/C18:2/C16:2 TAG- C22:6/C18:3/C16:1	36	10

Fraction #	[M+Na]	MS/MS $[M+Na-RCOOH]^+$ $[M+Na-RCOO-Na]^+$	Identification TAG	PN	DB
	895.7	645.6/C16:3 623.0/C16:3-Na 643.4/C16:2 621.3/C16:2-Na 639.2/C16:0 617.5/C16:0-Na 613.2/C18:1 590.8.3/C18:1-Na 593.2/C20:5 571.4/C20:5-Na	TAG- C20:5/C18:1/C16:4	34	10
	973.7	693.4/C18:2 671.3/C18:2-Na 670.9/C20:5 649.4/C20:5-Na 643.3/C22:5 621.3/C22:5-Na	TAG- C18:2/C20:5/C22:5	36	12
6	923.6	699.4/C16:1 647.3/C16:1-Na 643.4/C18:2 621.3/C18:2-Na 621.5/C20:5 599.4/C20:5-Na 595.3/C22:6 573.3/C22:6-Na	TAG- C16:1/C18:2/C22:6 TAG-2C18:2/C20:5	38	9
	973.6	691.4/C18:1 668.9/C18:1-Na 671.3/C20:5 649.5/C20:5-Na 645.3/C22:6 623.1/C22:6-Na	TAG- C18:1/C20:5/C22:6	36	12
	897.7	643.3/C16:1 621.3/C16:1-Na 617.4/C18:2 595.4/C18:2-Na 595.0/C20:5 573.3/C20:5-Na	TAG- C16:1/C18:2/C20:5	36	9
	949.7	669.3/C18:2 647.3/C18:2-Na 667.4/C18:1 645.1/C18:1-Na 621.4/C22:6 599.4/C22:6-Na 619.2/C22:5 597.5/C22:5-Na	TAG-2C18:2/C22:6	38	10

Fraction #	[M+Na]	MS/MS $[M+Na-RCOOH]^+$ $[M+Na-RCOO-Na]^+$	Identification TAG	PN	DB
8/7	925.9	671.3/C16:1 649.4/C16:1-Na 645.3/C18:2 623.2/C18:2-Na 623.8/C20:5 601.5/C20:5-Na 597.3/C22:6 575.2/C22:6-Na	TAG-C16:1/C18:2/C22:6 TAG-2C18:2/C20:5	38	9
	949.7	671.3/C18:3 649.3/C18:3-Na 667.3/C18:1 645.3/C18:1-Na 621.3/C22:6 599.6/C22:6-Na	TAG-C18:3/C18:1/C22:6	40	9
	977.7	697.6/C18:2 675.0/C18:2-Na 695.4/C18:1 673.2/C18:1-Na 671.2/C20:3 649.8/C20:3-Na 649.0/C22:6 627.6/C226-Na	TAG-C20:3/C22:6/C18:1	42	9
	875.7	647.3/C14:0 625.4/C14:0-Na 621.3/C16:1 599.2/C16:1-Na 595.3/C18:2 573.3/C18:2-Na 597.3/C18:3 575.6/C18:3-Na 593.3/C18:1 571.2/C18:1-Na	TAG-C18:3-C18:1-C16:1	42	5
	951.7	671.3/C18:2 649.3/C18:2-Na 669.3/C18:1 647.4/C18:1-Na 623.4/C22:6 601.3/C22:6-Na	TAG-2C18:2/C22:6	38	10
9	927.8	673.3/C16:1 651.2/C16:1-Na 647.3/ C18:2 625.3/ C18:2-Na 645.3/ C18:1 623.2/ C18:1-Na 622.9/C20:4 601.5/C20:4-Na 597.4/C22:5 575.6/C22:5-Na	TAG-C16:1/C18:1/C22:5 TAG-C18:1/C18:2/C20:4	42	7

Fraction #	[M+Na]	MS/MS $[M+Na-RCOOH]^+$ $[M+Na-RCOO-Na]^+$	Identification TAG	PN	DB
	951.7	673.3/C18:3 651.3/C18:3-Na 671.3/C18:2 649.3/C18:2-Na 669.2/C18:1 647.6/C18:1-Na 623.4/C22:6 601.3/C22:6-Na	TAG- C18:2/C18:1/C22:6	40	9
	899.9	645.3/C16:1 623.4/C16:1-Na 617.3/C18:1 595.1/C18:1-Na 597.4/C20:5 575.4/C20:5-Na	TAG- C16:1/C18:1/C20:5	40	7
	847.7	597.3/C16:2 575.3/C16:2-Na 595.3/C16:1 573.1/C16:1-Na 569.3/C18:2 547.1/C18:2-Na 567.1/C18:1 545.2/C18:1-Na	TAG-2C16:2/C18:1	40	5
	901.7	625.3/C18:4 603.4/C18:4-Na 623.5/C18:3 601.5/C18:3-Na 621.4/C18:2 599.3/C18:2-Na 619.4/C18:1 597.3/C18:1-Na	TAG- C18:3/C18:2/C18:1 TAG-3C18:2 TAG/2C18:1/C18:4	42	6
	979.7	725.2/C16:1 702.4/C16:1-Na 699.5/C18:2 677.6/C18:2-Na 697.3/C18:1 675.5/C18:1-Na 673.3/C20:3 651.8/C20:3-Na 651.3/C22:6 629.8/C22:6-Na 649.5/C22:5 626.8/C22:-Na	TAG- C22:6/C20:3/C18:1	42	9
10	849.7	595.3/C16:1 573.1/C16:1-Na 569.3/C18:2 547.1/C18:2-Na 567.1/C18:1 545.2/C18:1-Na	TAG-2C16:1/C18:2	42	4

Fraction #	[M+Na]	MS/MS $[M+Na-RCOOH]^+$ $[M+Na-RCOO-Na]^+$	Identification TAG	PN	DB
	877.6	623.3/C16:1 601.5/C16:1 597.3/C18:2 575.2/C18:2-Na 595.3/C18:1 573.5/C18:1-Na	TAG- C16:1/C18:1/C18:2	44	4
	901.7	625.3/C18:4 603.4/C18:4-Na 623.5/C18:3 601.5/C18:3-Na 621.4/C18:2 599.3/C18:2-Na 619.4/C18:1 597.3/C18:1-Na	TAG- C18:3/C18:2/C18:1 TAG/2C18:1/C18:4 TAG-3C18:2	48	6
	927.6	645.3/C18:1 623.2/C18:1-Na 625.4/C20:5 603.4/ C20:5-Na	TAG-2C18:1/ C20:5	42	7
	903.5	623.4/C18:2 601.3/C18:2-Na 621.3/C18:1 599.3/C18:1-Na	TAG-2C18:2/C18:1	44	5
	953.7	673.2/C18:2 651.5/C18:2-Na 571.3/C18:1 549.5/C18:1-Na 625.3/C22:6 603.4/C22:6-Na 623.2/C22:5 601.4/C22:5-Na	TAG-2C18:1/C22:6 TAG- C18:2/C18:1/C22:5	42	8
	947.5	691.3/C16:0 669.5/C16:0-Na 645.6/C20:5 623.5/C20:5-Na 619.3/C22:6 597.1/C22:6-Na	TAG- C16:0/C22:6/C20:5	36	11
11	955.7	673.3/C18:1 651.3/C18:1-Na 625.3/C22:1 603.4/C22:1-Na	TAG-2C18:1/C22:1	50	4
	851.5	597.3/C16:1 575.5/C16:1-Na 569.4/C18:1 547.4/C18:1-Na	TAG-2C16:1/C18:1	44	3
	877.7		TAG- C16:1/C18:1/C18:2	44	4

Fraction #	[M+Na]	MS/MS $[M+Na-RCOOH]^+$ $[M+Na-RCOO-Na]^+$	Identification TAG	PN	DB
	877.8	621.3/C16:0 599.3/C16:0-Na 597.2/C18:1 575.3/C18:1-Na	TAG-2C18:2/C16:0	44	4
	851.8	623.3/C14:0 601.3/C14:0-Na 597.3/C16:1 575.5/C16:1-Na 595.4/C16:0 573.5/C16:0-Na 571.3/C18:2 549.4/C18:2-Na 569.4/C18:1 547.4/C18:1-Na	TAG-C18:1/C18:2/C14:0 TAG-C16:0/C16:1/C18:2	44	3
	901.8	645.4/C16:0 623.1/C16:0-Na 619.2/C18:1 596.9/C18:1-Na 599.4/C20:5 577.2/C20:5-Na	TAG-C16:0/C18:1/C20:5	42	6
	933.7	679.3/C16:1 657.4/C16:1-Na 653.3/C18:2 631.4/C18:2-Na 651.5/C18:1 629.5/C18:1-Na 625.3/C20:2 603.3/C20:2-Na 623.4/C20:1 601.1/C20:1-Na 595.3/C22:1 573.5/C22:1-Na	TAG-2C18:1/C20:2 TAG-C18:2/C18:1/C20:1 TAG-C16:1/C20:1/C20:2	48	4
	825.6	597.5/C14:0 575.2/C14:0-Na 571.5/C16:1 548.9/C16:1-Na 543.1/C18:1 521.1/C18:1-Na	TAG-C14:0/C16:1/C18:1	44	2
14	825.7	571.5/C16:1 549.3/C16:1-Na 569.3/C16:0 547.5/C16:0-Na	TAG-2C16:1/C16:0	44	2

Fraction #	[M+Na]	MS/MS $[M+Na-RCOOH]^+$ $[M+Na-RCOO-Na]^+$	Identification TAG	PN	DB
	929.8	673.3/C16:0 651.5/C16:0-Na 647.4/C18:1 625.3/C18:1-Na 599.4/C22:5 577.3/C22:5-Na	TAG- C16:0/C18:1/C22:5	44	6
	935.8	681.3/C16:1 659.5/C16:1-Na 653.3/C18:1 631.3/C18:1-Na 625.5/C20:1 603.3/C20:1-Na 597.3/C22:1 575.4/C22:1-Na	TAG- 2C18:1/C20:1 TAG- C16:1/C18:1/C22:1	48 50	4 3
	961.3	681.4/C18:2 659.4/C18:2-Na 679.4/C18:1 657.3/C18:1-Na 623.4/C22:1 601.4/C22:1-Na	TAG- C22:1/C18:2/C18:1	50	4
	853.8	625.3/C14:0 603.3/C14:0-Na 571.3/C18:1 549.3/C18:1-Na	TAG-2C18:1/C14:0	46	2
15	853.9	599.4/C16:1 577.3/C16:1-Na 597.3/C16:0 575.3/C16:0-Na 571.3/C18:1 549.5/C18:1-Na	TAG- C16:1/C16:0/C18:1	46	2
	879.8	623.3/C16:0 601.3/C16:0-Na 599.3/C18:2 577.2/C18:2-Na 597.3/C18:1 575.2/C18:1-Na	TAG- C16:0/C18:2/C18:1	46	3
	963.7	681.4/C18:1 659.3/C18:1-Na 625.2/C22:1 603.3/C22:1-Na	TAG-2C18:1/C22:1	50	4
16	881.9	627.1/C16:1 605.4/C16:1-Na 625.3/C16:0 603.4/C16:0-Na 599.3/C18:1 577.4/C18:1-Na 597.4/C18:0 575.1/C18:0-Na 571.3/C20:1 549.6/C20:1-Na	TAG- C18:0/C18:1/C16:0 TAG2C16:0/C20:1	50	1

Fraction #	[M+Na]	MS/MS $[M+Na-RCOOH]^+$ $[M+Na-RCOO-Na]^+$	Identification TAG	PN	DB
	901.6	645.4/C16:0 623.4/C16:0-Na 619.6/C18:2 597.5/C18:2-Na 573.4/C22:6 551.4/C22:6-Na	TAG-2C16:0/C22:6 TAG-3C18:2	42	6
	907.9	629.3/C18:3 627.3/C18:2 605.4/C18:2-Na 625.4/C18:1 603.4/C18:1-Na 623.3/C18:0 601.5/C18:0-Na	TAG- C18:0/C18:1/C18:2	48	3
	855.9	559.3/C16:0 577.4/C16:0-Na 573.2/C18:1 551.5/C18:1-Na	TAG-2C16:0/C18:1	48	1

Table 2. Peak identification of skin oil TAGs using 2D off-line RP-HPLC/ESI(+)/MS

Fraction #	$[M+Na]^+$	MS/MS $[M+Na-RCOOH]^+$ $[M+Na-RCOO-Na]^+$	Identification TAG	PN	DB
1					
2					
3	841.8	593.3/C16:4 571.3/C16:4-Na 591.5/C16:3 569.3/C16:3-Na 589.4/C16:2 567.3/C16:2-Na 563.5/C18:3 541.3/C18:3-Na 561.2/C18:2 539.3/C18:2-Na	TAG- C18:2/C16:4/C16:3	32	9
	855.9	605.7/C16:3 583.2/C16:3-Na 599.3/C16:0 577.3/C16:0-Na 573.2/C18:2 550.3/C18:2-Na	TAG- C16:0/C16:0/C18:2	2	48
	865.9	636.5/C14:0 617.3/C16:4 595.6/C16:4-Na 611.2/C16:1 589.3/C16:1-Na 609.6/C16:0 563.4/C20:5 541.3/C20:5-Na	TAG- C16:4/C16:4/C22:5	28	13
	917.8	669.5/C16:4 647.2/C16:4-Na 643.5/ C18:4 621.3/C18:4-Na 637.5/C18:2 615.5/C18:2-Na 635.2/C18:1 614.8/C18:1-Na 593.3/C21:1 589.4/C22:6 567.3/C22:6-Na	TAG-C22:6-C18:4- C16:4 TAG- C18:1/C16:4/C21:1	28	14
	943.7	691.3/C16:2 668.9/C16:2-Na 689.3/ C16:1 667.1/C16:1-Na 665.1/C18:3 663.2/ C18:3-Na 661.5/C18:2 639.3/C18:2-Na 641.4/C20:5 619.6/C20:5-Na	TAG- C20:5/C20:5/C18:3	32	13

Fraction #	$[M+Na]^+$	MS/MS $[M+Na-RCOOH]^+$ $[M+Na-RCOO-Na]^+$	Identification TAG	PN	DB
	995.8	693.4/C20:5 671.2/C20:5-Na 669.0/C20:0 645.2/C20:0-Na	TAG- C20:0/C20:5/C20:0	50	10
	891.6	643.3/C16:4 621.1/C16:4-Na 637.5/C16:1 615.0/C16:1-Na 611.4/C18:2 589.4/C18:2-Na 563.4/C22:6 541.6/C22:6-Na	TAG- C22:6/C16:4/C16:1	32	11
4	997.7	717.3/C18:2 695.4/C18:2-Na 669.3/C22:6 647.5/C22:6-Na	TAG-2C22:6/C18:2	34	14
	893.7	645.6/C16:4 623.0/C16:4-Na 643.4/C16:2 621.3/C16:2-Na 639.2/C16:1 617.5/C16:1-Na 611.2/C18:1 589.3/C18:1-Na 591.2/C20:5 569.4/C20:5-Na	TAG- C20:5/C18:1/C16:4	34	10
	971.7	691.4/C18:2 669.1/C18:2-Na 669.4/C20:5 647.4/C20:5-Na 643.3/C22:6 621.3/C22:6-N	TAG- C18:2/C20:5/C22:6	34	13
5					
	921.8	693.5/C14:0 671.8/C14:0-Na 669.1/C16:2 647.3/C16:2-Na 667.1/C16:1 645.4/C16:1-Na 643.4/C18:3 621.3/C18:3-Na 641.3/C18:2 619.3/C18:2-Na 638.9/C18:0 617.3/C18:0-Na 597.3/ C21:1 595.2/C21:0 592.9/C22:6	TAG- C22:6/C18:2/C16:2 TAG- C22:6/C18:3/C16:1	36	10

Fraction #	[M+Na]$^+$	MS/MS [M+Na-RCOOH]$^+$ [M+Na-RCOO-Na]$^+$	Identification TAG	PN	DB
	895.7	645.6/C16:3 623.0/C16:3-Na 643.4/C16:2 621.3/C16:2-Na 639.2/C16:0 617.5/C16:0-Na 613.2/C18:1 590.8.3/C18:1-Na 593.2/C20:5 571.4/C20:5-Na	TAG- C20:5/C18:1/C16:4	34	10
6	923.6	699.4/C16:1 647.3/C16:1-Na 643.4/C18:2 621.3/C18:2-Na 621.5/C20:5 599.4/C20:5-Na 595.3/C22:6 573.3/C22:6-Na	TAG- C16:1/C18:2/C22:6 TAG-2C18:2/C20:5	38	9
	973.6	691.4/C18:1 668.9/C18:1-Na 671.3/C20:5 649.5/C20:5-Na 645.3/C22:6 623.1/C22:6-Na	TAG- C18:1/C20:5/C22:6	36	12
	897.7	643.3/C16:1 621.3/C16:1-Na 617.4/C18:2 595.4/C18:2-Na 595.3/C20:5 573.3/C20:5-Na	TAG- C16:1/C18:2/C20:5	38	8
	949.8	669.3/C18:2 647.3/C18:2-Na 621.4/C22:6 599.4/C22:6-Na	TAG-2C18:2/C22:6	38	10
	999.6	723.5/ C18:4 701.4/ C18:4-Na 717.2/ C18:1 715.3/C18:1-Na 671.3/ C22:6 649.1/ C22:6-Na	TAG-2C18:1/C22:6	42	8

Fraction #	[M+Na]	MS/MS $[M+Na-RCOOH]^+$ $[M+Na-RCOO-Na]^+$	Identification TAG	PN	DB
	975.7	693.4/C18:2 671.3/C18:2-Na 673.9/C20:5 651.4/C20:5-Na 645.3/C22:4 623.3/C22:4-N	TAG- C18:2/C20:5/C22:5	36	12
	899.9	645.3/C16:1 623.4/C16:1-Na 643.3/C16:0 621.4/C16:0-Na 619.3/C18:2 597.4/C18:2-Na 615.2/C18:0 593.2/C18:0-Na 597.0/C20:5 575.4/C20:5-Na	TAG- C16:1/C18:1/C20:5	40	7
8	925.9	671.3/C16:1 649.5/C16:1-Na 645.3/C18:2 623.2/C18:2-Na 643.3/C18:1 621.5/C18:1-Na 623.4/C20:5 601.5/C20:5-Na 597.4/C22:6 575.6/C22:6-Na	TAG- C16:1/C18:2/C22:6 TAG- C18:1/C18:2/C20:5	38	9
	951.8	671.3/C18:2 649.3/C18:2-Na 669.3/C18:1 647.3/C18:1-Na 623.4/C22:6 601.3/C22:6-Na	TAG- C18:1/C18:2/C22:6	40	9
	899.9	645.3/C16:1 623.4/C16:1-Na 617.3/C18:1 595.1/C18:1-Na 597.4/C20:5 575.4/C20:5-Na	TAG- C16:1/C18:1/C20:5	40	7
9	849.7	597.3/C16:2 595.3/C16:1 573.1/C16:1-Na 569.3/C18:2 547.1/C18:2-Na 567.1/C18:1 545.2/C18:1-Na 565.3/C18:0	TAG-2C16:1/C18:2	42	4

Fraction #	[M+Na]	MS/MS $[M+Na-RCOOH]^+$ $[M+Na-RCOO-Na]^+$	Identification TAG	PN	DB
	901.7	625.3/C18:4 603.4/C18:4-Na 623.5/C18:3 601.5/C18:3-Na 621.4/C18:2 599.3/C18:2-Na 619.4/C18:1 597.3/C18:1-Na	TAG-C18:3/C18:2/C18:1 TAG/2C18:1/C18:4 TAG-3C18:2	42	6
	953.7	673.2/C18:2 651.5/C18:2-Na 571.3/C18:1 549.5/C18:1-Na 625.3/C22:6 603.4/C22:6-Na 623.2/C22:5 601.4/C22:5-Na	TAG-2C18:1/C22:6 TAG-C18:2/C18:1/C22:5	42	8
10	953.7	671.3/C18:1 649.5/C18:1-Na 625.3/C22:6 603.4/C22:6-Na	TAG-2C18:1/C22:6	42	8
	851.5	597.3/C16:1 575.5/C16:1-Na 569.4/C18:1 547.4/C18:1-Na	TAG-2C16:1/C18:1	44	3
	877.7	623.3/C16:1 601.5/C16:1 597.3/C18:2 575.2/C18:2-Na 595.3/C18:1 573.5/C18:1-Na	TAG-C16:1/C18:1/C18:2	44	4
	903.8	623.4/C18:2 601.3/C18:2-Na 621.3/C18:1 599.3/C18:1-Na	TAG-2C18:2/C18:1	44	5
	927.6	645.3/C18:1 623./ C18:2-Na 625.4/C20:5 603.4/ C20:5-Na	TAG-2C18:1/C20:5	42	7
	899.9	671.4/C14:0 649.3/C14:0-Na 621.5/C18:3 599.6/C18:3-Na 619.2/C18:2 597.2/C18:2-Na 617.3/C18:1 595.1/C18:1-Na 571.3/C22:6 549.3/C22:6-Na	TAG-C14:/C22:6/C18:1	40	7

Fraction #	[M+Na]	MS/MS $[M+Na-RCOOH]^+$ $[M+Na-RCOO-Na]^+$	Identification TAG	PN	DB
11	925.7	669.4/C16:0 647.4/C16:0-Na 645.4/C18:2 623.3/C18:2-Na 597.4/C22:6 575.2/C22:6-Na	TAG-C16:0/C18:2/C22:6	40	8
	851.8	623.3/C14:0 601.3/C14:0-Na 571.3/C18:2 549.4/C18:2-Na 569.4/C18:1 547.4/C18:1-Na	TAG-C18:1/C18:2/C14:0	44	3
	879.7	625.3/C16:1 603.3/C16:1-Na 597.3/C18:1 575.2/C18:1-Na	TAG-2C18:1/C16:1	46	3
	905.8	625.3/C18:2 603.5/C18:2-Na 623.4/C18:1 601.4/C18:1-Na	TAG-2C18:1/C18:2	48	4
	931.7	651.5/C18:2 629.9/C18:2-Na 649.5/C18:1 627.6/C18:1-Na 625..5/C20:3 603.4/C20:3-Na 623.4/C20:2 601.2/C20:2-Na 621.6/C20:1 599.2/C20:1-Na	TAG-C18:1/C18:2/C20:2	46	5
12	927.3	671.4/C16:0 649.4/C16:0-Na 645.3/C18:1 623.2/C18:1-Na 599.3/C22:6 577.4/C22:6-Na	TAG/C16:0/C18:1/C22:6	42	7
	907.5	625.3/C18:1 603.5/C18:1-Na	TAG-3C18:1	48	3
	983.5	703.6/C18:1 681.2/C18:1-Na 681.3/C20:5 659.8/ C20:5+Na 645.3/C22:1 623.1/ C22:1+ Na	TAG-C20:5/C22:1/C18:1	46	7
	877.8		TAG-2C18:2/C16:0	44	4

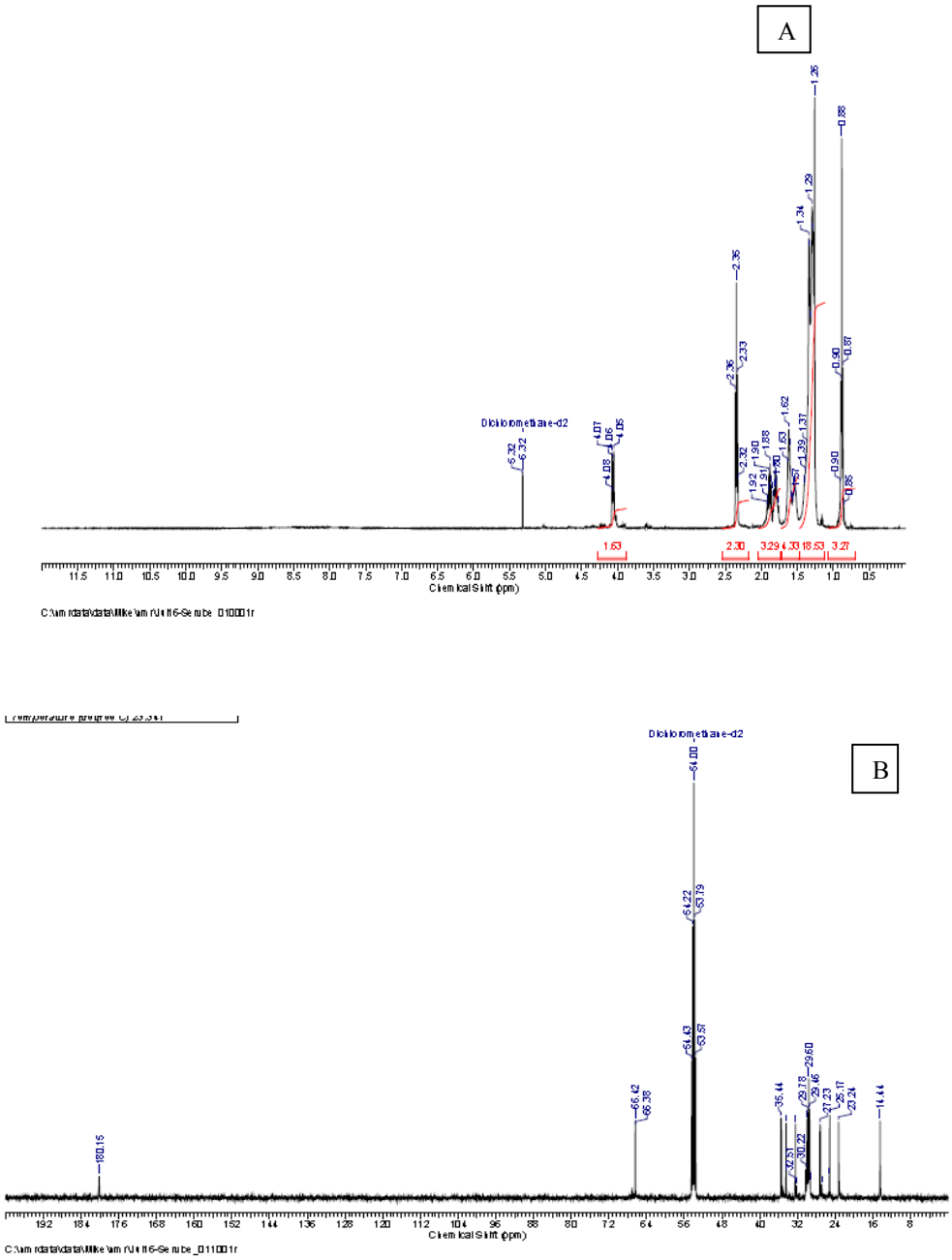
Fraction #	[M+Na]	MS/MS $[M+Na-RCOOH]^+$ $[M+Na-RCOO-Na]^+$	Identification TAG	PN	DB
13	825.7	571.5/C16:1 549.3/C16:1-Na 569.3/C16:0 547.5/C16:0-Na	TAG-2C16:1/C16:0	44	2
	877.8	621.3/C16:0 599.4/C16:0-Na 599.4/C18:3 577.3/C18:3-Na 595.3/C18:1 573.5/C18:1-Na	TAG- C18:3/C16:0/C18:1	44	4
	903.9	647.4/C16:0 625.4/C16:0-Na 621.3/C18:1 599.3/C18:1-Na 599.3/C22:4 577.4/C22:4-Na	TAG- C22:4/C18:2/C16:0	44	6
	929.8	673.3/C16:0 651.5/C16:0-Na 647.4/C18:1 625.3/C18:1-Na 599.4/C22:5 577.3/C22:5-Na	TAG- C16:0/C18:1/C22:5	44	6
	935.8	653.3/C18:1 631.3/C18:1-Na 625.5/C20:1 603.3/C20:1-Na	TAG- 2C18:1/C20:1	50	3
	961.3	681.4/C18:2 659.4/C18:2-Na 679.4/C18:1 657.3/C18:1-Na 623.4/C22:1 601.4/C22:1-Na	TAG- C22:1/C18:2/C18:1	50	4

Fraction #	[M+Na]	MS/MS $[M+Na-RCOOH]^+$ $[M+Na-RCOO-Na]^+$	Identification TAG	PN	DB
14	853.9	599.4/C16:1 577.3/C16:1-Na 597.3/C16:0 575.3/C16:0-Na 571.3/C18:1 549.5/C18:1-Na	TAG- C16:1/C16:0/C18:1	46	2
	879.8	623.3/C16:0 601.3/C16:0-Na 599.3/C18:2 577.2/C18:2-Na 597.3/C18:1 575.2/C18:1-Na	TAG- C16:0/C18:2/C18:1	46	3
	905.6	649.4/ C16:0 627.2/ C16:0-Na 627.5/ C18:3 605.3/ C18:3-Na 623.3/ C18:2 601.2/ C18:2-Na 621.3/ C18:0 599.3/ C18:0-Na	TAG- C18:0/C18:2/C18:3	44	5
	963.7	681.4/C18:1 659.3/C18:1-Na 625.2/C22:1 603.3/C22:1-Na	TAG-2C18:1/C22:1	52	3
15	881.6	625.3/C16:0 603.5/C16:0-Na 599.4/C18:1 577.4/C18:1-Na	TAG-2C18:1/C16:0	50	1
	955.7	673.2/C18:1 651.3/C18:1-Na 671.3/C18:0 649.2/C18:0-Na 627.3/C22:6 605.4/C22:6-Na	TAGC18:1- C18:0/C22:6	44	7
	907.7	651.4/C16:0 629.5/C16:0-Na 627.3/C18:2 605.4/C18:2-Na 625.4/C18:1 603.4/C18:1-Na 623.3/C18:0 601.5/C18:0-Na 597.1/C20:1 575.3/C20:1-Na	TAG- C18:0/C18:1/C18:2 TAG- C18:2/C16:0/C20:1	48	3
	929.7	647.5/C18:1 625.2/C18:1-Na 645.5/C18:0 623.5/C18:0-Na	TAG- C18:1/C18:0/C20:5	44	6

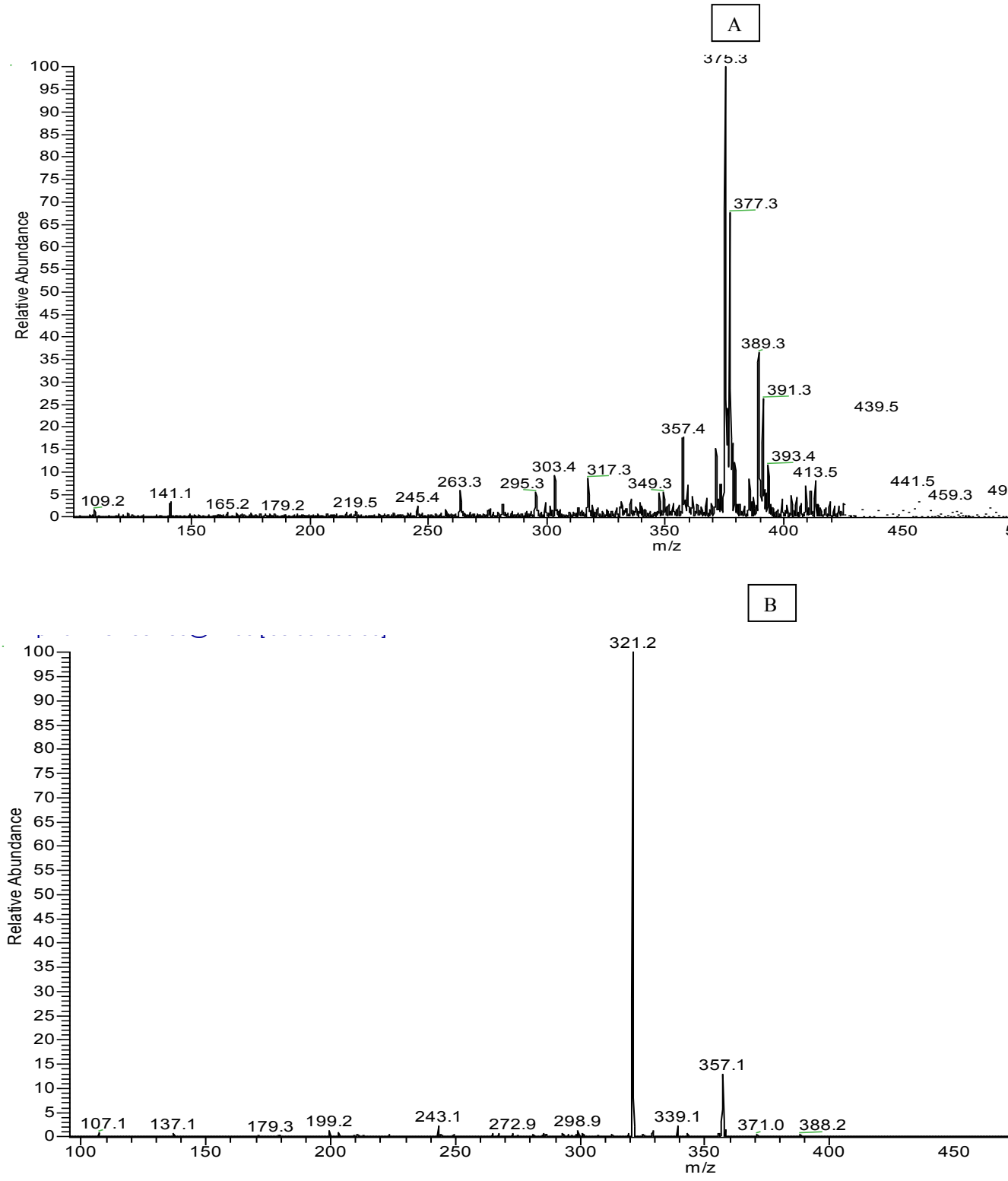
Fraction #	[M+Na]	MS/MS $[M+Na-RCOOH]^+$ $[M+Na-RCOO-Na]^+$	Identification TAG	PN	DB
16	800.1	571.3/C14:0 549.1/C14:0-Na 543.2/C16:0 521.2/C16:0-Na 545.2/C16:1 523.4/C16:1-Na	TAG- C14:0/C16:0/C16:1	44	1
	827.5	599.3/C14:0 577.2/C14:0-Na 573.5/C16:1 551.3/C16:1-Na 571.3/C16:0 549.3/C16:0-Na 573.3/C16:1 551/C16:1-Na 545.1/C18:0 523.3/C18:0-Na 525.3/C20:5			
	875.9	619.3/C16:0 597.5/C16:0-Na 573.4/C20:5 551.5/C20:5-Na	TAG-2C16:0/C20:5	42	5
	881.9	627.1/C16:1 605.4/C16:1-Na 625.3/C16:0 603.4/C16:0-Na 599.3/C18:1 577.4/C18:1-Na 597.4/C18:0 575.1/C18:0-Na 571.3/C20:1 549.6/C20:1-Na	TAG- C18:0/C18:1/C16:0 TAG2/C16:0/C20:1	50	1
	901.6	645.4/C16:0 623.4/C16:0-Na 573.4/C22:6 551.4/C22:6-Na	TAG-2C16:0/C22:6	40	6
	907.9	627.3/C18:2 605.4/C18:2-Na 625.4/C18:1 603.4/C18:1-Na 623.3/C18:0 601.5/C18:0-Na	TAG- C18:0/C18:1/C18:2	48	3

Fraction #	[M+Na]	MS/MS $[M+Na-RCOOH]^+$ $[M+Na-RCOO-Na]^+$	Identification TAG	PN	DB
17	827.8	599.3/C14:0 577.2/C14:0-Na 571.3/C16:0 549.3/C16:0-Na 573.3/C16:1 551/C16:1-Na 545.1/C18:0 523.3/C18:0-Na 525.3/C20:5			
	855.9	559.3/C16:0 577.4/C16:0-Na 573.2/C18:1 551.5/C18:1-Na	TAG-2C16:0/C18:1	48	1
	883.1	627.3/C16:0 605.3/C16:0-Na 601.3/C18:1 579.4/C18:1-Na 599.3/C18:0 577.4/C18:0-Na	TAGC16:0- C18:1/C18:0	50	1
	909.9	627.3/C18:1 605.3/C18:1-Na 625.3/C18:0 603.5/C18:0-Na 653.4/C16:0 631.5/C16:0-Na 599.4/C20:0 577.2/C20:0-Na	TAG2C18:1-C18: TAG- C18:1/C16:0/C20:0	50	2
	903.6	647.4/C16:0 625.3/C16:0-Na 573.3/C22:5 551.4/C22:5-Na	TAG-2C16:0/C22:5	44	5
	935.8	651.4/C18:0 629.6/C18:0-Na 653.4/C18:1 631/C18:1-Na 627.5/C20:2 605.3/C20:2-Na 625.2/C20:1 603.3/C20:1-Na	TAG-2C18:1/C20:1 TAG- C18:0/C18:1/C20:2	50	3
18	883.1	627.3/C16:0 605.3/C16:0-Na 601.3/C18:1 579.4/C18:1-Na 599.3/C18:0 577.4/C18:0-Na	TAGC16:0- C18:1/C18:0	50	1

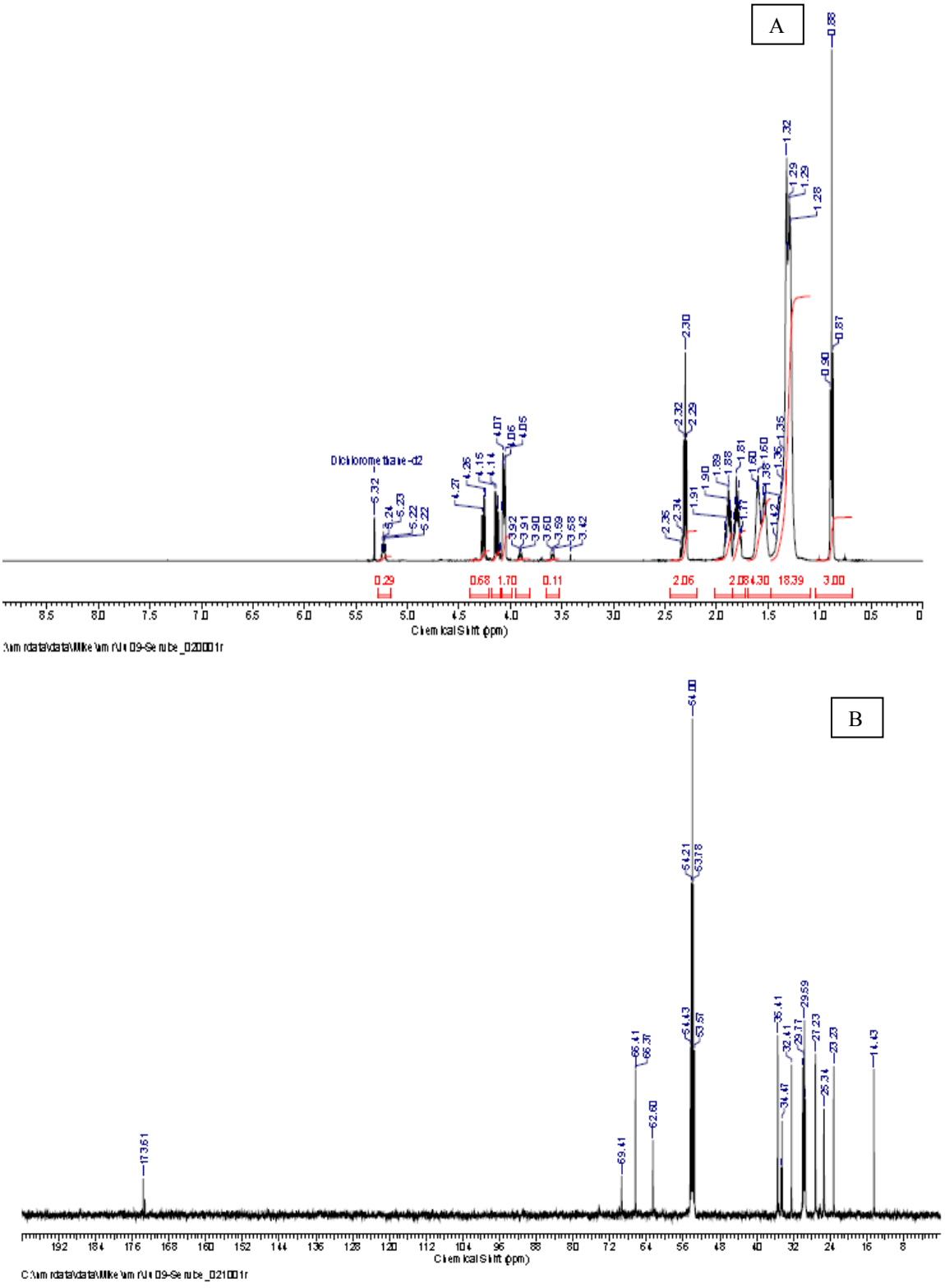
Appendix 2



App-2-Fig 1. (A) ^{1}H NMR spectrum; (B) ^{13}C NMR spectrum of FA-C18Cl_2 [Compound 2].

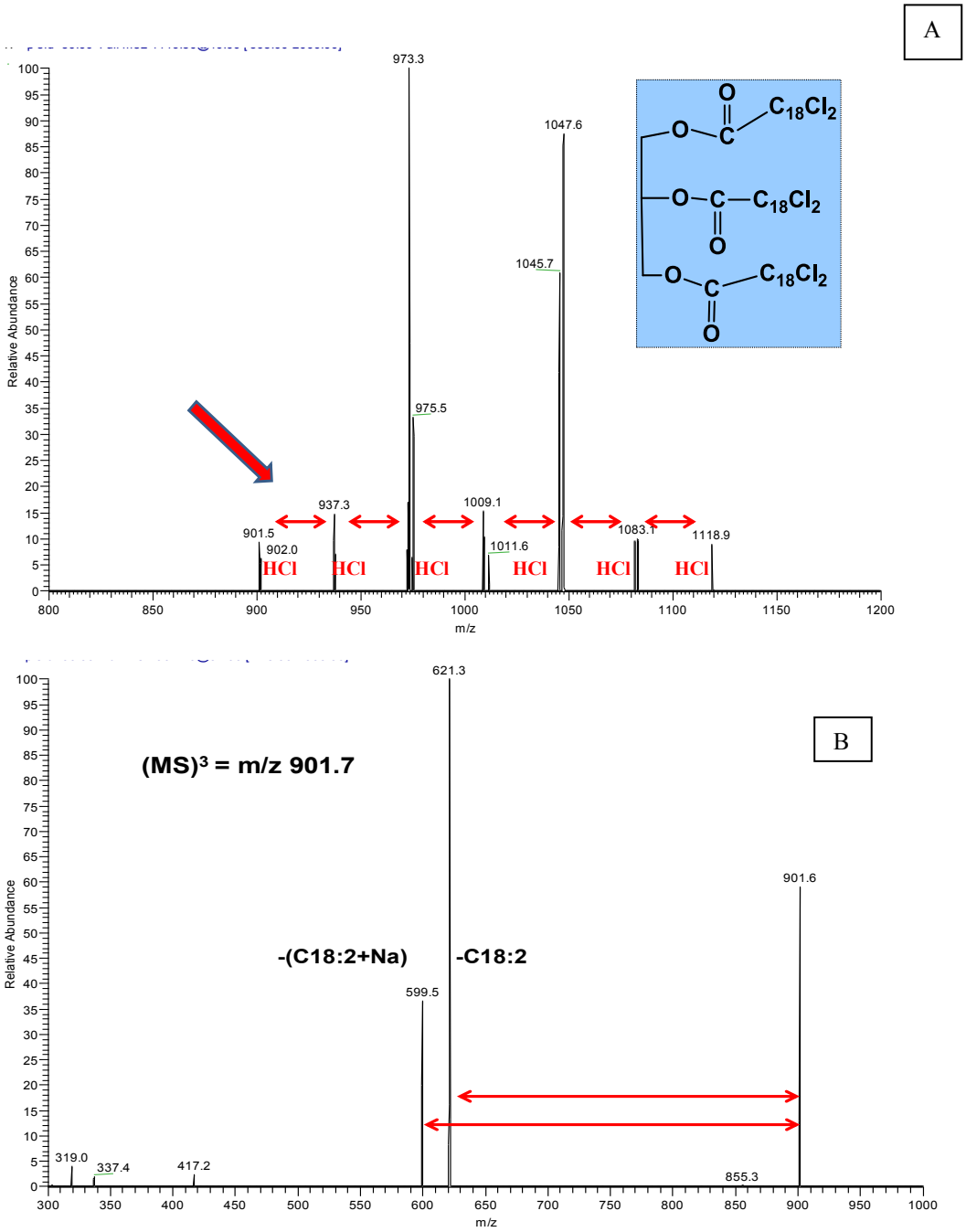


App-2-Fig.2. (A). ESI-MS spectrum of FA-C18Cl$_2$ [Compound 2] at *m/z* 375.7; (B) MS/MS spectrum at *m/z* 375.7

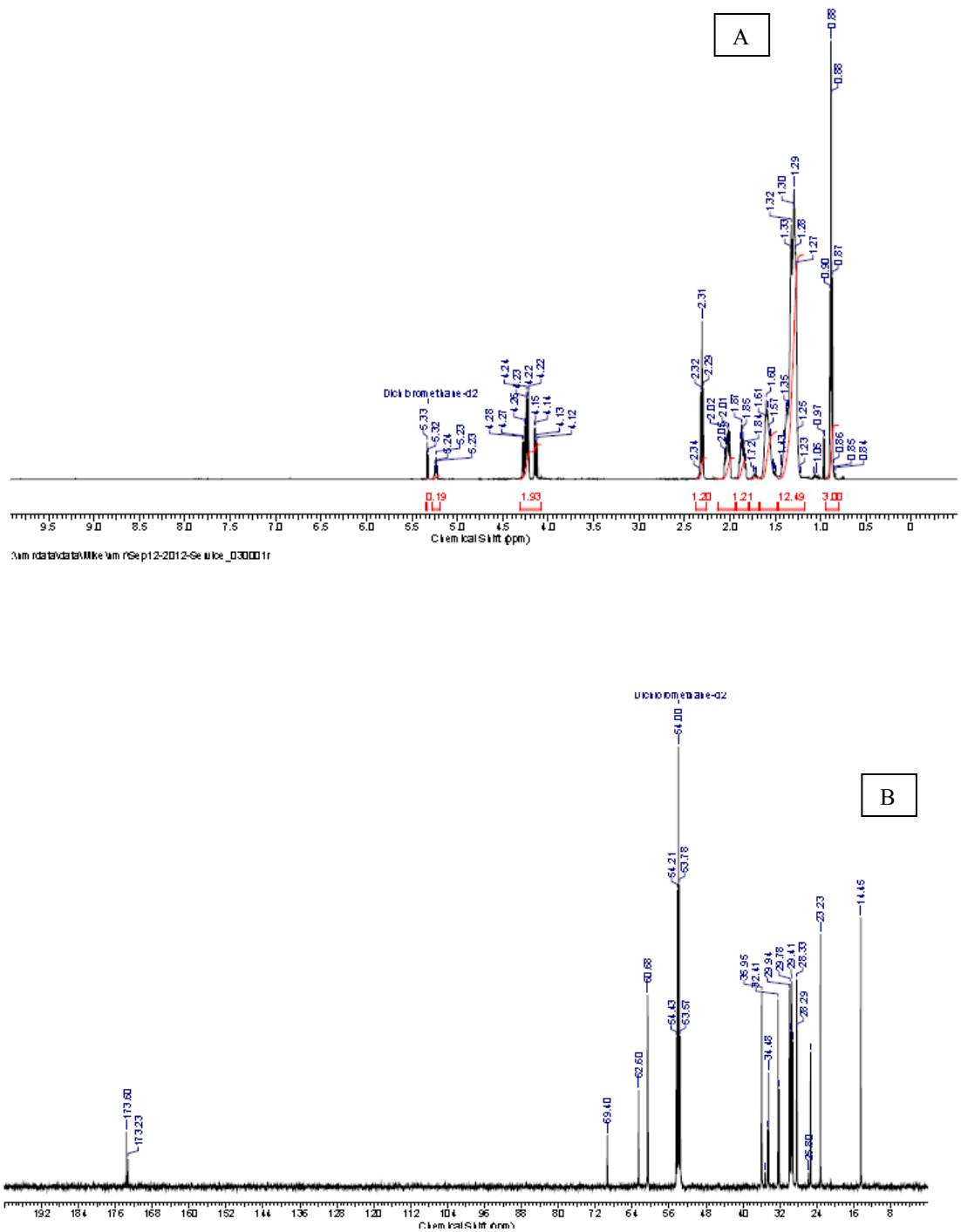


App-2-Fig 3. (A)H^1 NMR spectrum; (B) C^{13}NMR spectra of TAG-3C18Cl_6

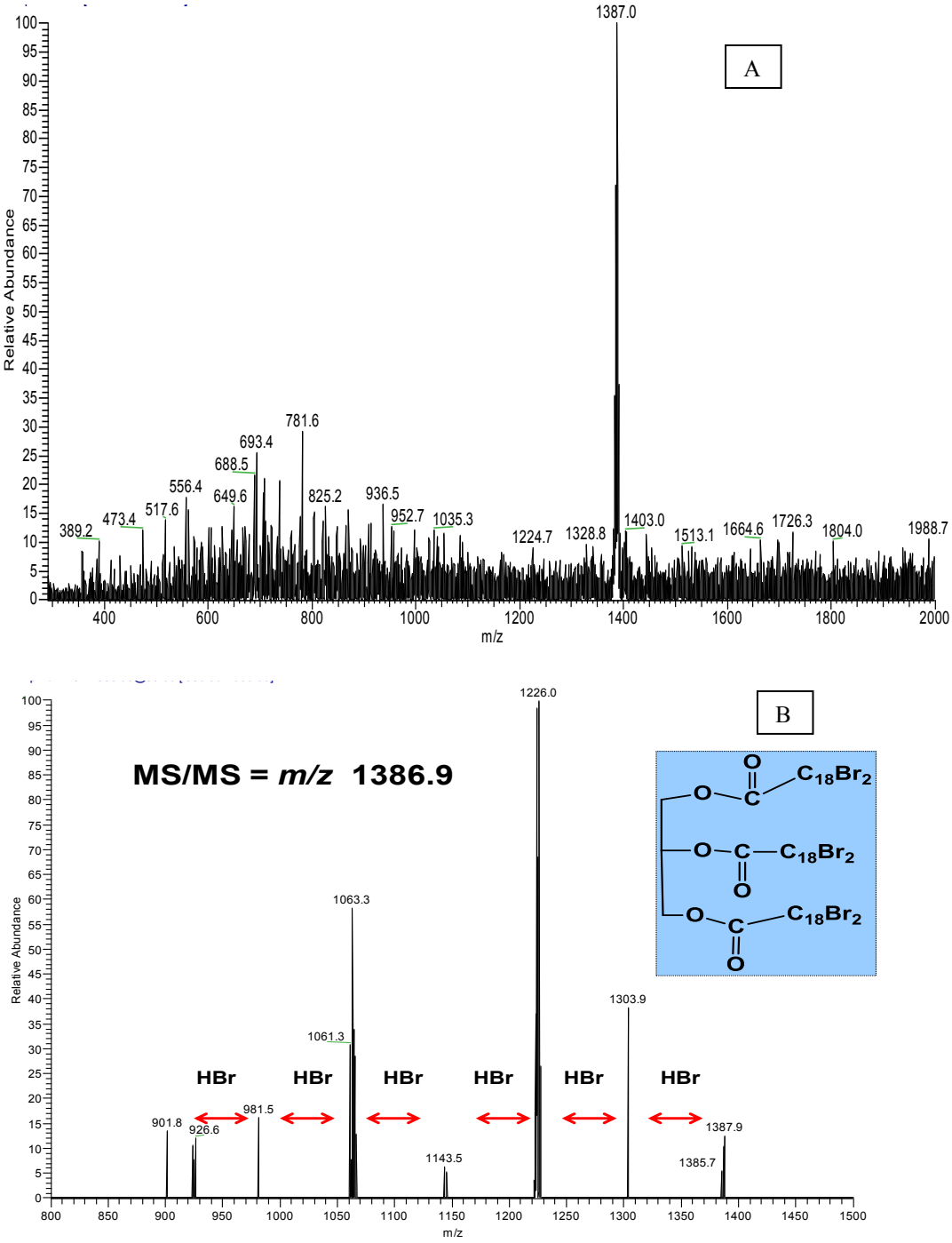
[Compound 16]



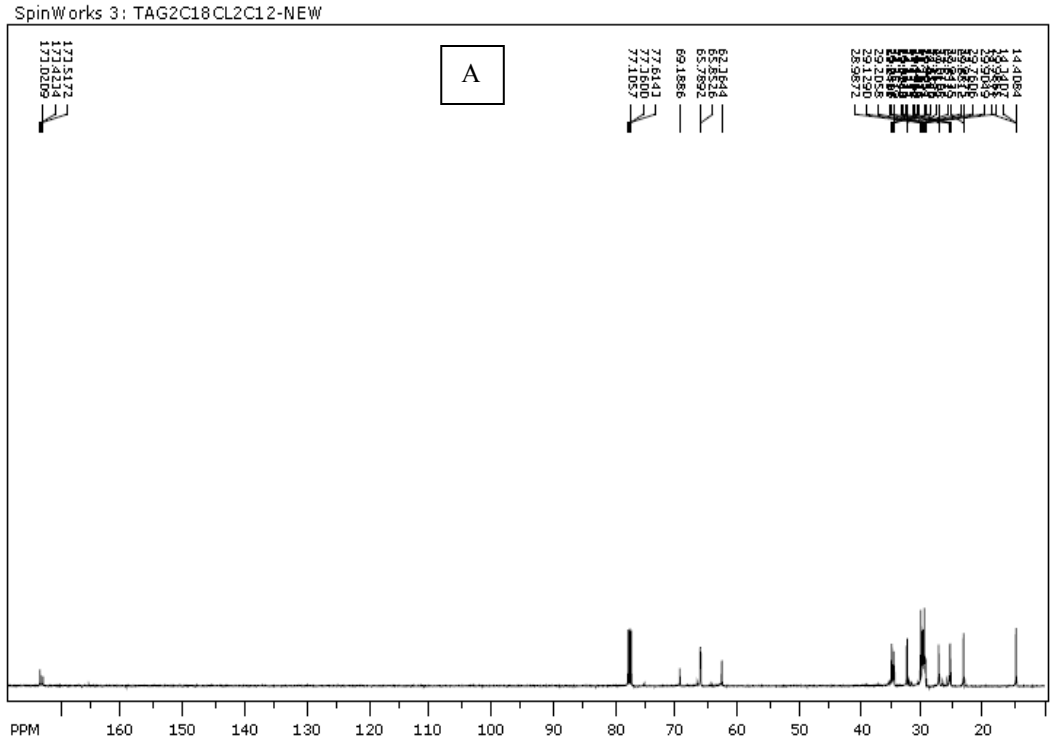
App-2-Fig 4.(A).CID spectrum of TAG-3C18Cl$_6$ [Compound 16] at *m/z* 1118.9;(B) MS^3 spectrum of *m/z* 1118.9

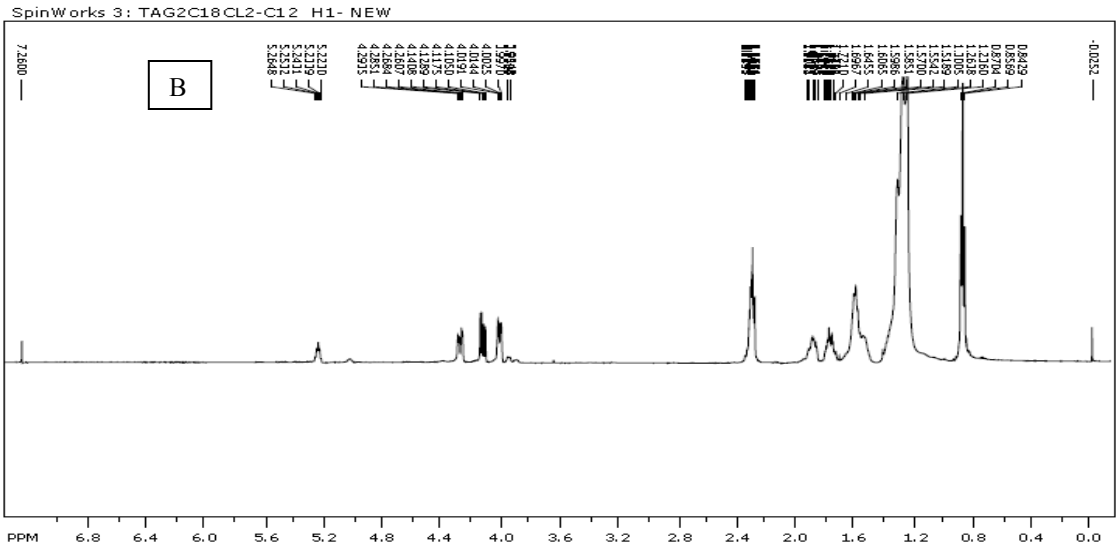


App-2-Fig 5. (A)H^1 NMR spectrum; (B) C^{13}NMR spectra of TAG-3C18Br_6 [Compound 17].

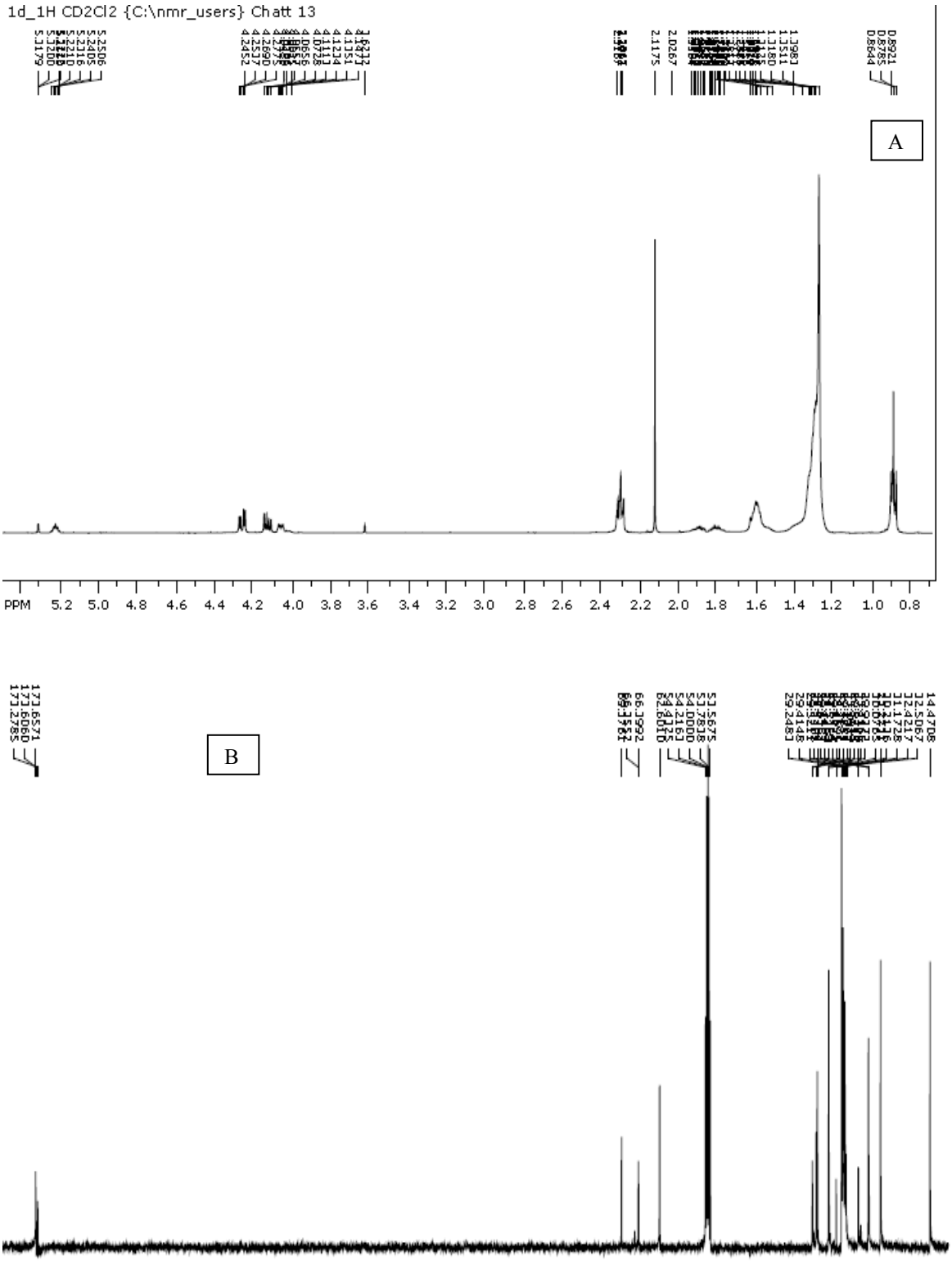


App-2-Fig 6.(A). ESI-MS spectrum of TAG-3C18Br$_6$ [Compound 17] at *m/z* 1387.9 ;(B) CID spectrum at *m/z* 1387.9.

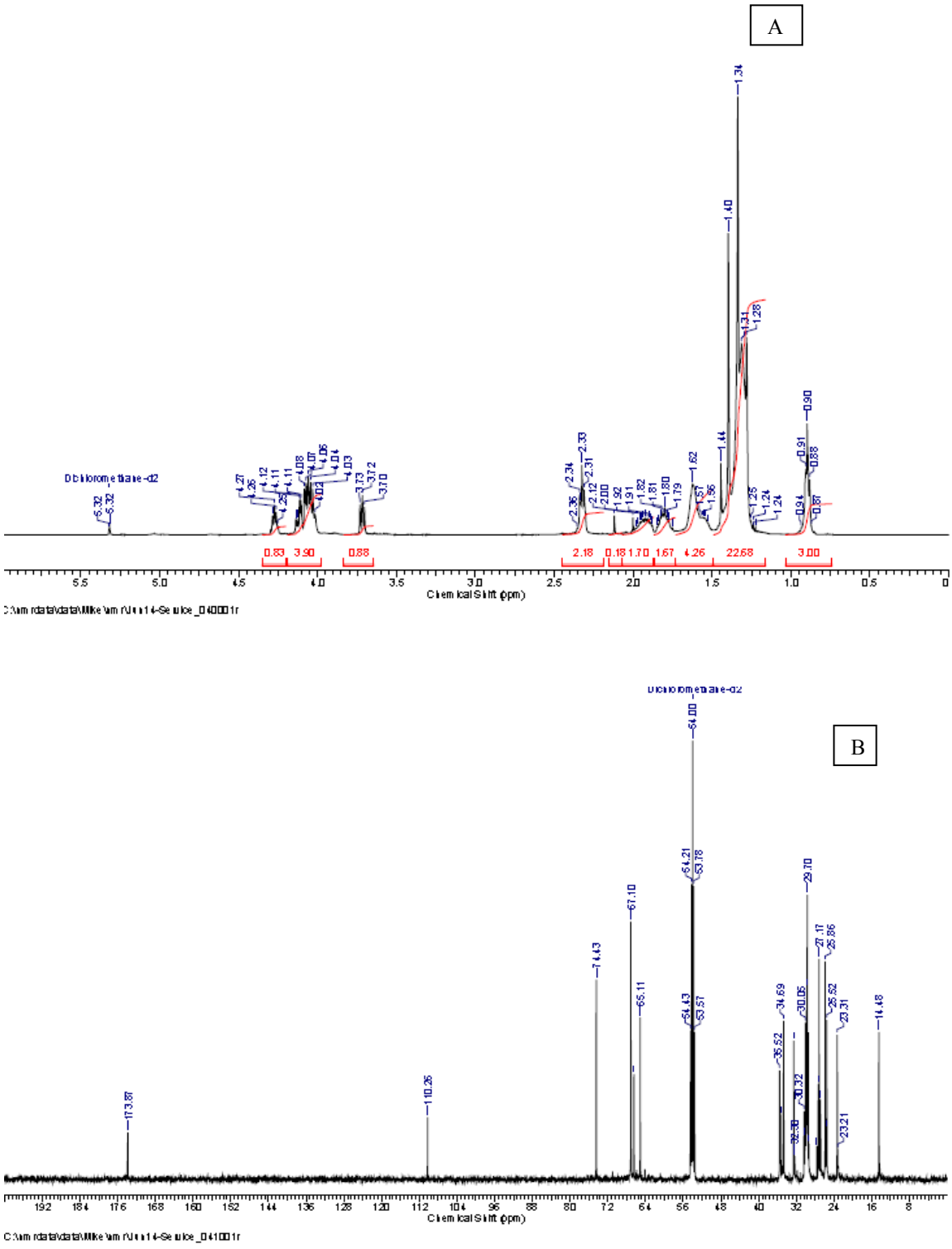




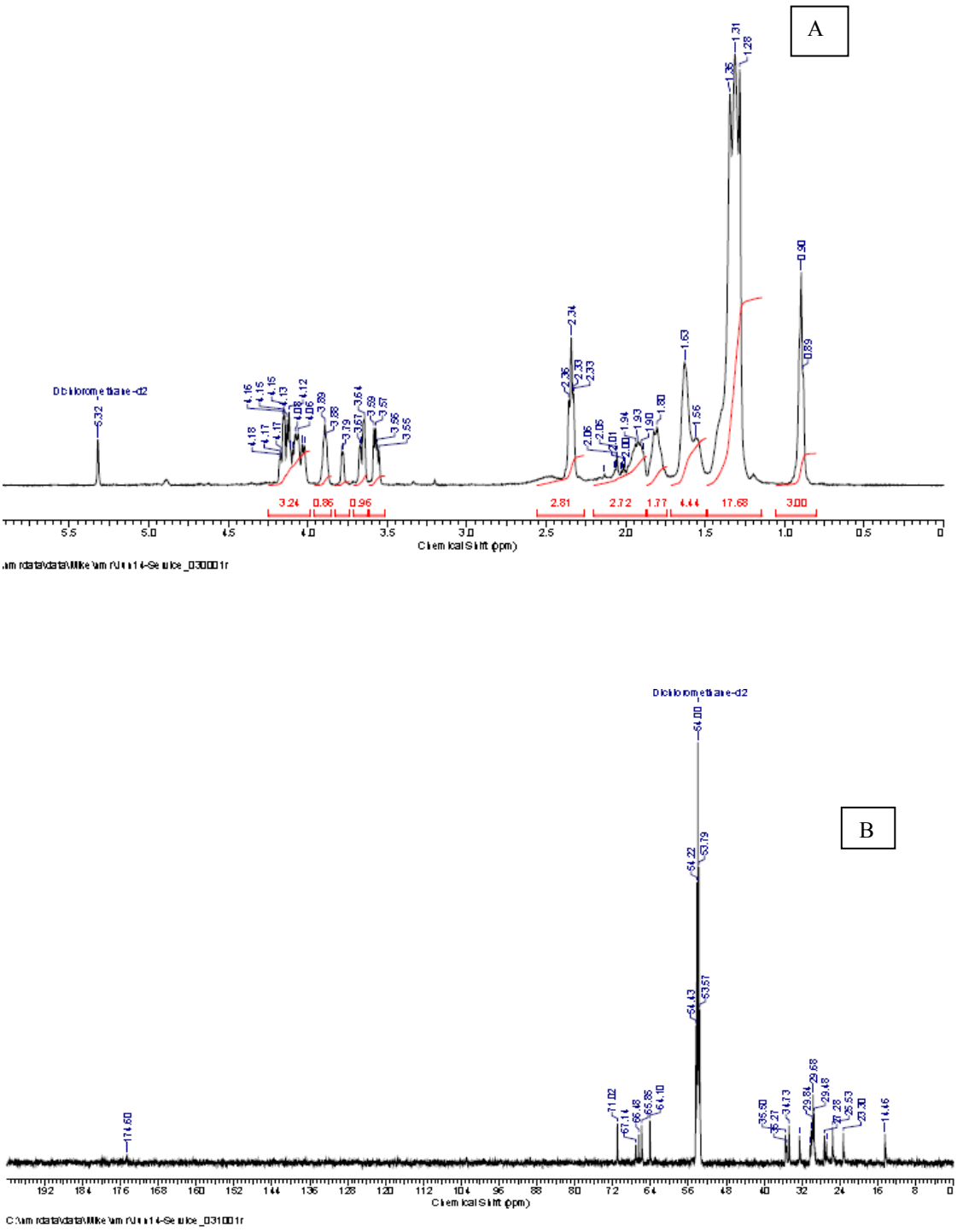
App-2-Fig 7. (A)H^1 NMR spectrum; (B) C^{13}NMR spectra of TAG$C18Cl_2$-$C18Cl_2$-C12:0 [Compound 14]



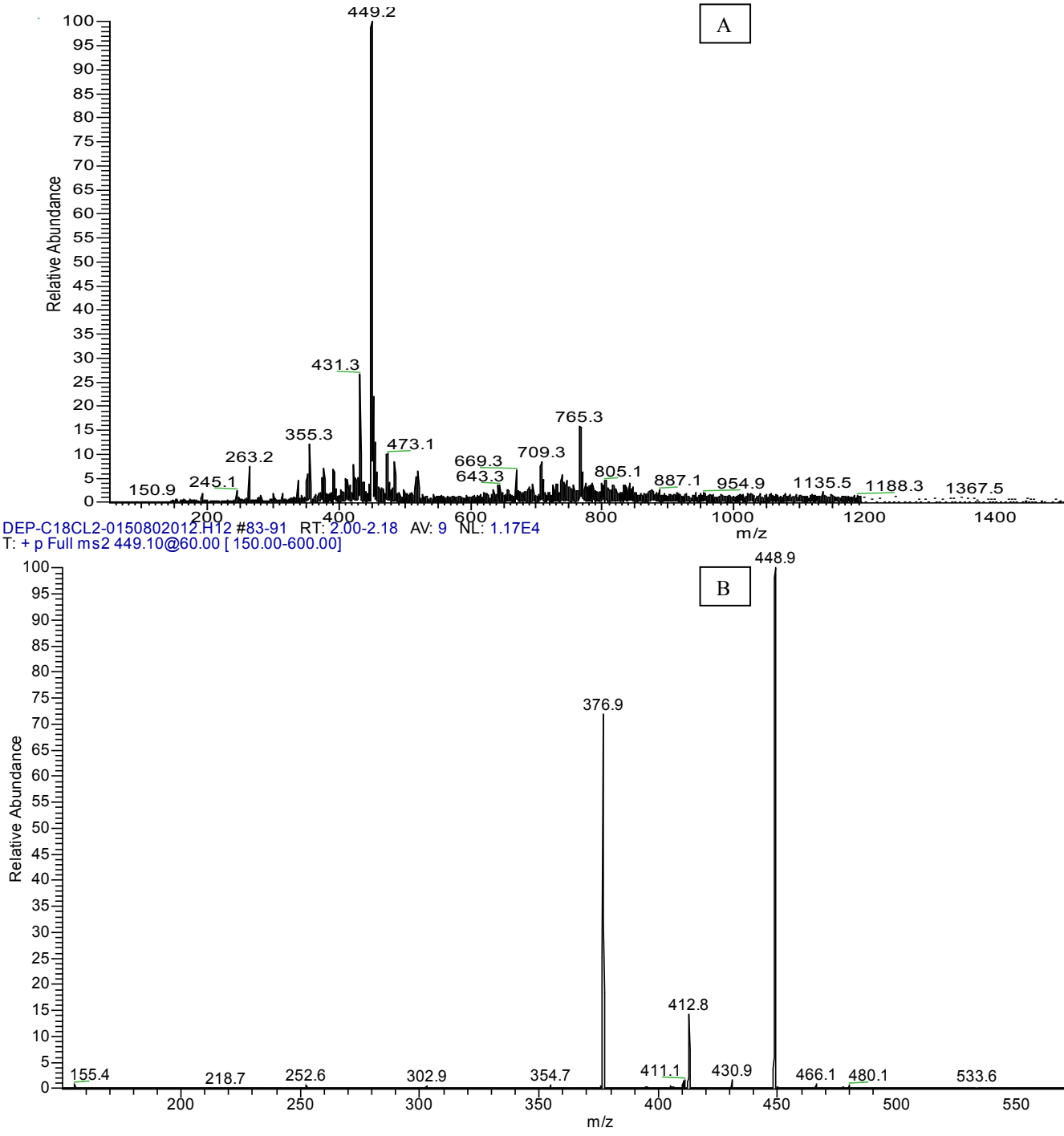
App-2-Fig 8. (A)H^1 NMR spectrum; (B) C^{13}NMR spectra of TAGC12:0-C18Cl_2-C12:0 [Compound 6]



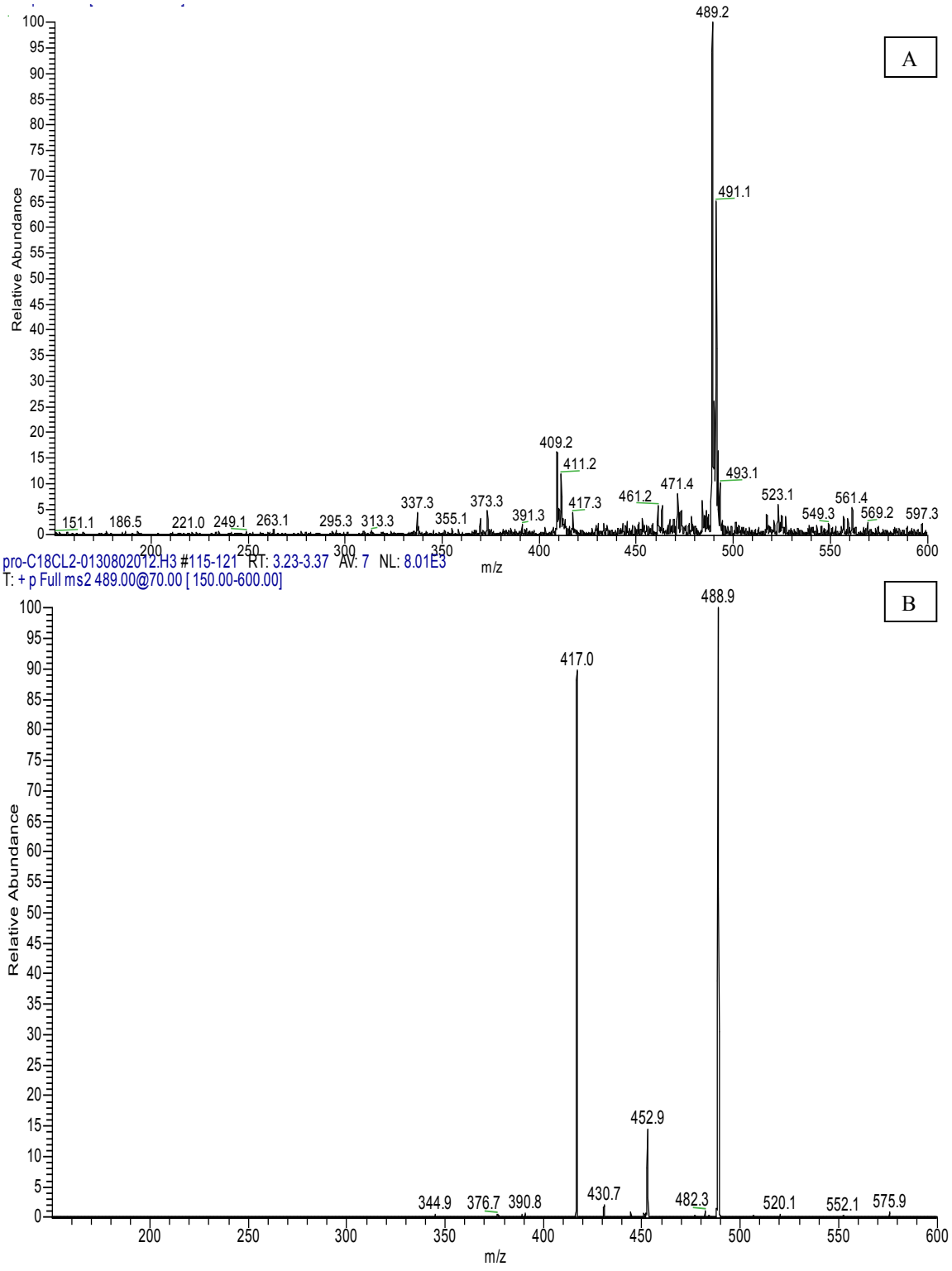
App-2-Fig 9 (A) H^1 NMR spectrum; (B) C^{13}NMR spectra of $C_{24}H_{44}Cl_2O_4Na$ [Compound9]



App-2-Fig10. (A) H^1 NMR spectrum; (B) C^{13}NMR spectra of $C_{21}H_{40}Cl_2O_4Na$ [compound 11]



App-2-Fig 11.(A). ESI-MS spectrum of $C_{21}H_{40}Cl_2O_4Na$ [compound 11] at *m/z* 449.2 ;(B). CID spectrum of *m/z* 449.2.



App-2-Fig 12.(A). ESI-MS spectrum of $C_{24}H_{44}Cl_2O_4Na$ [compound 9] at *m/z* 489.2; (B). CID spectrum of *m/z* 489.2

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APPENDIX 3

Copyright release request letter

From: Permissions Helpdesk <permissionshelpdesk@elsevier.com>
Sent: January 11, 2016 6:37 PM
To: Abir Lefsay
Subject: RE: copyright release request

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