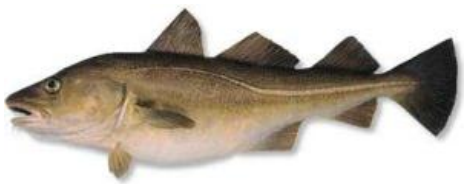
Exploring the capabilities of gas chromatography and liquid chromatography single and tandem mass spectrometry for discriminating and characterizing marine oils by using chemometric tools

by

Yingxu Zeng

Thesis for the degree of European Master in Quality in Analytical Laboratories



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List of Publications

[1] P. Araujo, **Y.X. Zeng**, Z.Y. Du, T.T. Nguyen, L. Frøyland, B. Grung, Discrimination of omega-3 rich marine animal oils by gas chromatography, *Journal of Chromatography A* (Accepted).

[2] **Y.X. Zeng**, P. Araujo, B. Grung, Evaluation of liquid chromatography and mass spectrometry profiles as potential tools for fingerprinting marine oils. (Manuscript).

[3] **Y.X. Zeng**, P. Araujo, Z.Y. Du, T.T. Nguyen, L. Frøyland, B. Grung, Structural characterization of triacylglycerols in cod liver oil using liquid chromatography electrospray tandem mass spectrometry, *Talanta* (Submitted).

List of Abbreviations

APCI	Atmospheric pressure chemical ionization
CODA	Component detection algorithm
DAG	Diacylglycerols
DHA	Docosahexaenoic acid (22:6n-3)
DPA	Docosapentaenoic acid (22:5n-3)
ECN	Equivalent carbon number
EIC	Extracted ion chromatogram
EPA	Eicosapentaenoic acid (20:5n-3)
ESI	Electrospray ionization
FAME	Fatty acid methyl esters
FFA	Free fatty acids
FID	Flame ionization detector
GC	Gas chromatography
GC-MS	Gas chromatography-mass spectrometry
HPLC	High performance liquid chromatography
LC-ESI-MS	Liquid chromatography electrospray single mass spectrometry
LC-ESI-MS^2	Liquid chromatography electrospray tandem mass spectrometry
MAG	Monoacylglycerols
MCQ	Mass chromatographic quality
MS	Mass spectrometry
NMR	Nuclear magnetic resonance
PCA	Principal component analysis
PUFA	Polyunsaturated fatty acids
SFA	Saturated fatty acids
TIC	Total ion chromatogram
TAG	Triacylglycerols

Abstract

Assessing the capabilities of instrumental techniques for discriminating marine oils and studying the positional distribution of fatty acids on the backbone of triacylglycerols (TAG) are of vital importance from commercial, nutritional, biochemical and technological points of view. This represents a great challenge for analysts due to the wide variety of fatty acids and the complexity of naturally occurring TAG species.

In this thesis, the potential of gas chromatography (GC) for discriminating full fatty acid methyl ester (FAME) profiles of marine oils (cod liver, salmon, seal and whale oils) is evaluated by means of principal component analysis (PCA). The FAME profiles from plant oils such as rapeseed, linseed and soy oils and seven different brands of omega-3 (ω-3) fatty acids supplements are also used in the discrimination process. The results from the PCA plots can reliably distinguish between plant, ω-3 fatty acids supplements, fish and marine mammal oils. By removing the contribution of the ω-3 fatty acids supplements and plant oils, it is possible to discriminate within every type of fish and marine animal oils. GC offers a rapid, simple and convenient means of discriminating marine oils from different species, brands and grades.

The thesis also studies the feasibility of fingerprinting and discriminating marine oils based on their TAG profiles using liquid chromatography electrospray single and tandem mass spectrometry (LC-ESI-MS and LC-ESI-MS^2) in conjunction with chemometric tools. Four kinds of profiles, including total ion chromatogram (TIC) and mass spectral profiles derived from LC-ESI-MS and LC-ESI-MS^2 experiments, are examined prior to data pretreatment by component detection algorithm (CODA) to reduce the noise and background. These profiles are subsequently subjected to PCA to evaluate their performance for discriminating marine oils and plant oils. The results show that the TIC profiles derived from both LC-ESI-MS and LC-ESI-MS^2 experiments turn out to be inadequate for discrimination of complex marine oils. Although the classification results are remarkably improved by using single mass spectral profiles derived from LC-ESI-MS experiments, the differentiation among seal oils of different species and qualities is not achieved. In comparison, the use of tandem mass spectral profiles from LC-ESI-

MS^2 experiment is demonstrated to be the best strategy for discrimination of marine oils which enables the differentiation not only between marine oils and plant oils but also among the seal oils of different species and qualities. The tandem mass spectral profiles could preferably represent the characteristics of TAG patterns, and could be used as an alternative approach for fingerprinting and detecting of adulteration of marine oils.

The final aspect studied in the present thesis is the structural characterization of TAG by using LC-ESI-MS^2 for identifying the positional distribution of fatty acids on the glycerol backbone in cod liver oil. A computational algorithm is developed to characterize rapidly and interpret automatically the mass spectra of the various detected TAG species. Three different solvent mixtures are used to dissolve the sample prior to the instrumental analysis. The discrepancies between the results indicate that the choice of the solvent system influences the identification of the TAG species. The results obtained by the proposed LC-ESI-MS^2 approach are in agreement with those from the well established lipase method. LC-ESI-MS^2 provides a suitable and powerful strategy for the structural characterization of TAG in cod liver oil.

1 Introduction

1.1 Background

Marine oils are the important sources of omega-3 (ω-3) polyunsaturated fatty acids (PUFA), which have attracted extensive interests due to the accumulating scientific evidences supporting their potential health benefits in improving chronic cardiovascular diseases [1-3] and inflammatory pathologies [4, 5], thus leading to an escalating consumer demand for ω-3 fatty acids rich functional food, dietary supplements and pharmaceuticals.

There are two critical issues for marine oils studies which constitute great challenges for analysts: discrimination of various marine oils and characterization of the positional distribution of fatty acids on the triacylglycerols (TAG) backbone in marine oils.

A literature overview of the different instrumental techniques used for the analysis of plant, fish and marine animal oils shows that the current literatures on nutritional oil discrimination and characterization has been mainly focused on plant oils, while the studies on marine oils represents a meagre proportion. It is surprising that the capabilities of gas chromatography (GC) and liquid chromatography electrospray single and tandem mass spectrometry (LC-ESI-MS and LC-ESI-MS^2) techniques has not been explored yet in the discrimination of marine oils by using the fatty acid composition and TAG profiles. Although the importance of developing techniques aiming at detecting adulteration of marine oils has been emphasised more than 100 years ago [6, 7], it has been much neglected by practitioners during the course of a century and it is a topic of contemporary relevance that needs attention.

In addition to marine oils discrimination, there is a pressing need accordingly for analysts to develop reliable analytical methods with the capacity to characterize the positional distribution of fatty acids on the backbone of TAG in marine oils in order to gain a better knowledge of their various lipids properties and structural composition and thereby utilize their nutritional values further. Although LC-ESI-MS^2 has become increasingly popular for characterizing TAG in plant

oils due to the high degree of information derived from its implementation, the application of LC-ESI-MS^2 in characterization of TAG in marine oils has not been investigated yet, which might be ascribed to the high complexity of naturally occurring TAG present in marine oils.

1.2 Lipids generalities

1.2.1 Lipids

Lipids can be defined mainly as fatty acids, their derivatives, and substances related biosynthetically or functionally to these compounds, which are generally soluble in organic solvents such as chloroform, and are most commonly found in the tissues of plants, animals and microorganisms [8]. Generally, the classification of lipids are based on their physical properties at room temperature (oils are liquid and fats are solid), their polarity (polar and neutral lipids), their essentiality for humans (essential and nonessential fatty acids), or their structure (simple or complex). For example, based on structure, lipids can be classified as derived, simple or complex lipids. Derived lipids include fatty acids and alcohols, which are the building blocks for the simple and complex lipids. Simple lipids (usually neutral), compose of fatty acids and alcohol components, include acylglycerols, ether acylglycerols, sterols, and their esters and wax esters, which can be hydrolyzed to an alcohol and an acid. Complex lipids (usually polar) include glycerophospholipids (phospholipids), glyceroglycolipids (glycolipids), and sphingolipids [9].

Dietary lipids are essential component for human body since they function as sources of metabolic energy, carrier of fat-soluble vitamins, and contribute to the formation of cell and tissue membranes. Among all the dietary lipids, those derived from fish and marine mammals have recently become a research focus due to their significant contents of essential ω-3 PUFA, which have been recognized to be important in human health and nutrition. Marine lipids are composed of neutral lipids comprising TAG, phospholipids, sterols, wax esters, and some unusual lipids such as glycerol esters, glycolipids, sulfolipids, and hydrocarbons. Most of the variations in lipid are found in the TAG fractions, whereas the phospholipids show fewer variations [10].

1.2.2 Fatty acids

Fatty acids are straight chain carboxylic acids that constitute the starting point in lipid structures. Fatty acids with a chain length of 10 carbon atoms or less are referred as short-chain fatty acids, and they are all saturated; while fatty acids having up to 14 carbon atoms are medium-chain fatty acids. Those with more than 14 carbon atoms are long-chain fatty acids, and may be saturated or unsaturated. These kinds of compounds are critical for the normal development and function of all organisms, and in particular, very long chain PUFA are necessary for the health and maintenance of higher organisms such as mammals.

Fatty acid nomenclature

Fatty acids trivial names are commonly used, for example, palmitic, stearic, or oleic acids, as shown in Table 1.1[10]. Nowadays, more meaningful systematic names defined by standard IUPAC terminology are encouraged by naming fatty acid after its parent hydrocarbon (Table 1.1).

Table 1.1: Terms and symbols designating major fatty acids [10]

Fatty acids trivial name	Chain length	Double bonds	Symbol I	Symbol II	Symbol III	Fatty acids systematic name
Myristic	14	0	$C_{14:0}$	$C_{14:0}$	14:0	*n*-Tetradecanoi acid
Palmitic	16	0	$C_{16:0}$	$C_{16:0}$	16:0	*n*-Hexadecanoic acid
Palmitoleic	16	1	$C_{16:1}$	$C_{16:1n-7}$	16:1 *n*-7	*cis*-9-Hexadecanoic acid
Stearic	18	0	$C_{18:0}$	$C_{18:0}$	18:0	*n*-Octadecanoic acid
Oleic	18	1	$C_{18:1}$	$C_{18:1n-9}$	18:1 *n*-9	*cis*-9-Octadecanoic acid
Linoleic	18	2	$C_{18:2}$	$C_{18:2n-6}$	18:2 *n*-6	*cis*,*cis*-9,12-Octadecadienoic acid
α-Linolenic	18	3	$C_{18:3}$	$C_{18:3n-3}$	18:3 *n*-3	All *cis*-9,12,15-Octadecatrienoic acid
γ-Linolenic	18	3	$C_{18:3}$	$C_{18:3n-6}$	18:3 *n*-6	All *cis*-6,9,12-Octadecatrienoic acid
Gadoleic	20	1	$C_{20:1}$	$C_{20:1n-9}$	20:1 *n*-9	*cis*-9-Eicosenoic acid
Arachidonic	20	4	$C_{20:4}$	$C_{20:4n-6}$	20:4 *n*-6	All *cis*-5,8,11,14-Eicosatetraenoic acid
EPA	20	5	$C_{20:5}$	$C_{20:5n-3}$	20:5 *n*-3	*cis*-5,8,11,14,17-Eicosapentaenoic acid
Cetoleic	22	1	$C_{22:1}$	$C_{22:1n-11}$	22:1 *n*-11	*cis*-11-Docosaenoic acid
DHA	22	6	$C_{22:6}$	$C_{22:6n-3}$	22:6 *n*-3	*cis*-4,7,10,13,16,19-Docosahexaenoic acid

For example, oleic acid is *cis*-9-octadecenoic acid, a carboxylic acid (oic) with 18 carbon atoms (octadec) and one olefinic centre (en) which lies between carbon 9 and 10 (counting from the carboxyl end) and has *cis* configuration, i.e. $CH_3(CH_2)_7CH=CH(CH_2)_7COOH$. Symbol designations (Table 1.1) are also found in common usage, which are composed of the carbon number in the fatty acid chain followed by a colon, then the number of double bonds and the position of the first double bond counting from the methyl end of the fatty acid (usually denoted by the letter *n* minus an integer number such as 3, 6, 9, 12 and 15). For example, *n*-3 and *n*-6 (also referred as ω-3 and ω-6 fatty acids) denote fatty acids with the first double bond at carbon 3 and 6 from the methyl end, respectively. The symbol, 20:5 *n*-3 identifies a fatty acid, i.e., eicosapentaenoic acid (EPA), with 20 carbon atoms and 5 double bonds, the first double bond occurring after the third carbon atom.

1.2.3 Polyunsaturated fatty acid families

1.2.3.1 Classification of polyunsaturated fatty acid families

PUFA are important structural components that confer membrane fluidity and selective permeability. They also serve as precursors for eicosanoids, growth regulators and hormones, and are constituents of membrane phospholipids involved in signal transduction[11, 12]. They are fatty acids of 18 carbons or more in length with two or more double bonds. PUFA can be classified into two major groups, ω-3 and ω-6 families, depending on the position of the first double bond proximate to the methyl end of the PUFA. The ω-6 family, such as linoleic and arachidonic acid, is mainly found in most of the vegetable oils, while the ω-3 family, such as α-linolenic acid (ALA, $C_{18:3n-3}$) is mostly found in vegetable oils (principally soybean and canola), EPA and docosahexaenoic acid (DHA, $C_{22:6n-3}$) are mainly found in marine oils.

The recommended intake of ω-6/ω-3 PUFA ratio is 2.3:1, while the present dietary pattern indicates a much higher ratio of ω-6/ω-3 PUFA ratio (8-12:1) [13]. Imbalance in ω-3 and ω-6 PUFAs metabolism has been implicated in hypertension as well as chronic diseases such as diabetes, hypercholesterolemia, rheumatoid arthritis, autoimmune disorders, Crohn's disease, and cancer, etc[14, 15] .

1.2.3.2 Elongation and desaturation of polyunsaturated fatty acids

PUFA are obtained either through diet or synthesized from dietary essential fatty acids (EFA). Through a common desaturase/elongase system (Fig. 1.1 [16]), linoleic acid (LA, $C_{18:2n-6}$) is metabolized to arachidonic acid (AA, $C_{20:4n-6}$) and ALA is metabolized to EPA and DHA.

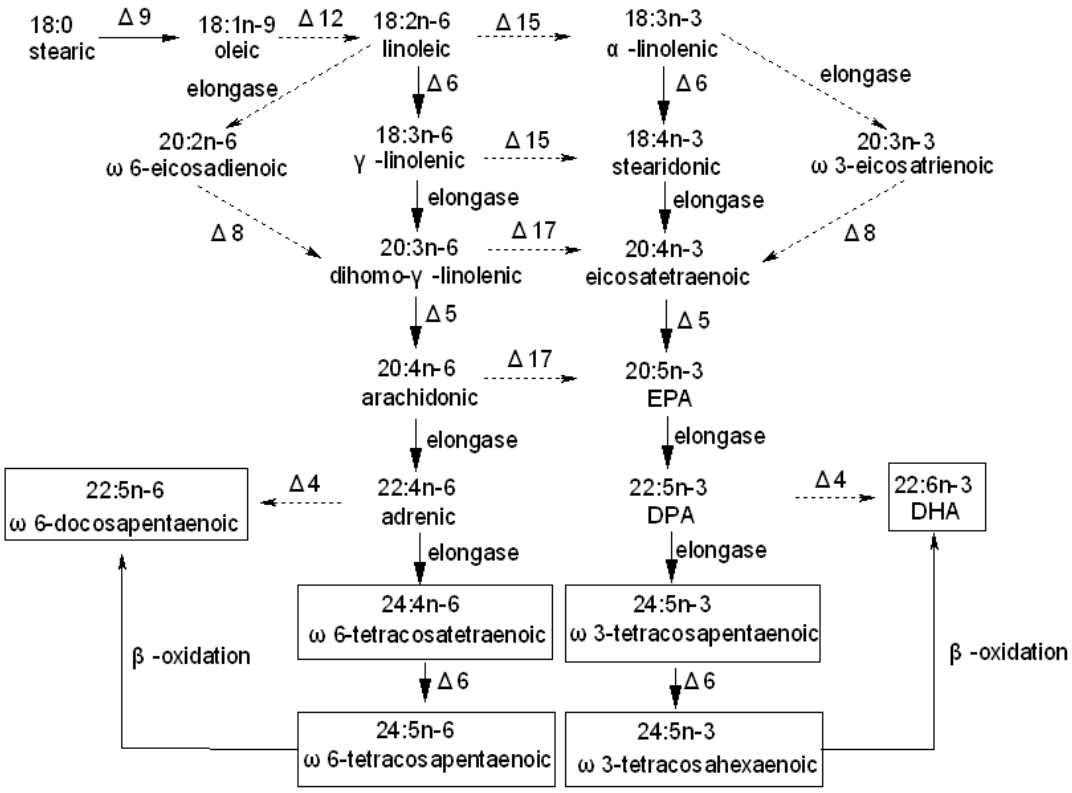


Figure 1.1: Elongation and desaturation of long-chain PUFA. Arrows with solid line are found both in mammals and lower eukaryotes, while arrows with dotted line are exclusively for lower eukaryotes. Fatty acids in square frame indicate the pathway is exclusively in mammals [16].

A detailed description of the biosynthesis of PUFA by increasing the carbon chain length and degree of unsaturation through the addition of extra double bonds to the carboxyl group is shown in Fig. 1.1. For example, ALA undergoes desaturation to form stearidonic acid (SDA, $C_{18:4n-3}$) by a Δ6-desaturase, and SDA is elongated to eicosatetraenoic acid (ETA, $C_{20:4n-3}$). EPA is synthesized through the addition of another double bond by a Δ5-desaturase to ETA, which is subsequently converted to docosapentaenoic acid (DPA, $C_{22:5n-3}$) through the further elongation. The synthesis of DHA from DPA occurs through two different mechanisms in eukaryotes. In

higher eukaryotes such as mammals, DPA is firstly elongated to tetracosapentaenoic acid (TPA, $C_{24:5n-3}$), which is desaturated to tetracosahexaenoic acid (THA, $C_{24:6n-3}$) by a Δ6-desaturase. The THA is then oxidized to DHA in peroxisomes; while in lower eukaryotes, DHA is synthesized by direct addition of a double bond to DPA by a Δ4-desaturase [16]. Similarly, LA undergoes the same desaturation and elongation steps as ALA to form long chain ω-6 PUFA. Normally, only a very small proportion of dietary LA and ALA can be converted to PUFA, as most of them are β-oxidized to provide energy. The already low formation of PUFA can be further depressed by various nutritional and hormonal factors [17].

1.2.3.3 Importance of ω-3 polyunsaturated fatty acids

Increasing evidences have shown that ω-3 PUFA have a range of potentially favourable effects on cardiovascular diseases, inflammatory diseases, brain function and mental health[18]. The nutraceutical potentials of ω-3 PUFA include protection against arrhythmias, diabetes, coronary heart disease, stroke and hypertension, beneficial effects in Crohn disease, asthma and chronic obstructive pulmonary diseases, alleviation of symptoms of cystic fibrosis, cancers of the breast, colon, and prostate, prevention of inflammatory and autoimmune disorders (rheumatoid arthritis, psoriasis) and improvement in growth and development[10, 14, 19].

1.2.4 Triacylglycerols

1.2.4.1 Structure and composition

Dietary oils are complex mixtures containing a wide range of compounds, including TAG, diacylglycerols (DAG), free fatty acids (FFA), phospholipids, and other minor components. TAG are the main components of dietary oils (> 98 %), which are made up of three fatty acid molecules esterified to a glycerol backbone. The molecular structure of each individual TAG species can be described basically by three main attributes [20]:

– the total carbon number (CN) defined as the sum of the alkyl chain lengths of the three fatty acids,
– the degree of unsaturation in each fatty acid, and
– the position and configuration of the double bonds in each fatty acid.

Moreover, each TAG species may be differentiated in regiospecific/stereospecific isomers by determining the positioning of three fatty acids on the glycerol backbone, since the trihydric alcohol glycerol itself has a plane of symmetry. When the two primary hydroxyl groups are esterified with different fatty acids, the resulting TAG can be asymmetric and thus can display optical activity. The stereochemistry of TAG can be represented by a Fischer projection and the "stereospecific numbering" (*sn*) system as recommended by the IUPAC-IUB commission on the nomenclature of glycerolipids[21]. A Fischer projection of a natural *L*-glycerol derivative is shown in Fig. 1.2, the secondary hydroxyl group is labelled as position *sn*-2. The carbon atom above this then becomes *sn*-1 position while the below becomes *sn*-3 position. A single molecular species is identified by listing the *sn*-1, *sn*-2 and *sn*-3 positions in this particular order [20].

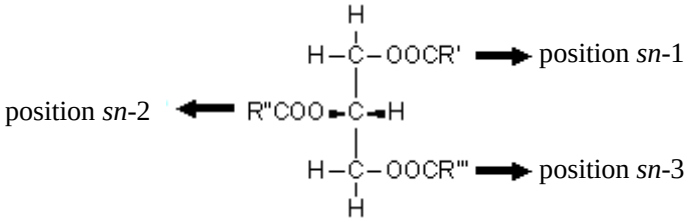


Figure 1.2: Schematic structure of a Fischer projection of a TAG molecule

The potential number of TAG is quite large and it rises very quickly with the number of fatty acids present in the pool (Table 1.2). For example, a fat containing only 2 different fatty acids results in the theoretical number of 8 possible TAG; or 6 TAG if stereoisomers are discounted and 4 TAG if all the isomers are excluded.

AAA	AAB	ABA	BAA	ABB	BAB	BBA	BBB
A_3	A_2B			AB_2			B_3

Most vegetable and plant oils contain normally 5-10 different fatty acids, which may give 125-1000 individual TAG molecules as shown in Table 1.2. However, the situation is even more complex with samples such as oils derived from fish or marine mammals containing 20-40 fatty acids.

Table 1.2: Relation between number of fatty acids (n) and number of TAG

Fatty acids	Number of TAG		
n	All TAGs n^3	Excluding stereoisomers $(n^3+ n^2)/2$	Excluding all isomers $(n^3+ 3n^2+2n)/6$
2	8	6	4
3	27	18	10
4	64	48	20
5	125	75	35
10	1000	550	220
20	8000	4200	1540
40	64000	32800	11480

1.2.4.2 Digestion, absorption and metabolism

The digestion, absorption, and metabolism of TAG are efficient, relatively well-defined processes. The major phases of these processes are listed in Table 1.3 [22]. Generally, fatty acid chain length and degree of unsaturation as well as the positional distribution of fatty acids in dietary TAG profoundly affect digestion, absorption, and metabolism of dietary fats [23, 24].

Table 1.3: Major phases of TAG digestion, absorption, and metabolism

intraluminal digestion
micellar solubilization
permeation across cell membrane
chylomicron formation
chylomicron release into lymphatics
transport in lymph
lipolysis at tissue sites
fatty acid oxidation

The first step in the digestion of TAG which takes place in the stomach, is a partial enzymatic hydrolysis into DAG and FFA, performed by lingual lipase and possibly gastric lipase [25, 26]. Both lipases preferentially hydrolyze the *sn*-3 ester bond resulting in formation of *sn*-1,2-DAG [25, 27]. Approximately 30 % of total dietary TAG may be digested in the stomach [28]. The products remaining in the stomach after hydrolysis, i.e., FFA, DAG, and monoacylglycerols (MAG) contained in emulsion droplets, are propelled through the pylorus into the duodenum (Fig. 1.3).

The major digestion of TAG results from hydrolysis with pancreatic lipase in the intestine. Pancreatic lipase acts in conjunction with co-lipase and bile salts to digest TAG (Fig. 1.3). The process of hydrolysis is regiospecific since pancreatic lipase preferentially hydrolyses fatty acids from *sn*-1 or *sn*-3 positions of the TAG, with the release of *sn*-2-MAG and FFA [29]. Isomerization of the *sn*-2-MAG to *sn*-1 or *sn*-3-MAG occurs to some extent, and these can be degraded completely to glycerol and FFA [27].

In the human adult, most of the fatty acid in the *sn*-2 position remain intact as *sn*-2-MAG during digestion and absorption as the rate of hydrolysis at the *sn*-2 position of the TAG is very slow [29]. The lipolysis products including FFA, MAG and DAG are solubilised together with phospholipids and cholesterol by lysophospholipids and bile salts into micelles cells and thus absorbed (Fig. 1.3). However, most of these products have specific melting points above body temperature, which may influence subsequent digestion, absorption, and metabolism. MAG can readily form mixed micelles and are subsequently absorbed, while FFA have variable incorporation into mixed micelles. For example, digestion and absorption of long chain saturated fatty acids (SFA) occurs less readily than for shorter chain or more highly unsaturated fatty acids since the latter require lower concentrations of bile salts to achieve emulsification into micellar form. Besides, the unesterfied long chain SFA tend to form hydrated acid-calcium soaps that are insoluble in aqueous media at the pH of the intestine, which will also hamper the absorption of long chain SFA. Evidences also indicate the better absorption of SFA as *sn*-2-MAG rather than as FFA[29-31].

Within the intestine, the fatty acids in the *sn*-2 position of the MAG and the fatty acids released from the *sn*-1 or *sn*-3 position of the TAG are absorbed in mucosal cells and re-synthesized into TAG, whereby the fatty acids at the *sn*-2 position in dietary TAG are conserved. The newly synthesized TAG are incorporated into lipoprotein complexes termed chylomicrons (CM), consisting mainly of TAG (86-92 %) and cholesterol esters (0.8-1.4 %), free cholesterol (0.8-1.6 %), phospholipids (6-8 %), and apoprotein (1-2 %) [22]. CM are secreted into the lymph and then exported into the plasma in the form of very-low-density lipoproteins (VLDL). These particles are transported to the peripheral tissues, where they are hydrolysed, releasing FFA, most of which are absorbed into the adjacent adipocytes and re-utilized for TAG synthesis within the cell [32]. Eventually, the CM remnants are returned to the liver, where the remaining lipids are

hydrolysed and absorbed (Fig. 1.3). The FFA within the liver can be utilized for a variety of purposes, from oxidation to the synthesis of structural lipids, but a proportion is re-converted into TAG, and some of this is stored as lipid droplets within the cytoplasm of the cells. Excessive accumulation of storage TAG is associated with fatty liver, insulin resistance and type 2 diabetes.

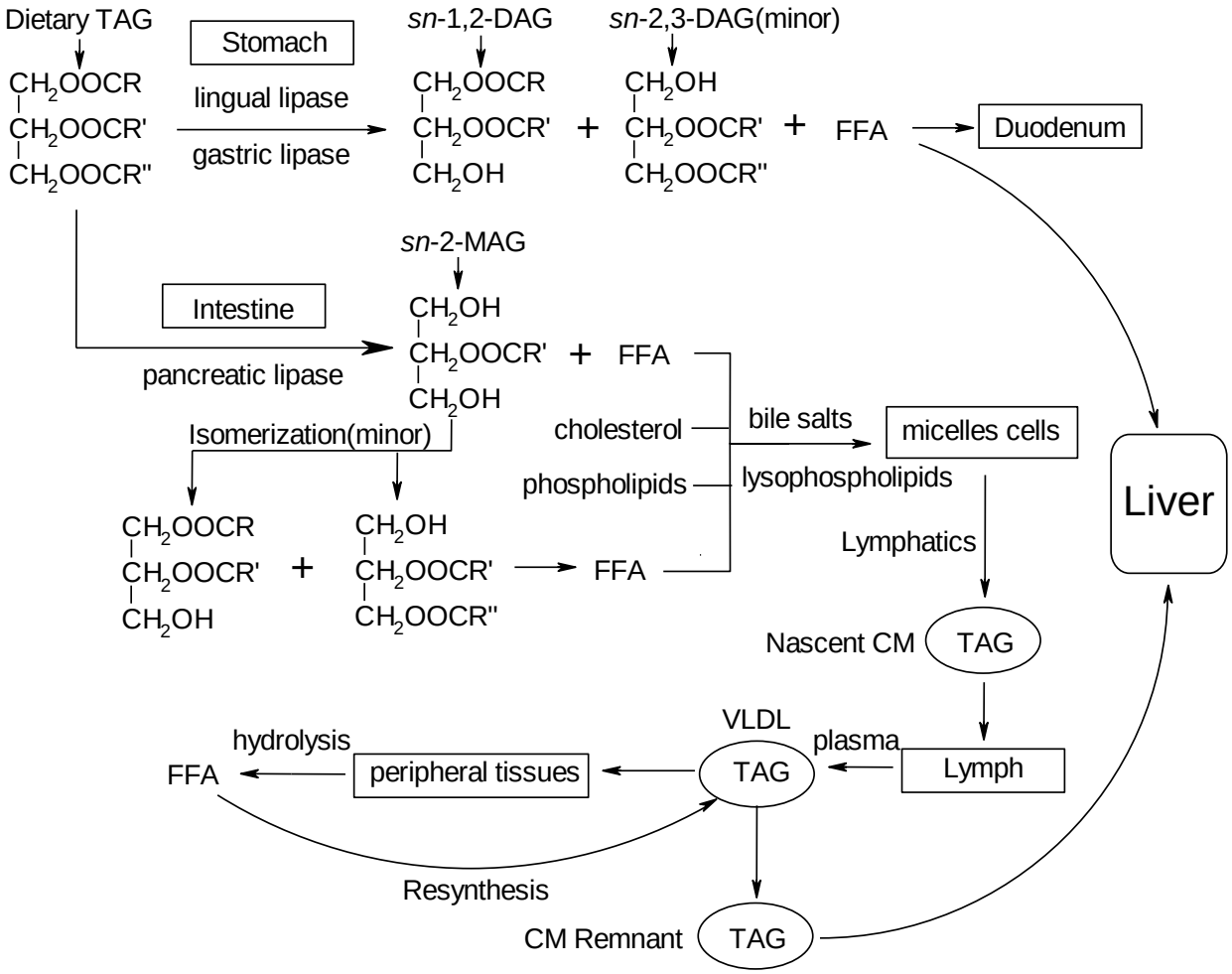


Figure 1.3: A representation of the digestion, absorption and metabolism of TAG

1.3 Dietary oils discrimination

Dietary oils authenticity has become an important subject from both a commercial and nutritional perspective. Authenticity covers many aspects, including adulteration, mislabeling, discrimination, classification, characterization and misleading origin.

Dietary oils possess a characteristic and more or less unique pattern of fatty acids and TAG. These patterns can be used as a means of identification for batch-consistency testing, detection of adulteration and possibly as a basis for a chemotaxonomy, revealing relationships between species [33]. Fatty acids composition of dietary oils has been traditionally used in the food industry as an indicator of purity and adulteration, although this function might be limited in the interpretation of data due to the wide variation in dietary oils from different geographical origins. FAO/WHO Codex Committee on Fats and Oils specified the routine testing of the purity of dietary oils and fats, which is primarily based on the determination of the fatty acids composition by gas liquid chromatography (GLC) and comparison of the obtained values with purity criteria [34]. In addition to fatty acids composition, TAG composition has also been established as a measurement of the quality and purity of dietary oils, which is used increasingly in the food industry to confirm the authenticity of dietary oils [35]. Although TAG are directly related to the fatty acids composition of dietary oils, the stereospecific positional distribution of fatty acids on the glycerol backbone remains preserved during analysis. As a consequence of that, TAG patterns of dietary oils usually provide a larger amount of information than a simple fatty acids profiling.

Numerous instrumental techniques have been developed to monitor the authenticity of dietary oils. A literature overview of the different instrumental techniques used for the analysis of plant, fish and marine mammal oils in studies is given in Table 1.4, where the terms discrimination, adulteration, classification, profiling, differentiation, authentication or characterization have been a vital and important component of the various studies. It can be observed that most of the current discrimination studies are based on chromatographic analysis by using GC and high performance liquid chromatography (HPLC). These techniques have been complemented with many other modern techniques, such as silver ion chromatography, mass spectrometry (MS), nuclear magnetic resonance spectrometry (NMR), near-infrared spectroscopy (NIR), Raman spectroscopy, etc. The conjunction of chromatography and mass spectrometry methods, such as gas chromatography-mass spectrometry (GC-MS), HPLC coupled to atmospheric pressure chemical ionization-MS (HPLC/APCI-MS), are growing in popularity for dietary oils discrimination as much more useful information could be derived from them than a single technique.

Table 1.4: Literature overview of the different analytical techniques used in the discrimination of oils derived from plants, fish and marine mammals. The numbers under every technique-column indicate how many times a technique has implemented for a particular oil.

Oil	Chromatography							IR		NMR			MS					Other				References
	Gas			Liquid			Other															
	GC	GCMS	GCIRMS	HPLC	HPLC APCI-MS	Ag-HPLC APCI-MS	Ag-TLC	NIR	MIR	^{1}H	^{13}C	^{31}P	ESI	APPI	HS	MaldiToF	F	E	R	N	
Almond	1			1	2					1		1	1								36-39
Amaranth					1								1								37
Apricot	1																				40
Avocado					2																36, 41
Blackcurrant					1																41
Borage					1																41
Bran		1											1								42
Brazilnut					1								1								37
Camellia	1	1						1	1				1								40, 42, 43
Canola	1			1				1													44, 45
Castor	1	1											1								40, 42
Cayenne		1											1								42
Cocoa butter		1											1								42
Coconut	1	1			1					1		1	1								39, 42, 46
Corn	3	1	2	2	2			4		1		1	4	1			1	1	1		37, 41, 42, 44, 47 39, 45, 48 49-59
Corn germ					1																36
Cottonseed	2	1		1	1			2					1								41, 42, 44, 49, 50, 59
Dragon head					1								1								37
Egoma seed		1											1								42
Evening primrose		1			2								2								37, 41, 42
Grape seed		1			2						1		1				1				36, 41, 42, 53, 60
Grapestone													1								51
Groundnut			1							1		1									39, 48
Hazelnut	2	1		2	1					2		1	3	1	1					1	37, 39, 44, 51, 52, 56, 61-63

Table 1.4 Continued

Husk													1									51
Jojoba		1											1									42
Kukui					1																	41
Linseed		1			3								2				1					36, 37, 42, 53
Macadamia		1			1								2									37, 42
Milk Thistle					1								1									42
Mustard seed	1			1																		44
Olive	8	2		4	1		1	6	1	3	1	1	3	1	1		1	1	1	1		39-45, 49-59, 61-69
Onion																1						70
Oregano													1									71
Palm	1	1	1		2					1		1	2									37, 39, 41, 42, 46, 48
Peanut	3	1		2	2			1					2				1					36, 40-42, 44, 45, 51, 53, 55, 56
Pepper seed	1																					40
Perilla		1		1									1									42, 72
Pistachio					1								1									37
Poppy seed		1			1								1			1						37, 73
Prikachberry	1																					40
Pumpkinseed	1				1						1											36, 55, 60
Rapeseed	4	2	1	1	3			1		1		1	2				1		1			37, 39-42, 44, 48, 49, 53, 55, 58, 68, 74
Redcurrant					1																	41
Rice						1																75
Rosemary													1									71
Safflower	2	1		1	1					1		1	1									39-42, 44, 76
Sesame	2	1		3	1			1		1		1	1									39-42, 44, 45, 56, 72, 76
Soybean	4	1		2	3			2	1	1	1	1	3				1		1			36, 37, 39-42, 44, 45, 50, 51, 53, 55, 56, 58, 60, 76
Sunflower	4	2	1	2	3			4		1	1	1	4	1		1	1	1	1			36, 37, 39, 41, 42, 44, 45, 48-60, 68, 73
Tall		1											1									42
Tea seed		1											1									42

Table 1.4 Continued

Tung		1							1	42
Turkey red		1							1	42
Walnut	2		2	1	1	1	1	1		36, 39, 40, 44, 45, 56
Wheat germ				2			1			36, 41, 60
Cod liver oil	1	1			1		2		1	42, 77-79
Cuttlefish		1							1	42
Fish					1					80
Herring							1			81
Mackerel		1					1		1	42, 81
Salmon	1						3			77, 81, 82
Saury		1							1	42
Seal							1			77
Shark liver oil		1							1	42
Sardine		1							1	42
Whale		1							1	42

*Abbreviation*s: GCIRMS: GC-isotope radio MS; HPLC: High performance liquid chromatography; APCI-MS: Atmospheric pressure chemical ionization-MS; Ag-HPLC: Silver-HPLC; Ag-TLC: Silver-thin layer chromatography; NMR: Nuclear magnetic resonance; NIR: Near infrared spectroscopy; MIR: Mid infrared spectroscopy; ESI-MS: Electrospray ionization; HS: Headspace; APPI: Atmospheric pressure photospray ionization; MaldiToF: Matrix-assisted laser desorption ionization time-of-flight; F: Fluorescence; E: Carbon paste electrodes; R: Raman spectroscopy; N: Electronic nose.

Another main conclusion that can be drawn from Table 1.4 is that the current literature on nutritional oil discrimination has been mainly focused on plant oils and among them olive, sunflower and corn oil (either pure or mixed) are the most frequently investigated oils, representing 64, 50 and 43 % of the total number of references given in Table 1.4, respectively; while for the marine oils which are nutritionally important, only a meagre 3 % were represented and moreover the potentiality of popular techniques such as GC and LC-ESI-MS^2 on the discrimination and adulteration of marine oils has not been explored yet. Consequently, it is quite significant to establish reliable strategies for the discrimination and detection of adulteration of marine oils and related products.

1.4 Triacylglycerols characterization

Characterization of TAG from oils and fats has long been a difficult task due to the enormous number of individual TAG species formed by various fatty acids as described in Section 1.2.4. A variety of analytical methods have been employed for the analysis of molecular species of TAG, which mainly include indirect and direct methods. The former are the traditional methods to determine the positional distributions of fatty acids on the backbone of TAG, such as enzymatic hydrolysis method using pancreatic lipase and stereospecific analysis by using Grignard reagent [83-85]. The drawback of these approaches lies in the involvement of tedious analysis procedures and the occurrence of possible isomerization of glycerides. Direct methods mainly consist of NMR [77, 86, 87] and MS [36, 37, 88] techniques, while the latter is favored especially when it is coupled to chromatographic techniques. HPLC is the most commonly employed separation technique coupled to MS for the analysis of TAG, which offers significant advantages over GC and thin-layer chromatography (TLC), as the conjunction of LC and MS techniques gives information both on molecular species compositions and on regiospecific distributions of fatty acids in the TAG molecules.

The MS technique has demonstrated its ability in the complete characterization of the TAG structure through the detailed information obtained, such as molecular weight, carbon number, the degree of unsaturation and the positions of acyl groups on the glycerol backbone [76, 89, 90]. The analytical methods utilizing the MS technique for the TAG analysis from oils and fats mainly employed the following ionization techniques, electron ionization (EI) [91], chemical ionization

(CI) [92-94], desorption chemical ionization (DCI) [95, 96], fast atom bombardment (FAB) [89, 97, 98], atmospheric pressure chemical ionization (APCI) [37, 99-102]and electrospray ionization (ESI) [76, 103-106], among which APCI and ESI are the most popular techniques. Table 1.5 lists the literatures of TAG analysis in various matrices using APCI and ESI techniques in recent years.

Table 1.5: Literatures of TAG analysis from various matrices by APCI and ESI techniques

	APCI	
Samples	Techniques	References
93 plant oils (olive oil, sunflower oil, rice oil etc.)	(NARP)-HPLC/APCI-MS	107
Berry oils	SFC/APCI-MS	108, 109
Black currant, alpine currant, cloudberry seed oil	Ag-HPLC/APCI-MS	88
Canola oil	RP-HPLC/APCI-MS, RP-HPLC/ESI-MS^2, MS^3	90
Conifer seed oils	HPLC/APCI-MS, GC-FID	110
Corn oil	Ag-LC×RP-LC/APCI-MS, HPLC-ELSD, HPLC-UV	111
Highly saturated fatty acid fats(coconut, cocoa butter, palm oils etc.)	RP-HPLC/APCI-MS	112
Olive, hazelnut oil mixtures	RP-HPLC/APCI-MS, GC-FID	113
Rape seed oil	LC/APCI-MS	74
Rapeseed oil, sunflower seed oil	LC/APCI-MS, LC/ESI-MS/MS, direct inlet ammonia NICI-MS/MS	114
Rice oil	Ag-HPLC/APCI-MS	75
Seed oil of *Momordica charantia L. var. abbreviata Ser*	(NARP)-HPLC/APCI-MS, ^{13}C-NMR	115
Soybean oil	RP-HPLC/APCI-MS	116
Soybean, rapeseed, hazelnut, olive, evening primrose, blackcurrant, blue poppy seed, maize germ oil	HPLC/APCI-MS	117
Soybean,linseed oil	Ag-LC×RP-LC/APCI-MS	118
Structured lipid sample produced by rape seed oil with capric acid	HPLC/APCI-MS	119
Walnut, hazelnut, cashew nut, almond, poppy seed, yellow melon, mango, date oil	(NARP)-HPLC/APCI-MS, HPLC-ELSD, HPLC-UV	120
Palm oil, cocoa butter, beef, pork, chicken fats	RP-HPLC/APCI-MS	121
Beef, lamb, pork, chicken fat	HPLC/APCI-MS	122
Lard, mutton tallow	RP-HPLC/APCI-MS	123
Fat body of bumblebees	Ag-LC×RP-LC/APCI-MS	124
Milk fat	SFC/APCI-MS	125
Donkey milk	RP-LC×Ag-LC /APCI-MS	126, 127
Bovine milk fat	HPLC/APCI-MS	100
Margarine Base Stocks	RP-HPLC/APCI-MS, HPLC-FID	128
Hydrogenated soybean oil margarine basestock	HPLC/APCI-MS	129

Table 1.5 Continued

	ESI	
Samples	Techniques	References
Botswana seed oils	ESI-MS, ^{13}C-NMR, GC-MS	130
Canola oil	RP-HPLC/ESI-MS^2, MS^3, RP-HPLC/APCI-MS	90
Castor Oil	LC/ESI-MS, LC/ESI-MS^2,LC/ESI-MS^3	131
Coffee Beans (*Coffea canephora P.*)	LC/ESI-MS^2, GC-FID, HPLC-PDA	105
Corn oil	LC/ESI-MS	132
Interesterified palm oil	On-line Ag-HPLC/ESI-MS, Ag-HPLC-FID, Ag-SPE-SFC-FID	104
Malaysian cocoa butter	LC/ESI-MS^2, HPLC-PDA	133
Olive oil	ESI-MS^2 and MS^3 of lithiated adducts	134
Ouricuri oil	HPLC-ESI/ MS^2, HPLC-ELSD	135
Pequi (*Caryocar brasiliensis Camb.*) oil	LC/ESI-MS, LC/ESI-MS^2, GC-MS, HPLC-ELSD	106
Rapeseed, butter oil	LC/ESI-MS^2, GC-FID, HPLC-ELSD	136
Tuna, microalgal, microbial micelia	Chiral-phase HPLC/ESI-MS	103
Mouse liver, white adipose tissue	RP high resolution LC/ESI-QTOF MS/MS	137
Butterfat	NP-HPLC/ESI-MS^2	138
Animal ganglia lipids	ESI-MS	139
Archaeological samples	High resolution nanoESI, FT-ICR MS, IRMPD MS/MS	140
Cell lipids from mice tumor	ESI-MS, ESI-MS^2, ESI-MS^3	141
Molecular species	ESI-MS, ESI-MS^2	142
TAG mixtures	CAD-MS^2, ESI-MS^2	143
Synthetic TAG mixtures	(NARP)-HPLC/ESI-MS^2	144
Synthetic short-chain TAGs	LC/ESI-MS, MS^2 , ^{1}H NMR	145
TAG isomer mixtures	ESI-MS^2, ESI-MS^3	146

Abbreviations: SFC: Supercritical fluid chromatography; RP: Reversed-phase; NARP: Non-aqueous reversed-phase; IRMPD: Infrared multiphoton dissociation; FID: Flame ionization detector; ELSD: Evaporative light scattering detector; FTICR: Fourier-transform ion cyclotron resonance; PDA: Photodiode array detector; SPE: Solid phase extraction; CAD: collisionally activated dissociation.

Since the first report of the application of RP-HPLC coupled to APCI-MS in the analysis of TAG standards mixture by Byrdwell and Edward [101], APCI-MS has been used in TAG analysis of various oils until now, as can be seen from Table 1.5. However, most of the studies are mainly concerned with plant oils, representing 67.9 % of the total number of references.

APCI-MS has exhibited significant advantages for TAG analysis in different matrices: 1) compatibility with non-polar solvents, facilitating the TAG analysis; 2) capability of producing mostly protonated molecules and DAG fragments; 3) enabling the differentiation of positional isomers through the evaluation of the relative abundance ratios of DAG fragments. However, the

identification of relatively saturated TAG species could be difficult, since APCI does not produce significant abundance of protonated molecules [147].

Analysis of TAG by ESI-MS was first introduced by Duffin *et al.* [76] for the analysis of mixtures containing MAG, DAG, and TAG dissolved in chloroform:methanol (70:30, v:v) with ammonium acetate or sodium acetate as the modifiers by direct infusion into the ESI interface. Several years later this technique for analysis of TAG became widespread. As is observed from Table 1.5, the majority of the investigations have been focused on the analysis of TAG from various plant oils, where only one study is concerned with marine oil, and 40.9 % of the studies applied the direct infusion of TAG into the ESI-MS without coupling to HPLC.

ESI-MS offers an excellent strategy for the analysis of high molecular weight compounds and an efficient interface for liquid chromatography. Although single ESI-MS derived spectra only contain quasimolecular ions with no fragmentation, the introduction of tandem ESI-MS (usually referred as ESI-MS^2) can satisfactorily solve this problem, since ESI-MS^2 derived spectra of TAG are mainly characterized by the presence of simple yet abundant DAG fragments from precursor ions. Moreover, studies shows that differentiation can be achieved between *sn*-2 and *sn*-1 or *sn*-3 fatty acyl groups based on the relative intensities of DAG fragments, which provides valuable information for the structural characterization of TAG [144, 146]. Therefore, it is worth exploring the potential of ESI-MS^2 for the analysis of TAG in marine oils.

1.5 Chemometric techniques

1.5.1 Multivariate analysis

Multivariate analysis is the term used to describe the analysis of data where numerous observations or variables are obtained for each object studied [148]. It is used for a number of distinct and different purposes which are divided into three main groups, i.e., description (explorative data structure modeling), discrimination and classification, and regression and prediction. The commonly employed multivariate techniques include principal component analysis (PCA), partial least squares (PLS) and soft independent modeling of class analogy (SIMCA) [149]. In the present thesis, emphasis is placed on PCA for "discrimination and classification" of various dietary oils.

PCA is a well-documented multivariate method for reducing the dimensionality of a data set by rotating and constructing orthogonal linear combinations of the original variables and projecting the maximum variability onto new axis also known as principal components (PCs). Each PC can be considered as a new variable that represents some underlying feature of the data. The first PC is the major axis of the points in the *p*-dimensional space that accounts for maximum amount of variance in the data. The second PC is perpendicular to the first PC and it defines the next largest amount of variation accounts, and so on. Once obtained, the PCs can be graphically plotted in order to distinguish and classify different samples.

1.5.2 Chemometric applications in liquid chromatography-mass spectrometry

Liquid chromatography-mass spectrometry (LC-MS) employing ESI or APCI ionization techniques is a powerful analytical tool for the specific detection and potential identification of compounds in complex mixtures. However, analysts often encounter several problems when analyzing LC-MS data. First, the amount of data obtained from LC-MS has increased a lot over the years as a result of instrumental developments and applications dealing with increasingly complex multi-component samples and matrices. Interpretation of large data sets has thus become a formidable challenge. Second, the combination of LC with MS, particularly using ESI as an ionization method, can result in chromatograms and mass spectra with a high level of background and noise, which comes from a number of sources, such as the LC mobile phase and buffers [150]. The contributions of solvent and background will often dominate the chromatogram. As a consequence of this, the resulting total ion chromatograms do not exhibit a clear separation of the mixture sample. Even though great improvements have been made in the instrumentation, the output signals still contain a great deal of noise embedding the useful information together with false peaks and spikes [151]. Due to the above mentioned problems, the data interpretation is actually much more time-consuming than the data acquisition and has thus become a bottleneck in LC-MS method development and analysis.

Currently, several chemometric techniques are available to assist the data analysis generated from LC-MS, which are mainly focused on the preprocessing of data, such as reducing the noise and background in the data, improving the signal to noise ratio (S/N), extracting high quality

chromatograms, etc. Singular value decomposition (SVD) is a commonly used data reduction algorithm to improve the S/N in the LC-MS data [152]. Sequential paired covariance (SPC), higher order-sequential paired covariance (HO-SPC), windowed mass selection method (WMSM), and component detection algorithm (CODA) are the chemometric methods developed specifically for chromatography/MS data [153].

The algorithm of SPC performs an un-scaled, un-normalized correlation between two adjacent mass spectra by multiplying their intensities together mass by mass. If the adjacent spectra have common features, it will lead to large intensities of these scanning points, whereas the noise will be suppressed by the operation as it is un-correlated between neighboring spectra [154]. SPC has been applied to LC-MS data obtained on a triple quadrupole instrument. It was demonstrated that application of SPC on the data enhances the resolution of the eluting peaks of a complex peptide mixture and the S/N ratio of both chromatographic peaks and mass spectra [155]. HO-SPC is one of the variations of SPC, in which the intensities of more than two mass spectra are multiplied together at a time. It is shown to be useful in eliminating coincidental noise in sequential mass spectra, giving the potential to extract broad, low intensity analyte peaks [155].

WMSM is an extension of the HO-SPC algorithm that removes random and high background noise based on the assumption that analytes can be distinguished from noise by means of differences in peak width [153]. It consists of two steps: 1) random noise is eliminated by choosing a time window which corresponds to the analyte peak width. The ion that has a non-zero signal over the length of the window is retained. The random noises that displays zero intensities intermittently will be eliminated since a zero signal will be produced by multiplication of ion intensities at a particular *m/z* value over a series of scans; 2) the high background caused by the mobile phase and column bleed is removed by setting a much larger window size than the maximum expected elution time of an analyte ion. The ion that has a consistent signal over a long period results from a high background and thus eliminated. WMSM has been shown to be useful in eliminating random noise and improving S/N in both chromatographic and mass spectra profiles [153].

CODA was specifically developed for LC-MS data in order to reduce random noise and high background by selecting only high-quality (low noise and background) chromatograms from complex LC-MS data quickly [150, 156], which has demonstrated to be effective in the analysis of

LC-MS data from urine samples, human serum and peptides [83, 153, 157]. CODA is based on two factors: 1) background mass chromatograms will have a high mean value compared to good mass chromatograms; 2) noisy, spiky mass chromatograms will be more affected by smoothing than good mass chromatograms. Consequently, by calculating a similarity coefficient (so-called mass chromatographic quality, MCQ) between the original and mean centered chromatogram for each mass chromatogram, the algorithm will distinguish spiky chromatograms and solvent chromatograms from the mass chromatograms of potential components. The spiky and solvent chromatograms will be easily eliminated by selecting peaks with a high similarity coefficient thus significantly increasing the productivity in the LC-MS data.

Briefly, the process of CODA consists of the calculation of the similarity index by the following equation:

$$c_j = \frac{1}{\sqrt{r-w}} \sum_{i=1}^{r-w+1} \mathrm{a}(\lambda)_{ij}\, \mathrm{a}(w,s)_{ij} \qquad (1.1)$$

Where c_j is the similarity index (also referred as MCQ) for jth mass chromatogram, i.e., mass chromatographic quality value, r is the number of rows (scans) in the original dataset, w is the width of a rectangular smoothing window, $\mathrm{a}(\lambda)_{ij}$ stands for an element of data matrix at time point i and mass channel j, $\mathrm{a}(w, s)_{ij}$ represents the data matrix which is first smoothed with a window w and then standardized.

The MCQ in Eq. (1.1) is calculated for each mass chromatogram. A high similarity index indicates that the mass chromatogram in question contains information of eluting components, while a low similarity index shows that any signal at this mass might be due to the noise or background. By specifying a MCQ threshold (between 0 and 1), the high-quality mass chromatograms will be retained by selecting the mass chromatograms with MCQ values higher than the defined threshold, while other mass chromatograms with lower MCQ values will be discarded.

WMSM and CODA are optimal tools for LC-MS data analysis. The former is more suitable for mass chromatograms with frequent and high intensity noise peaks, and the latter performs well for data with high background signal [153].

1.6 Aims of the study

The main objectives of the present study are:

1. To assess the potential of GC for discriminating ω-3 fatty acids rich oils derived from marine animals (cod liver, salmon, seal and whale) of different species, brands and grades by using three different data analysis strategies based on fatty acid methyl esters (FAME) profiles and PCA.
2. To fingerprint and discriminate marine oils based on their total ion chromatogram (TIC) and mass spectral profiles derived from LC-ESI-MS and LC-ESI-MS^2 experiments by using chemometric tools including CODA and PCA.
3. To establish a reliable LC-ESI-MS^2 strategy for the detection and characterization of TAG species from cod liver oil and develop a computational algorithm for the rapid and automatic interpretation of TAG species based on their structural features and mass spectral behaviour. The well-established lipase method is used to confirm the results of the proposed LC-ESI-MS^2 approach.

2 Discrimination study based on fatty acids composition

2.1 Background

Dietary oils authenticity has become a focal point attracting the attention of producers, consumers, and policy makers. In particular, the need for quality assessment of marine oils has been intensified due to the emerging bodies of evidence supporting their roles in counteracting inflammatory processes in various parts of the body[4, 5, 158, 159], decreasing the concentrations of harmful cholesterol [14, 19], preventing the formation of blood clots and fatty deposits on the arterial walls in people with coronary heart disease [160, 161], etc. These health benefits are ascribed to the high content of ω-3 fatty acids in marine oils such as EPA and DHA.

Marine oils as well as their by-products have been used as the important sources of ω-3 fatty acids for humans in the form of food ingredients, dietary supplements and medicines, etc. For example, microencapsulated fish oil has been introduced for enrichment of foodstuff including bread, infant formulas, baby food, soups, and prepared food, such as pizza [87]. Adulteration of these products involves the deliberate or accidental addition of adulterants, i.e., cheaper oils including various animal fats and plant oils: mutton, beef, pork, chicken, lard and soy, linseed, rapeseed oil, etc [79]. The importance of developing techniques for detecting adulteration of marine oils can be traced back to the late 19th and early 20th century, when a great scarcity of cod liver oil accompanied by famine prices of the market brought about adulteration of genuine cod liver oil with low-grade shark oil [6, 7]. Nowadays, these products carry a premium price into the market which brings out the serious problems of adulteration in the trade of those products. Besides, the quality of these products depends on the origin and quality of raw materials as well as the extraction process, subsequent purification and storage conditions. As a consequence, it is of vital importance to establish a feasible approach for the quality assessment of ω-3 fatty acids rich marine oil.

The literature overview of the different instrumental techniques used for the analysis of plant, fish and marine mammal oils shown in Table 1.4 indicates that dietary oils discrimination are

mainly based on fatty acids composition through GC and GC-MS. However, the current studies have been mainly focused on plant oils, while the studies on fish and marine mammal oils constitute a meagre 3 % of the total number of references. It is surprising, that the potentiality of less complex techniques such as GC has not been explored yet in the discrimination of nutritionally important marine oils by using the conventional FAME profiles.

Considering that discrimination studies of ω-3 fatty acids rich oils derived from fish and marine mammals have not been previously reported, in the present thesis, the capability of GC analysis for discriminating marine oils of different species, brands and grades was assessed for authentication purposes by using the FAME profiles and PCA. The GC FAME profiles from plant oils such as rapeseed, linseed and soy oils and seven different brands of ω-3 supplements were also used in the discrimination process. The discrimination between and within animal oils was studied by using three different data analysis strategies: 1) the analysis of the full FAME profiles from plant, supplement and animal oils; 2) the analysis of selected FAME profiles from plant, supplement and animal oils with levels higher than 0.5 % of the total composition; 3) the analysis of the full FAME profiles from animal oils.

2.2 Experimental

2.2.1 Reagents and samples

Sodium hydroxide, hexane, methanol, boron trifluoride in methanol (20 % w/v) and chloroform were purchased from Merck (Darmstadt, Germany). Butylated hydroxytoluene (BHT) and boron trichloride in methanol (14 %) were purchased from Sigma-Aldrich Co. USA. FAME standards were purchased from Nu-Chek Prep (Elysian, MN), the nonadecanoic acid methyl ester ($C_{19:0}$) internal standard was from Fluka (Buchs, Switzerland). De-ionized water was purified in a Milli-Q system (Milli-Q system Millipore, Milford, MA). The fish oils were cod liver oil from Peter Möller, Lysaker, Norway and salmon oil from Havnegater, Sortland, Norway. The two brands of harp seal oils (*Phagophilus groenlandicus*) were from Rieber Skinn A/S, Bergen, Norway (two refined samples from different batches, designated as RSA1 and RSA2, and one crude sample designated as CSA were provided) and from JFM Sunile A/S, Os, Norway (one refined sample designated as RSB was provided). Whale oil (*Balaenoptera acutorostrata*) conventionally (WC)

and molecularly (WM) distilled were from Myklebust Trading AS, Myklebost, Norway. The plant oils analysed were soy oil (Mills DA, Sofienberg, Norway), linseed and rapeseed oils (Kinsarvik Naturkost, Bergen, Norway). The seven commercial ω-3 supplements obtained from a local pharmacy were Fri Flyt (Vesterålens Naturprodukter AS, Sortland, Norway), Natur-Omega (Naturhuset AS, Vøyenenga, Norway) Møllers dobbel (Peter Möller, Lysaker, Norway), Pikasol (Axellus A/S**,** Oslo, Norway) Omega-3 Forte (Vitamed, Sarpsborg, Norway), Omega-3 høykonsentrert (Sunkost, Oslo, Norway), El Dorado (Probio Nutraceuticals, Tromsø, Norway). The supplements were designated as K1, K2, K3, K4, K5, K6 and K7 respectively.

2.2.2 Fatty acid methyl esters preparation

The FAME preparation protocol has been published elsewhere [162]. Briefly, 50 mg of sample are mixed with 2 ml BF_3/CH_3OH and 5 mg of $C_{19:0}$ internal standard. The mixture is heated at 100 °C for 1 h and cooled down to room temperature. Aliquots of 1 ml of hexane and 2 ml of H_2O are added, vortex-mixed for 15 seconds, placed in a centrifuge at 3000 rpm for 2 min and the FAME are then extracted from the upper hexane phase. Depending on the fat content the sample is either concentrated under nitrogen or diluted with hexane and subsequently subjected to GC analysis.

2.2.3 Gas chromatography instrumentation

Analysis of the FAME was performed on a Perkin-Elmer AutoSystem XL gas chromatograph (Perkin-Elmer, Norwalk, Connecticut) equipped with a liquid autosampler and a flame ionisation detector. The FAME samples were analysed on a CP-Sil 88 capillary column (50 m × 0.32 mm I.D. 0.2 μm film thickness, Varian, Courtaboeuf, France). Data collection was performed by the Perkin-Elmer TotalChrom Data System software version 6.3. The temperature program was as follows: the oven temperature was held at 60 °C for 1 min, ramped to 160 °C at 25 °C /min, held at 160 °C for 28 min, ramped to 190 °C at 25 °C /min, held at 190 °C for 17 min, ramped to 220 °C at 25 °C /min and finally held at 220 °C for 10 min. Direct on-column injection was used. The injector port temperature was ramped instantaneously from 50 to 250 °C and the detector temperature was 250 °C. The carrier gas was ultra-pure helium at a pressure of 82 KPa. The analysis time was 60 min. This time interval was sufficient to detect FAME with chains from 10

to 24 carbons in length. The FAME peaks were identified by comparison of their retention times with the retention times of highly purified FAME standards.

2.2.4 Principal component analysis

Multivariate data analysis has been extensively used in oil discrimination. For instance, 66 % of the total number of articles reported in Table 1.4 used multivariate approaches of some sort, while 43 % used PCA. The data was normalized by using internal standards. Two-dimensional PCA score plots were created on the normalized data in order to reduce the number of variables and eliminate the redundancy of information. The PCA score and loading plots of the FAME profiles from the various oils were computed with the software package Statgraphics Plus 5.1 (Statistical Graphics Corp.).

2.3 Results and discussion

The plant, fish, marine mammals oils were analyzed in triplicate and the ω-3 supplements in duplicate. The triplicate and duplicate lipid profiles of the various injected oil samples, expressed as mg-FAME/g-sample, are presented in Tables 2.1-2.4.

Table 2.1: FAME concentrations (mg/g) for different plant oils

Sample	Plant oil								
Name	Soy			Rapeseed			Linseed		
Designation	SO			RP			LN		
Replicate	i	ii	iii	i	ii	iii	i	ii	iii
$C_{16:0}$	89.53	90.28	89.02	35.84	36.16	34.52	40.51	41.28	39.65
$C_{16:1n-7}$	0.00	0.00	0.00	1.68	1.73	1.64	0.00	0.00	0.00
$C_{18:0}$	28.17	28.52	27.91	15.03	15.39	14.53	33.92	34.88	32.86
$C_{18:1n-9}$	225.35	225.59	225.39	565.29	567.52	562.97	194.60	195.76	193.34
$C_{18:1n-7}$	13.64	13.68	13.41	24.65	25.19	24.09	5.45	5.53	5.36
$C_{18:2n-6}$	494.03	496.11	493.95	172.65	176.78	168.41	133.41	134.25	132.48
$C_{20:0}$	4.47	4.64	4.51	5.38	5.51	5.15	3.10	3.10	2.99
$C_{18:3n-3}$	51.93	51.96	51.92	84.59	85.52	83.55	506.11	507.27	504.89
$C_{20:1n-9}$	2.09	2.10	2.00	10.14	10.44	9.74	0.00	0.00	0.00
$C_{22:0}$	4.37	4.42	4.38	2.74	2.75	2.73	0.89	0.92	0.85
$C_{24:0}$	1.53	1.58	1.47	0.00	0.00	0.00	0.00	0.00	0.00

Table 2.2: FAME concentrations (mg/g) for different brands and grades of seal oil

Sample	Seal oil											
Manufacturer	A									B		
Quality	Crude			Refined						Refined		
Designation	CSA			RSA1 (batch 1)			RSA2 (batch 2)			RSB		
Replicate	i	ii	iii	i	ii	iii	i	ii	iii	i	ii	iii
$C_{14:0}$	42.80	44.34	41.46	42.15	42.49	41.61	45.37	45.89	45.86	40.17	40.31	40.14
$C_{14:1n-9}$	6.33	6.67	6.19	6.94	7.08	6.59	7.10	7.25	6.99	5.96	6.03	5.99
$C_{15:0}$	2.51	2.71	2.51	2.64	2.65	2.43	2.79	2.91	2.77	2.84	2.87	2.85
$C_{16:0}$	72.84	75.50	70.39	69.57	70.41	68.52	66.17	66.72	65.92	75.48	75.75	75.32
$C_{16:1n-9}$	3.54	3.77	3.50	4.25	4.29	4.02	3.82	3.88	3.86	4.59	4.79	4.49
$C_{16:1n-7}$	143.46	148.59	138.54	149.93	151.00	148.66	155.90	157.14	155.66	147.83	148.27	147.49
$C_{17:0}$	0.50	0.54	0.49	0.38	0.39	0.37	0.67	0.70	0.65	1.66	1.73	1.68
$C_{16:2n-4}$	6.50	6.81	6.39	5.70	5.81	5.39	5.66	5.69	5.65	4.59	4.66	4.62
$C_{18:0}$	10.52	10.77	10.08	9.96	10.04	9.67	8.41	8.82	8.51	12.21	12.59	11.93
$C_{16:3n-3}$	2.39	2.57	2.37	2.01	2.09	1.96	2.01	2.12	1.97	1.56	1.58	1.55
$C_{18:1n-11}$	30.22	32.00	29.64	39.88	41.28	38.29	39.73	40.46	39.14	44.81	45.09	44.73
$C_{18:1n-9}$	157.99	163.52	152.27	153.14	156.24	149.84	149.57	150.11	149.13	152.61	153.30	152.00
$C_{18:1n-7}$	38.25	38.38	38.20	38.50	38.59	38.21	35.47	35.85	35.18	42.35	42.66	42.45
$C_{16:4n-3}$	4.33	4.51	4.20	2.99	3.00	2.78	3.82	3.98	3.75	3.40	3.56	3.35
$C_{18:2n-6}$	17.54	17.84	16.44	16.52	16.67	16.56	18.02	18.23	17.93	15.43	15.84	15.14
$C_{20:0}$	0.31	0.32	0.30	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
$C_{18:3n-3}$	4.99	5.11	4.73	4.55	4.71	4.45	4.97	5.04	4.95	4.49	4.66	4.38
$C_{20:1n-11}$	17.18	17.77	16.38	21.00	21.84	20.17	18.88	19.37	18.79	25.84	26.03	25.75
$C_{20:1n-9}$	85.17	89.05	81.09	85.46	86.42	84.43	70.51	72.27	69.76	99.62	100.33	99.91
$C_{20:1n-7}$	5.37	5.53	5.01	5.16	5.26	5.06	4.23	4.41	4.15	6.20	6.46	6.00
$C_{18:4n-3}$	12.96	13.32	12.40	11.55	12.03	10.88	12.44	13.02	12.86	9.27	9.37	9.27
$C_{20:2n-6}$	1.69	1.75	1.63	1.73	1.83	1.71	1.60	1.63	1.59	2.08	2.06	2.00
$C_{20:4n-6}$	4.91	5.10	4.71	4.24	4.50	4.19	4.85	4.96	4.84	3.60	3.65	3.45
$C_{22:1n-11}$	18.89	19.50	18.24	19.73	20.51	18.76	17.24	17.60	17.09	23.47	23.64	23.20
$C_{22:1n-9}$	4.67	4.66	4.23	4.73	4.83	4.42	3.98	4.07	3.97	6.10	6.23	5.88
$C_{20:4n-3}$	4.53	4.64	4.21	4.25	4.26	4.04	4.60	4.65	4.63	3.83	3.87	3.80
$C_{20:5n-3}$	67.19	70.68	65.71	61.25	64.45	58.24	64.54	64.93	64.26	53.66	54.82	53.51
$C_{24:1n-9}$	4.68	4.77	4.39	4.84	4.94	4.55	5.01	5.14	4.98	4.26	4.31	4.25
$C_{22:5n-3}$	38.06	40.32	37.20	38.58	39.95	37.01	38.14	38.47	37.90	36.63	36.84	36.51
$C_{22:6n-3}$	82.69	86.38	78.61	76.78	81.50	74.05	88.21	90.58	86.85	57.45	58.32	57.58

Table 2.3: FAME concentrations (mg/g) for whale (different grades) and fish (different species) oils

Sample	Whale oil						Fish oil					
Quality	Conventionally distilled			Molecularly distilled			Cod liver			Salmon		
Designation	WC			WM			CL			SA		
Replicate	i	ii	iii	i	ii	iii	i	ii	iii	i	ii	iii
$C_{14:0}$	50.54	50.59	50.40	47.82	48.10	47.52	33.67	33.64	33.59	41.05	41.83	40.47
$C_{14:1n-9}$	3.89	3.82	3.86	3.45	3.71	3.40	0.00	0.00	0.00	0.00	0.00	0.00
$C_{15:0}$	3.43	3.47	3.29	3.61	3.68	3.33	3.11	3.17	3.01	3.85	3.90	3.76
$C_{16:0}$	74.45	75.18	72.73	95.29	95.76	94.62	91.89	92.61	90.97	130.47	133.40	127.50
$C_{16:1n-9}$	3.22	3.24	3.09	3.54	3.57	3.53	4.33	4.59	4.25	2.81	2.87	2.63
$C_{16:1n-7}$	74.00	75.19	71.81	65.05	65.70	64.60	60.69	62.80	58.56	39.62	39.94	39.09
$C_{17:0}$	4.93	5.01	4.84	3.74	3.99	3.68	5.77	5.79	5.65	2.66	2.76	2.54
$C_{16:2n-4}$	3.55	3.56	3.46	2.75	2.95	2.75	4.21	4.29	3.98	3.73	3.88	3.69
$C_{18:0}$	16.35	16.07	15.62	23.54	23.91	23.38	18.47	19.08	17.75	30.53	31.41	29.78
$C_{16:3n-3}$	0.00	0.00	0.00	0.00	0.00	0.00	3.38	3.39	3.37	1.93	1.99	1.84
$C_{18:1n-11}$	18.23	18.02	17.44	19.67	19.76	19.38	13.63	13.66	13.56	3.89	4.00	3.76
$C_{18:1n-9}$	147.00	147.26	146.13	136.56	137.06	135.87	122.70	123.80	121.41	242.10	243.16	240.88
$C_{18:1n-7}$	21.36	21.33	20.90	25.54	25.59	25.30	34.87	35.05	34.52	29.48	30.15	28.61
$C_{16:4n-3}$	2.55	2.57	2.45	0.00	0.00	0.00	4.61	4.69	4.33	3.22	3.33	3.04
$C_{18:2n-6}$	19.44	19.66	19.19	17.39	17.63	16.95	18.47	18.50	18.42	81.37	82.28	80.39
$C_{20:0}$	0.50	0.51	0.48	0.66	0.68	0.64	0.00	0.00	0.00	1.83	1.90	1.74
$C_{18:3n-3}$	12.58	12.71	12.35	10.91	11.09	10.71	7.78	7.83	7.54	30.43	31.01	29.92
$C_{20:1n-11}$	20.94	21.58	20.21	21.66	21.76	21.36	10.17	10.25	10.04	4.63	4.76	4.59
$C_{20:1n-9}$	96.94	98.11	94.76	128.41	129.07	127.55	77.90	78.18	77.74	43.11	43.46	42.69
$C_{20:1n-7}$	2.09	2.06	2.03	2.65	2.82	2.68	3.24	3.28	3.15	2.53	2.70	2.44
$C_{18:4n-3}$	27.59	27.88	26.30	19.38	19.84	18.72	24.49	25.05	23.73	9.17	9.25	8.78
$C_{20:2n-6}$	2.97	3.05	2.84	3.47	3.55	3.37	2.57	2.61	2.48	6.88	7.10	6.64
$C_{20:3n-6}$	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.60	1.67	1.51
$C_{22:0}$	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.72	1.79	1.67
$C_{20:3n-3}$	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	3.25	3.39	3.15
$C_{20:4n-6}$	3.76	3.91	3.58	2.79	2.99	2.80	6.52	6.53	6.50	3.40	3.64	3.37
$C_{22:1n-11}$	94.43	96.59	91.27	120.77	122.09	119.38	49.95	50.00	49.88	39.23	39.90	38.76
$C_{22:1n-9}$	6.98	7.07	6.69	10.53	10.92	10.11	6.02	6.14	5.88	7.46	7.86	7.26
$C_{20:4n-3}$	14.09	14.13	13.95	11.14	11.34	10.74	7.97	7.99	7.88	10.74	11.35	10.32
$C_{20:5n-3}$	46.45	47.18	45.32	35.12	36.14	33.90	106.67	108.09	105.05	39.12	40.69	37.72
$C_{24:0}$	0.00	0.00	0.00	0.00	0.00	0.00	1.72	1.78	1.64	0.00	0.00	0.00
$C_{24:1n-9}$	6.94	7.06	6.80	6.48	6.95	6.41	7.91	7.91	7.87	8.11	8.40	7.77
$C_{22:5n-3}$	22.99	23.02	22.86	20.51	21.07	20.34	16.39	16.58	15.99	21.87	22.35	21.28
$C_{22:6n-3}$	76.72	76.31	75.14	48.30	50.72	47.27	145.64	149.56	141.24	52.29	54.01	50.37

Table 2.4: FAME concentrations (mg/g) for different brands of ω-3 supplements

Sample	Supplements													
Designation	K1		K2		K3		K4		K5		K6		K7	
Replicate	i	ii	i	ii	i	ii	i	ii	i	ii	i	ii	i	ii
$C_{14:0}$	2.64	2.52	0.00	0.00	22.16	22.22	1.25	1.24	3.01	3.05	2.53	2.56	2.67	1.23
$C_{15:0}$	0.00	0.00	0.00	0.00	1.39	1.45	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
$C_{16:0}$	22.21	22.38	44.67	44.35	58.50	58.77	24.22	23.93	22.73	22.71	12.38	12.55	22.69	15.33
$C_{16:1n-9}$	0.00	0.00	0.00	0.00	1.16	1.13	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
$C_{16:1n-7}$	8.71	8.46	0.00	0.00	26.34	25.92	8.58	8.33	8.40	8.35	7.16	7.35	8.67	7.87
$C_{17:0}$	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	2.67	2.13
$C_{16:2n-4}$	1.59	1.55	0.00	0.00	4.42	4.75	0.99	1.06	1.06	1.13	1.08	1.16	1.86	0.98
$C_{18:0}$	29.72	29.87	22.76	22.69	18.83	19.16	31.64	31.46	26.98	26.54	14.23	14.61	29.82	22.05
$C_{16:3n-3}$	2.40	2.28	0.00	0.00	6.20	5.96	1.52	1.60	1.68	1.59	1.51	1.40	1.95	1.15
$C_{18:1n-9}$	62.24	62.59	175.01	174.10	46.33	45.41	60.07	59.29	51.92	50.98	39.50	39.96	62.65	50.65
$C_{18:1n-7}$	18.30	18.39	8.50	8.57	14.49	13.85	21.90	21.45	20.17	19.92	13.65	13.71	18.72	16.47
$C_{18:2n-6}$	6.87	7.00	259.92	258.84	5.66	5.31	8.22	8.24	6.99	6.70	4.97	5.37	7.21	7.21
$C_{20:0}$	3.28	3.42	1.89	1.98	2.48	2.33	4.83	4.79	9.29	9.22	4.30	4.29	3.40	6.56
$C_{18:3n-3}$	3.68	3.74	312.52	312.51	3.49	3.30	5.81	5.67	4.51	4.52	3.28	3.14	3.73	3.85
$C_{20:1n-11}$	1.76	1.71	2.93	2.82	2.01	1.85	2.50	2.39	1.15	1.13	1.35	1.32	1.78	1.07
$C_{20:1n-9}$	13.50	13.51	0.00	0.00	13.87	13.69	24.22	23.84	23.17	22.97	19.20	19.48	13.94	23.69
$C_{20:1n-7}$	4.47	4.48	0.00	0.00	3.02	3.06	4.29	4.25	3.18	3.05	3.37	3.47	4.46	2.79
$C_{18:4n-3}$	16.38	16.52	0.00	0.00	13.71	13.61	21.09	21.00	20.61	20.27	13.14	12.80	16.37	16.72
$C_{20:2n-6}$	2.08	2.12	0.00	0.00	1.55	1.61	2.50	2.57	2.12	2.18	2.10	2.23	2.19	1.97
$C_{20:3n-6}$	1.84	1.95	0.00	0.00	1.37	1.45	2.32	2.30	2.48	2.52	2.36	2.56	1.78	2.21
$C_{22:0}$	0.96	0.90	0.00	0.00	0.85	0.81	0.00	0.00	2.39	2.35	0.00	0.00	0.00	1.23
$C_{20:4n-6}$	12.86	13.10	0.00	0.00	9.38	8.86	17.70	17.55	15.74	15.40	15.33	14.86	12.89	14.01
$C_{22:1n-11}$	11.19	11.56	0.00	0.00	20.61	19.40	15.46	15.33	8.93	8.53	10.78	10.73	11.51	8.20
$C_{22:1n-9}$	1.48	1.46	0.00	0.00	2.79	2.63	3.31	3.19	5.22	5.05	4.72	4.62	1.54	5.49
$C_{20:4n-3}$	10.47	10.50	0.00	0.00	7.67	7.97	14.66	14.53	16.19	16.01	14.66	14.53	10.54	15.08
$C_{20:5n-3}$	242.64	244.97	0.00	0.00	174.87	174.23	283.79	281.47	276.42	273.09	260.27	250.08	239.80	238.08
$C_{24:0}$	0.00	0.00	0.00	0.00	1.12	1.21	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
$C_{24:1n-9}$	9.27	9.36	0.00	0.00	5.04	5.56	11.35	11.34	12.21	12.09	16.26	17.17	11.91	14.51
$C_{22:5n-3}$	24.93	25.15	0.00	0.00	25.57	26.89	28.87	28.71	35.82	35.24	42.12	40.79	24.72	36.55
$C_{22:6n-3}$	168.02	169.44	0.00	0.00	167.82	182.44	175.10	173.61	175.31	173.04	198.61	182.92	162.81	172.44

The individual profiles were arranged in a data matrix consisting of 47 rows representing the various analyzed oils with their respective replicates and 34 columns representing the individual FAME detected by GC. The 34 individual FAME profiles were: $C_{14:0}$, $C_{14:1n-9}$, $C_{15:0}$, $C_{16:0}$, $C_{16:1n-9}$, $C_{16:1n-7}$, $C_{17:0}$, $C_{16:2n-4}$, $C_{18:0}$, $C_{16:3n-3}$, $C_{18:1n-11}$, $C_{18:1n-9}$, $C_{18:1n-7}$, $C_{16:4n-3}$, $C_{18:2n-6}$, $C_{20:0}$, $C_{18:3n-3}$,

$C_{20:1n-11}$, $C_{20:1n-9}$, $C_{20:1n-7}$, $C_{18:4n-3}$, $C_{20:2n-6}$, $C_{20:3n-6}$, $C_{22:0}$, $C_{20:3n-3}$, $C_{20:4n-6}$, $C_{22:1n-11}$, $C_{22:1n-9}$, $C_{20:4n-3}$, $C_{20:5n-3}$, $C_{24:0}$, $C_{24:1n-9}$, $C_{22:5n-3}$, $C_{22:6n-3}$.

2.3.1 Full fatty acid methyl esters profiles discrimination

The 47×34 matrix was submitted to PCA as a data exploration technique and a total of six PCs grouped in decreasing order of variance extracted. The first component (PC1) explains 41.91 % of the total variation and the information retained by this component can discriminate the oils according to their nature (Fig. 2.1).

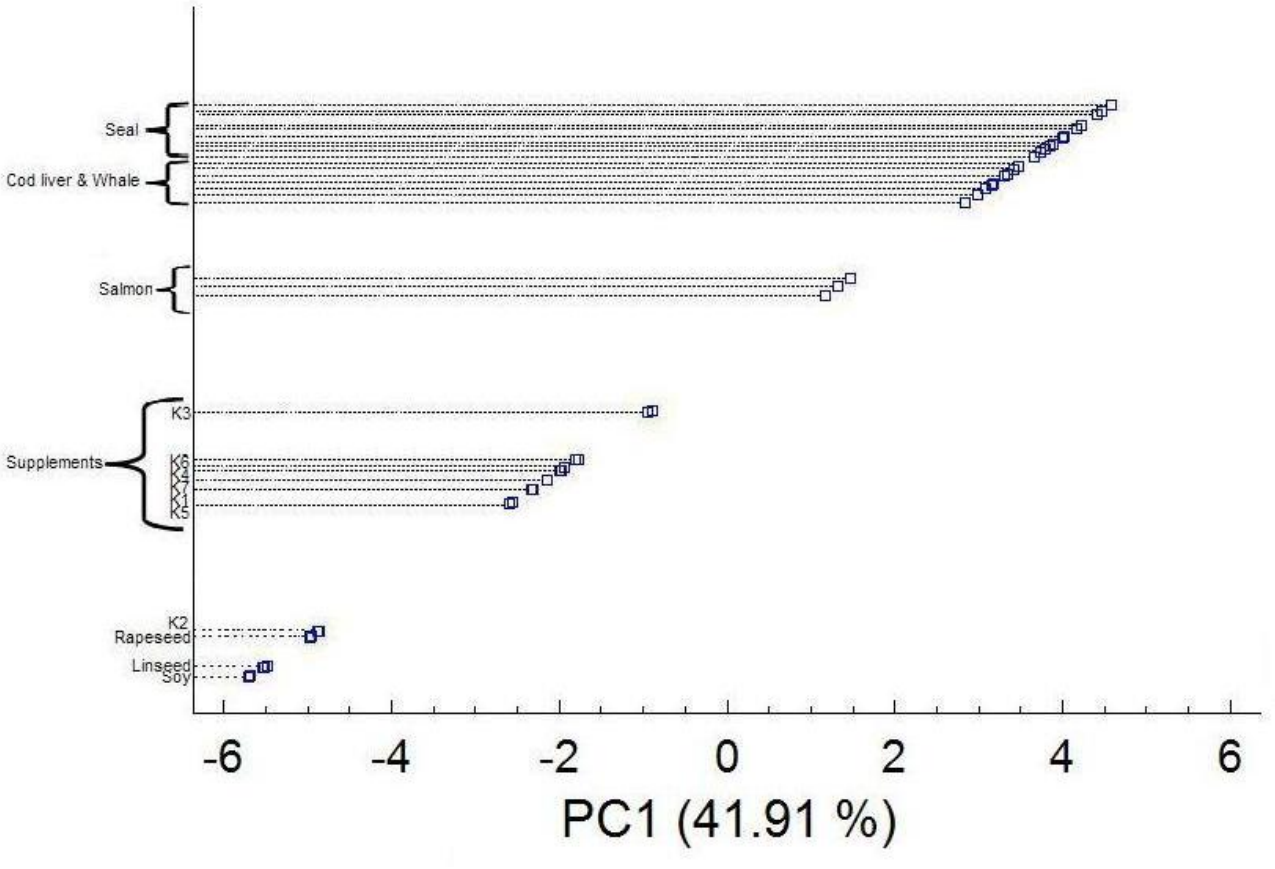


Figure 2.1: PC1 score plot for the different oil samples by using the full FAME profiles

The scores of the analyzed plant and animal oils grouped themselves at opposite ends of the PC1 axis while the scores of six supplements (K1 and K3-7) in the middle of this axis. The scores for K2 overlaps those from rapeseed oil, hence it is likely that this supplement contains this particular oil. In addition, the supplement group and the animal group exhibit some sub-groups

which discriminate supplement K3 in the former and salmon oil in the latter. These latent sub-structures are attributed to the consistently high levels of $C_{16:0}$ and $C_{16:1n-7}$ in supplement K3 (on average 3 times higher compared to the others supplements of this group) and the high levels of $C_{18:1n-9}$, $C_{18:2n-6}$ and $C_{18:3n-3}$ in the salmon oil (on average 1.7, 4.7 and 5.0 times higher than the rest of marine animal oils respectively). A plot of the scores of the two first components (Fig. 2.2), which explain 66.35 % of the data variation, differentiates basically the same number of groups and sub-groups found in Fig. 2.1 and allowed to conclude that PC1 discriminates in effect between animal and plant oils while PC2 differentiates between supplements and plant oils. Besides, this score plot revealed that in addition to rapeseed oil, supplement K2 also contains linseed oil due to the proximity of their scores. This proximity was constantly observed when the scores of any of the six PCs were plotted against each other. The presence of rapeseed and linseed oils in the composition of K2 was confirmed by searching the webpage of the manufacturer of this particular supplement. The PC1versus PC2 plot (Fig. 2.2) revealed some variability in the individual scores for K7 which could be attributed to experimental errors, indicating the importance of replication in discrimination studies.

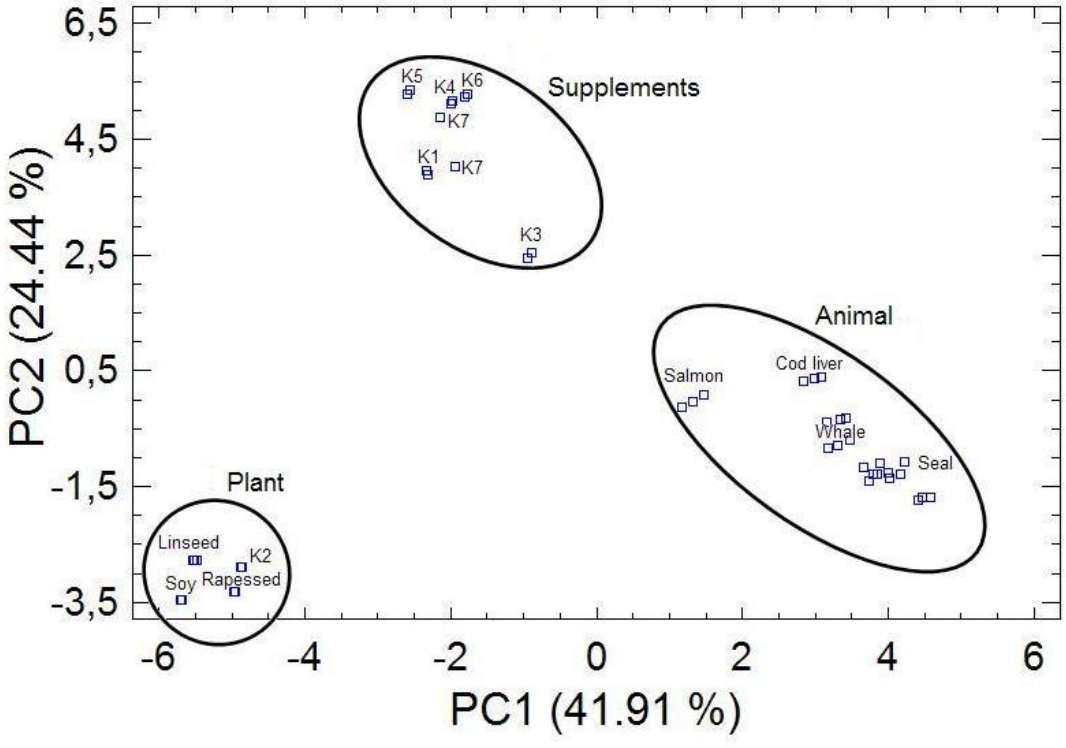


Figure 2.2: PC1 and PC2 score plot for the different oil samples by using the full FAME profiles

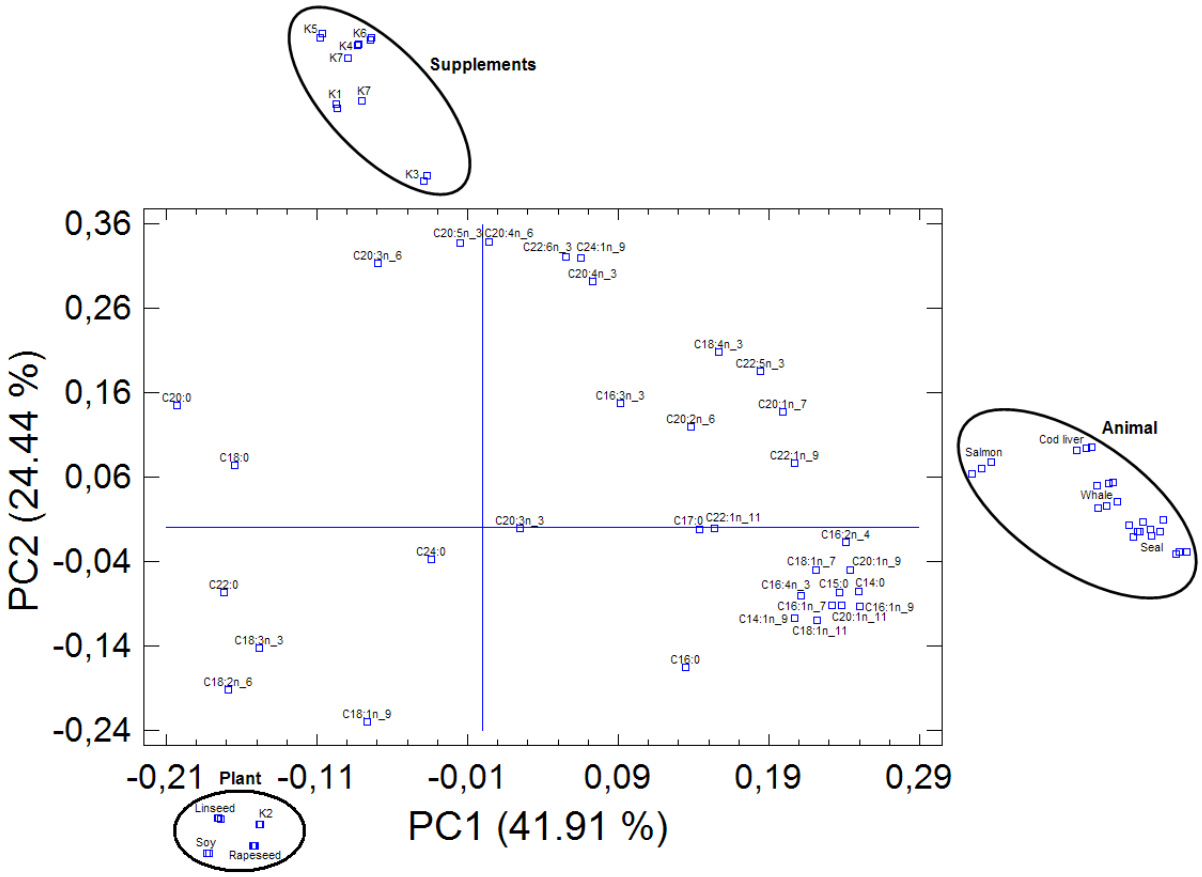


Figure 2.3: FAME loading plot for PC1 and PC2 and its relationship to the scores portrayed in Fig. 2.2

The plot of the loadings of the two first components, expressing the relationship between the various FAME (Fig. 2.3) showed the lack of correlation between $C_{20:5n-3}$ and $C_{18:1n-9}$ on the PC2 axis, indicating that any of the plant oils studied in the present investigation are present in the composition of supplements K1, K3, K4, K5, K6 and K7. These observations are in agreement with the various manufacturers who have reported some special developed oil (name not disclosed), refined fish oil (from non-specified origin), gelatin from pork, etc, among the various constituents of their supplements rather than plant oils. The superimposition of the three main clusters from Fig. 2.2 on the loading plot (Fig. 2.3) demonstrates unequivocally that the observed anti-correlation between $C_{20:5n-3}$ (positive PC2 loading value) and $C_{18:1n-9}$ (negative PC2 loading value) is responsible for the discrimination between supplements (with scores highly associated to $C_{20:5n-3}$) and plant oils (with scores highly associated to $C_{18:1n-9}$). Similarly, the lack of correlation between $C_{16:1n-9}$, $C_{14:0}$, $C_{20:1n-9}$, $C_{16:2n-4}$ (positive PC1 loading values) and $C_{22:0}$, $C_{18:2n-6}$

(negative PC1 loading values) is responsible for discriminating between animal and plant oils. The six PCs computed by using the 47×34 data matrix were plotted against each other to produce two and three dimensional PC scores graphs and consequently explore the capability of these PCs to discriminate confidently within the marine animal oils. Unfortunately, clear and well-defined patterns that allow differentiating the various oils and grades were not observed in any of the graphical representations, hence a data reduction was implemented.

2.3.2 Selected fatty acid methyl esters profiles discrimination

FAME data reduction has been used in the discrimination of oils derived from one fish species (cod liver oil) by selecting the 15 FAME with levels higher than 1 % of the total composition [78]. Considering that in the present study the 34 FAME or variables are given in mg/g and arranged in columns for PCA purposes (47×34), it was decided to discard all the FAME columns with average values < 5 mg/g (< 0.5 % of the total averaged FAME profile). In that way 19 FAME profiles were retained ($C_{14:0}$, $C_{16:0}$, $C_{16:1n-7}$, $C_{18:0}$, $C_{18:1n-11}$, $C_{18:1n-9}$, $C_{18:1n-7}$, $C_{18:2n-6}$, $C_{18:3n-3}$, $C_{20:1n-11}$, $C_{20:1n-9}$, $C_{18:4n-3}$, $C_{20:4n-6}$, $C_{22:1n-11}$, $C_{20:4n-3}$, $C_{20:5n-3}$, $C_{24:1n-9}$, $C_{22:5n-3}$, $C_{22:6n-3}$). A new data matrix of size 47×19 was submitted to PCA and three PCs, explaining 85.29 % of the total data variability, extracted. The three PCs were used to generate various two and pseudo-three dimensional score plots which essentially showed a clear discrimination between plant, supplements and marine animal oils as already observed in Fig. 2.1 and Fig. 2.2. However, the graphs consistently misclassified supplement K2 as containing a mixture of the three plant oils (soy, linseed and rapeseed oil), while in fact only linseed and rapeseed oil are present in this particular supplement. This result indicates that PCA on full FAME profiles outperforms the proposed data reduction approach for supplement classification. The various plots generated with the aforementioned three PCs were also unable to establish a clear distinction between the various species, brands and grades of animal oils; hence a further discrimination study was carried out by using only the full FAME profiles derived from marine oils.

2.3.3 Full fatty acid methyl esters profiles of marine oils discrimination

The contribution of the supplement and plant oils was removed as an alternative approach to discriminate between the various marine oils and a 24×34 data matrix generated (Tables 2.2 and 2.3) and subjected to PCA. By using this data matrix, 97.58 % of its variability was explained by

four extracted PCs (49.80, 22.65, 20.08 and 5.05 %). The PC1 and PC4 score plot (Fig. 2.4) shows that PC1 can discriminate between the four different types of animal oils, namely seal oil, cod liver oil, whale oil and salmon oil and that PC4 can discriminate effectively within every animal oil specie. For instance, the unmistakable differentiation between whale oil molecularly distilled (WM) from whale oil conventionally distilled (WC) which is mainly due to the lack of $C_{16:4n-3}$ in WM and the slightly higher levels of $C_{22:6n-3}$ in WC (1.6 times). In addition, Fig. 2.4 shows a clear discrimination between seal oils samples from different manufacturers. It is also observed that the two different batches of refined seal oil from manufacturer-A (RSA1 and RSA2 in Fig. 2.4) display positive and negative PC4 score values respectively indicating some differences between them. The crude and the refined seal oil from the same manufacturer and designated as CSA and RSA2 in Fig. 2.4, exhibit negative scores values, indicating a correlation between these two oils regardless their alleged quality.

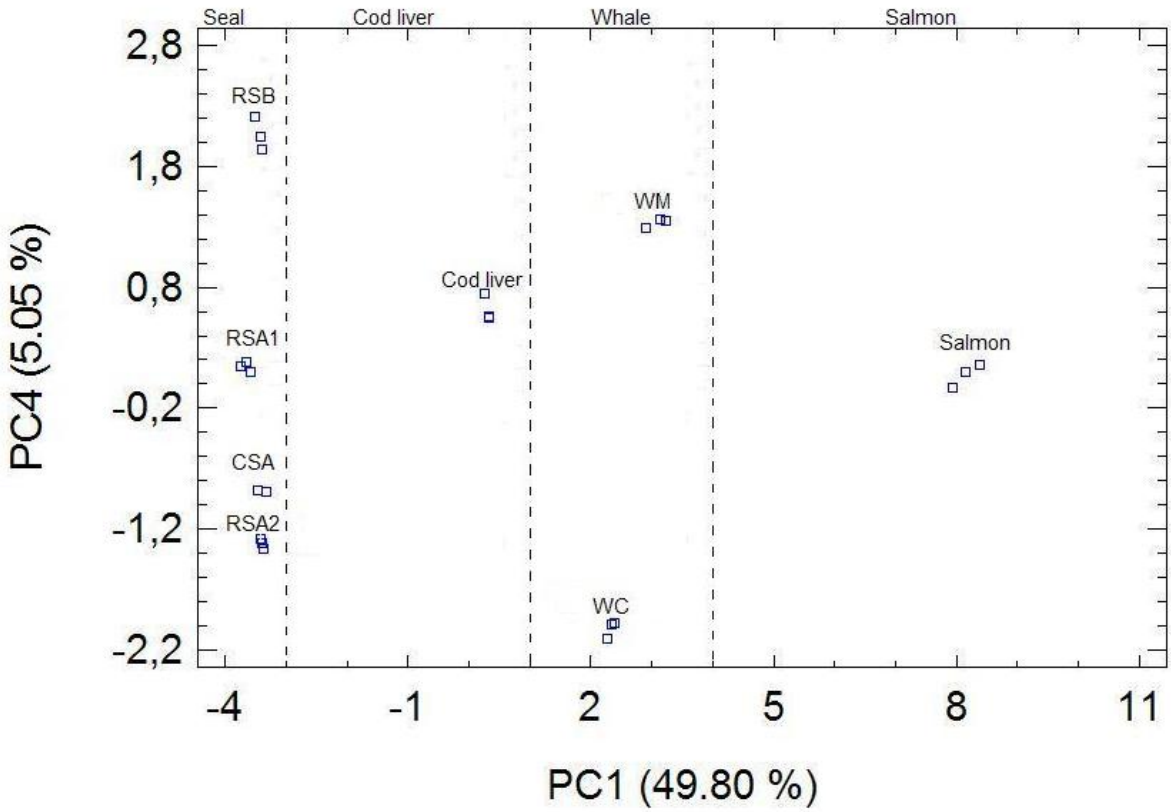


Figure 2.4: PC1 and PC4 score plot for the marine oils by using the full FAME profiles. For abbreviations see section 2.2.1

2.4 Conclusions

Compared to the most popular, complex, and sophisticated analytical techniques presented in Table 1.4, GC might offer a rapid, simple and convenient means of discriminating between ω-3 rich oils derived from fish and marine mammals. The different approaches used in the discrimination process indicated that PCA on the full FAME profiles is the best strategy to discriminate between the various oils considered in this study. On the basis of Table 1.4 and considering that ω-3 rich oils derived from fish and marine mammals are highly regarded as alternative medicines worldwide, the potentiality of unexplored single or coupled techniques for authentication and discrimination of these kinds of oils should be investigated to prevent fraudulent practices.

3 Discrimination study based on triacylglyerol profiles

3.1 Background

Marine oils have drawn more and more attention due to their high nutritional value as described in Chapter 2. TAG are the primary components in marine oils (> 98 %). The fatty acids mainly exist in the form of TAG, in which they are distributed on the backbone of TAG according to certain stereospecific positioning patterns described in Section 1.2.4. By using proper analytical instrumentation for TAG analysis such as LC-MS and NMR, the positioning patterns of TAG derived from the intact TAG can be determined. The TAG patterns provide information not only on fatty acids composition but also on the stereospecificity of fatty acids on TAG molecules. Compared to simple fatty acids composition analysis, TAG patterns usually carry more information and could be used for fingerprinting purposes.

Fingerprinting has been extensively studied and internationally accepted as a feasible means for the quality control of traditional Chinese medicines due to their ability to reveal the inherent relationship of multiple compounds and determine the characteristic pattern of samples [163, 164]. By combining fingerprinting methods with multivariate techniques, such as PCA, similarity analysis or other chemometric methods, the identity, consistency and authenticity of samples can be determined based on the similarities and dissimilarities of their profiles. Chromatographic profiles such as total ion chromatogram (TIC) or base peak chromatogram (BPC) are widely employed in fingerprinting. They may work well for the simple samples provided the time and one-dimensional information used for alignment is similar across the samples, but could be inappropriate for complex samples containing closely eluting components with varying intensities in different samples. The absence of mass spectral information might lead to the inaccuracy of classification and discrimination.

Although the feasibility of dietary oils discrimination studies based on the TAG profiling has been evaluated in several investigations by using the lipase method[165], HPLC[33, 166], ^{13}C- NMR[81, 87], matrix-assisted laser desorption ionization-MS (MALDI-MS) [73] and fast atom bombardment-MS (FAB-MS) [98], the current studies have not explored the marine oils discrimination by means

of LC-ESI-MS and LC-ESI-MS^2 which are becoming increasing popular for TAG analysis in various vegetable oils [105, 106, 135]. The lack of interest in applying these techniques could be due to the high complexity of naturally occurring TAG present in marine oils, and also the great amount of data generated by LC-ESI-MS and LC-ESI-MS^2 with the useful information embedded in noise and background which inevitably provide great interferences for the TAG analysis. Consequently, it is essential to perform data pre-processing with the help of chemometric approaches to extract the useful information from the original data prior to multivariate analysis such as PCA.

This chapter evaluated the capabilities of fingerprinting and discriminating several marine oils (seal, whale, cod liver and salmon) and plant oils (soy, rapeseed and linseed) based on their TAG profiles from LC-ESI-MS and LC-ESI-MS^2 experiments by means of PCA. Four TAG profiles, namely, TIC and mass spectral profiles from LC-ESI-MS and LC-ESI-MS^2experiments were constructed based on the pre-processed data by CODA in order to eliminate the noise and background. The pre-processed profiles are subsequently submitted to PCA to investigate their performance on the marine oils discrimination and find the most appropriate profiles that represent the characteristics of TAG patterns.

3.2 Experimental

3.2.1 Materials

1-Arachidin-2-Olein-3-Palmitin-glycerol (AOP), 1-Arachidin-2-Palmitin-3-Olein-glycerol (APO) with an impurity of 1-Behenin-2-Palmitin-3-Olein-glycerol (BPO), 1-Arachidin-2-Linolein-3-Olein-glycerol (ALO) with an impurity of 1-Behenin-2-Linolein-3-Olein-glycerol (BLO) are from Larodan Fine Chemicals (Malmö, Sweden), and mixtures of these TAG standards were prepared in a chloroform:methanol (2:1, v/v). The description and sources of samples were listed in Table 3.1. They have been tabulated for the sake of clarity on the discrimination section where the oils were defined by numbers (No. 1-12)

Table 3.1: Description and sources of samples

No.	Sample	Description and Sources
1	Refined seal oil	*Phagophilus groenlandicus*, Rieber Skinn A/S, Bergen, Norway
2	Seal carbon oil	*Phagophilus groenlandicus*, Rieber Skinn A/S, Bergen, Norway
3	Seal oil A	*Phagophilus groenlandicus*, Rieber Skinn A/S, Bergen, Norway
4	Seal oil B	*Phagophilus groenlandicus*, Rieber Skinn A/S, Bergen, Norway
5	Seal oil C	JFM Sunile A/S, Os, Norway
6	Seal oil D	JFM Sunile A/S, Os, Norway
7	Whale oil	*Balaenoptera acutorostrata*, Myklebust Trading AS, Myklebost, Norway
8	Cod liver oil	Peter Möller, Lysaker
9	Salmon oil	Havnegater, Sortland, Norway
10	Soy oil	Mills DA, Sofienberg, Norway
11	Rapeseed oil	Kinsarvik Naturkost, Bergen, Norway
12	Linseed oil	Kinsarvik Naturkost, Bergen, Norway

3.2.2 Liquid chromatography ion-trap mass spectrometry

An aliquot of sample (1 ml) was dissolved in 1 ml of chloroform:methanol (2:1, v/v) and vortex-mixed for 30 s. The products were then individually subjected to LC-ESI-MS^2 analysis. The TAG analysis were carried out by using an Agilent 1100 series LC/MSD trap, SL model with an electrospray interface, a quaternary pump, degasser, autosampler, thermostatted column compartment, variable-wavelength UV detector and 10 µl injection volume. The Zorbax Eclipse-C_8 RP 150 × 4.6 mm, 5 µm (Agilent Technologies, Palo Alto, CA) was kept in the column compartment at 40 °C and the solvent system in gradient mode consisted of 10 mM isopropanol:ammonium acetate 90:10 v/v (A), acetone (B) and acetonitrile (C) at a flow rate of 0.2 ml/min and UV detection at 254 nm. After testing different delivered LC solvent programs, the following gradient was selected: an initial 5 min condition 90 % A and 10 % C that was ramped in 5 min to 65 % A and 5 % C and returned to the initial condition in 15 min and subsequently ramped in 5 min to 65 % A and 5 % C and returned to the initial condition in 30 min. By using this gradient program, reproducible retention times and peak areas from sample to sample were monitored. Nitrogen was used as nebulizing (50 psi) and drying gas (8 L/min) at 350 °C. The ESI source was operated in positive ion mode and the ion optics responsible for getting the ions in the ion-trap such as capillary exit, skimmer, lens and octapoles voltages were controlled by using the Smart View option with a resolution of 13000 *m*/*z*/sec (FWHM/*m*/*z* =

0.6-0.7). Complete system control, data acquisition and processing were done using the ChemStation for LC/MSD version 4.2 from Agilent.

3.2.3 Chemometric data analysis

All the data was exported to netCDF file by DataAnalysis for LC/MSD Trap Version 3.3 (Bruker Daltonik GmbH Inc., Billerica, MA, USA). The netCDF file was then exported to a Matlab file and the mass spectra bins with the size of a single *m/z* unit in order to reduce the amount and complexity of the data and to allow subsequent data analysis. The data pre-treatment was performed by CODA coded in MATLAB 7.9 R2009b (The Math Works Inc., Natick, Massachusetts, USA). The pre-processed data was used to construct the different TAG profiles and then subjected to PCA (coded in MATLAB 7.9). The corresponding computation was carried out on a Microsoft Windows XP® 2003 operating system (Microsoft Corporation, Redmond, WA, USA).

3.3 Results and discussion

3.3.1 Data treatment by component detection algorithm

The performance of CODA was tested and verfied through the data of TAG standards mixture. Examples of three typical mass chromatograms with different MCQ values from LC-ESI-MS data set of TAG standards mixture are shown in Fig. 3.1. For the mass chromatogram with noise and background, the process of data smoothing and standardization will make the values of $a(\lambda)_{ij}$ and $a(w,s)_{ij}$ (both values are calculated by Eq. (1.1)) significantly different, leading to a low MCQ value (Fig. 3.1 c), while the high-quality mass chromatogram remains nearly unaffected during the process, thus gives rise to a similar $a(w,s)_{ij}$ value as the original $a(\lambda)_{ij}$ value, resulting a MCQ value close to 1 (Fig. 3.1 a). Therefore, only mass chromatograms with MCQ value higher than an assigned threshold will be selected, while others chromatograms are discarded. The typical MCQ value used is 0.89. However, the MCQ threshold needs to remain variable when processing different data. For example, when the concentrations of the components in a sample are very low, the mass chromatograms will have a lower quality, which indicates in this case a lower MCQ threshold could be defined in order to detect all components present. Therefore, the

assignment of MCQ threshold needs to be examined individually according to the feature of each data.

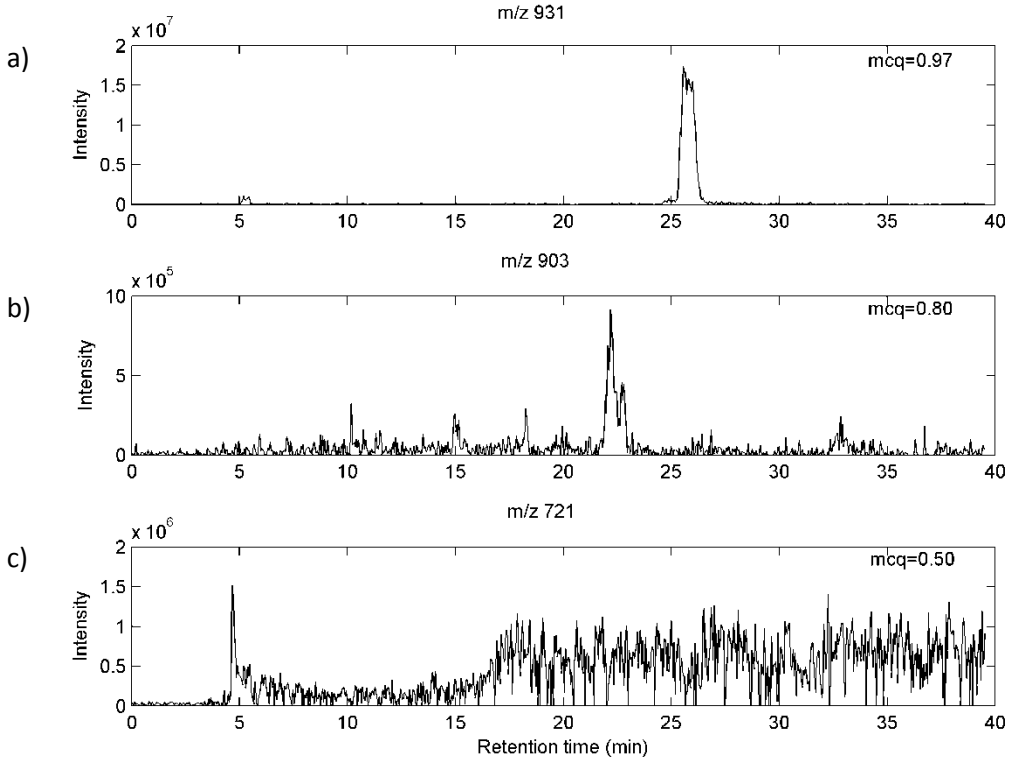


Figure 3.1: A selection of typical mass chromatograms of TAG standards mixture from LC-ESI-MS experiments in decreasing order of MCQ

By applying the CODA, the useful information will be extracted effectively from the complex LC-ESI-MS and LC-ESI-MS^2 data sets, which greatly reduces the noise and background thus satisfactorily eliminating their interferences. The following examples will show how CODA perform pre-treatment of the complex dataset derived from the LC-ESI-MS experiments.

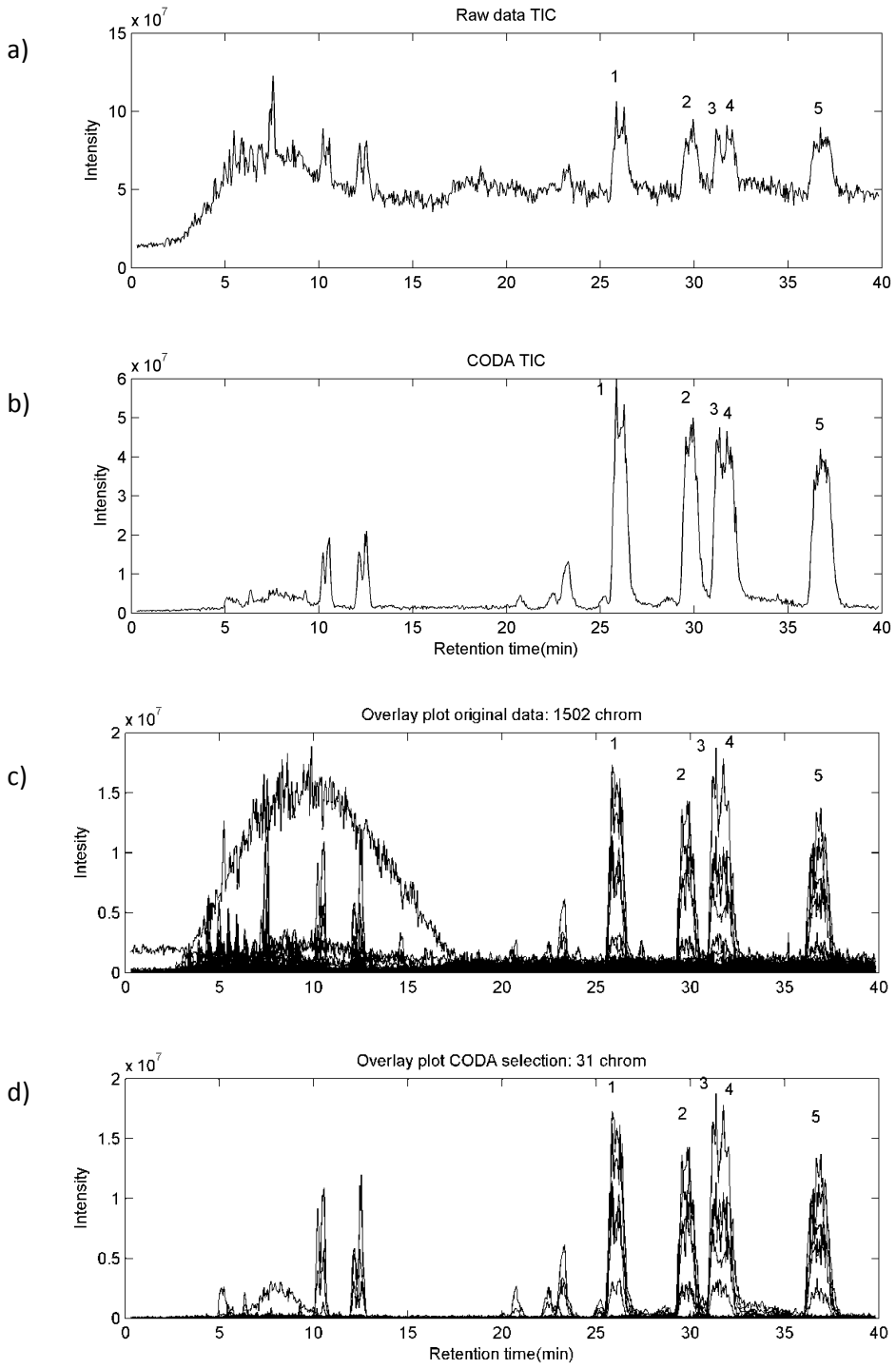


Figure 3.2: LC-ESI-MS analysis of TAG standards mixture (1. ALO; 2. BLO; 3. AOP; 4. APO; 5. BPO)
a) The original TIC profile; b) The TIC profile after CODA; c) The overlay plot of all 1502 mass chromatograms from original data; d) The overlay plot of 31 chromatograms selected by CODA (w = 7, MCQ = 0.9).

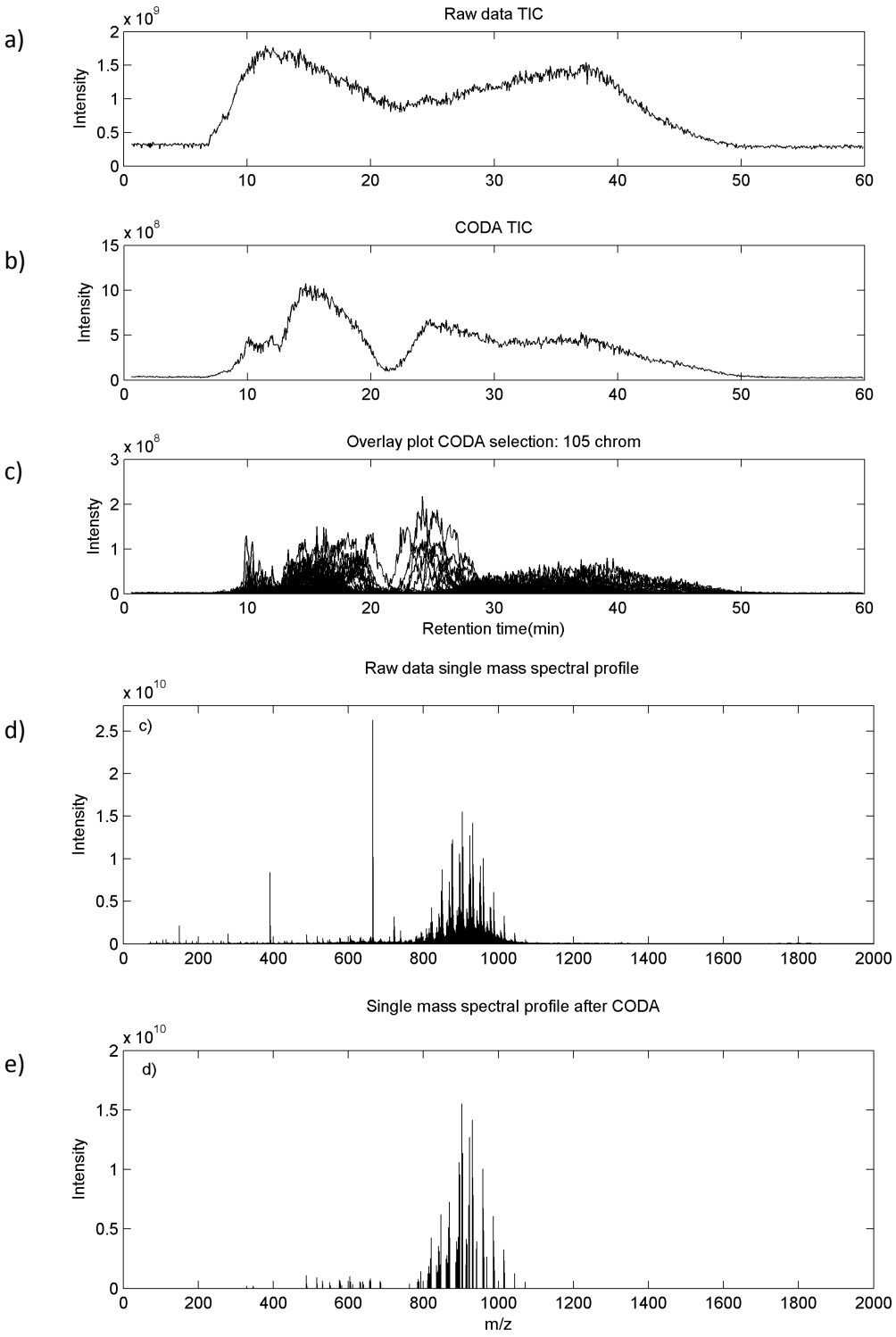


Figure 3.3: LC-ESI-MS analysis of seal oil (No. 5)
a) The original TIC profile; b) The TIC profile after CODA; c) The overlay plot of 105 chromatograms selected by CODA; d) The original single mass spectral profile; e) The single mass spectral profile after CODA (w = 7, MCQ = 0.8).

The original TIC and the overlay plot of all 1502 mass chromatograms derived from TAG standards mixture by using LC-ESI-MS are shown in Fig. 3.2 a and c, respectively. The original TIC resulting from all the chromatograms is very nosiy (Fig. 3.2 a), which is ascribed to the noise and high level of background from the low-quality chromatograms as can be clearly visualized by overlaying the 1502 mass chromatograms (Fig. 3.2 c). After the application of CODA, only 31 high-quality mass chromatograms (MCQ $\geq$ 0.9) are selected from a total of 1502 chromatograms. The noise and high background are greatly eliminated as can be observed in both TIC (Fig. 3.2 b) and mass chromatograms overlay plot (Fig. 3.2 d) after data treatment by CODA.

Another example of application of CODA on TIC and single mass spectral profiles from LC-ESI-MS experiments of seal oil (No. 5) is shown in Fig. 3.3. The original TIC of seal oil is displayed based on the total of 2202 mass chromatograms (Fig. 3.3 a). However, the resulting TIC (Fig. 3.3 a) does not exhibit a clear separation of the TAG mixtures present in the sample, as it is dominated by the contribution of the mobile phase solvent and background noise ions. Instead, only two broad peaks arising from the severe overlapping of many chromatographic peaks are observed. After applying CODA, the mass chromatograms with noise and background are sufficiently cleaned up, and only 105 mass chromatograms are retained (Fig. 3.3 c). The high level of background observed in original TIC profile is also greatly eliminated (Fig. 3.3 b). The corresponding single mass spectral profiles will be processed by CODA simultaneously. The original single mass spectral profile (Fig. 3.3 d) is obtained from the sum of 2202 mass spectra, while the processed single mass spectral profile (Fig. 3.3 e) only represents the sum of 105 high-quality mass spectra, which greatly facilitates and simplifies the subsequent multivariate analysis.

3.3.2 Principal component analysis

Different profiles including TIC and mass spectral profiles derived from LC-ESI-MS and LC-ESI-MS^2 experiments are obtained and pre-processed by CODA. These profiles were subjected to PCA to evaluate their performance on discrimination of the marine oils and plant oils. The corresponding score plots were obtained by using the first three principal components (PCs) for each kind of data profile in order to classify and discriminate the oil samples investigated in this chapter.

3.3.2.1 LC-ESI-MS and LC-ESI-MS^2 TIC profiles

TIC profiles were obtained by plotting the summed current from each mass spectrum against the retention time, which reduces the two dimensional data into one signal, and consequently only chromatographic information is retained in the TIC profiles.

PCA analysis of TIC profiles from LC-ESI-MS experiments

Fig. 3.4 a shows the typical TIC profiles derived from seven different and representative oils. It may appear that these profiles are quite simple and only characterized with several broad peaks, however, they are the results of severe overlapping chromatographic peaks. The slight differences observed among these seven samples might fail to represent their characteristic TAG patterns. PCA was performed based on these TIC profiles, and the corresponding 3D score plot was presented in Fig. 3.4 b, which explains 56.9 % of the total data variation. Clearly, this plot neither allows a differentiation of the samples nor reveals the presence of specific clusters. This result indicates that the TIC profiles derived from LC-ESI-MS experiments are not suitable for discriminating marine oils, since the chromatographic information often become useless when many peaks eluted over the chromatographic run and cannot be resolved. Nevertheless, efforts were made to discriminate the various marine oils by using their TIC profiles from LC-ESI-MS^2 experiments.

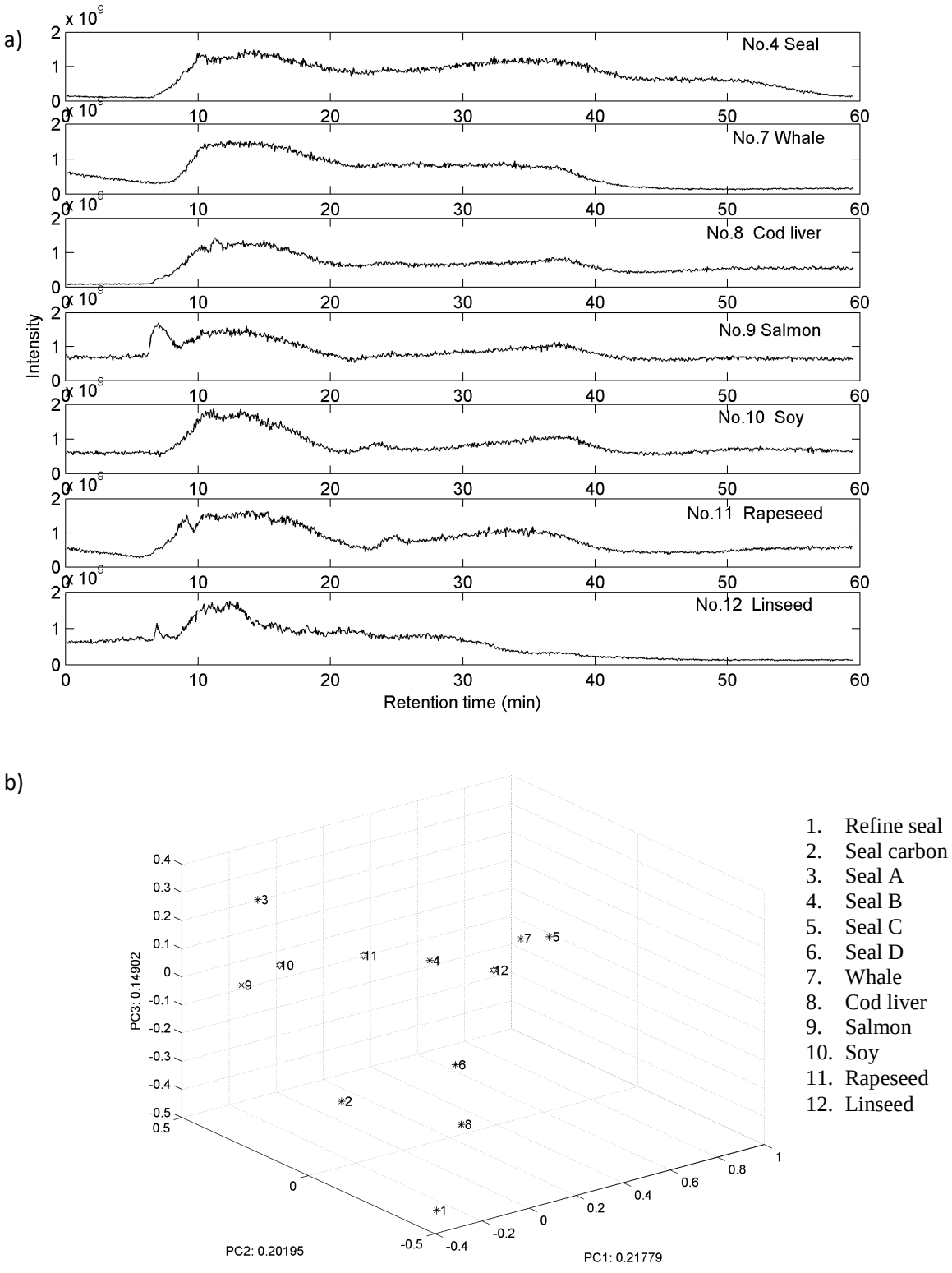


Figure 3.4: a) Typical TIC profiles of seven oils derived from LC-ESI-MS experiments; b) 3D score plot of PCA by using the above TIC profiles

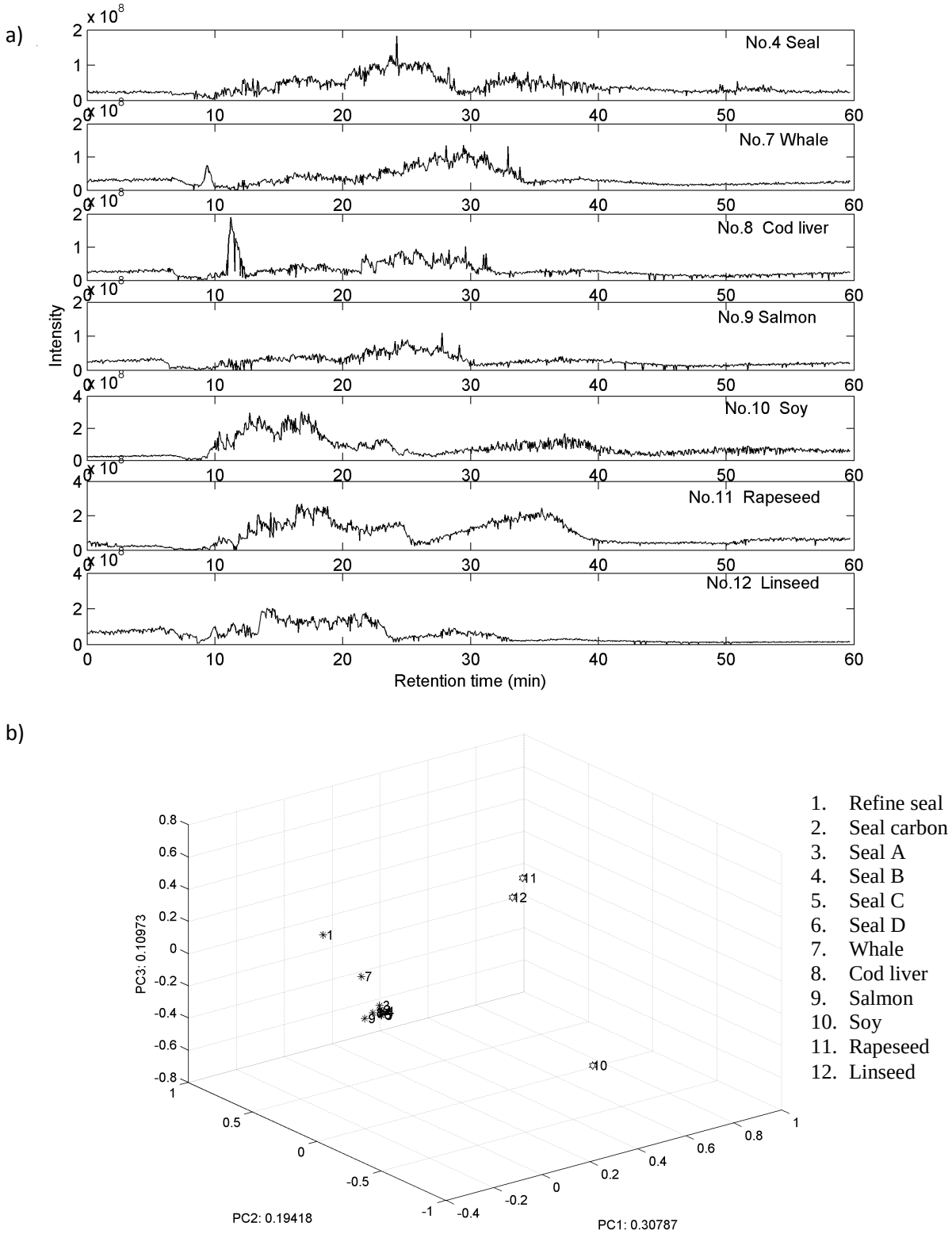


Figure 3.5: a) Typical TIC profiles of seven oil samples from LC-ESI-MS^2 experiments; (b) 3D score plot of PCA by using the above TIC profiles

PCA analysis of TIC profiles from LC-ESI-MS^2 experiments

The typical TIC profiles from the oils derived from LC-ESI-MS^2 experiments are shown in Fig. 3.5 a. Although the noise and background from the data set was reduced by CODA, the resulting TIC profiles still did not exhibit a clear separation of peaks. In spite of that, the TIC profiles of plant oils between 8-25 min were found to be significantly different from marine oils, as the former are dominated by abundant peak clusters. PCA was subsequently carried out by using these profiles. The 3D score plot (Fig. 3.5 b) which explains 61.2 % of the data variation displays a better classification pattern than obtained in Fig. 3.4 b. The two specific clusters observed in Fig. 3.5 b allow discriminating marine oils from plant oils. However, the cod liver oil (No.8) and salmon oil (No. 9) were apparently misclassified into the cluster of seal oils (No. 1-6), indicating the characteristic TAG patterns are not well represented by TIC profiles from LC-ESI-MS^2 experiments.

3.3.2.2 Mass spectral profiles from LC-ESI-MS and LC-ESI-MS^2 experiments

The single and tandem mass spectral profiles obtained by plotting the sum of all the ion intensities (0-1600 *m/z*) between 0-60 min (total analysis time) were pre-processed by CODA (Fig. 3.6 a and Fig. 3.7 a).

PCA analysis of single mass spectral profiles from LC-ESI-MS

The pre-processed single mass spectral profiles of seven oils were shown in Fig. 3.6 a. The ions are almost concentrated in the region between 800 and 1100 *m/z*, which mainly correspond to the abundant ammoniated adduct TAG ions, $[M + NH_4]^+$, acting as the base peak of most of the mass spectra. The three plant oils can be visually recognized to be different from the other marine oils, as their single mass spectral profiles have a much narrower range of *m/z* region compared to marine oils. The single mass spectral profile from whale oil appears to exhibit some dissimilarities as compared to the other three marine oils. The discrepancies among the profiles from different samples are well reflected in 3D score plot (Fig. 3.6 b) by applying PCA technique. A total of 56.5 % of data variation was explained by using three PCs.

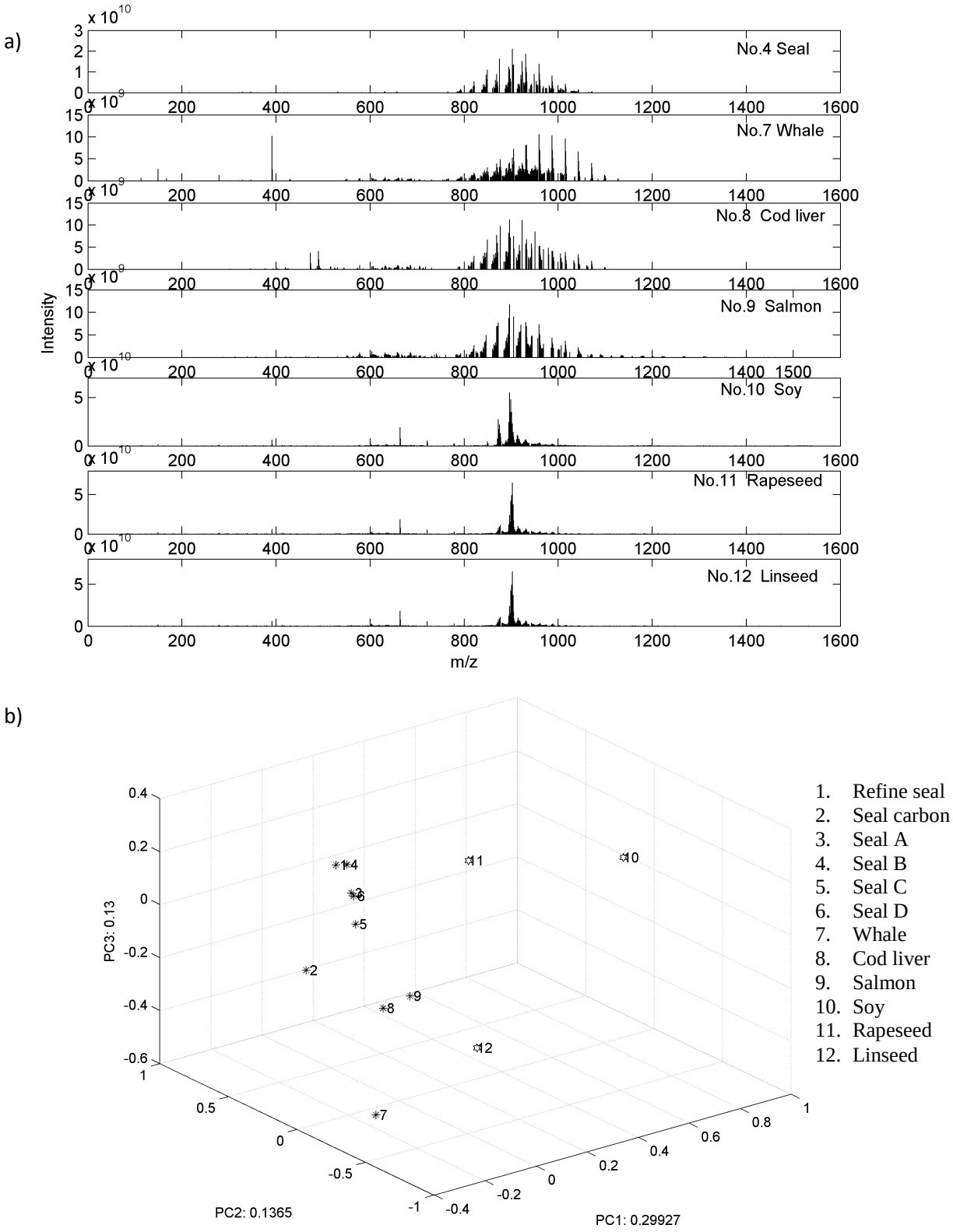


Figure 3.6: a) Typical single mass spectral profiles of seven oils derived from LC-ESI-MS experiments; b) 3D score plot of PCA by using the above single mass spectral profiles

The 3D score plot (Fig. 3.6 b) shows a clear classification pattern where specific groups and sub-groups are distinguished. The marine oils were generally located in one domain which allows differentiating from the other three plant oils. In particular, the six seal oils (No. 1-6) were nearly clustered together, while the other marine oils had a certain distance to this sub-cluster, in which whale oil (No. 7) was the most distant object. The three plant oils (No. 10-12) were scattered in another domain, however, a close correlation was not observed among them, as they were located far away from each other. Although the PCA score plot based on single mass spectral profiles enables basic differentiation between marine oils and plant oils, the discrimination among seal oils from different species and qualities were not observed. Therefore, tandem mass spectral profiles from LC-ESI-MS^2 experiments were examined further.

PCA analysis of tandem mass spectral profiles from LC-ESI-MS^2 experiments

The processed tandem mass spectral profiles of seven oils are shown in Fig. 3.7 a. As previously reported [76, 144, 146], the ammoniated ESI-MS^2 spectra are mainly characterized with the abundant DAG fragments in the form of $[M + NH_4 - RCOONH_4]^+$, resulting from the loss of fatty acids from the ammoniated TAG precursor ions, $[M + NH_4]^+$. This phenomenon was well represented in the tandem mass spectral profiles presented in Fig. 3.7 a, as most of the ions were accumulated in the *m/z* range from 500 to 750 corresponding to the molecular weight of various DAG ions. Similar to the single mass spectral profiles in Fig. 3.6 a, the tandem mass spectral profiles (Fig. 3.7 a) of marine oils show a wider region of DAG ions than the plant oils due to the higher complexity of TAG species present in marine oils confirming the observations in Table 1.2 regarding the total theoretical number of TAG species. The ions observed between 800 and 1000 *m/z* stand for the protonated TAG ions, which are especially intense in the tandem mass spectral profiles of plant oils such as soy oil. These profiles were subjected to PCA and the resulting 3D score plot explains 78.9 % of variation (Fig. 3.7 b). A clear classification pattern was observed where the marine and plant oils fall into different domains, leading to their differentiation. The marine oils are basically divided into two clusters, one formed by seal oils and another by the combination of cod liver, salmon and whale oils. It is worth noting that the seal oils (No. 1-6) were nearly arrayed in one straight line parallel to the PC3 axis, indicating they could be distinguished along the PC3 axis. This might be ascribed to the more detailed information provided by tandem mass spectral profiles, as they show the presence of both DAG ions and

protonated TAG ions. Compared to the single mass spectral profiles which are only characterized by abundant ammoniated TAG ions, tandem mass spectral profiles can represent the characteristics of TAG patterns more satisfactorily.

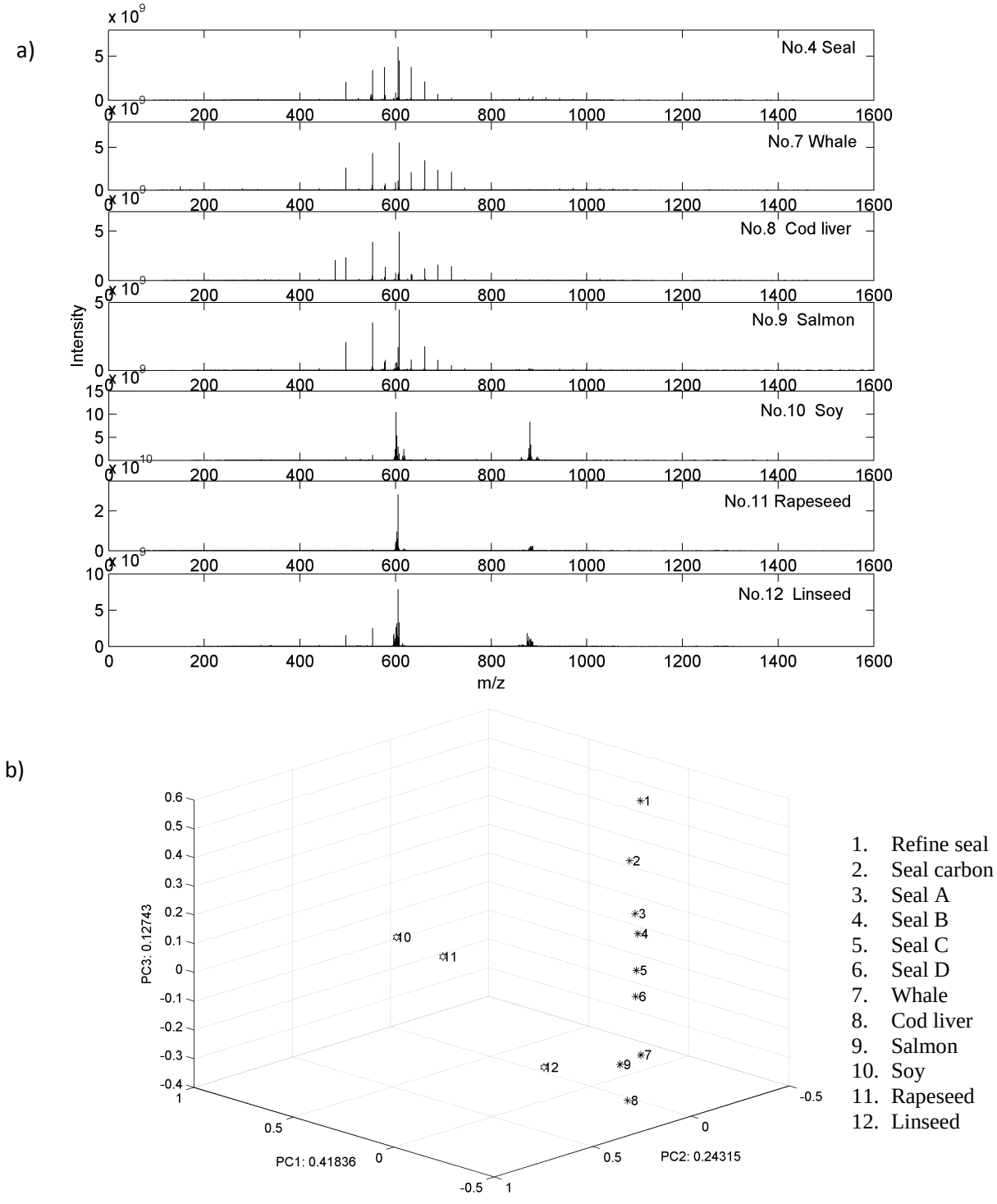


Figure 3.7: a) Typical tandem mass spectral profiles from seven oils derived from LC-ESI-MS^2 experiments; b) 3D score plot of PCA by using the above tandem mass spectral profiles

3.4 Conclusions

In this chapter, the potential of TAG profiles developed by using LC-ESI-MS and LC-ESI-MS^2 techniques in conjunction with chemometric methods was evaluated by means of PCA for discrimination purposes. The raw data obtained were processed by CODA and were subsequently used to construct four kinds of TAG profiles, namely, 1). TIC profiles from LC-ESI-MS experiments; 2). TIC profiles from LC-ESI-MS^2 experiments; 3). single mass spectral profiles from LC-ESI-MS experiments; 4). tandem mass spectral profiles from LC-ESI-MS experiments. The four constructed profiles were individually studied by PCA. The results show that TIC profiles derived from both LC-ESI-MS and LC-ESI-MS^2 experiments are inappropriate for marine oils discrimination, since the presence of many unresolved TAG peaks in the TIC profiles that in turn leads to the inaccuracy of classification. Although single mass spectral profiles yielded a basic discrimination, it was unable to distinguish between seal oils of different species and qualities due to the absence of DAG ions. The use of tandem mass spectral profiles was the best strategy for discrimination of marine oils, as differentiation was achieved not only between marine oils and plant oils but also among the seal oils of different species and qualities.

4 Characterization study: Triacylglycerols

4.1 Background

The positioning of fatty acids on the backbone of TAG molecules is of vital importance since it could affect many lipid properties such as physical properties, nutritional properties, oxidative stability, lipid absorption, metabolism and atherogenesis [29, 167-169]. For these reasons, national and international consumer protection organisations have encouraged and supported the development of reliable analytical methods with the capacity to characterize the positional distribution of fatty acids on the backbone of TAG in order to gain a better knowledge of their various properties and structural composition and thereby utilize their nutritional values further [74, 170]. Besides, characterization of TAG can be used to confirm the authenticity and adulteration further [35, 171].

In this chapter, cod liver oil was selected to study the characterization of TAG. Cod liver oil has been traditionally consumed as a dietary supplement in Norway. Recent dietary surveys have shown that cod liver oil supplements were used by around 35 % of the population in Norway and more than half of the eldest age group of the population surveyed [172]. Nowadays, cod liver oil has attracted extensive interests due to the rising scientific evidences and increasing consumer awareness of its nutritional advantages. It is indicated that cod liver oil is beneficial in cardiovascular diseases (e.g. coronary heart disease) [1, 2, 173] and various inflammatory pathologies (e.g. rheumatoid arthritis, bowel disease, chronic bronchitis, laryngitis, angina, ophthalmia) [4, 5, 174, 175], which have been attributed to the abundant content of EPA and DHA present in the form of TAG.

Cod liver oil is mainly composed of TAG (> 98 %) esterified with various fatty acids. Analysis of TAG in cod liver oil is quite challenging due to the presence of a large number of positional and structural TAG isomers with very similar chemical and physical properties. Indirect methods such as Grignard degradation method have been used for the analysis of cod liver oil [176], but it is not always applicable since it is often accompanied by some isomerization of glycerides. Besides, the requirements of several operational steps and long sample preparation make it quite

laborious and time-consuming. Direct methods such as high resolution ^{13}C-NMR and ^{1}H-NMR have also been implemented for the positional distribution of ω-3 fatty acids on TAG in cod liver oil [78, 177, 178]. In addition, the ability of NMR techniques for authentication of cod liver oil adulteration has been recently demonstrated [78, 86, 87]. Silver-ion HPLC-MS detection was used to study the range and variations in molecular species of TAG in cod liver oil [179], but only several TAG were identified in the profiles by comparing the retention times of TAG with several TAG standards.

Currently, ^{13}C-NMR and ^{1}H-NMR are the most widely employed techniques for TAG analysis in cod liver oil. It is surprising that the current literature on cod liver oil analysis has not explored yet the potentiality of LC-ESI-MS^2 for the analysis of TAG. LC-ESI-MS^2 has become increasingly popular for characterizing TAG in vegetable oils due to the high degree of information derived from its implementation. For instance, molecular weight, carbon number, degree of unsaturation and positional distribution of acyl groups on the glycerol backbone have been unambiguously determined in studies involving a wide variety of plant oils [52, 105, 106, 114, 135]. However, the advantages of this technique are often lost due to the tedious and time-consuming manual interpretation of mass spectra. It can be foreseen that LC-ESI-MS^2 in conjunction with an automated interpretation algorithm might offer a powerful means of analysis of TAG in cod liver oil and thereby provide valuable information on the quality control and adulteration of cod liver oil products further.

The objective of the present study was to develop a LC-ESI-MS^2 strategy assisted by an automated interpretation algorithm that enables identifying the relative arrangement of the acyl groups on the glycerol backbone of cod liver oil. To this aim, selected pure TAG standards and cod liver oil dissolved in different solvent mixtures were analysed. The computational algorithm based on the structural features and mass spectral behaviour of TAG molecule was developed, which allows the automatic interpretation and rapid prediction of the positional distribution of the various acyl groups on TAG molecules. The validity of the LC-ESI-MS^2 approach proposed in this investigation was assessed by comparing the results against the well-established lipase method. To our knowledge, this is the first study on the structural characterization of TAG in cod liver oil by means of LC-ESI-MS^2 assisted by the developed computational algorithm.

4.2 Experimental

4.2.1 Materials and reagents

Cod liver oil was from Peter Möller (Lysaker, Norway). All solvents were HPLC grade. Lipase from *Rhizopus arrhizus* was obtained from Sigma-Aldrich (Schnelldorf, Germany). FAME standards were purchased from Nu-Chek Prep (Elysian, MN). Nonadecanoic acid methyl ester ($C_{19:0}$) internal standard and formic acid were from Fluka (Buchs, Switzerland). 1-Arachidin-2-Olein-3-Palmitin-glycerol (AOP), 1-Arachidin-2-Palmitin-3-Olein-glycerol (APO) with an impurity of 1-Behenin-2-Palmitin-3-Olein-glycerol (BPO), 1-Arachidin-2-Linolein-3-Olein-glycerol (ALO) with an impurity of 1-Behenin-2-Linolein-3-Olein-glycerol (BLO) are from Larodan Fine Chemicals (Malmö, Sweden), and mixtures of these TAG standards were prepared in a chloroform:methanol (2:1, v/v).

4.2.2 Sample protocols

4.2.2.1 Sample preparation for LC-ESI-MS^2 analysis

Threes aliquots of cod liver oil (2 ml) designated as CL1, CL2 and CL3 were dissolved in 2 ml of chloroform:methanol (2:1, v/v), 2 ml of hexane and vortex-mixed for 30 s. The hexane phases were collected and dried under a gentle stream of nitrogen at room temperature. The dried residues CL1, CL2 and CL3 were dissolved into 0.5 ml of chloroform:methanol (2:1, v/v), acetonitrile:acetone (2:1, v/v) and isopropanol:acetonitrile (1:2, v/v) respectively. The final products were then individually submitted to LC-ESI-MS^2 analysis. The above described procedure was also applied to TAG standards dissolved in chloroform:methanol (2:1, v/v).

4.2.2.2 Lipase method

The protocol was slightly modified from the procedure described elsewhere [83]. Briefly, 1 ml of Tris-HCl buffer (40 mM, pH 7.2) containing 50 mM of sodium borate was added to a nitrogen-dried oil sample (1 ml) and the mixture sonicated for 10 min. 60 µl of lipase (150 units) were added to the sonicated mixture and incubated at 22 °C for up to 60 min with continuous shaking. The reaction was stopped by adding 0.8 ml of acetic acid (0.1 M) and the total lipids extracted by adding 3 ml of chloroform/methanol (2:1, v/v). The lipid solution was divided into two equal

portions (I and II), dried under nitrogen and methylated for 30 and 2 min at room temperature and in a microwave oven by using 1 ml methanolic solutions of NaOH (0.1 N) and HCl (0.2 N) for portion I and II respectively. The FAME in each methylation reactor were extracted into hexane after the addition of 0.2 ml of water to the reaction mixture. The hexane extracts of the NaOH reaction were washed once with water to remove any trace of NaOH before drying under nitrogen. The dried FAME extracts were redissolved in hexane and analyzed by GC. The FAME were estimated quantitatively by using $C_{19:0}$ internal standard. The lipase method was also applied to the TAG standards dissolved in chloroform:methanol (2:1, v/v). The calculation, determination of positional distribution and the enhancement of data were based on a protocol described in the literature [83].

4.2.3 Instrumentation

4.2.3.1 Liquid Chromatography Ion-Trap Mass Spectrometry

The LC-ESI-MS^2 used in this study was an Agilent 1100 series LC/MSD trap, SL model with an electrospray interface, a quaternary pump, degasser, autosampler, thermostatted column compartment, variable-wavelength UV detector and 10 µl injection volume. The reversed phase Ultrasphere® 5 µm Spherical 80 Å pore C-18 analytical column (250 mm × 4.6 mm i.d., Beckman Coulter, Kolbotn, Norway) was kept in the column compartment at 30 °C and the solvent system in gradient mode consisted of 10 mM isopropanol:ammonium acetate 90:10 v/v (A), acetone (B) and acetonitrile (C) at a flow rate of 0.8 ml/min and UV detection at 254 nm. After testing different delivered LC solvent programs, the following gradient was selected: an initial 5 min condition 90 % A and 10 % C that was ramped in 5 min to 65 % A and 5 % C and returned to the initial condition in 15 min and subsequently ramped in 5 min to 65 % A and 5 % C and returned to the initial condition in 30 min where it was held for 30 min.

By using this gradient program, reproducible retention times and peak areas from sample to sample were monitored. Nitrogen was used as nebulizing (50 psi) and drying gas (8 L/min) at 350 °C. The ESI source was operated in positive ion mode and the ion optics responsible for getting the ions in the ion-trap such as capillary exit, skimmer, lens and octapoles voltages were controlled by using the Smart View option with a resolution of 13000 *m*/z/sec (FWHM/*m*/*z* =

0.6-0.7). Complete system control, data acquisition and processing were done using the ChemStation for LC/MSD version 4.2 from Agilent.

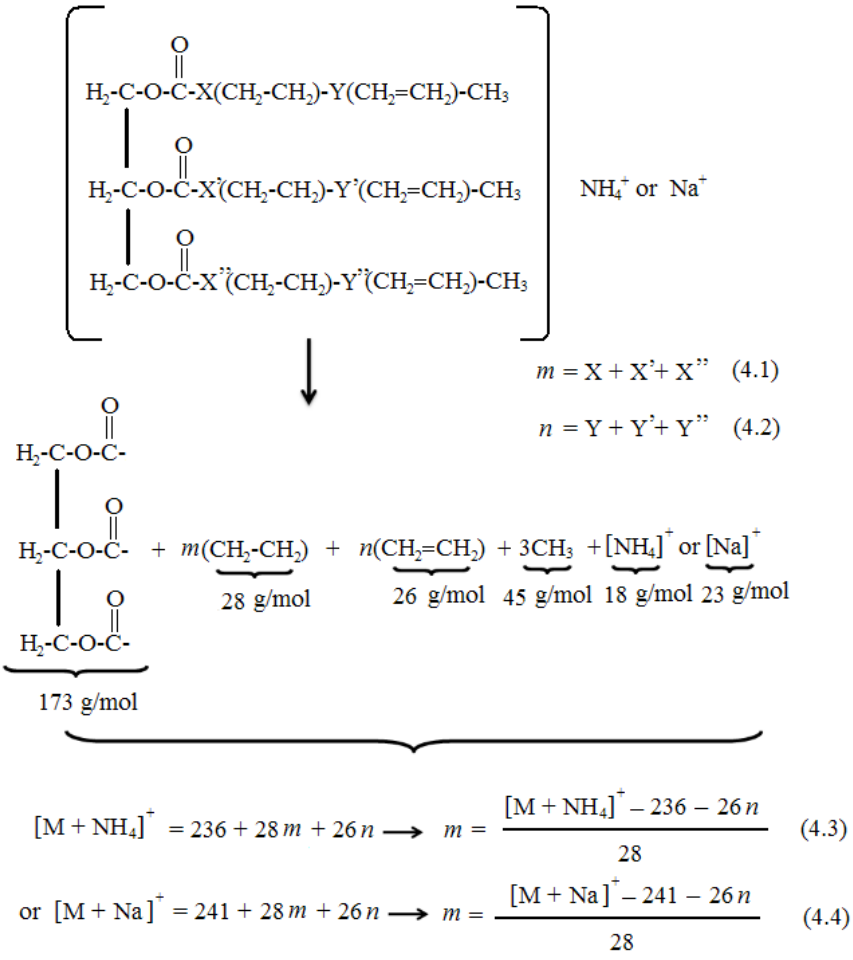
4.2.3.2 Gas chromatography

The GC analyses of the FAME prepared by the lipase method were performed on a Perkin-Elmer AutoSystem XL gas chromatograph (Perkin-Elmer, Norwalk, Connecticut) equipped with a liquid autosampler and a flame ionisation detector. The FAME samples were analysed on a CP-Sil 88 capillary column (50 m × 0.32 mm I.D. 0.2 μm film thickness, Varian, Courtaboeuf, France). Data collection was performed by the Perkin-Elmer TotalChrom Data System Software version 6.3. The temperature program was as follows: the oven temperature was held at 60 °C for 1 min, ramped to 160 °C at 25 °C /min, held at 160 °C for 28 min, ramped to 190 °C at 25 °C /min, held at 190 °C for 17 min, ramped to 220 °C at 25 °C /min and finally held at 220 °C for 10 min. Direct on-column injection was used. The injector port temperature was ramped instantaneously from 50 to 250 °C and the detector temperature was 250 °C. The carrier gas was ultra-pure helium at a pressure of 82 KPa. The analysis time was 60 min. This time interval was sufficient to detect FAME with chains from 10 to 24 carbons in length. The FAME peaks were identified by comparison of their retention times with the retention times of highly purified FAME standards.

4.2.4 Computation

4.2.4.1 General algebraic expression for TAG identification

The basic features of a TAG molecule (Fig. 4.1) were used to unambiguously identify various fatty acid combinations on the TAG backbone. The total number of single ethylene (–CH_2–CH_2–) and double ethenyl (–CH=CH–) bonds in a TAG molecule (labelled as *m* and *n* respectively) were calculated according to Eq. (4.1) or (4.2) given in Fig. 4.1.



Example: Calculation of *m* and *n* for ammoniated TAG (LnLnLn)

$[M + NH_4]^+$ *m/z* 890

n-value	*m*-calculated
0	23.26
1	22.43
2	21.50
3	20.57
4	19.64
5	18.71
6	17.79
7	16.86
8	15.93
9	15.00
10	15.07
11	13.14
12	12.21
13	11.29
14	10.36
15	9.43
16	8.50
17	7.57
18	6.64

Figure 4.1: Basic features of a TAG molecule used to derive a general algebraic expression for TAG identification based on the total number of ethylene ($-CH_2-CH_2-$) and ethenyl ($-CH_2=CH_2-$) bonds

By introducing the experimental *m/z* value of the precursor adduct ion and substituting automatically integral values of *n* from 0 to 18 (the total possible range number of double ethenyl bonds), it is possible to estimate the total number of single ethylene bonds (*m*) by using Eq. (4.3) or (4.4) described in Fig. 4.1. It is important to highlight that Eq. (4.3) or (4.4) will yield a positive TAG identification if and only if *n* (introduced as an integral number) is able to generate an integral *m* value. For example, when a TAG ammoniated adduct (*m/z* 890) containing three linolenic acids (18:3n) is analysed, the only possible *m* and *n* integral values derived from Eq. (4.3) are 15 and 9 respectively (Fig. 4.1).

4.2.4.2 Computational algorithm for TAG interpretation

The computational algorithm for the automatic interpretation of TAG molecules from mass spectra was developed by MATLAB 7.9 R2009b (The Math Works Inc., Natick, Massachusetts, USA) and the corresponding computation was performed on a Microsoft Windows XP® 2003 operating system (Microsoft Corporation, Redmond, WA, USA). For the single mass spectrum interpretation, the mass spectrum data was exported to ASCII spectrum file and MassList file; while for all the mass spectra interpretation in one run, the whole chromatogram data was exported to ASCII file and netCDF file. All the above files can be exported by DataAnalysis for LC/MSD Trap Version 3.3, and were then used as the input files for the algorithm, which could automatically give the interpretation results of TAG without manually introducing data into the algorithm.

The computational theory was based on the behavior of ESI-MS^2 mass spectra of TAG as demonstrated in previous studies [89, 144, 146]. Briefly, the precursor adduct ions from the ESI-MS^2 mass spectrum of TAG, produces very abundant DAG fragment ions due to the loss of fatty acyl moieties from the glycerol backbone. In view of the above information, the following rules were applied in the computation of TAG from the mass spectra.

1. All the observed adduct ions are of the ammoniated $[M + NH_4]^+$ or the sodiated $[M + Na]^+$ form.
2. The major product ions generated from $[M + NH_4]^+$ or $[M + Na]^+$ adducts are DAG fragments in the form of $[M + NH_4 - RCOONH_4]^+$ or $[M + Na - RCOOH]^+$ respectively, which correspond to the loss of particular fatty acids from the TAG backbone.
3. Only the product ions with *m/z* values exhibiting intensities higher than 10,000 icps (ions count per second) are screened and subjected to computation.
4. The positional distribution of the fatty acids on the TAG molecule is based on the relative intensities of its DAG fragments. The fatty acid which corresponds to the least abundant DAG fragment (lowest intensity) will be assigned in the *sn*-2 position on the TAG backbone. All the *m/z* values of possible DAG fragments observed from the mass spectrum are designated as $Frag_1$, $Frag_2$, …, $Frag_i$, and the molecular weight (MW) of corresponding fatty acids are designated as FA_1, FA_2, …, FA_i.

5. The FA_i is calculated by subtracting $Frag_i$ from its observed precursor adduct (either $[M + NH_4]^+$ or $[M + Na]^+$) as follows:

For $[M + NH_4]^+$ adducts:

$$FA_i = [M + NH_4]^+ - [M + NH_4 - RCOONH_4]^+ - [NH_4]^+ + [H]^+$$

$$FA_i = [M + NH_4]^+ - Frag_i - 17 \quad (4.5)$$

For $[M + Na]^+$ adducts:

$$FA_i = [M + Na]^+ - [M + Na - RCOOH]^+$$

$$FA_i = [M + Na]^+ - Frag_i \quad (4.6)$$

The potential fatty acids identified by Eq. (4.5) or (4.6) are compared against their nominal MW with a tolerance of ± 0.5 *m/z*.

6. All the possible fatty acid candidates are combined on the TAG backbone and their *m* and *n* values are obtained by Eq. (4.1) and (4.2) respectively (Fig. 4.1). A positive TAG identification is achieved when the previously calculated *m* and *n* values from a particular TAG candidate are equal to those estimated by Eq. (4.3) or (4.4).

7. The equivalent carbon number (ECN) of each identified TAG is calculated by the following equation:

$$ECN = CN - 2n \quad (4.7)$$

Where CN is the total carbon number of a TAG molecule and *n* is calculated by Eq. (4.2).

In summary, the algorithm offers the automatic interpretation of TAG in two ways, namely, the interpretation of single mass spectrum or all mass spectra from the full chromatogram in one run. The user only needs to load the exported files into the algorithm respectively which in turn will determine all the possible TAG molecules fulfilling the criteria defined above.

4.3 Results and discussion

4.3.1 LC-ESI-MS^2 analysis of TAG standards

The algorithm was tested and verified by using TAG standards. The interpretation function as well as the behaviour of TAG mass spectrum will be explained by the following examples.

A TAG molecule with the same fatty acid on its backbone, such as LnLnLn, exhibits a very simple mass spectrum (Fig. 4.2 a) with only a single DAG fragment ion ($[LnLn]^+$ at *m/z* 595.4) resulting from the dissociation of linolenic acid (Ln) from the LnLnLn . A different pattern arises from a TAG molecule containing three different acyl groups such as AOP. The AOP ammoniated precursor $[M + NH_4]^+$ at *m/z* 907 (Fig. 4.2 b) gives rise to three DAG fragments $[PO]^+$, $[AP]^+$ and $[AO]^+$ at *m/z* 577.5, 607.6 and 633.6 respectively. The least abundant DAG fragment ion, at *m/z* 607.6, corresponds to the loss of oleic acid (18:1n) from the middle position (*sn*-2), indicating that the cleavage from this particular position is energetically less favoured than the outer positions (*sn*-1 and *sn*-3).

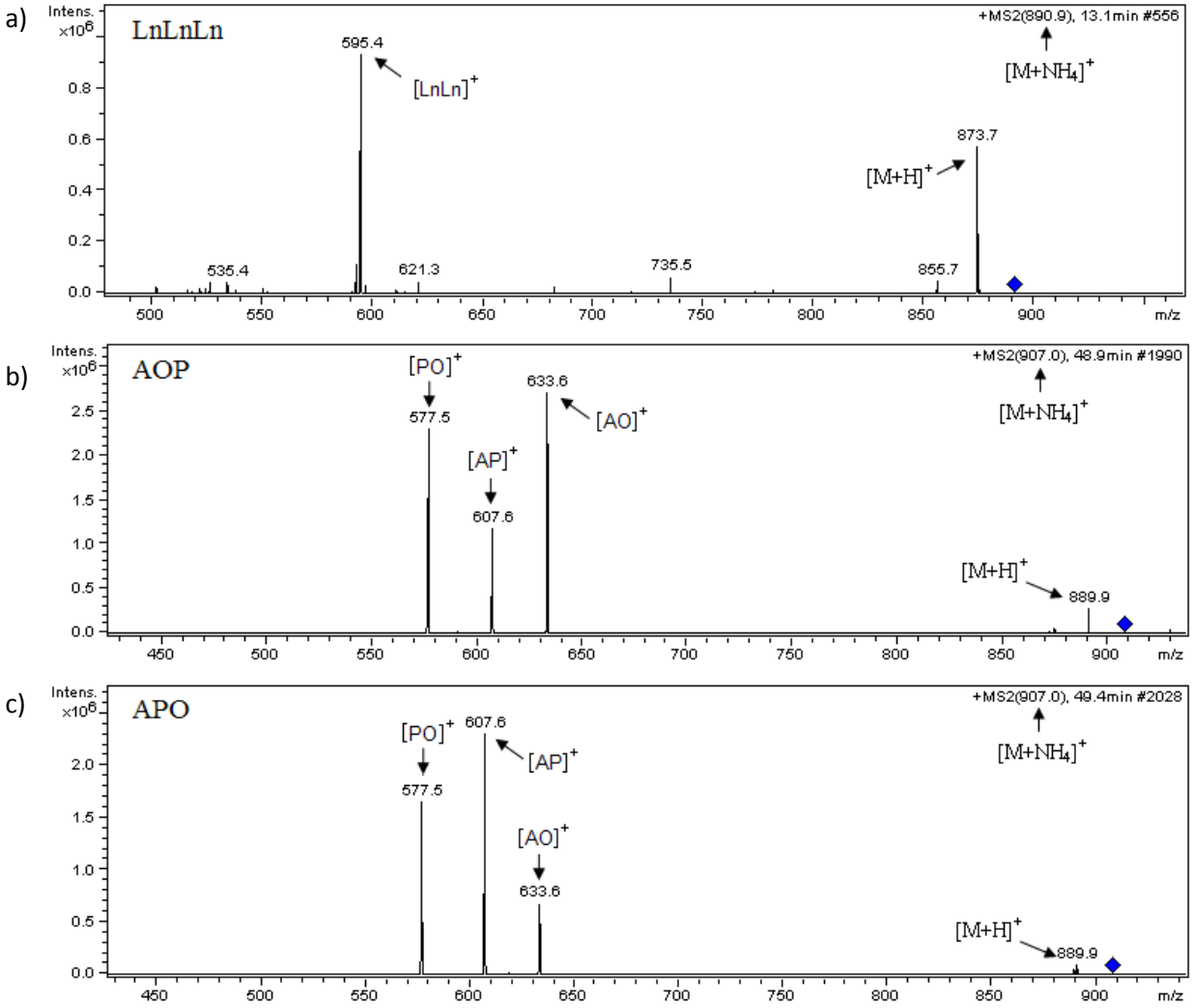


Figure 4.2: ESI-MS^2 spectra of the ammoniated TAG standards (a) LnLnLn (b) AOP and (c) APO

Similarly, the mass spectrum of APO (Fig. 4.2 c) displays the same three DAG fragment ions observed in the mass spectrum of its stereoisomer AOP, however the relative intensities of the generated DAG fragments are different in both spectra. In the case of APO (Fig. 4.2 c), the DAG fragment $[AO]^+$ at *m/z* 633.6 displays the lowest intensity, indicating the loss of palmitic acid (16:0) from the *sn*-2 position. The observed preferential cleavage of the fatty acids from the outer positions and the relative low intensity at the middle position of the DAG fragments which enables assigning a particular fatty acid to the *sn*-2 position have been previously reported [89, 144, 146]. After confirming the preferential cleavage phenomenon in our investigation by using TAG standards, the computation rules and interpretation capability of the proposed algorithm were proposed. The automatic positional arrangement of fatty acids on TAG molecules derived from ESI-MS^2 spectra was also verified through the use of TAG standards.

4.3.2 LC-ESI-MS^2 analysis of TAG in cod liver oil

The algorithm was applied to the identification of TAG species in three cod liver oil samples labelled as CL1, CL2 and CL3 dissolved in three different solvent systems chloroform:methanol (2:1, v/v), acetonitrile:acetone (2:1, v/v) and isopropanol:acetonitrile (1:2, v/v) respectively. The identified TAG species in every sample (Table 4.1) are listed in increasing order of ECN and carbon number of the individual fatty acyl groups, along with their *sn*-2 and *sn*-1/3 positions (no distinction is made between the outer positions). The total numbers of identified TAG species were 319, 555 and 434 for CL1, CL2 and CL3, respectively. The total number of common TAG species in the three solvent systems was 199 and the major TAG fraction components were 16:0, 16:1n, 18:1n, 20:1n, 22:1n, EPA and DHA.

The results clearly revealed that the solvent mixture influences the characterization of TAG in cod liver oil to some extent, which may be due to the different solubility of TAG species in the various solvent systems used in the sample preparation.

Table 4.1: TAG species identified by LC-ESI-MS^2 in cod liver oil from sample CL1, CL2 and CL3 dissolved in chloroform: methanol (2:1, v/v), acetonitrile: acetone (2:1, v/v) and isopropanol: acetonitrile (1:2, v/v) respectively. Note that *sn*-1 and *sn*-3 are given separately, however no distinction is made between them.

ECN	*sn*-1	*sn*-2	*sn*-3	CL1	CL2	CL3	ECN	*sn*-1	*sn*-2	*sn*-3	CL1	CL2	CL3
30	EPA	18:4n	DHA		*		34	EPA	14:0	DHA		*	
30	EPA	DHA	EPA		*	*	34	DHA	16:1n	DHA	*	*	*
30	EPA	DHA	DHA			*	36	14:0	18:3n	DHA	*	*	
30	DHA	18:4n	DHA		*	*	36	14:0	18:4n	DPA		*	
34	14:0	18:4n	EPA	*			36	14:0	20:4n	EPA	*	*	
34	14:0	18:4n	DHA	*	*	*	36	14:0	20:4n	DHA	*	*	*
34	14:0	EPA	EPA			*	36	14:0	DPA	18:4n		*	
34	14:0	DHA	18:4n		*		36	14:0	DPA	EPA	*	*	*
34	16:0	16:4n	DHA			*	36	14:0	DHA	20:4n			*
34	16:1n	16:3n	DHA		*		36	16:0	18:4n	EPA	*	*	
34	16:1n	18:4n	EPA	*	*	*	36	16:0	18:4n	DHA	*	*	*
34	16:1n	18:4n	DHA	*	*	*	36	16:0	EPA	18:4n			*
34	16:1n	EPA	18:4n		*		36	16:0	EPA	EPA		*	
34	16:1n	EPA	EPA			*	36	16:0	EPA	DHA	*		
34	16:1n	EPA	DHA	*		*	36	16:0	DHA	16:3n	*	*	*
34	16:1n	DHA	16:3n		*	*	36	16:0	DHA	18:4n	*	*	
34	16:1n	DHA	18:4n			*	36	16:0	DHA	EPA	*	*	
34	16:1n	DHA	EPA	*	*		36	16:1n	18:3n	EPA	*	*	
34	16:2n	16:2n	DHA			*	36	16:1n	18:3n	DHA	*	*	
34	16:4n	16:0	DHA		*	*	36	16:1n	18:4n	20:4n	*	*	
34	16:4n	18:1n	EPA			*	36	16:1n	20:4n	EPA	*	*	*
34	16:4n	18:1n	DHA	*	*		36	16:1n	20:4n	DHA	*	*	
34	16:4n	20:2n	EPA			*	36	16:1n	DPA	16:3n	*	*	
34	16:4n	DHA	18:1n	*	*	*	36	16:1n	DPA	18:4n	*	*	
34	18:1n	16:4n	DHA		*	*	36	16:1n	DPA	EPA	*		
34	18:1n	16:4n	EPA	*	*	*	36	16:1n	DHA	18:3n	*	*	
34	18:2n	16:3n	EPA			*	36	16:2n	20:4n	20:4n			*
34	18:2n	16:3n	DHA			*	36	16:3n	18:1n	EPA		*	
34	18:2n	18:4n	DHA		*		36	16:3n	EPA	18:1n	*		
34	18:2n	DHA	18:4n		*		36	16:3n	DHA	18:1n	*	*	
34	18:2n	DHA	EPA	*	*	*	36	16:4n	20:1n	DHA			*
34	18:3n	18:3n	18:4n		*		36	16:4n	20:4n	18:1n		*	
34	18:3n	18:3n	DHA		*		36	18:1n	16:3n	DHA		*	*
34	18:3n	18:3n	EPA			*	36	18:1n	16:3n	EPA		*	*
34	18:3n	18:4n	DPA			*	36	18:1n	18:4n	18:4n	*	*	
34	18:4n	14:0	DHA		*	*	36	18:1n	18:4n	EPA	*	*	*
34	18:4n	16:1n	EPA	*	*	*	36	18:1n	18:4n	DHA	*	*	*
34	18:4n	16:1n	DHA		*	*	36	18:1n	EPA	18:4n	*	*	
34	18:4n	18:2n	18:4n		*	*	36	18:1n	EPA	EPA			*
34	18:4n	18:2n	EPA	*	*	*	36	18:1n	EPA	DHA	*		
34	18:4n	18:2n	DHA	*			36	18:1n	DHA	18:4n	*	*	
34	20:4n	16:3n	20:4n			*	36	18:1n	DHA	EPA	*	*	
34	20:4n	18:3n	EPA		*		36	18:2n	20:4n	18:4n	*	*	*
34	20:4n	18:4n	20:4n			*	36	18:3n	14:0	DHA	*		
34	20:4n	20:4n	EPA		*	*	36	18:3n	16:1n	DHA	*	*	*
34	EPA	14:0	EPA	*	*	*	36	18:3n	16:2n	DPA		*	
34	EPA	14:0	DHA	*	*	*	36	18:3n	18:2n	EPA	*	*	
34	EPA	16:1n	EPA	*	*	*	36	18:3n	18:3n	18:3n	*	*	
34	EPA	16:1n	DHA	*	*	*	36	18:3n	18:3n	20:4n		*	
34	EPA	18:2n	EPA	*	*	*	36	18:3n	20:4n	18:3n	*	*	*

Table 4.1 Continued

34	EPA	18:2n	DHA	*	*	*	36	18:3n	20:4n	20:4n	*	
36	18:3n	DPA	18:3n	*			38	18:1n	18:3n	18:4n		*
36	18:4n	16:0	DHA	*	*	*	38	18:1n	18:3n	DHA	*	*	*
36	18:4n	16:0	EPA	*	*		38	18:1n	20:4n	EPA	*	*	
36	18:4n	16:1n	DPA		*		38	18:1n	20:4n	DHA	*		
36	18:4n	18:1n	18:4n	*	*	*	38	18:1n	EPA	DPA	*		
36	18:4n	18:1n	DHA	*	*	*	38	18:1n	DPA	18:4n	*		
36	18:4n	18:1n	EPA	*	*	*	38	18:1n	DHA	18:3n		*	
36	18:4n	18:2n	20:4n		*		38	18:2n	14:0	DHA			*
36	18:4n	20:2n	18:4n	*			38	18:2n	18:2n	18:4n		*	*
36	20:4n	14:0	DHA	*		*	38	18:2n	18:2n	EPA	*		
36	20:4n	16:1n	EPA		*	*	38	18:2n	18:2n	DHA		*	*
36	20:4n	18:3n	20:4n	*			38	18:3n	16:0	EPA			*
36	20:4n	20:4n	20:4n		*		38	18:3n	18:1n	DHA	*		
36	EPA	14:0	DPA		*		38	18:4n	18:0	DHA	*		
36	EPA	16:0	DHA	*	*	*	38	18:4n	20:1n	EPA	*		
36	EPA	16:0	EPA	*	*	*	38	20:1n	16:3n	DHA		*	
36	EPA	18:1n	EPA	*	*	*	38	20:4n	16:0	DHA		*	*
36	EPA	18:1n	DHA	*	*	*	38	20:4n	16:1n	DPA	*		
36	EPA	20:2n	EPA		*		38	20:4n	18:1n	EPA		*	*
36	DHA	16:0	DHA	*	*	*	38	20:4n	18:2n	20:4n		*	*
36	DHA	18:1n	DHA	*	*	*	38	EPA	16:0	DPA		*	
38	14:0	16:1n	DHA	*		*	38	EPA	18:0	EPA	*	*	*
38	14:0	18:2n	18:4n			*	38	EPA	18:0	DHA	*		
38	14:0	18:2n	EPA	*	*		38	EPA	18:1n	DPA			*
38	14:0	18:2n	DHA		*		38	DPA	16:0	DHA			*
38	14:0	18:3n	18:3n		*	*	40	14:0	18:1n	18:4n	*		*
38	14:0	18:4n	18:2n			*	40	14:0	18:1n	EPA	*	*	*
38	14:0	EPA	16:1n	*	*	*	40	14:0	18:1n	DHA	*	*	*
38	14:0	DHA	16:1n		*	*	40	14:0	20:2n	EPA	*		
38	16:0	16:2n	18:4n	*			40	14:0	20:2n	DHA			*
38	16:0	16:3n	18:3n		*		40	14:0	20:4n	16:1n	*	*	*
38	16:0	18:3n	DHA	*			40	14:0	20:4n	18:2n	*	*	
38	16:0	18:4n	16:2n	*			40	14:0	EPA	18:1n	*	*	
38	16:0	20:4n	EPA	*			40	14:0	DPA	14:0		*	*
38	16:0	20:4n	DHA	*	*	*	40	14:0	DPA	16:1n	*	*	*
38	16:0	EPA	16:2n	*			40	14:0	DPA	18:2n		*	
38	16:0	DPA	EPA	*	*	*	40	14:0	DHA	16:0		*	*
38	16:0	DPA	DHA			*	40	14:0	DHA	18:1n	*	*	*
38	16:0	DHA	20:4n		*		40	16:0	14:0	EPA		*	
38	16:1n	14:0	EPA	*	*		40	16:0	16:1n	18:4n		*	
38	16:1n	14:0	DHA	*	*	*	40	16:0	16:1n	EPA	*	*	*
38	16:1n	16:1n	EPA		*		40	16:0	16:1n	DHA	*	*	*
38	16:1n	18:1n	16:4n	*			40	16:0	16:4n	18:1n	*		
38	16:1n	18:2n	18:4n	*			40	16:0	18:2n	18:4n	*		
38	16:1n	18:2n	EPA		*	*	40	16:0	18:2n	EPA	*	*	*
38	16:1n	18:3n	18:3n	*	*		40	16:0	18:2n	DHA	*	*	
38	16:1n	18:3n	20:4n			*	40	16:0	18:3n	18:3n		*	
38	16:1n	18:4n	16:1n	*	*	*	40	16:0	18:4n	16:1n		*	*
38	16:1n	18:4n	18:2n	*			40	16:0	18:4n	18:2n		*	
38	16:1n	EPA	16:1n	*	*	*	40	16:0	20:4n	20:4n		*	
38	16:1n	DHA	16:1n		*	*	40	16:0	EPA	16:1n	*		*
38	16:2n	18:1n	DHA	*			40	16:0	EPA	18:2n			*
38	16:3n	18:3n	18:1n		*		40	16:0	DHA	16:1n	*	*	*
38	16:4n	18:1n	18:2n		*		40	16:0	DHA	18:2n	*	*	*

Table 4.1 Continued

38	18:0	18:4n	DHA	*	*	*	40	16:1n	16:0	18:1n		*	
38	18:0	EPA	DHA			*	40	16:1n	16:0	18:4n	*		
40	16:1n	16:0	EPA	*	*	*	42	14:0	20:1n	EPA	*	*	*
40	16:1n	16:0	DHA	*	*	*	42	14:0	20:1n	DHA	*	*	*
40	16:1n	16:3n	18:1n	*	*	*	42	14:0	20:4n	18:1n	*	*	*
40	16:1n	18:1n	EPA		*		42	14:0	EPA	18:0		*	
40	16:1n	18:1n	DHA	*	*	*	42	14:0	DPA	16:0	*	*	*
40	16:1n	18:3n	18:2n	*		*	42	14:0	DPA	18:1n	*	*	*
40	16:1n	18:4n	18:1n	*	*	*	42	15:0	15:0	DPA		*	
40	16:1n	20:2n	18:4n			*	42	16:0	14:0	DPA	*	*	
40	16:1n	20:2n	EPA	*		*	42	16:0	16:1n	20:4n		*	*
40	16:1n	20:4n	16:1n	*	*	*	42	16:0	18:1n	18:4n	*	*	*
40	16:1n	20:4n	18:2n		*		42	16:0	18:1n	EPA	*	*	*
40	16:1n	EPA	18:1n	*	*	*	42	16:0	18:1n	DHA	*		*
40	16:1n	DPA	16:1n	*	*	*	42	16:0	18:2n	20:4n		*	*
40	16:1n	DPA	18:2n	*	*	*	42	16:0	18:2n	DPA			*
40	16:1n	DHA	18:1n	*	*	*	42	16:0	18:4n	18:1n	*	*	*
40	16:2n	18:0	18:4n		*		42	16:0	20:1n	16:4n		*	*
40	16:2n	20:1n	DHA		*		42	16:0	20:1n	18:4n	*		*
40	16:3n	20:0	EPA		*		42	16:0	20:2n	EPA		*	
40	18:0	16:2n	DHA	*			42	16:0	20:4n	16:1n	*	*	*
40	18:0	20:4n	18:4n	*			42	16:0	EPA	16:0		*	
40	18:0	DPA	EPA	*			42	16:0	EPA	18:1n	*	*	*
40	18:1n	14:0	18:4n	*	*		42	16:0	DPA	16:1n	*	*	*
40	18:1n	14:0	EPA	*	*	*	42	16:0	DPA	18:2n	*	*	
40	18:1n	14:0	DHA	*	*	*	42	16:0	DHA	16:0		*	*
40	18:1n	16:1n	EPA	*	*	*	42	16:0	DHA	18:1n	*	*	*
40	18:1n	16:1n	DHA	*	*	*	42	16:1n	16:0	DPA		*	
40	18:1n	16:2n	DPA		*		42	16:1n	16:3n	20:1n			*
40	18:1n	16:3n	18:2n			*	42	16:1n	18:0	18:4n	*	*	*
40	18:1n	16:4n	18:1n	*	*	*	42	16:1n	18:0	EPA			*
40	18:1n	18:2n	18:4n		*		42	16:1n	18:0	DHA		*	*
40	18:1n	18:2n	EPA		*		42	16:1n	18:2n	18:2n		*	*
40	18:1n	18:3n	18:3n	*	*	*	42	16:1n	18:4n	20:1n		*	
40	18:1n	18:4n	18:2n		*	*	42	16:1n	20:1n	18:4n	*	*	*
40	18:1n	20:4n	18:3n			*	42	16:1n	20:1n	EPA		*	*
40	18:1n	20:4n	20:4n	*			42	16:1n	20:1n	DHA	*	*	*
40	18:1n	EPA	18:2n	*	*	*	42	16:1n	20:2n	20:4n		*	
40	18:1n	DHA	18:2n	*	*		42	16:1n	20:4n	18:1n	*	*	*
40	18:2n	16:0	18:2n			*	42	16:1n	DPA	18:1n	*	*	*
40	18:2n	16:0	EPA	*	*	*	42	16:2n	16:2n	20:1n		*	
40	18:2n	16:0	DHA	*	*	*	42	16:2n	20:4n	20:1n		*	
40	18:2n	18:1n	18:4n		*		42	16:4n	18:0	18:1n			*
40	18:2n	18:1n	DHA	*			42	18:0	14:0	EPA		*	
40	18:2n	18:2n	18:3n	*	*		42	18:0	14:0	DHA		*	*
40	18:2n	18:2n	20:4n	*			42	18:0	16:1n	18:4n		*	*
40	18:2n	18:3n	18:2n		*	*	42	18:0	16:1n	DHA		*	
40	18:2n	20:4n	18:2n	*	*	*	42	18:0	16:4n	18:1n			*
40	18:2n	EPA	20:1n		*		42	18:0	18:2n	DHA	*		*
40	18:2n	DPA	18:2n		*		42	18:0	18:3n	18:3n		*	*
40	18:3n	18:0	18:4n			*	42	18:0	20:4n	18:3n		*	
40	18:4n	20:1n	20:4n	*			42	18:0	DHA	18:2n	*		
40	20:0	16:4n	20:4n		*		42	18:1n	14:0	20:4n		*	*
40	20:1n	16:1n	DHA	*	*		42	18:1n	14:0	DPA		*	*
40	20:2n	14:0	DHA	*			42	18:1n	16:0	18:4n	*	*	*

Table 4.1 Continued

42	14:0	18:0	EPA		*		42	18:1n	16:0	EPA	*	*	*
42	14:0	18:0	DHA		*		42	18:1n	16:0	DHA	*	*	
42	14:0	20:1n	18:4n	*	*	*	42	18:1n	16:1n	18:3n		*	*
42	18:1n	16:1n	20:4n	*			44	17:0	17:0	DHA		*	
42	18:1n	16:1n	DHA	*			44	18:0	16:0	DHA		*	
42	18:1n	16:3n	18:1n			*	44	18:0	16:1n	20:4n		*	
42	18:1n	16:4n	20:1n	*	*		44	18:0	16:1n	DPA		*	*
42	18:1n	18:4n	18:1n	*	*	*	44	18:0	16:4n	20:1n		*	
42	18:1n	20:4n	18:2n		*		44	18:0	18:1n	18:4n		*	
42	18:1n	EPA	18:1n	*	*	*	44	18:0	18:1n	EPA	*	*	*
42	18:1n	DHA	18:1n	*	*	*	44	18:0	18:1n	DHA	*	*	*
42	18:2n	16:3n	20:1n			*	44	18:0	18:4n	18:1n		*	*
42	18:2n	18:2n	18:2n		*	*	44	18:0	EPA	18:1n		*	
42	18:2n	18:4n	20:1n			*	44	18:0	DHA	18:1n	*	*	*
42	18:2n	20:1n	EPA			*	44	18:1n	16:0	20:4n	*	*	*
42	18:3n	18:0	20:4n			*	44	18:1n	16:0	DPA		*	*
42	18:3n	20:1n	18:3n		*	*	44	18:1n	16:3n	20:1n	*	*	*
42	18:4n	14:0	20:1n		*	*	44	18:1n	16:4n	22:1n		*	*
42	18:4n	16:1n	20:1n	*	*	*	44	18:1n	18:0	18:4n	*	*	
42	20:1n	14:0	EPA	*	*	*	44	18:1n	18:0	EPA		*	*
42	20:1n	14:0	DHA	*	*		44	18:1n	18:0	DHA	*	*	*
42	20:1n	16:1n	EPA			*	44	18:1n	18:3n	18:1n		*	*
42	20:1n	16:1n	DHA		*	*	44	18:1n	18:4n	20:1n	*	*	*
44	14:0	20:0	DHA		*		44	18:1n	20:1n	18:4n	*	*	*
44	14:0	20:1n	20:4n		*	*	44	18:1n	20:1n	DHA	*	*	*
44	14:0	22:1n	18:4n	*	*	*	44	18:1n	20:4n	18:1n	*	*	*
44	14:0	22:1n	EPA			*	44	18:1n	EPA	20:1n		*	*
44	14:0	22:1n	DHA	*	*	*	44	18:1n	22:1n	18:4n			*
44	14:0	DPA	18:0		*	*	44	18:1n	DPA	18:1n			*
44	14:0	DPA	20:1n		*	*	44	18:1n	DHA	20:1n	*	*	*
44	16:0	18:0	DHA		*		44	18:3n	18:3n	22:1n	*	*	*
44	16:0	18:1n	20:4n		*	*	44	18:3n	20:0	18:3n		*	
44	16:0	18:4n	20:1n	*	*	*	44	18:4n	16:1n	22:1n		*	*
44	16:0	20:1n	18:4n		*	*	44	18:4n	18:1n	20:1n	*		*
44	16:0	20:1n	EPA		*	*	44	20:0	14:0	DHA		*	
44	16:0	20:1n	DHA	*	*	*	44	20:1n	14:0	20:4n	*	*	
44	16:0	20:4n	18:1n	*	*	*	44	20:1n	14:0	DPA	*	*	*
44	16:0	EPA	16:0			*	44	20:1n	16:0	EPA	*	*	*
44	16:0	EPA	18:0		*		44	20:1n	16:0	DHA	*	*	*
44	16:0	EPA	20:1n		*	*	44	20:1n	16:1n	20:4n		*	*
44	16:0	DPA	16:0	*	*	*	44	20:1n	16:1n	DPA			*
44	16:0	DPA	18:1n	*	*	*	44	20:1n	16:4n	20:1n		*	*
44	16:0	DHA	18:0	*	*	*	44	20:1n	18:1n	DHA	*	*	*
44	16:0	DHA	20:1n	*	*	*	44	EPA	14:0	22:1n		*	*
44	16:1n	14:0	18:1n			*	44	22:1n	14:0	DHA	*	*	*
44	16:1n	16:0	16:1n			*	44	22:1n	16:1n	DHA	*	*	*
44	16:1n	18:0	20:4n			*	46	14:0	16:2n	22:1n		*	
44	16:1n	18:0	DPA		*	*	46	14:0	20:1n	16:1n	*	*	*
44	16:1n	18:1n	16:1n		*		46	14:0	20:1n	18:2n		*	
44	16:1n	18:4n	22:1n		*	*	46	14:0	20:2n	18:1n		*	
44	16:1n	20:1n	18:3n			*	46	14:0	22:0	DHA		*	
44	16:1n	20:1n	20:4n		*		46	14:0	22:1n	16:2n		*	
44	16:1n	20:4n	20:1n		*	*	46	14:0	22:1n	DPA		*	
44	16:1n	22:1n	16:3n			*	46	14:0	24:1n	EPA		*	
44	16:1n	22:1n	18:4n		*	*	46	14:0	24:1n	DHA	*		

Table 4.1 Continued

44	16:1n	22:1n	DHA		*	*	46	14:0	DPA	22:1n		*	
44	16:1n	DPA	18:0		*	*	46	16:0	16:1n	18:1n		*	
44	16:3n	20:1n	18:1n			*	46	16:0	16:1n	20:2n			*
44	16:3n	22:1n	18:2n			*	46	16:0	16:2n	20:1n		*	*
46	16:0	18:0	18:3n		*	*	46	20:1n	16:4n	22:1n		*	*
46	16:0	18:1n	18:2n		*	*	46	20:1n	18:0	EPA	*	*	*
46	16:0	18:2n	18:1n		*	*	46	20:1n	18:0	DHA	*	*	*
46	16:0	18:3n	18:0		*		46	20:1n	18:4n	20:1n	*	*	*
46	16:0	18:4n	22:1n			*	46	20:1n	20:1n	DHA	*	*	*
46	16:0	20:0	DHA		*		46	20:1n	EPA	20:1n		*	
46	16:0	20:1n	16:2n		*		46	20:1n	DHA	20:1n	*	*	*
46	16:0	20:1n	20:4n		*		46	20:4n	16:1n	22:1n	*		
46	16:0	20:1n	DPA		*	*	46	EPA	16:0	22:1n	*	*	*
46	16:0	20:2n	16:1n		*	*	46	22:0	14:0	DHA		*	
46	16:0	20:4n	20:1n		*	*	46	22:1n	14:0	DPA		*	*
46	16:0	EPA	22:1n	*	*	*	46	22:1n	16:0	DHA	*	*	*
46	16:0	22:1n	18:4n		*	*	46	22:1n	18:1n	DHA	*		
46	16:0	22:1n	EPA	*	*	*	48	14:0	22:1n	16:1n		*	*
46	16:0	22:1n	DHA	*	*	*	48	16:0	16:1n	20:1n	*	*	*
46	16:0	DPA	18:0		*	*	48	16:0	16:2n	22:1n		*	*
46	16:0	DPA	20:1n	*	*	*	48	16:0	20:1n	16:1n	*		
46	16:0	DHA	22:1n	*	*	*	48	16:0	20:1n	18:2n			*
46	16:1n	16:0	18:1n	*	*	*	48	16:0	20:2n	18:1n		*	*
46	16:1n	16:1n	20:1n		*	*	48	16:0	DPA	22:1n		*	*
46	16:1n	18:0	16:1n		*		48	16:0	24:1n	EPA		*	*
46	16:1n	18:0	18:2n		*	*	48	16:0	24:1n	DHA		*	*
46	16:1n	20:0	DPA		*		48	16:1n	14:0	22:1n	*	*	
46	16:1n	20:1n	16:1n		*	*	48	16:1n	20:0	18:2n		*	
46	16:1n	20:4n	22:1n		*	*	48	16:1n	20:1n	18:1n	*	*	*
46	16:1n	22:1n	20:4n		*		48	16:1n	20:2n	18:0		*	*
46	16:1n	24:1n	DHA		*		48	16:1n	22:1n	16:1n		*	*
46	16:2n	18:0	18:1n		*	*	48	18:0	16:2n	20:1n		*	*
46	18:0	16:1n	18:2n		*	*	48	18:0	18:1n	18:2n		*	*
46	18:0	16:2n	18:1n		*		48	18:0	18:2n	18:1n	*	*	
46	18:0	18:1n	DPA		*	*	48	18:0	18:3n	18:0		*	*
46	18:0	20:1n	18:4n		*		48	18:0	EPA	22:1n		*	*
46	18:0	20:1n	EPA		*	*	48	18:0	22:1n	DHA	*	*	*
46	18:0	20:1n	DHA	*	*	*	48	18:0	DPA	20:1n		*	
46	18:0	20:2n	20:4n		*		48	18:0	DHA	22:1n	*	*	*
46	18:0	20:4n	18:1n			*	48	18:1n	14:0	20:1n	*	*	*
46	18:0	EPA	20:1n		*		48	18:1n	16:0	18:1n	*	*	*
46	18:0	DPA	18:1n		*	*	48	18:1n	16:0	20:2n	*		
46	18:0	DHA	18:0	*	*	*	48	18:1n	16:1n	20:1n		*	
46	18:1n	14:0	18:1n	*	*	*	48	18:1n	18:0	18:2n		*	*
46	18:1n	16:0	18:2n		*	*	48	18:1n	18:1n	18:1n	*	*	*
46	18:1n	16:1n	18:1n	*	*	*	48	18:1n	20:0	DPA			*
46	18:1n	18:0	DPA		*	*	48	18:1n	20:4n	22:1n	*	*	*
46	18:1n	18:4n	22:1n	*	*		48	18:1n	DPA	22:1n			*
46	18:1n	20:0	EPA		*		48	18:1n	24:1n	18:4n	*	*	*
46	18:1n	20:4n	20:1n		*	*	48	18:1n	24:1n	DHA	*	*	
46	18:1n	22:1n	18:4n	*	*	*	48	18:2n	14:0	22:1n	*	*	*
46	18:1n	22:1n	DHA	*	*	*	48	18:2n	16:0	20:1n	*	*	*
46	18:1n	DHA	22:1n		*	*	48	18:4n	18:1n	24:1n			*
46	18:2n	14:0	20:1n		*		48	18:4n	22:1n	20:1n	*	*	*
46	18:3n	24:1n	18:3n	*	*	*	48	20:0	20:1n	DHA		*	

Table 4.1 Continued

46	18:4n	18:0	20:1n		*	*	48	20:1n	14:0	20:2n	*	*	
46	18:4n	20:1n	20:1n		*		48	20:1n	16:4n	24:1n		*	
46	20:0	16:0	DHA	*	*	*	48	20:1n	18:0	DPA		*	*
46	20:0	18:1n	EPA		*		48	20:1n	18:4n	22:1n		*	*
46	20:1n	16:0	DPA		*	*	48	20:1n	20:0	EPA			*
48	20:1n	20:0	DHA		*		50	18:4n	20:1n	24:1n		*	*
48	20:1n	20:4n	20:1n	*	*	*	50	18:4n	24:1n	20:1n		*	*
48	20:1n	EPA	22:1n			*	50	20:0	20:0	DHA		*	
48	20:1n	22:1n	DHA	*	*	*	50	20:1n	14:0	20:1n		*	*
48	20:1n	DPA	20:1n	*		*	50	20:1n	16:1n	20:1n	*	*	*
48	20:1n	DHA	22:1n	*	*	*	50	20:1n	20:4n	22:1n		*	*
48	20:4n	16:0	22:1n		*		50	20:1n	24:1n	DHA	*	*	*
48	20:4n	18:1n	22:1n		*	*	50	20:2n	14:0	22:1n	*		
48	22:1n	16:0	DPA		*		50	20:4n	18:1n	24:1n		*	*
48	22:1n	16:4n	22:1n		*	*	50	20:4n	20:1n	22:1n		*	*
48	22:1n	18:0	DHA	*	*	*	50	22:1n	18:0	DPA			*
48	22:1n	20:1n	DHA	*	*	*	50	22:1n	18:4n	22:1n	*	*	*
48	DPA	14:0	24:1n		*	*	50	22:1n	22:1n	DHA		*	
48	DPA	16:1n	24:1n	*			50	22:1n	DHA	22:1n	*	*	*
48	DHA	16:0	24:1n	*	*		50	DPA	16:0	24:1n		*	*
48	DHA	18:1n	24:1n	*	*	*	50	DHA	20:1n	24:1n	*	*	
50	14:0	20:2n	22:1n		*		52	14:0	20:0	20:1n		*	
50	14:0	22:1n	18:1n		*	*	52	14:0	22:1n	20:1n		*	
50	16:0	14:0	22:1n		*		52	14:0	24:1n	18:1n		*	*
50	16:0	16:1n	22:1n		*	*	52	16:0	14:0	24:1n		*	
50	16:0	18:0	18:1n		*		52	16:0	16:1n	24:1n		*	*
50	16:0	18:1n	18:0	*	*	*	52	16:0	18:1n	22:1n	*	*	*
50	16:0	18:1n	20:1n	*			52	16:0	20:0	18:1n		*	
50	16:0	18:2n	22:1n		*		52	16:0	20:1n	18:0		*	
50	16:0	20:1n	16:0	*	*	*	52	16:0	20:1n	20:1n	*	*	*
50	16:0	20:1n	18:1n	*	*	*	52	16:0	22:1n	16:0		*	
50	16:0	20:1n	20:2n		*		52	16:0	22:1n	18:1n	*	*	*
50	16:0	20:2n	20:1n	*			52	16:0	22:1n	20:1n		*	
50	16:0	22:1n	16:1n		*	*	52	16:1n	22:1n	20:1n			*
50	16:0	22:1n	18:2n		*	*	52	16:1n	24:1n	18:1n			*
50	16:0	DPA	24:1n		*		52	16:1n	24:00	16:1n		*	
50	16:0	24:1n	DPA		*	*	52	18:0	14:0	22:1n		*	
50	16:1n	14:0	20:1n		*		52	18:0	18:1n	18:0		*	
50	16:1n	18:0	20:1n		*	*	52	18:1n	14:0	20:1n			*
50	16:1n	18:1n	22:1n		*	*	52	18:1n	16:0	22:1n	*	*	*
50	16:1n	20:0	20:2n		*		52	18:1n	16:1n	24:1n		*	
50	16:1n	20:1n	20:1n		*	*	52	18:1n	18:0	20:1n		*	*
50	16:1n	22:1n	18:1n	*	*	*	52	18:1n	20:1n	20:1n	*	*	*
50	16:1n	24:1n	16:1n		*	*	52	18:1n	22:1n	18:1n	*	*	*
50	18:0	14:0	20:1n	*	*	*	52	20:1n	14:0	22:1n		*	*
50	18:0	16:1n	20:1n		*	*	52	20:1n	16:0	20:1n	*	*	*
50	18:0	16:2n	22:1n		*		52	20:1n	16:1n	22:1n		*	*
50	18:0	18:1n	20:2n		*		52	20:1n	18:1n	20:1n	*	*	
50	18:0	20:1n	18:2n		*	*	52	22:1n	DPA	22:1n	*		
50	18:0	DPA	22:1n		*		52	22:1n	DHA	24:1n		*	
50	18:1n	14:0	22:1n		*	*	52	22:1n	24:1n	DHA		*	
50	18:1n	16:0	20:1n	*	*	*	52	DPA	20:1n	24:1n	*		
50	18:1n	16:1n	22:1n	*	*	*	54	14:0	20:0	22:1n		*	
50	18:1n	18:0	18:1n		*	*	54	14:0	20:1n	22:0		*	
50	18:1n	18:1n	20:1n	*	*		54	14:0	24:1n	20:1n			*

Table 4.1 Continued

50	18:1n	20:0	18:2n		*		54	16:0	18:1n	22:0		*	
50	18:1n	20:1n	18:1n	*	*	*	54	16:0	18:1n	24:1n		*	*
50	18:2n	14:0	24:1n	*	*		54	16:0	20:1n	20:0		*	
50	18:2n	16:0	22:1n	*	*	*	54	16:0	22:1n	18:0		*	
50	18:2n	18:0	20:1n		*	*	54	16:0	22:1n	20:1n	*	*	*
50	18:2n	18:1n	20:0		*		54	16:0	22:1n	18:1n		*	
54	16:0	24:1n	18:1n	*	*	*	54	18:0	16:0	22:1n		*	
54	18:0	18:1n	22:1n		*		56	14:0	24:1n	22:1n		*	
54	18:0	20:0	18:1n		*		56	16:0	20:1n	24:1n		*	
54	18:0	20:1n	18:0		*		56	16:0	24:1n	20:1n	*	*	*
54	18:1n	18:0	22:1n		*	*	56	16:1n	24:1n	22:1n		*	
54	18:1n	18:1n	22:0	*			56	18:0	20:1n	22:1n			*
54	18:1n	20:1n	22:1n	*	*		56	18:1n	24:1n	20:1n	*	*	*
54	18:1n	22:1n	20:1n	*	*	*	56	20:1n	18:0	22:1n	*	*	*
54	18:1n	24:1n	18:1n		*	*	56	20:1n	20:1n	22:1n	*	*	*
54	20:1n	14:0	24:1n		*	*	56	20:1n	22:1n	20:1n	*	*	*
54	20:1n	16:0	22:1n	*	*	*	56	22:1n	16:0	22:1n	*	*	*
54	20:1n	16:1n	24:1n			*	56	22:1n	16:1n	24:1n		*	
54	20:1n	18:0	20:1n		*	*	56	22:1n	18:1n	22:1n	*	*	*
54	20:1n	18:1n	22:1n	*	*	*	56	22:1n	20:1n	18:0	*	*	
54	20:1n	20:1n	20:1n	*	*	*	58	18:1n	24:1n	22:1n		*	*
54	22:1n	14:0	22:1n		*	*	58	20:1n	24:1n	20:1n		*	*
54	22:1n	16:1n	22:1n		*	*	58	22:1n	20:1n	22:1n		*	*
54	22:1n	24:1n	DPA	*			58	22:1n	18:1n	24:1n		*	*

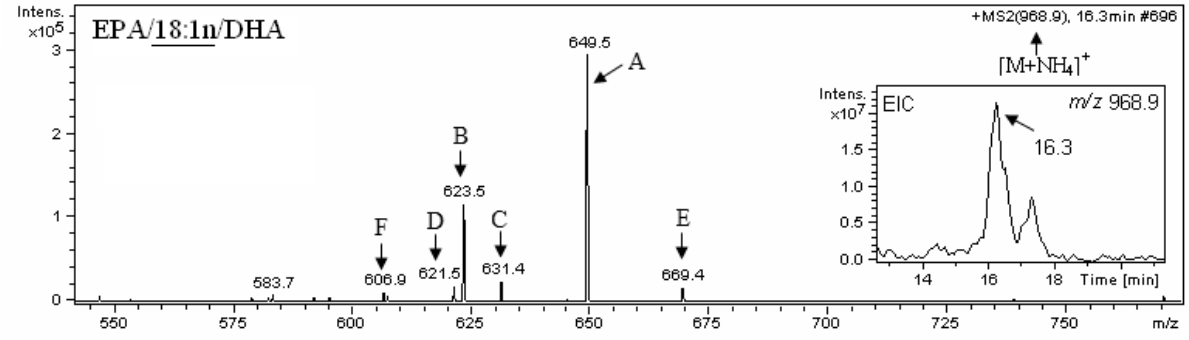
Several examples for the identification of TAG species in cod liver oil are given to illustrate the algorithm interpretation process.

4.3.2.1 Single TAG structure in cod liver oil

The extracted ion chromatogram (EIC) of a precursor ammoniated adduct at *m/z* 968.9 for CL1 and its corresponding ESI-MS^2 spectrum at 16.3 min is showed in Fig. 4.3 a. The mass spectrum was converted into an ASCII spectrum and MassList files and then used as input for the algorithm. Several fragments were visualized in the mass spectrum. The algorithm outcomes (Fig. 4.3 b) indicate the fragments at *m/z* 649.5, 623.4, 669.4 and 621.5 result from the loss of EPA, DHA, 18:1n and DPA from potential TAG ammoniated precursors respectively, while the MW of 320.49 and 344.99 (estimated from the fragments at *m/z* 631.4 and 606.9 respectively) do not match any saturated or unsaturated fatty acid containing between 14 and 35 carbon molecules. The algorithm identified the combination EPA, DHA and 18:1n as a TAG molecule. This combination fulfils all the requirements described in Section 4.2.4. In addition, the algorithm assigned the *sn*-2 position to 18:1n as a result of the low intensity of the corresponding fragment at *m/z* 669.4. Although a fragment corresponding with the loss of DPA is observed in

this spectrum, the presence of this particular fatty acid does not comply with the general requirements for a positive TAG identification described in Section 4.2.4.

a)



b)

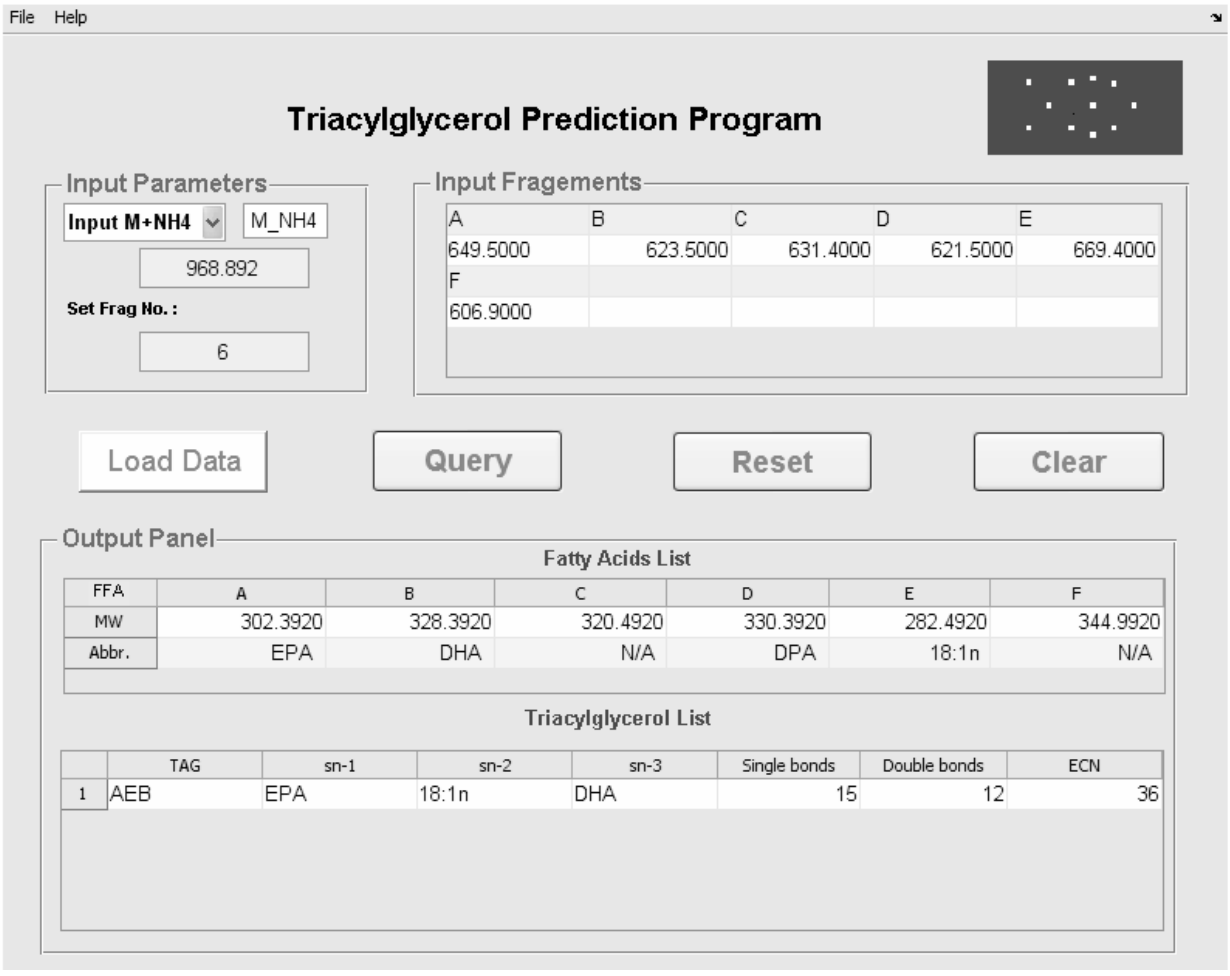


Figure 4.3: a) ESI-MS^2 spectrum of the ammoniated EPA/18:1n/DHA from sample CL1 at 16.3 min and corresponding embedded extracted ion chromatogram (EIC) at *m/z* 968.9. b) Algorithm outcomes after introducing the spectral data at 16.3 min.

4.3.2.2 TAG positional isomers in cod liver oil

The sodiated precursor ion at *m/z* 927.9 in sample CL2 exhibits two overlapping chromatographic peaks at 22.6 and 22.8 min (Fig. 4.4). Although the mass spectra of these peaks display similar fragmentation patterns at *m/z* 577.5, 599.5, 623.4, 645.4, 671.5 and 699.5, their relative intensities are different, indicating the presence of stereoisomers. The algorithm revealed that only the combination of 16:0, 18:1n and DHA constitutes a positive TAG molecule in both spectra (Fig. 4.4 a-b) and that the fatty acids 16:0 and DHA (the least intense fragments) are located in the *sn*-2 position of the identified TAG stereoisomers at 22.6 and 22.8 min respectively. It is important to mention that the sodiated adducts observed in Fig. 4.4 might be ascribed to some sodium impurities in the solvents which have been reported elsewhere [76, 133, 180].

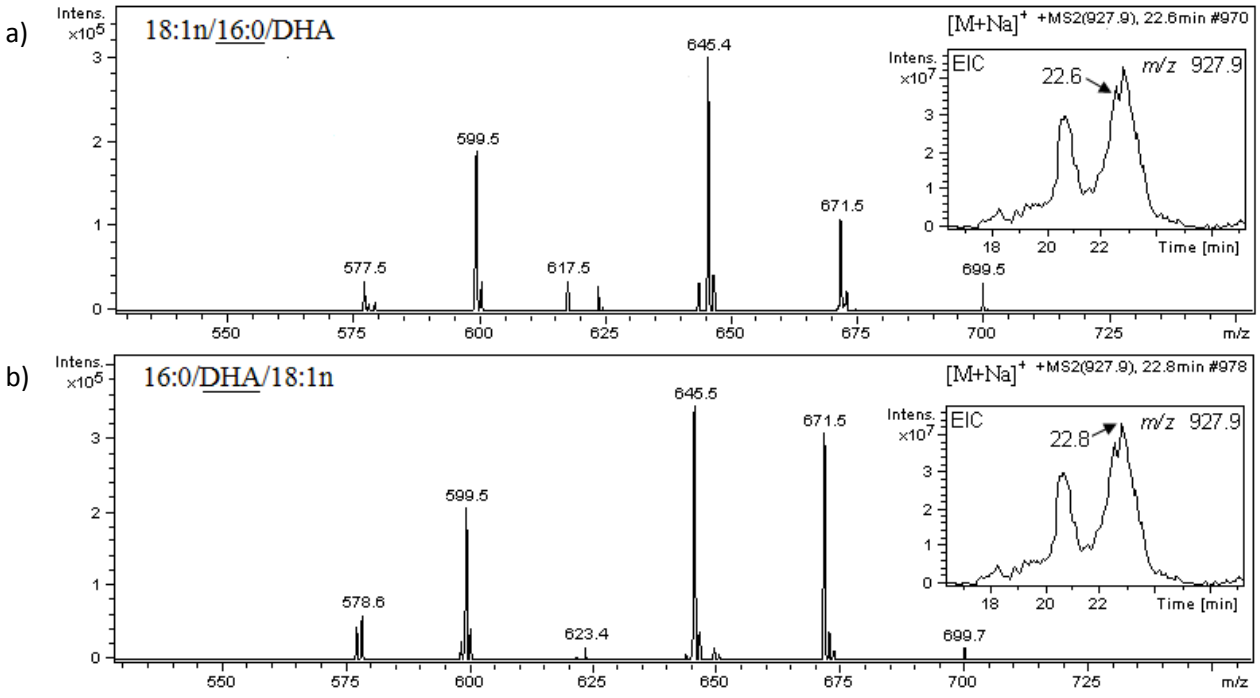


Figure 4.4: ESI-MS^2 spectra of the sodiated adducts from sample CL2. a) 18:1n/16:0/DHA at 22.6 min and b) 16:0/DHA/18:1n at 22.8 min and their corresponding embedded EIC at *m/z* 927.9.

4.3.2.3 TAG structural isomers in cod liver oil

Although only one peak was observed in the EIC at 32.8 min with a *m/z* 877.0 (Fig. 4.5 a), the algorithm shows firstly, that the four DAG fragment ions (*m/z* 577.5, 603.5, 605.6 and 549.5) derived from the precursor ion $[M + NH_4]^+$ at *m/z* 877 (Fig. 4.5 a) result from the loss of 18:1n, 16:0, 16:1n and 20:1n from TAG molecules and secondly that with these identified fatty acids only two TAG species fulfil the program criteria, namely 18:1n/16:0/18:1n and 16:0/20:1n/16:1n (*sn*-2 positions are underlined). Similarly, the ability of the algorithm to identify co-eluting sodiated TAG isomers from a single chromatographic peak was showed in Fig. 4.5 b where the two TAG molecules fulfilling the program criteria were 18:1n/DHA/20:1n and 16:1n/22:1n/DHA.

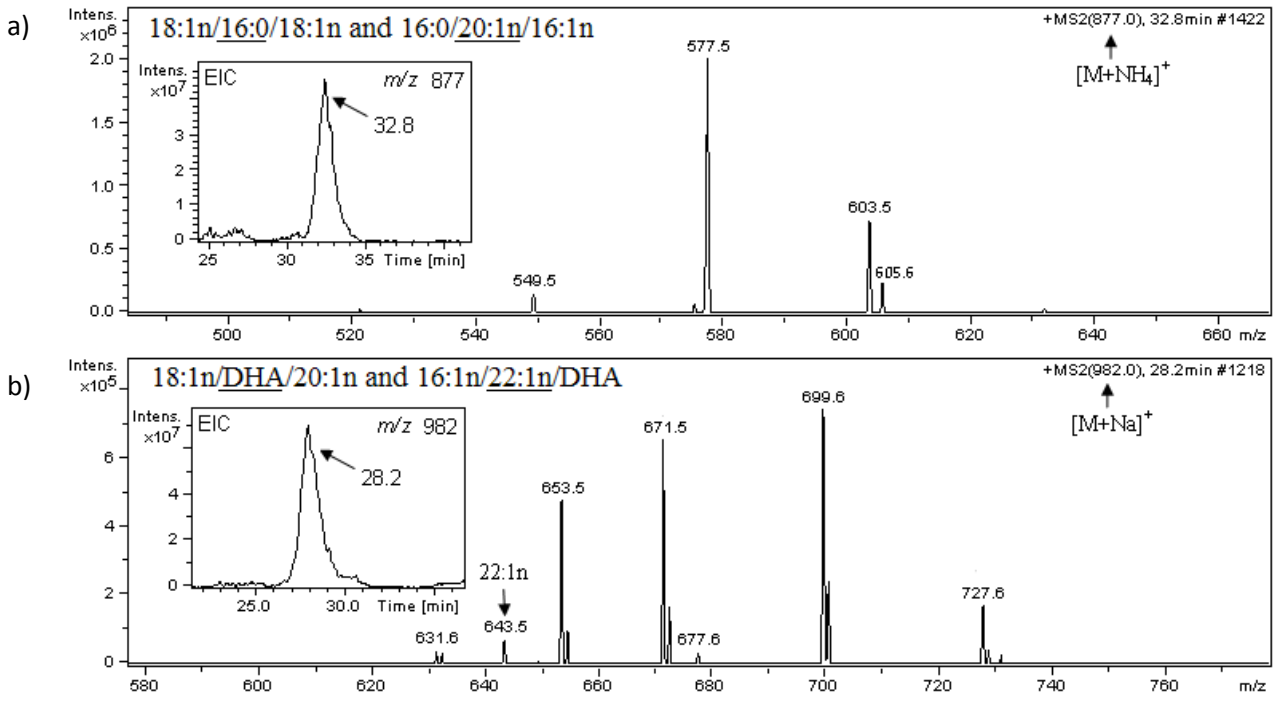


Figure 4.5: a) ESI-MS^2 spectrum of the ammoniated adducts from sample CL2 18:1n/16:0/18:1n and 16:0/20:1n/16:1n at 32.8 min and their corresponding embedded EIC at *m/z* 877.0; b) ESI-MS^2 spectrum of the sodiated adducts from sample CL2 18:1n/DHA/20:1n and 16:1n/22:1n/DHA at 28.2 min and their corresponding embedded EIC at *m/z* 982.0.

4.3.3 Comparison with the lipase method

The benchmark lipase method was used to select the best solvent system (CL1, Cl2 and CL3) for dissolving the cod liver oil samples. The systems CL1 and CL3 resembled the results obtained by the lipase method. However, a detailed inspection of the results revealed that they failed to detect 17:0 and 24:0 in the *sn*-2 position and 15:0 and 24:0 in the outer positions (*sn*-1/3). A total positional agreement was achieved when the results portrayed in Table 4.1 for the CL2 system were compared against the lipase method, indicating that acetonitrile:acetone (2:1, v/v) is the best solvent mixture for dissolving cod liver oil samples to be submitted to LC-ESI-MS^2 for TAG structural characterization.

4.4 Conclusions

A LC-ESI-MS^2 strategy in conjunction with an automated interpretation algorithm was successfully established in order to identify the relative arrangement of the acyl groups on the glycerol backbone of cod liver oil. The developed computational algorithm facilitated the rapid interpretation and prediction of the positional distribution of the various acyl groups on TAG molecules based on the information obtained from the ESI-MS^2 spectra. The mixture acetonitrile:acetone (2:1, v/v) was the best choice among the three assayed mixtures and it enables the characterization of TAG in cod liver oil in agreement with the lipase method. It is concluded that LC-ESI-MS^2 is a suitable and powerful strategy for the structural characterization of TAG in cod liver oil and a useful means to help the understanding of its properties and nutritional value as well as the detection of adulteration for these kinds of products.

5 Concluding remarks

There is a strong emphasis on the nutritional value of marine oils and a substantial interest in introducing marine oils as functional food, dietary supplements and pharmaceuticals. The need to determine the authenticity of marine oils and characterize the triacylglycerols (TAG) in marine oils is a matter of great importance for both authorities and industries which has been highlighted over 120 years.

Three main aspects concerned with discrimination and characterization of marine oils were explored for the first time in the present thesis. Firstly, the capability of gas chromatography (GC) for discriminating marine oils was established by using fatty acids methyl esters (FAME) profiles and principal component analysis (PCA). Secondly, the use of tandem mass spectral profiles in conjunction with chemometric tools for fingerprinting and discriminating marine oils was demonstrated. Thirdly, the potential of liquid chromatography electrospray tandem mass spectrometry (LC-ESI-MS^2) assisted by the developed computational algorithm for the characterization and interpretation of TAG molecules in cod liver oil was proved and the results generated from this approach were in agreement with the well established benchmark lipase method.

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