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)
TABLE OF CONTENTS

LIST OF TABLES .................................................................................................................................................. viii
LIST OF FIGURES ................................................................................................................................................. ix
ABSTRACT ............................................................................................................................................................ xv
LIST OF ABBREVIATIONS USED .................................................................................................................. xvi
ACKNOWLEDGEMENTS ................................................................................................................................. xix

CHAPTER 1   Introduction ...................................................................................................................................  1

CHAPTER 2   Literature Survey ..........................................................................................................................  8
  2.1. Classification of organohalogen compounds (OCs)...............................................................................  8
  2.1.1. Anthropogenic sources of OCs ...........................................................................................................  8
  2.1.2. Natural sources of OCs .................................................................................................................... 11
  2.2. Methods of solvent extraction .............................................................................................................. 12
  2.3. Methods of analysis .................................................................................................................................. 13
  2.4. Chlorinated Fatty Acids (Cl-FA) ............................................................................................................. 14
  2.5. Triacylglycerols (TAG) .......................................................................................................................... 19
    2.5.1. Synthesis of Triacylglycerol (TAG) ................................................................................................. 19
      2.5.1.1. Enzymatic Method ...................................................................................................................... 19
      2.5.1.2. Chemo-enzymatic method ........................................................................................................ 19
      2.5.1.3. Chemical method ..................................................................................................................... 20
    2.5.2. Identification of Triacylglycerol ....................................................................................................... 20
      2.5.2.1. Nuclear Magnetic Resonance spectroscopy (NMR) ................................................................ 21
      2.5.2.2. Mass Spectrometry (MS) ......................................................................................................... 21
      2.5.2.3. Gas Chromatography .............................................................................................................. 23
      2.5.2.4. High Pressure Liquid Chromatography (HPLC) .................................................................. 23

CHAPTER 3      Separation and identification of free fatty acids in oils by GC/MS and separation of triacylglycerols by HPLC ...................................................................................................................... 26
  3.1 Objectives ................................................................................................................................................... 26
  3.2. Introduction ............................................................................................................................................. 26
  3.3. Experimental .......................................................................................................................................... 28
    3.3.1. Chemicals ....................................................................................................................................... 28
    3.3.2. Instrument operation conditions ................................................................................................... 28
3.3.2.1. GC/M……………………………………………………………………………28
3.3.2.2. RP-HPLC……………………………………………………………………28
3.3.3. Procedures………………………………………………………………………29
3.3.3.1. Preparation of Ag-HPLC columns………………………………………29
3.3.3.2. Calibration curve for fatty acid ethyl ester (FAEE)…………..30
3.3.3.3. Calibration curve of the standard mixtures of FAME……..31
3.3.3.4. Esterification procedures for FFA and oils using BF$_3$-MeOH…………………………………………………………………………………………31
3.3.3.4.1. Esterification of FFA using BF$_3$-MeOH…………………………….31
3.3.3.4.2. Esterification of oil samples using BF$_3$-MeOH………………32
3.3.3.5. Preparation of oil samples for HPLC analysis…………………..32
3.4. Results and Discussion…………………………………………………………32
3.4.1. GC/MS separation of FAEE…………………………………………………..32
3.4.2. GC/MS separation of FAME of the standard mixture………………37
3.4.3. GC/MS separation of FAME of FFA……………………………………..40
3.4.4. GC/MS separation of FAME of oil samples…………………………41
3.4.5. Separation of TAGs in oils by RP-HPLC…………………………………43
3.4.6. Separation of TAGs in oils by Ag-HPLC…………………………………46
3.4.6.1. Optimization of separation of TAGs in corn and salmon oil………………….46
3.4.6.2. Separation of grape seed and canola oils by Ag-HPLC………………49
3.5. Conclusions……………………………………………………………………….50
CHAPTER 4 Identification of triacylglycerols in corn oil………………………………………..51
4.1. Objectives………………………………………………………………………….51
4.2. Introduction……………………………………………………………………….51
4.3. Experimental……………………………………………………………………..53
4.3.1. Chemicals…………………………………………………………………………53
4.3.2. Instruments and operating conditions……………………………………..53
4.3.2.1. RP-HPLC………………………………………………………………………53
4.3.2.2. ESI/MS…………………………………………………………………………53
4.4. Results and discussion……………………………………………………………53
4.4.1. Off-line 2D RP-HPLC/GC/MS derivatization of triglyceride mixture and corn oil…………………………………………………………………………….54
4.4.1.1. Off-line 2D RP-HPLC/GC/MS derivatization method for the commercial triglyceride mixture .......................54

4.4.1.2. Off-line 2D RP-HPLC/GC/MS derivatization method for the corn oil ........................................................................................................56

4.4.2. On-line and off-line Ag-HPLC/RP-HPLC methods for the separation of TAGs in corn oil .................................................................59

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 ...................60

4.4.2.2. Off-line 2D Ag-HPLC/RP-HPLC method for the separation of the TAGs in corn oil .........................................................63

4.4.2.3. On-line 2D Ag-HPLC/RP-HPLC method for separation of TAGs in ......................................................................................66

4.4.3. Off-line 3D Ag-HPLC/ESI(+)/MS/GC-MS for identification of TAGs in corn oil ..............................................................................69

4.4.4. Off-line 2D Ag-HPLC/ESI/MS and RP-HPLC/ESI/MS for identification of TAGs in corn oil .............................................................................74

4.4.4.1. Off-line 2D Ag-HPLC/ESI/MS for identification of the TAGs in corn oil ......................................................................................74

4.4.4.2. Off-line 2D RP-HPLC/ESI(+)/MS for the identification of TAGs in corn oil ......................................................................................89

4.5. Conclusions ..................................................................................................................104

CHAPTER 5 synthesis of chlorinated triacylglycerol compounds and their characterization in corn and salmon oils ........................................................................107

5.1. Objectives ..................................................................................................................107

5.2.1. Introduction ..........................................................................................................107

5.2.1.1. Experimental ..................................................................................................109

5.2.1.1. Chemicals ......................................................................................................109

5.2.1.2. Instruments and their operation conditions ..................................................110

5.2.1.2.1 Melting point measurements ......................................................................110

5.2.1.2.2. NMR ........................................................................................................110

5.2.1.2.3. ESI(+)MS .................................................................................................110

5.2.3. Synthesis procedures GC/MS .................................................................111

5.2.3.1. Synthesis of 9,10-Dichlorooctadecanoic acid (compound 2) ....111
5.2.3.2. Synthesis of 2-(9,10-dichlorooctadecanoyl)-1,3-didodecanoyl-glycerol (compound 6)……………………………………………………………………………..112

5.2.3.2.1. Synthesis of 1,3-didodecanoylglycerol (compound 5)…112

5.2.3.2.2. Synthesis of compound 6………………………………………..112

5.2.3.3. Syntheses of chlorinated unsymmetrical triacylglycerols (compounds 13 and 14)…………………………………………………….113

5.2.3.3.1. Synthesis of monoacilisopropylidenes…………………….113

5.2.3.3.1.1. Synthesis of 1,2-isopropylidene-3-(9,10-dichlorooctadecanoyl) glycerol (compound 9)…………………………………….113

5.2.3.3.1.2. Synthesis of rac-1,2-Isopropylidene-3-dodecanoylglycerol(compound 10)……………………………………………………..114

5.2.3.3.2. Syntheses of monoacylglcerols……………………………….114

5.2.3.3.2.1. Synthesis of 1-(9,10-Dichlorooctadecanoyl)glycerol (compound 11)…………………………………………………………114

5.2.3.3.2.2. Synthesis of rac-1-Dodecanoylglycerol (compound 12)……………………………………………………………..114

5.2.3.3.3. Coupling of monoacyl glycerol with two equivalent fatty acids...115

5.2.3.3.3.1. Synthesis of 1-(9,10-Dichlorooctadecanoyl)-2,3-didodecanoyl glycerol (compound 13)…………………………………………115

5.2.3.3.3.2. Synthesis of 1,2-Di-(9,10-dichlorooctadecanoyl)-3-dodecanoyl glycerol (compound 14)……………………………………115

5.2.3.4. Hexahalogenated triacylglycerols………………………………...116

5.2.3.4.1. Tri-(9,10-dichlorooctadecanoyl)glycerol (compound 16)……116

5.2.3.4.2. Tri-(9,10-dibromooctadecanoyl)glycerol (compound 17)……116

5.2.4. Results and Discussion………………………………………………117

5.2.4.1. Chlorination of oleic acid………………………………………….117

5.2.4.2. Discussion the synthesis of 2-(9,10-dichlorooctadecanoyl)-1,3-didodecanoylglycerol (compound 6)……………………………117

5.2.4.3. Discussion synthesis of two unsymmetrical chlorinated triacylglycerols (compound 13 and compound 14)…………………119

5.2.4.4. Discussion synthesis of hexahalogenated triacylglycerols……..120

5.2.4.5. Mass spectrometry………………………………………………121

5.3. Spiking corn oil with Cl-TAG compounds………………………………………127

5.4. Studies of Cl-TAG compounds in farmed Atlantic salmon oil…………136
LIST OF TABLES

Table 2.1. A literature survey on the type of anthropogenic OCs and their sources...9
Table 2.2. A literature survey of separation and determination methods for Cl-FAAs in fish and water.................................................................16
Table 3.1. The amounts of secondary stock solution, solvent, and internal standard for calibration curve of FAEE..................................................31
Table 3.2. Comparison of FAME in the oil samples and the standards...........43
Table 4.1. The fractions of the triglycerides mixture depend on the time........56
Table 4.2. Fatty acids found in the different fractions of corn oil by GC/MS......58
Table 4.3. Peak identification of corn oil TAGs using 3D off-line
Ag-HPLC/ESI(+)/MS/GC-MS.................................................................71
Table 4.4. Peak identification of corn oil TAGs using 2D off-line HPLC/ESI(+)/MS...77
Table 4.5. Peak identification of corn oil TAGs using PR-HPLC/ESI(+)/MS........90
Table 4.6. Oxidation products of TAGs in corn oil using RP-HPLC/ESI(+)/MS......103
Table 4.7. A summary of the corn oil TAGs identified by RP-HPLC/GC/MS and RP-HPLC/ESI(+)/MS.................................................................104
Table 4.8. A summary of TAGs identified in the corn oil by Ag-HPLC/ESI(+)/MS and RP-HPLC/ESI(+)/MS.................................................................106
Table 5.1. Isotope clusters observed upon ESI(+)MS of the chlorinated compounds.........................................................................................121
Table 5.2. The results of spiking the corn oil with different concentration of TAG-C12:0/C18Cl2/C12:0.................................................................131
Table 5.3. Peak identification of the possible oxidized TAG compounds in salmon skin oil...........................................................................156
Table 5.4. FAME in salmon tissue oil................................................................164
Table 5.5. FAME in salmon skin oil...............................................................166
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..............169
LIST OF FIGURES

Fig 2.1. Three common chlorinated fatty acids.................................................................14
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......................................................33
Fig 3.2. Mass spectra of the methyl ester of C14:0..............................................................34
Fig 3.3. Mass spectra of the ethyl ester of C14:0...............................................................34
Fig 3.4. Comparison of mass spectra of the methyl esters of (A) C18:0; (B) C18:1; (C) C18:2...........................................................................................................................35
Fig 3.5. Calibration curve for the ethyl esters of C14:0, C18:2, C18:1, and C18:0.................................................................36
Fig 3.6. Calibration curve for the ethyl esters using C8 as internal standard........36
Fig 3.7. (A) GC/MS chromatogram of methyl ester standard solutions; (B) expanded region (20.0-22.0 min).................................................................38
Fig 3.8. (A) Calibration curves for the methyl ester standard solutions; (B) expanded region (0.00-0.20 µg mL^{-1}).................................................................39
Fig 3.9. The GC/MS chromatogram for the lowest concentration (0.125 µg mL^{-1}) of standard mixture.................................................................40
Fig 3.10. Time study for the formation of the methyl esters using BF$_3$-MeO.41
Fig 3.11. GC/MS chromatogram of corn oil sample derivative using the BF$_3$-MeOH procedure............................................................................................42
Fig 3.12. RP-HPLC of corn oil.........................................................................................44
Fig 3.13. RP-HPLC of salmon oil..................................................................................44
Fig 3.14. RP-HPLC of grape seed oil.................................................................45
Fig 3.15. RP-HPLC of canola oil. .................................................................................45
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...........................47
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...........................48
Fig 3.18. Ag-HPLC chromatograms of grape seed using gradients mode [MeOH and MeOH with 20% of ACN with ratio of 100:50].................................49
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]..............49
Fig 4.1. Collection of 6 fractions of triglyceride mixture by the RP-HPLC method.................................................................................................................. 55

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)..............56

Fig 4.3. The RP-HPLC chromatogram of the corn oil fractions collected for GC/MS (the insert shows the complete chromatogram).............................57

Fig 4.4. GC/MS chromatogram of F2 of the RP-HPLC separation of corn oil.....58

Fig 4.5. GC/MS chromatograms of F3 & F4 of the RP-HPLC separation of corn oil........................................................................................................59

Fig 4.6. RP-HPLC isocratic separation of corn oil using MeOH:iso-propanol: (A) 70:30; (B) 100:100 (v/v)....................................................................................61

Fig 4.7. RP-HPLC isocratic separation of salmon oil using MeOH:iso-propanol (A) 70:30;(B) 100:100 (v/v).................................................................................62

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)..................................................63

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........64

Fig 4.10. The TAG fractions separated in second dimension RP-HPLC (Fig 4.9.); (A) F1, F2, F3; (B) F4, F5, F6........................................................................65

Fig 4.11. The set-up for the on-line 2D Ag-HPLC/RP-HPLC method.............63

Fig 4.12A. Different cutting positions of peak 2 and 5 in Ag-HPLC...............67

Fig 4.12B. Chromatograms of the 6 cut positions of peak 2 separated in RP-HPLC on-line method.................................................................68

Fig 4.12C. Chromatogram of F2 (Fig 4.8) separated in second dimension RP-HPLC (Fig 4.9.) off-line method.................................................68

Fig 4.13. Chromatograms of the 3 cutting positions of peak 5 separated in RP-HPLC.........................................................................................69

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)..................................................70
Fig 4.15. The ESI mass spectrum of (A) F5; (B) Expanded range m/z (920-1080)........................................................................................................72
Fig 4.16. The GC-MS chromatogram of the FAME of F5............................73
Fig 4.17. Collection of 6 fractions of corn oil sample from the first dimension (Ag-HPLC)........................................................................................................75
Fig 4.18. The ESI mass spectrum of F3............................................................78
Fig 4.19. The CID spectrum of [PLL+]Na+ at m/z 877.9.................................................................79
Fig 4.20A. The CID spectrum of [LOO+]Na+/[SLL+]Na+ at m/z 905.6.............80
Fig 4.20B. The expanded CID spectrum at m/z 580-650...............................80
Fig 4.21. The ESI mass spectrum of F5 of [OLnL+]Na+ ion at m/z 901.8............81
Fig 4.22A. The CID spectrum of F5 of [OLnL+]Na+ ion at m/z 901.8...............82
Fig 4.22B. The expanded CID spectrum at m/z 585-635................................82
Fig 4.23. The ESI mass spectrum of F5 of [LLL+]Na+ ion at m/z 901.9............83
Fig 4.24A. The CID spectrum of F5 of [LLL+]Na+ ion at m/z 901.9..................84
Fig 4.24B. The expanded CID spectrum at m/z 575-660...............................84
Fig 4.25. The ESI mass spectrum of F2 by the 2D off-line Ag-HPLC/ESI/MS method..................................................................................................................86
Fig 4.26. The ESI mass spectrum of F2 by 3D off-line Ag-HPLC/ESI/MS/GC/MS method..................................................................................................................86
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.................................................88
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........88
Fig 4.29. Seventeen fractions of corn oil collected form RP-HPLC.........................89
Fig 4.30. The CID spectrum of F16 of [ALL+]Na+ ion at m/z 933.5.....................92
Fig 4.31. The CID spectrum of F16 of [ALO+]Na+ ion at m/z 935.7.....................93
Fig 4.32. (A) The ESI mass spectrum of F9 corn oil using RP-HPLC/ESI(+)MS; (B) the CID spectrum of F9 [LLO+]Na+ ion at m/z 903.4...............................94
Fig 4.33. (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 903.7...............................94
Fig 4.34. 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. 95

Fig 4.35. RP-HPLC/ESI(+)MS using two Supelcosil LC-PAH (25 m x 4.6 mm x 3 μ particles) with gradient 100% acetonitrile (solvent A) to 100% isopropanol (solvent B). 97

Fig 4.36. The ESI mass spectrum of commercial TAG-C18:2 (m/z 901.8). 97

Fig 4.37. The expanded range at m/z (880-1050) showing possible oxidation at m/z 933.5 and 965.5. 99

Fig 4.38. The CID spectrum of the oxidized standard TAG-C18:2 ion at m/z 933.7. 100

Fig 4.39. The ESI mass spectrum of the oxidized TAG compounds in F4 of corn oil (Fig 4.29). 101

Fig 4.40. The CID spectrum of the oxidized TAG compounds in F4 of corn oil at m/z 933.5. 102

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. 119

Fig 5.2. Synthesis of the unsymmetrical triacylglycerols 13 and 14. Reagents and conditions: (A) EDCI, DMAP, CH₂Cl₂, 24 h; (B) cation-exchange resin, MeOH, 4 h; (C) EDCI, DMAP, CH₂Cl₂, 33 h. 120


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. 125

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 xii
possible proton abstractions from C11 in 19 are shown. Analogous routes follow from C8 proton abstractions in C19 and from initial binding of the sodium ion to the chlorine at C10, rather than C9 as shown in C18.

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

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

Fig 5.8. The calibration curve (1 nM- 6 µM) of the peak area study of TAG-3C18:1

Fig 5.9. TIC of TAG-C12:0/C18Cl₂/C12:0 (1 nM- 1 µM) range at m/z 813.5-815.5.

Fig 5.10. Calibration curve (1 nM- 1 µM) of the peak height of TAG-C12:0/C18Cl₂/C12:0

Fig 5.11. Calibration curve (1 nM- 1 µM) of the peak area of TAG-C12:0/C18Cl₂/C12:0

Fig 5.12. Seventeen fractions collected from the corn oil run RP-HPLC after spiked with Cl-TAGs (13, 14, and 16).

Fig 5.13(A). Fraction 1 of the corn oil by RP-HPLC/ESI(+/)+MS before spiking; (B) detected TAG-C12:0/C18Cl₂/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-C18Cl₂-C18Cl₂-C12 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-C18Cl₆ in F6 after spiking.

Fig 5.16. Preparation of salmon skin and tissue samples

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

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

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

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

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

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

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

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

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

Fig 5.26. The CID spectrum of F3 of salmon tissue oil at m/z 993.8
Fig 5.27. ESI(+) mass spectrum of F7 of salmon skin........................................152
Fig 5.28. The CID spectrum of F7 of salmon skin oil at m/z 871......................152
Fig 5.29. ESI(+) mass spectrum of F8 of salmon skin........................................154
Fig 5.30(A). The CID spectrum of F8 of salmon skin oil at m/z 873.5; (B) expanded range (m/z 560-665).................................................................155
Fig 5.31. $^1$H NMR spectrum of salmon skin oil................................................159
Fig 5.32. $^1$H NMR spectrum of salmon tissue oil..............................................159
Fig 5.33. $^{13}$C NMR spectrum of salmon skin oil..............................................161
Fig 5.34. $^{13}$C NMR spectrum of salmon tissue oil............................................161
Fig 5.35 (A). $^{13}$C NMR spectrum of salmon skin expanded (122-136 ppm); (B) tissue oils (126-133 ppm).................................................................162
Fig 5.36. GC/MS of the fatty acid methyl esters chromatogram for the salmon tissue.................................................................165
Fig 5.37. GC/MS of the fatty acid methyl esters chromatogram for the salmon skin oil.................................................................167
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

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>APCI/MS</td>
<td>atmospheric pressure chemical</td>
</tr>
<tr>
<td>AED</td>
<td>atomic emission detector</td>
</tr>
<tr>
<td>ALL</td>
<td>TAG-C20:0/C18:2/C18:2</td>
</tr>
<tr>
<td>ALO</td>
<td>TAG-C20:0/C18:2/C18:1</td>
</tr>
<tr>
<td>ALP</td>
<td>TAG-C20:0/C18:2/C16:0</td>
</tr>
<tr>
<td>ESI/MS</td>
<td>ionization mass spectrometry</td>
</tr>
<tr>
<td>BF$_3$-MeOH</td>
<td>boron trifluoride-methanol</td>
</tr>
<tr>
<td>C16:0</td>
<td>palmitic acid</td>
</tr>
<tr>
<td>C18:0</td>
<td>stearic acid, octadecanoic acid</td>
</tr>
<tr>
<td>C18:1</td>
<td>oleic acid, octadecenoic acid</td>
</tr>
<tr>
<td>C18:2</td>
<td>linoleic acid, octadecadienoic acid</td>
</tr>
<tr>
<td>C14:0</td>
<td>myristic acid, tetradecanoic acid</td>
</tr>
<tr>
<td>CID</td>
<td>collision-induced dissociation</td>
</tr>
<tr>
<td>CI</td>
<td>chemical ionisation</td>
</tr>
<tr>
<td>CI-TAG</td>
<td>chlorinated triacylglycerol</td>
</tr>
<tr>
<td>CIFAME</td>
<td>chlorinated fatty acid methyl ester</td>
</tr>
<tr>
<td>CI-FA</td>
<td>chlorinated fatty acid</td>
</tr>
<tr>
<td>DAG-C16:0</td>
<td>dipalmitin</td>
</tr>
<tr>
<td>DB</td>
<td>double bond</td>
</tr>
<tr>
<td>DDT</td>
<td>dichlorodiphenyltrichloroethane</td>
</tr>
<tr>
<td>DHA</td>
<td>docosahexaenoic acid</td>
</tr>
<tr>
<td>DUSR</td>
<td>dalhousie university SLOWPOKE-2 reactor</td>
</tr>
<tr>
<td>ECD</td>
<td>electron capture detector</td>
</tr>
<tr>
<td>EI</td>
<td>electron ionization</td>
</tr>
<tr>
<td>ESI</td>
<td>electrospray ionization</td>
</tr>
<tr>
<td>ELCD</td>
<td>electrolytic conductivity detector</td>
</tr>
<tr>
<td>ELSD</td>
<td>evaporative light scattering detector</td>
</tr>
<tr>
<td>EOX</td>
<td>extractable organic halogen</td>
</tr>
<tr>
<td>EPA</td>
<td>eicosapentaenoic acid</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>EPS</td>
<td>Canadian Environmental Protection Service</td>
</tr>
<tr>
<td>FFA</td>
<td>free fatty acid</td>
</tr>
<tr>
<td>FAME</td>
<td>fatty acid methyl ester</td>
</tr>
<tr>
<td>FABE</td>
<td>fatty acid butyl ester</td>
</tr>
<tr>
<td>FAEE</td>
<td>fatty acid ethyl ester</td>
</tr>
<tr>
<td>FID</td>
<td>flame ionization detection</td>
</tr>
<tr>
<td>LLE</td>
<td>liquid/liquid extraction</td>
</tr>
<tr>
<td>LLL</td>
<td>TAG-C18:2/C18:2/C18:2</td>
</tr>
<tr>
<td>LLLn</td>
<td>TAG-C18:2/C18:2/C18:3</td>
</tr>
<tr>
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<td>TAG-C18:2/C18:2/C18:1</td>
</tr>
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<tr>
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<td>TAG-C18:3/C18:1/C18:3</td>
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<td>TAG-C18:2/C18:1/C18:1</td>
</tr>
<tr>
<td>LSS</td>
<td>TAG-C18:2/C18:0/C18:0</td>
</tr>
<tr>
<td>GC</td>
<td>gas chromatography</td>
</tr>
<tr>
<td>HCH</td>
<td>hexachlorocyclohexanes</td>
</tr>
<tr>
<td>HPLC</td>
<td>high performance liquid chromatography</td>
</tr>
<tr>
<td>HTGC</td>
<td>high temperature gas chromatography</td>
</tr>
<tr>
<td>IARC</td>
<td>International Agency for Research on Cancer</td>
</tr>
<tr>
<td>INAA</td>
<td>instrumental neutron activation analysis</td>
</tr>
<tr>
<td>m/z</td>
<td>mass/charge ratio</td>
</tr>
<tr>
<td>MS</td>
<td>mass spectrometry</td>
</tr>
<tr>
<td>MAG-C18:1</td>
<td>1-oleoyl-glycerol</td>
</tr>
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<td>NAA</td>
<td>neutron activation analysis</td>
</tr>
<tr>
<td>NICI</td>
<td>negative ion chemical ionization</td>
</tr>
<tr>
<td>OC</td>
<td>organohalogen compounds</td>
</tr>
<tr>
<td>OCI</td>
<td>organochlorine compounds</td>
</tr>
<tr>
<td>OBr</td>
<td>organobromine compounds</td>
</tr>
<tr>
<td>OOO</td>
<td>TAG-C18:1/C18:1/C18:1</td>
</tr>
<tr>
<td>OLnO</td>
<td>TAG-C18:1/C18:3/C18:1</td>
</tr>
<tr>
<td>OLnLn</td>
<td>TAG-C18:1/C18:3/C18:3</td>
</tr>
<tr>
<td>PBBs</td>
<td>polybrominated biphenyls</td>
</tr>
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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 trioctanolate
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
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>UV</td>
<td>ultraviolet</td>
</tr>
<tr>
<td>XFA</td>
<td>halogenated fatty acids</td>
</tr>
<tr>
<td>XSD</td>
<td>halogen sensitive detector</td>
</tr>
</tbody>
</table>
ACKNOWLEDGEMENTS

My PhD thesis has been finally written after spending most of my youth in three graduate schools. An M.Sc. degree (2000) in biochemistry from the Department of Chemistry, Garyounis University, Benghazi, Libya; another M.Sc. degree (2007) in bioorganic chemistry from the Department of Chemistry, University of New Brunswick at Saint John, NB, Canada; and hopefully my PhD in analytical chemistry from the Department of Chemistry at Dalhousie University, Halifax, NS, Canada. I worked with three supervisors, and three unavoidable situations happened: Dr. Mohamed Frajany (my M.Sc. Supervisor, Libya) sadly passed away during my degree program. Dr. Margaret Kayser (my M.Sc. Supervisor, Canada) retired before I wrote my thesis. Finally, Dr. Robert Guy (my Co-Supervisor, Ph.D.) took early retirement before I could finish my Ph.D. work at Dalhousie.

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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 (OCI) and organobromine (OBr) compounds are used to designate the particular halogen atom under consideration. The OCI and extractable OCI (EOCI) compounds in marine samples have recently been investigated extensively. In this thesis emphasis has been placed on the characterization of EOCI 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 EOCI compounds account for most of the extractable organohalogen (EOX) in marine fish; only 10-15% of the EOCI has so far been identified [Kleijn et al., 2001]. The remaining 85-90% EOCI in fish lipids might contain chlorinated fatty acids (Cl-FA) which was investigated using gas chromatography (GC) with halogen-selective or electrolytic conductivity detector [Wesen 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 al., 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 CI-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 OCI compounds are of particular interest because of their stability, toxicity and carcinogenicity. It is therefore necessary to develop methods to detect, identify and measure OCIs in a given sample. A detailed literature survey of the work reported so far has been reviewed in Chapter 2. Briefly, the OCIs are classified to two groups depending on their source: natural or anthropogenic sometimes called unnatural. Most of the anthropogenic OCIs 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 OCIs, 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 OCIs 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 OCIs 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 OCIs 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 OCI profiles [Svendsen et al., 2008]. The salmon is exposed to OCIs 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 OCI 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 OCIs, in particular TAGs, CI-TAGs and CL-FA, in farmed Atlantic salmon.

Farmed Atlantic salmon (Salmo salar) was selected in this thesis for studying its OCIs 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 OCIs. Canadians generally consume large amounts of salmon. Finally, there is very little information reported in the literature on OCIs in Atlantic salmon.

In order to detect, identify and measure OCIs 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 OCI 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 OCIs 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; Bahrour, 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 OCI 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% ($\text{NH}_4$)$_2$HPO$_4$, 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 OCI compounds was considered the second important item for the characterization of OCI 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 [Bahrour, 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 glycercyl 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. Another 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 OCI compounds 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 OClIs. The most widely used method is GC because most of the OClIs 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 OClIs. Several GC and HPLC methods were developed in this thesis for the separation of OClIs. The most common detection technique used for studying OClIs 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 OClIs [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.

<table>
<thead>
<tr>
<th>Category</th>
<th>Common compounds</th>
<th>Source</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Halogenated Biphenyls</td>
<td>PCBs</td>
<td>Manufacturing facilities and equipment such as hydraulic fluid, brake fluid, recycled oil, paper ink Leakage from PCB-filled transformers and capacitors</td>
<td>Wolska et al., 2012</td>
</tr>
<tr>
<td></td>
<td>PBBs</td>
<td>Municipal and industrial solid waste (Landfills)</td>
<td>Alaee et al., 2003</td>
</tr>
<tr>
<td></td>
<td>PBBs</td>
<td>Flame retardants production waste incineration fossil fuel combustion Incineration of municipal hazardous waste sewage sludge Bleaching process of paper and pulp</td>
<td>Harrad, 1996</td>
</tr>
<tr>
<td>Halogenated fatty acids</td>
<td>CI-FA</td>
<td>Pulp mill effluent Bleaching and disinfection of foodstuffs Disinfection in municipal sewage plants and water</td>
<td>LeBel et al., 1997</td>
</tr>
<tr>
<td></td>
<td>Br-FA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Halogenated pesticides/herbicides</td>
<td>DDT, Dieldrin, Chloradane</td>
<td>Wood preservatives Pesticide formulations</td>
<td>Jorgenson, 2000</td>
</tr>
<tr>
<td>Halogenated aromatics</td>
<td>Chlorinated phenol</td>
<td>Municipal incinerators Electrochemical industry waste products Domestic and water waste Fluid in heat transfer Wood manufacture as anti-stain</td>
<td>Igbinosa et al., 2013</td>
</tr>
<tr>
<td>Halogenated alkanes</td>
<td>Chlorinated paraffin halogenated methane and ethane</td>
<td>Solvents and chemical in industry and chemical manufactures formulations in products such as paints, sealants, plastics and metalworking fluids</td>
<td>Zhoa et al., 2013</td>
</tr>
<tr>
<td>Halogenated polycyclic aromatics</td>
<td>Chlorinated terphenyls, chlorinated naphthalene</td>
<td>Indoor-outdoor air Emission sources include vehicle exhaust, evaporated gasoline, cigarette smoke, moth and pest repellants, and deodorizers (e.g., diaperpail and toilet</td>
<td>Batterman et al., 2012</td>
</tr>
<tr>
<td>Halogenated heterocyclic compounds</td>
<td>Chlorinated /brominated thiophenes</td>
<td>Incineration Bleaching process in pulp and paper manufactures Car exhaust</td>
<td>Sinkkonen, 1997</td>
</tr>
</tbody>
</table>
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:Cl2) 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 al., 2004].
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.

<table>
<thead>
<tr>
<th>Type of sample</th>
<th>Analytical technique</th>
<th>Type of Cl-FA</th>
<th>pre-concentration methods</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Drinking water</td>
<td>FAB/GC MS</td>
<td>C18:Cl2;OH2</td>
<td></td>
<td>Gibson (1986)</td>
</tr>
<tr>
<td>Eel (Anguillaanguilla)</td>
<td>GC-ELCD</td>
<td>C18:Cl2</td>
<td>Ag* &amp; Urea</td>
<td>Wesen et al., (1992)</td>
</tr>
<tr>
<td>Fresh water fish (white sucker) (Catostomus Commersoni)</td>
<td>GC/MS</td>
<td>DMOX C14:Cl2</td>
<td>Gel permeation HPLC</td>
<td>Zhwang et al., (2003)</td>
</tr>
<tr>
<td>American lobster (Homarus americanus)</td>
<td>GC/MS (NICI)</td>
<td>C14:Cl2</td>
<td>Sephadex LH-20</td>
<td>Milley et al., (1997)</td>
</tr>
<tr>
<td>Eel(Anguillaanguilla)</td>
<td>GC/FID</td>
<td>C14Cl4 C14Cl2 C16Cl2 C18Cl2</td>
<td>Ag*, Urea</td>
<td>Mu et al., (1996a)</td>
</tr>
<tr>
<td>Eel (Anguillaanguilla) Flounder (Platichthys Flesus) Atlantic salmon (Salmo Salar) Perch (Perca Fluviatilis) Pacific salmon (Oncorhynchus Nerka)</td>
<td>GC/FID GC/ELCD GC/MS (NICI)</td>
<td>C14Cl2 C16Cl2 C18Cl2</td>
<td>Ag*, Urea, TLC</td>
<td>Mu et al., (1996)</td>
</tr>
<tr>
<td>Eel (Anguillaanguilla)</td>
<td>GC/MS (EI)</td>
<td>C18:Cl2 C14:Cl2</td>
<td>SPE-NH₂ aminopropyl column</td>
<td>Akesson et al., (2004)</td>
</tr>
<tr>
<td>Chinook salmon (Oncorhynchus tshawytscha)</td>
<td>GC/MS GC/XSD</td>
<td>C16Cl2 C18Cl2 C14Cl2</td>
<td>SPE-NH₂ aminopropyl column</td>
<td>King et al., (2006)</td>
</tr>
<tr>
<td>perch(Perca fluviatilis), Lavaret (Coregonous lavaretus) Stickleback(Gasterosteus aculatus).</td>
<td>GC/XSD</td>
<td>C18Cl4</td>
<td>SPE-NH₂ aminopropyl column</td>
<td>Brown and Järskog (2015)</td>
</tr>
<tr>
<td>harbour porpoise (Phocoena phocoena) harbour seal (Phoca vitulina)</td>
<td>GC/ELD GC/MS (PICl)</td>
<td>C16Cl2 C18Cl2</td>
<td>Ag*, Urea, TLC</td>
<td>Clark, 2003</td>
</tr>
</tbody>
</table>
2.5.  **Triacylglycerols (TAG)**

This thesis project involved the identification of CI-TAGs in natural oils. No CI-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 CI-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*., 2005].
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 (EDCI) 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-dicyclohexyl-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 Me₂C=) is a wildly used protective group, and is best removed under acidic conditions. Several acids (acetic acid, HCl, CF₃COOH, H₂SO₄, 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 consists of vaporization with heated desolvation 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] allow 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)* 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 CI-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).
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 TAGs 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 Christie 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$_2$SO$_4$ 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 [Metcalf et al., 1961; Metcalf 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
The 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$_2$SO$_4$ in MeOH) and three base-catalyzed methods (MeONa, TMG/MeOH, and TMSN$_2$CH$_3$/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; Holčapek 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 [Holčapek 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$_2$Cl$_2$) 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 (CH₂Cl₂).

One (1.0) g of the fatty acid stock solution was mixed with 8 mL ethanol (EtOH) and 2 mL H₂SO₄ 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 (Na₂SO₄) and evaporated to dryness in a rotary evaporator. The residue (ethyl esters of the fatty acids) was dissolved in a small amount of CH₂Cl₂, transferred to a 50-mL volumetric flask and diluted to the mark with CH₂Cl₂. 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 CH₂Cl₂. 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 CH₂Cl₂. 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.

<table>
<thead>
<tr>
<th>Stock solution of FAEE (g)</th>
<th>Solvent (g)</th>
<th>Internal standard of C8:0 ethyl ester (g)</th>
<th>Concentration µg mL⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.00</td>
<td>0</td>
<td>0.250</td>
<td>0.608</td>
</tr>
<tr>
<td>1.00</td>
<td>1.00</td>
<td>0.250</td>
<td>0.338</td>
</tr>
<tr>
<td>0.50</td>
<td>1.50</td>
<td>0.250</td>
<td>0.168</td>
</tr>
<tr>
<td>0.10</td>
<td>1.75</td>
<td>0.250</td>
<td>0.036</td>
</tr>
<tr>
<td>0.05</td>
<td>1.95</td>
<td>0.250</td>
<td>0.0168</td>
</tr>
<tr>
<td>0.00</td>
<td>2.00</td>
<td>0.250</td>
<td>0.0</td>
</tr>
</tbody>
</table>

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 BF₃-MeOH

3.3.3.4.1 Esterification of FFA using BF₃-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 BF₃-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 BF₃-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 BF₃-MeOH procedure. The mass ratio of BF₃-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 H₂SO₄ 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.
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 [$\text{CH}_3\text{CH}_2\text{COOCH}_3$]$^+$. 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].
The pattern of fragmentation 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.
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 μg mL\(^{-1}\) by dilution. Fig. 3.8B shows the calibration at the low end of this range.

Fig 3.7 (A) GC/MS chromatogram of methyl ester standard solutions; (B) expanded region (20.0-22.0 min).
The lowest concentration that gave a significant response was obtained at 0.125 \( \mu \text{g mL}^{-1} \) 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 \( \mu g \text{ mL}^{-1} \) could be cross contamination of the samples by the GC syringe or possible problems with the liner in the injection port.

![Chromatogram of 125 ng/mL Standard](image)

**Fig 3.9.** The GC/MS chromatogram for the lowest concentration (0.125 \( \mu g \text{ mL}^{-1} \)) 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.
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.

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


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.

![Figure 3.18](image1.png)

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

![Figure 3.19](image2.png)

*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 triacylglycerols 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-HPLC/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 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.

<table>
<thead>
<tr>
<th>F #</th>
<th>Time (min)</th>
<th>Results (FAME / FA-isopro)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0-5</td>
<td>Nothing</td>
</tr>
<tr>
<td>2</td>
<td>5-10</td>
<td>C8:0</td>
</tr>
<tr>
<td>3</td>
<td>10-15</td>
<td>C8 &amp; C18:1</td>
</tr>
<tr>
<td>4</td>
<td>15-20</td>
<td>C16</td>
</tr>
<tr>
<td>5</td>
<td>20-25</td>
<td>C18:2</td>
</tr>
<tr>
<td>6</td>
<td>25-30</td>
<td>C18:1</td>
</tr>
</tbody>
</table>

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.

![Reversed Phase Chromatogram of Corn Oil](image)

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 BF₃-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.

<table>
<thead>
<tr>
<th>F#</th>
<th>Time (min)</th>
<th>Results (FAME)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>19.3</td>
<td>C16:0, C18:1</td>
</tr>
<tr>
<td>2</td>
<td>20.2</td>
<td>C18:2</td>
</tr>
<tr>
<td>3</td>
<td>21.7</td>
<td>C18:2, C18:1</td>
</tr>
<tr>
<td>4</td>
<td>23.3</td>
<td>C18:2, C18:1</td>
</tr>
<tr>
<td>5</td>
<td>26.3</td>
<td>C16:0, C18:2, C8:1</td>
</tr>
<tr>
<td>6</td>
<td>27.5</td>
<td>C16:0, C18:2, C8:1</td>
</tr>
<tr>
<td>7</td>
<td>29.7</td>
<td>C16:0, C18:2, C8:1, C18:0, C20:1</td>
</tr>
</tbody>
</table>

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.](image)

**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-isopropanol (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.

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.

![Figure 4.13. Chromatograms of the 3 cutting positions of peak 5 separated in RP-HPLC.](image)

**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

<table>
<thead>
<tr>
<th>F#</th>
<th>M</th>
<th>[M+107/109Ag]⁺</th>
<th>[M+Na]⁺</th>
<th>GC-MS</th>
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<tr>
<td>F2</td>
<td>856.7</td>
<td>963.7(965.6)</td>
<td>7</td>
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<td>991.7(993.6)</td>
<td>25</td>
<td>C18:2</td>
<td>SLO</td>
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<td>989.7(991.6)</td>
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<td>961.7(963.6)</td>
<td>52</td>
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<td>903.9</td>
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<td>C16:0</td>
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<td>4</td>
<td>C18:0</td>
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<td>F8</td>
<td>878.7</td>
<td>901.8</td>
<td>-</td>
<td>C16:0</td>
<td>LLL</td>
<td>6</td>
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<td>85</td>
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<td></td>
<td></td>
<td>-</td>
<td>C18:0</td>
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<tr>
<td>F9</td>
<td>876.7</td>
<td>899.7</td>
<td>-</td>
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<td>40</td>
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<td></td>
<td></td>
<td>C18:0</td>
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</table>

* 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 107Ag and 109Ag 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-RCOOH]* and [M+Na-RCOO-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; Holčapek 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

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

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<th>Na A-Na</th>
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<tr>
<td>F3</td>
<td>LOO</td>
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<td>905.7</td>
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<td>623.5</td>
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<td>601.4</td>
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<td>L O S L-Na O-Na S-Na</td>
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<tr>
<td>PLL</td>
<td>854.7</td>
<td>877.7</td>
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<td>597.4</td>
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<td>575.4</td>
<td>575.4</td>
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<td>623.3</td>
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<td></td>
<td>601.5</td>
<td>599.4</td>
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<td>Ln L O Ln-Na L-Na O-Na</td>
<td>5</td>
</tr>
<tr>
<td>F5</td>
<td>LLL</td>
<td>OLLn</td>
<td>878.7</td>
<td>901.8</td>
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<td></td>
<td></td>
<td></td>
<td>601.6</td>
<td>621.4</td>
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<tr>
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<td>LnOLn</td>
<td>876.7</td>
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<td>619.4</td>
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<td>Ln L O Ln-Na L-Na O-O Na</td>
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P (C16:0); S (C18:0); O (C18:1); L (C18:2); Ln (C18:3); A (C20:0)
$m/z$ 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 4.22A. 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 silver [M+ 107/109Ag]+ ions at m/z 963.7(965.6) and 991.7(993.6) corresponding to [PLO+107/109Ag] and [OOO+107/109Ag]/[SOL+107/109Ag]+, 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+107/109Ag]+, 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.

![Graph showing the absorbance at 210 nm for different time intervals.](image)

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

<table>
<thead>
<tr>
<th>F#</th>
<th>TAG</th>
<th>M</th>
<th>[M+ Na]^+</th>
<th>[M+ Na-RCOOH]^+</th>
<th>[M+ Na-RCOO-Na]^+</th>
<th>Loss</th>
<th>DB</th>
<th>PN</th>
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<tbody>
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<td>F1-F6</td>
<td>Oxidized TAG</td>
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<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>F7</td>
<td>LLL</td>
<td>878.7</td>
<td>901.7</td>
<td>621.2/599.3</td>
<td>L L-Na</td>
<td>6</td>
<td>42</td>
<td></td>
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<tr>
<td>F8</td>
<td>OLLn</td>
<td>878.8</td>
<td>901.7</td>
<td>623.2 621.3 619.4</td>
<td>Ln L O Ln Na L-Na O-Na</td>
<td>6</td>
<td>42</td>
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<tr>
<td></td>
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<td>909.6</td>
<td></td>
<td>601.3 599.4 597.3</td>
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<tr>
<td>F9</td>
<td>LLO</td>
<td>880.7</td>
<td>903.3</td>
<td>623.3 621.5 601.5 599.4</td>
<td>L O L-Na O-Na</td>
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<td>905.7</td>
<td>625.4 623.5 622.3 603.6 601.4 600.1</td>
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<td>879.7</td>
<td>623.4 599.4 597.5 601.4 577.5 575.5</td>
<td>P L O P-Na L-Na O-Na</td>
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<tr>
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<td>881.9</td>
<td>625.5 599.5 603.6 577.4</td>
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<td>SLO</td>
<td>POP</td>
<td>ALP</td>
<td>SLS</td>
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<td>F16</td>
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<tr>
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<td>884.7</td>
<td>907.9</td>
<td>627.5</td>
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<tr>
<td>F16</td>
<td>832.5</td>
<td>855.8</td>
<td>599.3</td>
<td>573.3</td>
<td>577.3</td>
<td>551.3</td>
<td>1</td>
<td>49</td>
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<td>909.8</td>
<td>653.3</td>
<td>629.2</td>
<td>625.3</td>
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<td>933.5</td>
<td>653.3</td>
<td>621.3</td>
<td>631.3</td>
<td>599.2</td>
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<td>A</td>
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<tr>
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<td>933.5</td>
<td>653.3</td>
<td>621.3</td>
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<td>605.4</td>
<td>603.4</td>
<td>O</td>
<td>S</td>
</tr>
<tr>
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<td>909.8</td>
<td>655.5</td>
<td>653.2</td>
<td>623.3</td>
<td>633.5</td>
<td>631.3</td>
<td>601.2</td>
</tr>
<tr>
<td>ALO</td>
<td>912.6</td>
<td>935.7</td>
<td>655.5</td>
<td>653.2</td>
<td>623.3</td>
<td>633.5</td>
<td>631.3</td>
<td>601.2</td>
</tr>
</tbody>
</table>

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 4.34 A, 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, epoxides, and hydroxyl epoxides. 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 oxidized 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$_2$O. Similarly, a loss of 88 u product ion at $m/z$ 845.5 corresponded to loss of C$_5$H$_{12}$O or C$_4$H$_8$O$_2$. 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.](image)

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 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+O2]⁺).
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

<table>
<thead>
<tr>
<th>F #</th>
<th>M</th>
<th>[M+Na]^+</th>
<th>The oxidized TAG</th>
<th>MS/MS</th>
<th>The identified of oxidized TAG</th>
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<tbody>
<tr>
<td>F1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F3</td>
<td>878.7</td>
<td>901.7</td>
<td>933.7</td>
<td>901.5/[M+ Na]^+ 653.4/C18:2</td>
<td>TAG3C18:2+O2</td>
</tr>
<tr>
<td>F4</td>
<td>878.7</td>
<td>901.7</td>
<td>915.9</td>
<td>635.3/ C18:2 613.2/ C18:2-Na</td>
<td>TAG-3C18:2+O</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>933.7</td>
<td>915.7/ H2O 653.3/ C18:2 599.2/C18:2+O2+Na</td>
<td>TAG-3C18:2+O2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>949.6</td>
<td>931.6/ H2O 17.2/ O2 901.4/[M+ Na]^+ 669.4/ C18:2 653.2/ C18:2+O 651.0/ C18:2+H2O 637.3/C18:2+O2</td>
<td>TAG-3C18:2+O3</td>
</tr>
<tr>
<td>F5</td>
<td>878.7</td>
<td>901.7</td>
<td>917.7</td>
<td>637.3/ C18:2 635.3/ C18:1</td>
<td>TAG- C18:2/C18:1/C18:3 +O</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>935.5</td>
<td>899.0/2 H2O 655.5/ C18:2 653.1/C18:1</td>
<td>TAG- C18:2/C18:1/C18:3 +O+ H2O</td>
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<tr>
<td>F6</td>
<td>876.7</td>
<td>899.7</td>
<td>899.8</td>
<td>621.2/ C18:3 599.3/ C18:3-Na 619.3/ C18:2 597.2/ C18:2-Na</td>
<td>TAG- C18:2/C18:2/C18:3</td>
</tr>
</tbody>
</table>
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

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

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.

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

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 CI-TAG compounds. The CI-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 CI-TAGs which are described in detail in chapter 5.
CHAPTER 5
Synthesis of chlorinated triacylglycerol compounds and their
coloration 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 CI-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; Höstmark et al., 1999; Lystad et al., 2001].

Three dichlorinated fatty acids, 9,10-dichlorooctadecanoic acid (9,10-dichlorostearic acid; \( \text{HO}_2\text{C(CH}_2\text{)}_7\text{(CHCl)}_2\text{(CH}_2\text{)}_7\text{CH}_3 \)), 7,8-dichlorohexadecanoic acid (7,8-dichloropalmitic acid; \( \text{HO}_2\text{C(CH}_2\text{)}_5\text{(CHCl)}_2\text{(CH}_2\text{)}_7\text{CH}_3 \)) and 5,6-dichlorotetradecanoic acid (5,6-dichloromyristic acid; \( \text{HO}_2\text{C(CH}_2\text{)}_3\text{(CHCl)}_2\text{(CH}_2\text{)}_7\text{CH}_3 \)), 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-Svård et al., 2001; Lawrence et al., 1984] and by the release of radioactive carbon dioxide when [1-\(^{14}\text{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-Svård et al., 2001] are consistent with the proposed uptake of 9,10-dichlorooctadecanoic acid from pulp mill effluents [Björn et al., 2004; Gustafson-Svård 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 Aspergillus 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 (^1H, 500 MHz) and carbon (^13C, 125 MHz) nuclear magnetic resonance (NMR) spectra were recorded on a Bruker AVANCE 500 MHz spectrometer. Samples were dissolved in either CDCl₃ or CD₂Cl₂ (30 mg mL⁻¹). Chemical shifts (δ) in parts per million (ppm) were referenced to solvent signals (CDCl₃: δ_H 7.26, δ_C 77.16; CD₂Cl₂: 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 (Supelco 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-dodecanoylglycerol (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-dichlorooctadeccanoyl)-1,3-didodecanoylglycerol (compound 6), 1,2-isopropylidene-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-dodecanoylglycerol (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-isopropylidenediglycerol (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 1 also 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$_2$Cl$_2$) 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-didodecanoyl glycerol (compound 5), as given below.

5.2.3.2.1. Synthesis of 1,3-didodecanoyl glycerol (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$_2$Cl$_2$) $\delta$: 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$_2$Cl$_2$) $\delta$: 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$_{48}$H$_{84}$Cl$_6$O$_6$Na).

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

5.2.3.3.1. Synthesis of monoacylisopropylidenedienes

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$_2$Cl$_2$) $\delta$: 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$_2$Cl$_2$) $\delta$: 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,
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-isopropylideneeglycerol (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$_2$Cl$_2$) 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$_2$Cl$_2$) δ: 174.6, 71.0, 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$_2$O$_4$Na).

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;
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; 1H 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); 13C 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 (35% 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$_{86}$Cl$_2$O$_6$Na).

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 (2.4 g, 73%). 1H 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); 13C 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
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%). 

\[ \text{1H NMR (CD}_2\text{Cl}_2) \delta: \]

- 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, \( J = 7.0 \));

\[ \text{13C NMR (CD}_2\text{Cl}_2) \delta: \]

- 173.6 (2C),
- 173.2, 69.4, 66.41 (3C),
- 66.37 (3C), 62.6 (2C),
- 34.7, 34.5 (2C), 32.4 (3C),
- 30.0 (3C), 29.8 (3C),
- 29.6 (2C), 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:
- 1119.5 (100), 1083.3 (24), 1047.4 (100), 1045.6 (40), 1009.6 (23), 973.5 (42); MS/MS (33% CID) of \( m/z \) 1119.5: 1117.5 (60, [M+Na]+);
- 1117.5 (100), 1083.3 (24), 1047.4 (100), 1045.6 (40), 1009.6 (23), 973.5 (42); MS/MS (33% CID) of \( m/z \) 1119.5: 1117.5 (60, [M+Na]+);

5.2.3.4.2. Tri-(9,10-dibromooctadecanoyl)glycerol (compound 17)

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 \(-10^\circ\text{C}\). 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%).

\[ \text{1H NMR (CD}_2\text{Cl}_2) \delta: \]

- 5.25–5.21 (m, 1H),
- 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, \( J = 7.5 \)),
- 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, \( J = 7.0 \));

\[ \text{13C NMR (CD}_2\text{Cl}_2) \delta: \]

- 173.6 (2C),
- 173.2, 69.4, 66.41 (3C),
- 66.37 (3C), 62.6 (2C),
- 34.7, 34.5 (2C), 32.4 (3C),
- 30.0 (3C), 29.8 (3C),
- 29.6 (2C), 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): \( m/z \) 1391.6 (17), 1390.7 (35), 1389.7 (42), 1388.7 (35), 1387.7 (59), 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_{2}O_{6}Na).

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 $^1$H 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 $^1$H NMR chemical shifts of methyl threo- and erythro-9,10-dichlorooctadecanoate [Wesén et al., 1995a], a distinct resonance ($\delta$ 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 ¹H 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 ¹³C NMR resonances and an (AB)_2X ¹H 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.
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.

![Chemical structures and reactions](image)

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].

<table>
<thead>
<tr>
<th>Comp’d</th>
<th>[M + Na]⁺ Formula</th>
<th>m/z (Observed Relative Intensity, Calculated Relative Intensity)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>A</td>
</tr>
<tr>
<td>2</td>
<td>C₁₈H₃₆Cl₂O₂Na</td>
<td>375.3 (100,100)</td>
</tr>
<tr>
<td>6</td>
<td>C₂₀H₄₀Cl₂O₄Na</td>
<td>813.6 (100,100)</td>
</tr>
<tr>
<td>9</td>
<td>C₂₀H₄₀Cl₂O₄Na</td>
<td>489.3 (100,100)</td>
</tr>
<tr>
<td>11</td>
<td>C₂₁H₄₂Cl₂O₄Na</td>
<td>449.2 (100,100)</td>
</tr>
<tr>
<td>13</td>
<td>C₂₁H₄₂Cl₂O₄Na</td>
<td>813.5 (100,100)</td>
</tr>
<tr>
<td>14</td>
<td>C₂₁H₄₂Cl₂O₄Na</td>
<td>965.7 (71,69)</td>
</tr>
<tr>
<td>16</td>
<td>C₂₂H₄₄Cl₂O₄Na</td>
<td>1117.5 (60,47)</td>
</tr>
</tbody>
</table>

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 dichlorooctadecanoate [Mu et al., 1996b] fragmented under chemical ionization
conditions, losing Cl₂, 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 H35Cl and H36Cl + H37Cl from the 35Cl37Cl 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.

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.
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 anemic 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.

![Figure 5.6. TIC of TAGC18:1 (1 nM- 6 μM) range m/z 907.7 -908.7.](image)
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-C18Cl2-C12 (1 nM- 1 µM) range m/z 813.5 -815.5.
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 thereby 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/C18Cl2/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/C18Cl2/C12:0 by ESI(+)/MS

<table>
<thead>
<tr>
<th>Concentration #</th>
<th>ESI(+) / MS observation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 nM</td>
<td>Not detected</td>
</tr>
<tr>
<td>2 nM</td>
<td>Not detected</td>
</tr>
<tr>
<td>5 nM</td>
<td>Not detected</td>
</tr>
<tr>
<td>0.01 µM</td>
<td>Not detected</td>
</tr>
<tr>
<td>0.02 µM</td>
<td>Not detected</td>
</tr>
<tr>
<td>0.05 µM</td>
<td>Not detected</td>
</tr>
<tr>
<td>0.1 µM</td>
<td>Not detected</td>
</tr>
<tr>
<td>0.2 µM</td>
<td>Detected</td>
</tr>
<tr>
<td>0.5 µM</td>
<td>Detected</td>
</tr>
<tr>
<td>1 µM</td>
<td>Detected</td>
</tr>
</tbody>
</table>

It is evident from the data in Table 5.2 that the concentration of TAG-C12:0/C18Cl2/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 concentration 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/C18Cl2/C12:0 (compound 13), TAG-C12:0/C18Cl2/C18Cl2 (compound 14) and TAG-C18Cl6 (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.

![HPLC chromatogram](image)

Fig 5.12. Seventeen fractions collected from the corn oil run RP-HPLC after spiked with Cl compounds (13, 14, and 16).

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-C18Cl2-C12:0 (compound 13) with 2 Cl atoms eluted at 2 min (F1, Fig 5.13A,B); the TAG-C12:0-C18Cl2-C18Cl2 (compound 14) with 4 Cl atoms eluted at 8 min (F3, Fig 5.14A,B); finally the TAG-C18Cl6 (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-C18Cl₂-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-C18Cl₂-C18Cl₂-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-C18Cl₆ 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\textsubscript{2}-C12 (compound 13) with low number of carbon (C\textsubscript{45}H\textsubscript{84}Cl\textsubscript{2}O\textsubscript{6}Na) eluted early followed by the TAG-C12-C18Cl\textsubscript{2}-C18Cl\textsubscript{2} (compound 14) containing more carbon (C\textsubscript{51}H\textsubscript{94}Cl\textsubscript{4}O\textsubscript{6}Na). Finally, the TAG-C18Cl\textsubscript{6} (compound 16) with highest carbon number C\textsubscript{57}H\textsubscript{104}Cl\textsubscript{6}O\textsubscript{6}Na 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 (\textit{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 \textit{et al.}, 2015; Nishizaki \textit{et al.}, 2014; Hara \textit{et al.}, 2013; von Schacky, 2009; and Lavie \textit{et al.}, 2009]. Several studies have reported that diet is one of the most important entry points for persistent organic pollutants into humans [Kiviranta \textit{et al.} 2004; Darnerud \textit{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 \textit{et al.}, 2004; Shaw \textit{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 \textit{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).

![Diagram of sample preparation](image)

Fig 5.16. Preparation 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$_2$SO$_4$. The excess solvent was evaporated under N$_2$ in a water bath to minimize any aerial oxidation that may occur (Fig 5.17).

![Extraction using Brinkman Polytron (sonication) Hexane : Acetone (1:1 v/v)](image)

**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 processor, 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.
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\textsubscript{2} technology and used UHPLC/ESI(+)\textsl{}/MS method to detect 180 TAGs and their fatty acids in wild Atlantic salmon (\textit{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]\textsuperscript{+} 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]\textsuperscript{+} by the ESI(+)\textsl{}/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 \textsl{m}/\textsl{z} 878.2, 904.1, 927.9, and 953.7 and TAG ions of lower abundance at \textsl{m}/\textsl{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 difference between the precursor [M+Na]⁺ ion and the product ions [M+Na-RCOOH]⁺ and [M+Na-RCOO-Na]⁺. For example, CID of the [M+Na]⁺ ion at m/z 927.9 (Fig 5.22) gave the product [M+Na-RCOOH]⁺ and [M+Na-RCOO-Na]⁺ ions at m/z 645.3 (623.3) and 625.4 (603.5); the mass difference 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 backbone, 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.
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 5.22. The CID spectrum of F10 of salmon tissue oil at \(m/z\) 927.9.
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).
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.
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.
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 C3H6O2 or C4H10O), 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+2H2O+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+3H2O 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$_2$O 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$_2$O 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$_2$O or TAG-C18:1/C16:1/C14:0+O$_3$. 153
Fig 5.29. ESI(+) mass spectrum of F8 of salmon skin.
Fig 5.30. (A) The CID spectrum of F8 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.

<table>
<thead>
<tr>
<th>F#</th>
<th>[M+Na]*</th>
<th>MS/MS</th>
<th>Identification</th>
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<td></td>
<td>[M+Na-RCOOH]+</td>
<td>[M+Na-RCOO-Na]+</td>
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<td>7</td>
<td>871.7</td>
<td>835.3/ O₂</td>
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<td>797.0/74 u (2H₂O+O+Na)</td>
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<td>623.2/C16:4</td>
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<td>601.3/C16:4-Na</td>
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<td>617.3/C16:1</td>
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<td>594.8/C16:1-Na</td>
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<td>615.4/C16:0</td>
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<td>593.2/C16:0-Na</td>
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<td>606.2/C16:4+HO</td>
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<td>569.4/C20:5</td>
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<td>547.2/C20:5-Na</td>
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<td>517.0/C20:5+2H₂O+O</td>
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<td>8</td>
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<td>799.3/74 u (2H₂O+O+Na)</td>
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<td>725.3/148 u</td>
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<td>644.9/C14:0</td>
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<td>622.2/C14:Na</td>
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<td>TAG-C18:1/C14:0/C16:1+ O₃</td>
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<td>TAG- C18:2/C18:3/C16:1</td>
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<td>9</td>
<td>875.5</td>
<td>801.3/74 u</td>
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<td>727.3/148 u</td>
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<td>647.5/C14:0</td>
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<td></td>
<td>621.5/C16:1</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>599.2/C16:1-Na</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>597.5/C18:3</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>575.3/C18:3-Na</td>
<td></td>
</tr>
<tr>
<td></td>
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<td>595.3/C18:2</td>
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<tr>
<td></td>
<td></td>
<td>573.0/C18:2-Na</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>593.3/C18:1</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>570.9/C18:1-Na</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>562.4/C16:1+2H₂O+Na</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>550.8/C16:2+2H₂O+O+Na</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>532.9/C16:1+2H₂O+O₂+Na</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>521.9/C18:2+4H₂O</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>505.3/C18:2+2H₂O+O₂+Na</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>TAG- C14:0/C16:2/C18:2+3H₂O</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>TAG-C18:1/C16:1/C14:0+ +H₂O+O₂</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>TAG- C18:1/C18:3/C16:1</td>
</tr>
</tbody>
</table>
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 13C 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 [Siddique et al., 2003; Falch et al., 2005]. 13C 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 et 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. $^{13}$C 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.

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

$\Sigma = 97.62$
Fig 5.36. GC/MS of the fatty acid methyl esters chromatogram for the salmon tissue.
Table 5.5. FAME in salmon skin oil

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

$\sum = 96.28$
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.

<table>
<thead>
<tr>
<th>FAMEs GC/MS</th>
<th>FA upon CID RP-HPLC/ESI(+)MS</th>
</tr>
</thead>
<tbody>
<tr>
<td>C14:0</td>
<td>C14:0</td>
</tr>
<tr>
<td>-</td>
<td>C16:4</td>
</tr>
<tr>
<td>-</td>
<td>C16:3</td>
</tr>
<tr>
<td>-</td>
<td>C16:2</td>
</tr>
<tr>
<td>C16:1</td>
<td>C16:1</td>
</tr>
<tr>
<td>C16:0</td>
<td>C16:0</td>
</tr>
<tr>
<td>-</td>
<td>C18:4</td>
</tr>
<tr>
<td>-</td>
<td>C18:3</td>
</tr>
<tr>
<td>C18:2</td>
<td>C18:2</td>
</tr>
<tr>
<td>C18:1</td>
<td>C18:1</td>
</tr>
<tr>
<td>C18:1</td>
<td>C18:1</td>
</tr>
<tr>
<td>C18:0</td>
<td>C18:0</td>
</tr>
<tr>
<td>C20:5</td>
<td>C20:5</td>
</tr>
<tr>
<td>C20:4</td>
<td>C20:4</td>
</tr>
<tr>
<td>C20:3</td>
<td>C20:3</td>
</tr>
<tr>
<td>C20:2</td>
<td>C20:2</td>
</tr>
<tr>
<td>C20:1</td>
<td>C20:1</td>
</tr>
<tr>
<td>C20:0</td>
<td>C20:0</td>
</tr>
<tr>
<td>C22:6</td>
<td>C22:6</td>
</tr>
<tr>
<td>-</td>
<td>C22:5</td>
</tr>
<tr>
<td>-</td>
<td>C22:4</td>
</tr>
<tr>
<td>C22:1</td>
<td>C22:1</td>
</tr>
<tr>
<td>C22:0</td>
<td>C22:0</td>
</tr>
</tbody>
</table>
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/C12:0, TAG-C12:0/C12:0/C18:Cl2, TAG-C12:0/C18:Cl2/C18:Cl2, and TAG-C18:Cl/C18:Cl/C18:Cl2 and one brominated TAG (TAG-C18:Br2/C18:Br2/ C18:Br2) 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 ^31P probe which indicated that there were no phospholipids in either tissue or skin oils. In addition, the ^1H NMR and ^13C 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 CI-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 CI-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 CI-TAGs be investigated with respect to the extraction OCls from salmon. Since the levels of CI-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 CI-TAGs. It has not been used by others for CI-TAGs for various reasons. Natural sample is a very complex mixture and finding low levels of CI-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


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


Brattberg, 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


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.


183


Ewald, G., and P. Sundin, 1993, ATP leakage from eel 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.


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


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


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


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.


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


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


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


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.


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


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.


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


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


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


## Appendix 1

Table 1. Peak identification of salmon tissue oil TAGs using 2D off-line RP-HPLC/ESI(+)/MS

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<th>PN</th>
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|               | TAG- C18:2/C18:1/C20:1                | 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  |
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627.3/C18:2  
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625.4/C18:1  
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601.5/C18:0-Na | TAG-C18:0/C18:1/C18:2 | 48 | 3 |
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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

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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-C18Cl₂ [Compound 2] at m/z 375.7; (B) MS/MS spectrum at m/z 375.7
App-2-Fig 3. (A) $^1$H NMR spectrum; (B) $^{13}$C NMR spectra of TAG-3C18Cl$_6$

[Compound 16]
App-2-Fig 4.(A). CID spectrum of TAG-3C18Cl [Compound 16] at m/z 1118.9; (B) MS^3 spectrum of m/z 1118.9

(ms^3) = m/z 901.7

-(C18:2+Na) -C18:2
App-2-Fig 5. (A) $^1$H NMR spectrum; (B) $^{13}$C NMR spectra of TAG-3C18Br₆

[Compound 17].
App-2-Fig 6. (A). ESI-MS spectrum of TAG-3C18Br [Compound 17] at \( m/z \) 1387.9; (B) CID spectrum at \( m/z \) 1387.9.
App-2-Fig 7. (A) $^1$H NMR spectrum; (B) $^{13}$C NMR spectra of TAGC18Cl$_2$-C18Cl$_2$-C12:0 [Compound 14]
App-2-Fig 8. (A)$^1$H NMR spectrum; (B) $^{13}$C NMR spectra of TAGC12:0-C18Cl$_2$-C12:0 [Compound 6]
App-2-Fig 9 (A) $^1$H NMR spectrum; (B) $^{13}$C NMR spectra of $C_{24}H_{44}Cl_2O_4Na$ [Compound9]
App-2-Fig10. (A) H\textsuperscript{1} NMR spectrum; (B) C\textsuperscript{13}NMR spectra of C\textsubscript{21}H\textsubscript{40}Cl\textsubscript{2}O\textsubscript{4}Na [compound 11]
App-2-Fig 11. (A). ESI-MS spectrum of C$_{21}$H$_{40}$Cl$_2$O$_4$Na [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_{2}O_{4}Na [compound 9] at m/z 489.2; (B). CID spectrum of m/z 489.2
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