DOCTORAL THESES IN FOOD SCIENCES AT THE UNIVERSITY OF TURKU

Strategies for Analyzing the Regio- and Stereospecific Structures of Individual Triacylglycerols in Natural Fats and Oils

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“Challenges are what make life interesting;
overcoming them is what makes life meaningful.”

Joshua J. Marine

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ABSTRACT

Food lipids are mainly found in the form of triacylglycerols (TAG) containing three structurally identical or different fatty acids. The positional distribution of fatty acids is under genetic control. In TAG biosynthesis, fatty acids are specifically esterified in glycerol in enzyme-catalyzed reactions resulting in distinctive TAG species that are not randomly built. The chiral nature of other macromolecules in food, such as proteins and carbohydrates, is well established in science but the chirality of lipids and their functions has been a long-standing question. The aim of this work is to contribute to this research area of chirality by giving fresh insights into new methods and chromatographic retention behavior of TAGs, and to study the detailed molecular structure of individual TAGs in natural fats and oils.

Novel chromatographic methods (RP- and chiral-phase HPLC) combined with mass spectrometry (MS) were used in the practical work for separation and identification of regioisomers (the positional distribution of fatty acids between the positions *sn*-2 and *sn*-1/3) and enantiomers (the positional distribution of fatty acids in the positions *sn*-1, *sn*-2, *sn*-3) of nutritionally important TAGs. ESI-MS/MS and NIAPCI-MS/MS were applied to study the regiospecific composition of TAGs in human milk, and ammonia NICI-MS and MS/MS to confirm the enantiospecific structures of TAGs of sea buckthorn pulp oil as the model food component. The studies included development of a chiral chromatographic method based on sample recycling.

The suitability of different MS/MS methods in the analysis of TAG regioisomers was investigated. Overall, the results revealed new information about the methodology and the chiral elution behavior of TAGs. The chiral-phase [2 × cellulose tris-(3,5-dimethylphenylcarbamate)] recycling HPLC method in a polar-organic phase enabled resolution of 17 out of the 21 enantiopairs of TAGs studied. Presence of both saturated and unsaturated fatty acid favored the separation by the chiral columns. A TAG enantiomer with an unsaturated FA in the *sn*-1 position eluted ahead of its enantiomer despite the length of carbon chains or the number of DBs. Within the TAG regioisomer 16:1-16:1-16:0 in sea buckthorn pulp oil, the enantiomeric ratio of *sn*-16:1-16:1-16:0 to TAG *sn*-16:0-16:1-16:1 was 70.5:29.5. Overall, the results clearly showed the non-random positional distribution of natural FAs, and the abundance of unsaturated FAs in the position *sn*-2, which is common in plant oils. The other most abundant TAG species included mainly TAGs with three different FAs, which were not possible to analyze with the current methodology. The method developed during the PhD project was also successfully applied to an enantiomeric purity assessment of synthesized TAGs.

Despite the promising progress in the stereospecific analysis of individual TAGs the elution order of TAG enantiomers is challenging to predict due to the complicated intermolecular forces involved in chiral recognition mechanisms. All new information concerning chiral retention behavior is essential for further development in the field. Chiral phase liquid chromatography combined with mass spectrometric analysis proved to be a reliable method for analysis of lipid stereoisomers in food and other biological systems. These results offer valuable information and a reliable tool to study specific positional distribution of fatty acids in natural samples and can be applied to studies of both food composition and nutritional aspects.

SUOMENKIELINEN ABSTRAKTI

Elintarvikkeiden lipidit koostuvat pääosin triasyyliglyseroleista, joissa on kolme samanlaista tai erilaista rasvahappoa. Rasvahappojen paikkajakauma on geneettisesti säädelty. Rasvahapot ovat esteröityneet glyseroliin entsyymikatalysoidussa triasyyliglyserolien biosynteesissä muodostaen kombinaatioita, jotka eivät ole sattumanvaraisia. Muiden ravinnon makromolekyylien kuten proteiinien ja hiilihydraattien kiraalisuus tunnetaan tieteessä jo varsin hyvin, ja myös lipidien kolmiulotteisia rakenteita ja niiden vaikutuksia on pyritty selvittämään jo vuosikymmenten ajan. Tutkimustyön tavoitteena oli kehittää rasvojen kiraalisuuden tutkimusta, soveltaa uusia menetelmiä triasyyliglyserolien kromatografisen retentiokäyttäytymisen selvittämiseen sekä tutkia luonnon rasvojen ja öljyjen yksittäisten triasyyliglyserolien yksityiskohtaista molekyylirakennetta.

Nykyaikaisia kromatografisia menetelmiä (käänteisfaasi- ja kiraalifaasinestekromatografia) yhdistettyinä massaspektrometriaan (MS) käytettiin ravitsemuksellisesti tärkeiden triasyyliglyserolien regioisomeerien (rasvahappojen paikkajakauma asemien *sn*-2 and *sn*-1/3 välillä) ja enantiomeerien (rasvahappojen paikkajakauma asemien *sn*-1, *sn*-2 and *sn*-3 välillä) erottamiseen ja tunnistamiseen. Regiospesifisessä analyysissä sovellettiin eri massaspektrometrimenetelmiä (ESI-MS/MS ja NIAPCI-MS/MS) äidinmaidon triasyyliglyserolien tutkimiseen. Ammoniakki NICI-MS- and MS/MS -menetelmien avulla varmistettiin mallielintarvikkeena tutkitun tyrnimarjan hedelmälihan öljyn triasyyliglyserolirakenteet enantiospesifisessä analyysissä. Lisäksi kehitettiin näytteen kierrätykseen perustuva kiraalikromatografinen menetelmä.

Tutkimuksessa tarkasteltiin eri massaspektrometristen menetelmien soveltuvuutta triasyyliglyserolien regiospesifiseen analyysiin. Kokonaisuudessaan tuloksena saatiin uutta tietoa menetelmistä ja yhdisteiden eluutiokäyttäytymisestä kiraalisessa ympäristössä. Tutkitusta 21:stä triasyyliglyserolista 17:sta saatiin enantiomeerit erotettua toisistaan käyttäen näytteen kierrätykseen perustuvaa nestekromatografiaa, jossa oli kiraalinen stationaarifaasi [2 × selluloosa tris-(3,5-dimetyylifenyylikarbamaatti)] ja eluenttina metanoli. Asymmetristen triasyyliglyserolien tyydyttynyt ja tyydyttymätön rasvahappo edistivät erottumista. Enantiomeeri, jossa tyydyttymätön rasvahappo oli asemassa *sn*-1, eluoitui toista enantiomeeria nopeammin hiiliketjun pituudesta tai kaksoissidosten lukumäärästä riippumatta. Yhdisteen *sn*-16:1-16:1-16:0 suhde sen *sn*-16:0-16:1-16:1 enantiomeeriin oli tyrnimarjan hedelmälihassa 70,5:29,5. Kokonaisuudessaan tulokset osoittivat selvästi luonnon rasvahappojen ei-sattumanvaraisen paikkajakauman ja tyydyttymättömien rasvahappojen runsauden asemassa *sn*-2, mikä on tyypillistä

kasviöljyille. Muut merkittävimmät jakeet koostuivat pääsiassa kolme erilaista rasvahappoa sisältävistä triasyyliglyseroleista, joiden analysoiminen ei ollut mahdollista tässä tutkimuksessa käytetyllä menetelmällä. Väitöskirjatyössä kehitetyn menetelmän osoitettiin olevan tehokas myös synteesituotteiden enantiopuhtauden määrittämisessä.

Vaikka yksittäisten triasyyliglyserolien yksityiskohtaisen molekyylirakenteen analysointi on viime vuosikymmenen aikana huomattavasti kehittynyt, on haasteellista ennustaa enantiomeerien eluutiokäyttäytymistä johtuen monimutkaisista molekyylien välisistä kiraalisista vuorovaikutuksista. Yhdisteiden retentiokäyttäytymisen perusteellinen selvittäminen on välttämätöntä jatkokehityksen kannalta. Kiraalinen nestekromatografia yhdistettynä massaspektrometriaan tarjoaa luotettavan menetelmän ravinnon ja muiden biologisten matriisien lipidien isomeerien analysoimiseen. Tuloksena saatiin arvokasta tietoa ja luotettava menetelmä luonnon rasvojen ja öljyjen yksityiskohtaisen paikkajakauman tutkimiseen. Tulevaisuudessa menetelmää voidaan soveltaa niin elintarvike- kuin ravitsemustutkimuksissa.

LIST OF ABBREVIATIONS

ACN	Acyl carbon number
APCI	Atmospheric pressure chemical ionization
DAG	Diacylglycerol
DB	Double bond
ELSD	Evaporative light-scattering detector
ECN	Equivivalent carbon number
ESI	Electrospray ionization
FA	Fatty acid
FAME	Fatty acid methyl ester
FID	Flame ionization detector
GC	Gas chromatography
HPLC	High-performance liquid chromatography
HRMS	High-resolution mass spectrometry
IT	Ion trap
LCFA	Long-chain fatty acid
MAG	Monoacylglycerol
MCFA	Medium-chain fatty acid
MD	Multidimensional
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry (quadrupole equipment)
MS^2	Tandem mass spectrometry (ion trap or similar equipment)
MUFA	Monounsaturated fatty acid
NMR	Nuclear magnetic resonance
NP	Normal-phase
PUFA	Polyunsaturated fatty acid
QqQ	Triple quadrupole
RF	Response factor
R-HPLC	Recycling high-performance liquid chromatography
RP	Reversed-phase
SAFA	Saturated fatty acid
SCFA	Short-chain fatty acid
SFC	Supercritical fluid chromatography
sn	Stereospecific numbering
TAG	Triacylglycerol
TLC	Thin-layer chromatography
TOF	Time-of-flight
UHPLC	Ultra high-performance liquid chromatography
UV/Vis	Ultraviolet-visible
VLCFA	Very long-chain fatty acid

SYSTEMATIC NAMES AND ABBREVIATIONS

Triacylglycerols are named according to the following fatty acid abbreviations (IUPAC-IUB Commission on Biochemical Nomenclature 1977).

ACN:DB*n*-x	Systematic name[a]	Trivial name[b]	Abbr.
4:0	Butano-	Butyr-	Bu
6:0	Hexano-	Capro-	Co
8:0	Octano-	Capryl-	Cy
10:0	Decano-	Capr-	Ca
12:0	Dodecano-	Laur-	La
14:0	Tetradecano-	Myrist-	M
16:0	Hexadecano-	Palmit-	P
16:1	9-Hexadeceno-	Palmitole-	Po
18:0	Octadecano-	Stear-	S
18:1*n*-9	9-Octadeceno-	Ole-	O
18:1*n*-7	11-Octadeceno-	Vaccen-	V
18:1*n*-9*trans*	*trans*-9-Octadeceno-	Elaid-	E
18:2*n*-6	9,12-Octadecadieno-	Linole-	L
18:3*n*-3	9,12,15-Octadecatrieno-	(9,12,15)-Linolen-	αLn
18:3*n*-6	6,9,12-Octadecatrieno-	(6,9,12)-Linolen-	γLn
20:0	Icosano-	Arachid-	A
20:5*n*-3	5,8,11,14,17-Eicosapentaeno-	Eicosapentaeno-	EPA
22:0	Docosano-	Behen-	B
22:6*n*-3	4,7,10,13,16,19-Docosahexaeno-	Docosahexaeno-	DHA

[a]Ending "-ic" for acid; "-yl" for acyl radical

[b]Ending "-ic" for acid; "-oyl" for acyl radical

A/B/C	TAG containing three different fatty acids in unknown steroisomeric (*sn*) positions
A-B-C	mixture containing regioisomeric pair, not necessarily in equimolar ratios
rac-ABC	racemic mixture containing both regioisomers in equimolar ratios
sn-ABC	the exact position of each fatty acid is known

LIST OF ORIGINAL PUBLICATIONS

I. Linderborg, KM.; Kalpio, M.; Mäkelä, J.; Niinikoski, H.; Kallio, HP.; Lagström, H. Tandem mass spectrometric analysis of human milk triacylglycerols from normal weight and overweight mothers on different diets. *Food Chemistry*. **2014**, 146, 583–590.

II. Kalpio, M.; Nylund, M.; Linderborg, KM.; Yang, B.; Kristinsson, B.; Haraldsson, GG.; Kallio H. Enantioselective chromatography in analysis of triacylglycerols common in edible fats and oils. *Food Chemistry*. **2015**, 172, 718–724.

III. Kalpio, M.; Magnússon, H.; Gudmundsson, HG; Linderborg, KM.; Kallio H.; Haraldsson, GG.; Yang, B. Synthesis and enantiospecific analysis of enantiostructured triacylglycerols containing n-3 polyunsaturated fatty acids. *Chemistry and Physics of Lipids.* **2020**, 231, 104937.

IV. Kalpio, M.; Linderborg, KM.; Kallio H.; Fabritius, M.; Yang, B. Strategy for stereospecific characterization of natural triacylglycerols using chiral chromatography and mass spectrometry. *Submitted.*

1 INTRODUCTION

Dietary lipids i.e. edible fats and oils have provoked wide interest among consumers as well as in the scientific community. Lipids are a vital part of a healthy diet by providing energy, essential fatty acids (FA) and fat-soluble vitamins, and also have significant impact on mouthfeel, palatability and texture of food due to their melting and crystallization properties. On the other hand, high fat intake increases the risks of obesity, diabetes and atherosclerosis. Even more important is the type of fat consumed. Based on the current data the European Food Safety Authority (EFSA) has concluded that a clear relationship has been established between the consumption of saturated FAs and an increase in blood LDL-cholesterol level. Accordingly, authorized health claims state that replacing saturated fats with unsaturated fats in the diet has been shown to lower blood cholesterol level, which in turn reduces the risk of cardiovascular diseases as high cholesterol is a risk factor in the development of coronary heart disease. (EFSA Panel on Dietetic Products, Nutrition and Allergies (NDA) 2011; European Commission Regulation (EU), No 1226/ 2014). Because of public concern about obesity and cardiovascular diseases, the lipid industry is keen to develop fats and fat-based ingredients with improved nutritional properties using technologies such as interesterification, hydrogenation and fractionation ('Fats and fatty acids in human nutrition. Report of an expert consultation' 2010). Processing of food always causes changes in its chemical composition due to various chemical reactions, which can be desirable or undesirable. People are increasingly aware of and interested in their diet and its effects on health and well-being. During the past decades, industrialization, urbanization, economic development and market globalization have largely changed diets and lifestyles ('Fats and fatty acids in human nutrition. Report of an expert consultation' 2010). At the same time with the growing consumption of different fats and oils, adulteration of biologically important oils such as olive oil, has grown. All these aspects make the improvement and development of highly sensitive and complex methods of structural analysis of lipids even more important in scientific, industrial and clinical applications (Carrasco-Pancorbo et al. 2009; García-Cañas et al. 2012).

Triacylglycerols (TAG) are the dominating form (approx. 97%) of natural food lipids both of plant and animal origins. From the chemical structure point of view, TAG is a simple molecule consisting of a glycerol backbone esterified with three FAs. However, a wide variety of TAGs can be formed with combinations of FAs differing in chain lengths, degrees of unsaturation, double bond (DB) positions, conformations of DBs, and the stereospecific positioning in the TAG molecules. For example, an oil containing only three different FAs may have 27 (3^3) different TAG molecular species. The species-specific

positional distribution of FAs is determined during TAG biosynthesis, in which acyltransferases specifically esterify each of the hydroxyl positions of the glycerol backbone resulting in non-random FA combinations (Coleman and Lee 2004). In addition to the structure and net content of the FAs, the specific molecular structure of the TAG must be considered, especially, in terms of nutritional, biochemical and technological aspects.

TAGs can be challenging molecules to study. They have a large structural variation and they are not easily volatilized. In addition, TAGs are prone to thermal decomposition and acyl migration. They lack chromophores that hinder spectrophotometric detection. Moreover, unsaturated FAs are susceptible to oxidation. Nevertheless, interest in the detailed composition of lipids has simultaneously increased with the increasing number of lipidomic studies being applied to their overall characterization. The rapid development of analytical methods has made it possible to analyze highly complex mixtures, and there has been some important progress in the stereospecific analysis of individual TAGs.

Typically, the core of lipid analysis is based on transesterification of the TAGs into methyl esters of FAs followed by gas chromatographic analysis. As a result, a large amount of information on the FA compositions of fats and of their nutritional characteristics is obtained. However, the method does not provide any information regarding the composition of individual TAG molecules. The marked advantage of using comprehensive TAG analysis together with the FA profiling is that the genetically controlled, species-specific positional distribution of FAs in the glycerol backbone is preserved. Consequently, the information obtained about the stereochemistry of intact TAGs is higher.

Foods are very complex matrices and so are lipids. The pretreatment methods need to be selected carefully depending on the aim of the study. However, the proper sampling, extraction and sample pre-treatment, although an essential part of the analytical workflow of lipid analysis, and, especially, in the case of mass spectrometric analysis to reduce the matrix effects, will not be discussed in this thesis. This is due to the fact that these methods have been covered in more detail by several reviews (Myher and Kuksis 1995; Ruiz-Gutiérrez and Barron 1995; Gallo and Ferranti 2016).

Despite the challenges related to stereospecific analysis of TAGs, there has been significant progress in the 21st century. The overall aim of the literature part of this thesis is to review different methods used for regiospecific and especially stereospecific analysis of TAGs. In the review of the literature, the positional distribution of FAs in TAGs and its nutritional, biochemical and technological, impacts are presented with examples. In addition, the current commonly used methods of studying the regio- and stereospecific composition

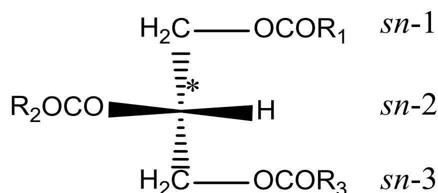
of individual TAGs are discussed without neglecting their roots in conventional enzymatic and chemical hydrolysis. The basic characteristics, advantages and limitations of each method are presented and their applicability is compared. Particular attention is allocated to recent works on stereospecific analysis of individual TAGs.

The original research presented in this thesis comprises one regioisomeric study of human milk TAGs, and three studies related to stereospecific analysis of synthesized and natural TAGs: one method development, one application of enantiomeric purity and one application with a natural sample matrix. The knowledge obtained about chiral retention behavior and the elution profile of 21 structured TAGs synthesized during this PhD project provide fundamental information on the chiral chromatography of TAGs for future research. The chiral liquid chromatographic method, which was developed and optimized, offered stereospecific information on TAG enantiomers from sea buckthorn pulp oil as an example of an analysis of a natural mixture. In the future, the chiral liquid chromatographic method can be applied to study the enantiomeric composition of individual TAGs, thus contributing to the field of lipidomics both in chemistry and biochemistry.

2 REVIEW OF THE LITERATURE

2.1 Triacylglycerols and their stereochemistry

Glycerol, as a trihydroxylic alcohol, can form triesters, i.e. TAGs with one, two or three different FAs. The Fischer projection of glycerol with the middle hydroxyl group positioned on the left side of the central carbon is presented in **Figure 1**. When there are different fatty acyl substituents in the two primary positions, the molecule is chiral. Thus, a diacid-TAG may be either chiral or achiral. For example, when palmitic acids (C16:0) occupy the positions *sn*-1 and *sn*-2 and oleic acid (C18:1*n*-9) the *sn*-3 position, the chiral acylglycerol is designated as 1,2-dipalmitoyl-3-oleoyl-*sn*-glycerol. The triacid-TAGs with three different acyl residues (such as C16:0, C18:1*n*-9 and C18:2*n*-6) are always chiral molecules (1-palmitoyl-2-oleoyl-3-linoleoyl-*sn*-glycerol).



sn-1

sn-2

sn-3

Figure 1. Fischer projection of a triacylglycerol.

Due to the number of different fatty acyl substituents the large variety of different TAG species exists depending on the source of oil. The fatty acyl substituents can 1) be saturated or not, 2) be short-chain C2–C6, SCFAs, medium-chain C8–C12, MCFAs, long-chain C14–C20, LCFAs or very long-chain C22 or more, VLCFAs), 3) differ in the degree of unsaturation, or 4) differ in the position and configuration of DBs. In addition, rare FAs with odd number of carbon atoms or branched skeletons or FAs with epoxy groups, OH groups or other substituents exist in special cases.

2.1.1 Nomenclature

There are different nomenclatures for TAGs depending whether the geometry or the chemical properties needs to be defined. The three FAs esterified to the glycerol backbone play an important role in the naming of TAGs. The positions of the FAs are defined by using stereospecific numbering (*sn*), as recommended by the IUPAC-IUB Commission (IUPAC-IUB Commission on Biochemical Nomenclature 1977).

In the s*ystematic nomenclature* of TAGs a complete identification of the FAs is provided i.e. the number of carbon atoms along with the DBs position and stereochemistry. Following this nomenclature, a TAG containing two C16:0 in the positions *sn*-1 and *sn*-2 and one C18:1*n*-9 in *sn*-3 is named as 1,2-hexadecanoyl-3-(9Z-octadecenoyl)-*sn*-glycerol (IUPAC-IUB Commission on Biochemical Nomenclature 1977).

If the *trivial system* is used, each FA is called by a common designation. E.g. a TAG containing two C16:0 in the positions *sn*-1 and *sn*-2 and one 18:3 in *sn*-3 is defined as 1,2-palmitoyl-3-linolenoyl-*sn*-glycerol. This naming does not provide detailed information about DB conformation (αLn or γLn) but the positional distribution of primary FAs is distinguished with the defined numbering.

To facilitate the TAG descriptions, FAs are *abbreviated using letters*. In the standard notation of TAGs the initial letter(s) of trivial names of FAs are used and can be arranged in the order of their position on the glycerol molecule. Thus, for example, P/P/O represents a TAG composed of two C16:0 and one C18:1*n*-9 in any stereospecific positions. In the numerical system, the corresponding TAG is marked 16:0/16:0/18:1 if the stereoisomeric positioning is unknown or 16:0-16:0-18:1 in the case of a mixture containing a regioisomeric pair, but not necessarily in equimolar ratios.

Structural system. A TAG can also be identified through the sum of carbon atoms in the FA residues (acyl carbon number, ACN) (e.g. ACN 50 in the case of 16:0/16:0/18:1) or also by the number of DBs that can be added (e.g. ACN:DB 50:1). This nomenclature provides only limited information about the FA composition and no information concerning the positions or stereochemistry of the DBs is provided.

Equivalent carbon number (ECN) defined as ACN minus twice the number of DBs, is based on the observation that ECNs are linearly related to the logarithm of the retention volume of a TAG eluted in isocratic liquid chromatography (Firestone 1994; Indelicato et al. 2017). E.g. PPO ECN is 48 $(= 2 * 16 + 18 - 2 * 1)$.

Regio- and stereospecific naming. If the TAG molecule consists of an equal amount of two enantiomers, prefix *rac* is used (e.g. *rac*-PPO = *sn*-PPO + *sn*-OPP or 1,2(2,3)-dipalmitoyl-3(1)-oleoyl-*rac*-glycerol) or if the molecule is a pure enantiomer, the prefix *sn* is marked (*sn*-PPO).

2.1.2 Stereospecific location of fatty acids in TAGs in oils and fats

In TAG biosynthesis, the fatty acyl groups are covalently attached by ester bonds to the glycerol backbone in one of three stereospecifically distinct locations. Several biosynthetic pathways for TAGs in both plant and animal tissues are known. For example, a dihydroxyacetone phosphate pathway predominates in liver and adipose tissue, and a monoacylglycerol (MAG) pathway in the intestinal enterocytes. A third pathway, in which a diacylglycerol transferase is involved, has been recognized in maturing plant seeds and some animal tissues. (Lehner and Kuksis 1996; Coleman and Lee 2004). The principal route in most living organisms is the Kennedy pathway (**Figure 2**, Weiss et al. 1960). The precursor, *sn*-glycerol-3-phosphate, is acylated at positions *sn*-1 and *sn*-2 in a reaction catalyzed by specific transferases to form a key intermediate in the biosynthesis of all glycerolipids, the phosphatidic acid. The phosphatide phosphatase removes the phosphate group resulting in 1,2-diacylglycerol (DAG). In the final step, a third FA is incorporated to the *sn*-3 position of DAG forming the TAG. In practice, the TAG synthesis is far more complex. For example, FAs can be desaturated or otherwise modified in phosphatidylcholines (Shanklin and Cahoon 1998) by fatty acid desaturase enzymes. The desaturated FAs can be brought to TAG by phospholipid:diacylglycerol acyltransferase directly from phosphatidylcholine or from phosphatidylcholine to the acyl-CoA pool through the activity of lysophosphatidylcholine acyltransferase as illustrated by Vuorinen et al. (2015).

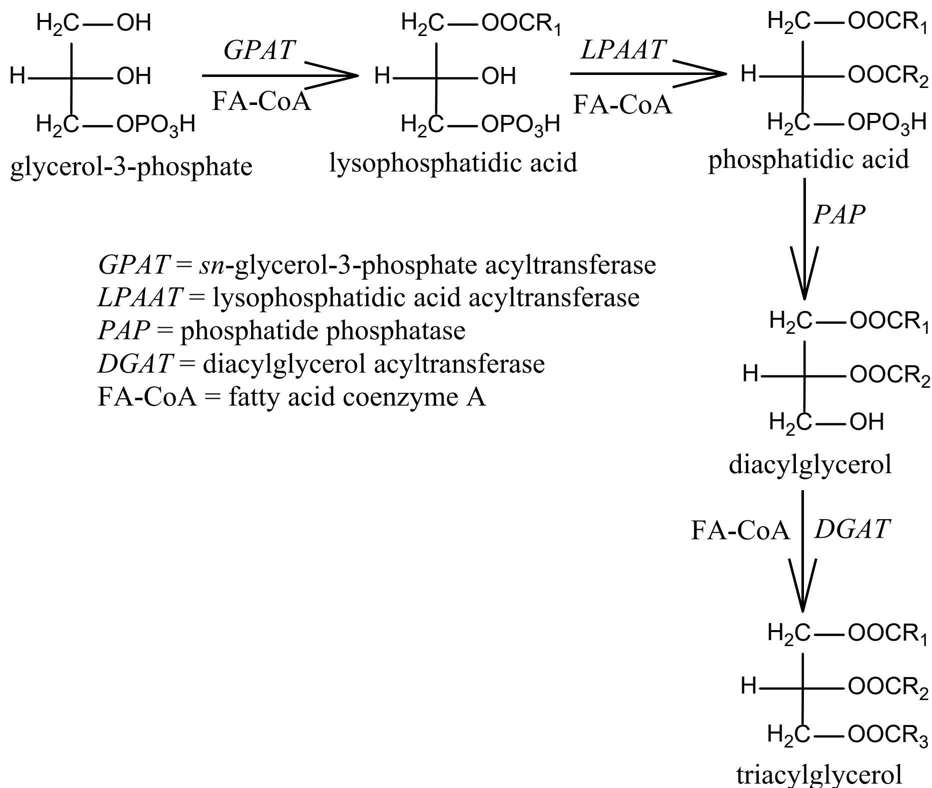


Figure 2. The Kennedy pathway from triacylglycerol biosynthesis. (Adapted from Maraschin et al. 2019)

The biosynthetic reactions catalyzed by unique enzymes result in non-random species-specific FA combinations (Coleman and Lee 2004), stereospecific distribution of FAs and distinctive TAG profiles as illustrated in **Table 1**. It has been demonstrated that fats and oils show different positional distribution of FAs in the *sn*-1 and *sn*-3 positions (Brockerhoff and Yurkowski 1966; Brockerhoff et al. 1966), and a particular TAG species in a dietary fats and oils occur in a specific ratio (Nagai et al. 2011a; Lísa and Holčapek 2013; Nagai et al. 2015, 2017), which implies that during TAG biosynthesis the positions *sn*-1 and *sn*-3 are distinguished. If the positional distribution of FAs was random, the regioisomeric composition of diacid-TAG would be A-A-B/A-B-A, 2:1. Likewise, the regioisomeric ratio in triacid-TAG should be 1:1:1 (A-B-C/B-A-C/A-C-B).

Table 1. Positional distribution of fatty acids in triacylglycerols of selected dietary fats and oils. Red shades indicate a higher percentage than a random distribution, whereas blue shades represent a lower percentage than a random distribution. (Data adapted from Jensen 1995; Hunter 2001; Lida et al. 2002; Karupaiah and Sundram 2007; Vichi et al. 2007; Berry 2009; Innis 2011; Michalski et al. 2013).

Fat or oil (main TAG species)	*Sn*-position	Percentage of major FAs on each *sn*-position[a]			
		C16:0	C18:0	C18:1	C18:2
Olive oil	*sn*-1	33	43	31	34
(O-O-O, O-O-P, O-L-O)[b]	*sn*-2	21	tr[c]	36	48
	sn-3	46	57	32	18
Cocoa butter	*sn*-1	47	48	11	10
(P-O-S, S-O-S, P-O-P)	*sn*-2	3	2	81	90
	sn-3	51	50	8	tr
Palm oil	*sn*-1	41	27	25	30
(P-O-P, P-O-O, P-L-P)	*sn*-2	9	tr	62	60
	sn-3	50	73	13	10
Lard	*sn*-1	23	54	43	35
(O-P-S, O-P-L, O-P-O)	*sn*-2	61	8	13	26
	sn-3	16	38	44	39
Beef tallow	*sn*-1	51	34	20	29
(P-O-O, P-O-P, P-O-S)	*sn*-2	21	18	42	36
	sn-3	28	48	38	36
Bovine milk	*sn*-1	44.5	56	59	22
(O-P-Bu, P-P-Bu, P-M-Bu)	*sn*-2	43	16	0	47
	sn-3	12.5	28	41	31
Human milk	*sn*-1	16	75	46	37
(O-P-O, L-P-O, P-P-O)	*sn*-2	68	15	12	21
	sn-3	16	10	32	42

[a]In this table, not all fatty acids are listed for each fat and oil. The sum of columns of each oil and fat is 100%.
[b]Fatty acid abbreviations are listed in NOMENCLATURE.
[c]tr = traces

The TAG composition of dietary plant oils, in many cases, can be considered quite simple compared to the TAGs of e.g. human milk, bovine milk, and fish oil. Plant oils are dominated by TAGs formed from five major FAs: palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1*n*-9), linoleic acid (C18:2*n*-6) and α-linolenic acid (C18:3*n*-3). Moreover, the regiospecific distribution of FAs in TAGs of the most common plant oils is known such as corn oil, palm oil, olive oil, rapeseed oil, soybean oil and sunflower seed oil

(Gunstone et al. 2007). For example, olive oil contains over 70% C18:1*n*-9 and it is mainly located in the position *sn*-2, while the percentage of saturated FAs (SAFA) in the *sn*-2 position does not exceed 2%. In olive oil the regioisomeric ratio for L-L-O/L-O-L is 100:0. (Vichi et al. 2007). Whereas, the regioisomeric ratio of the corresponding TAG in sunflower seed oil is 73:27 (Vichi et al. 2007) or 80:20 (Leskinen et al. 2007). In palm oil, the most abundant TAGs P-O-O and P-O-P are found in the regioisomeric ratio of 100:0 (P-O-O/O-P-O) and 22:78 (P-P-O/P-O-P), respectively (Kallio et al. 2001). In plant oils the monounsaturated FAs (MUFA) and polyunsaturated FAs (PUFA), such as C18:1*n*-9 and C18:2*n*-6, predominantly occupy the *sn*-2 position (Jakab et al. 2002; Holčapek et al. 2003; Leskinen et al. 2010a). SAFAs as well as FAs longer than C18 are predominantly esterified in the positions *sn*-1 and *sn*-3 of the glycerol (Brockerhoff 1971; Kallio et al. 2001; Jakab et al. 2002; Holčapek et al. 2003; Leskinen et al. 2007), and FA compositions in those two positions are very similar to each other (Brockerhoff and Yurkowski 1966). The last position to be esterified is s*n*-3 (**Figure 2**), containing less common FAs such as C18:3*n*-3.

The TAG composition in animal fats differs significantly between the animal species, location of the tissue and the digestive system of the animal (Kallio et al. 2001; Buchgraber et al. 2004b). Nevertheless, there are some common rules. In most species, the SAFAs are found in the position *sn*-1 and unsaturated FAs in the position *sn*-2 (Brockerhoff et al. 1966; Gunstone et al. 2007). For example, beef tallow contains TAGs *sn*-POO, *sn*-POP and *sn*-POS, where C16:0 and C18:1*n*-9 are located in the positions *sn*-1 and *sn*-2, respectively and FA composition of the position *sn*-3 varies (Brockerhoff et al. 1966; Karupaiah and Sundram 2007). Lard is a unique animal fat, because C16:0 is situated mainly in the *sn*-2 position (90%) (Mattson and Lutton 1958; Christie and Moore 1970; Kallio et al. 2001) unlike in tallow (15% of C16:0 in *sn*-2) (Kritchevsky et al. 1998). The two most abundant regioisomers in lard are O-P-S (P-O-S/O-P-S/P-S-O, 2:96:2) and O-P-O (P-O-O/O-P-O, 3:97). In tallow, among the three most abundant TAGs are P-O-S, P-P-S and P-O-O with the regioisomeric ratios 61:23:16 (P-O-S/O-P-S/P-S-O), 100:0 (P-P-S/P-S-P) and 86:14 (P-O-O/O-P-O). (Kallio et al. 2001).

Milk fat and fish oil are considered to be the most complex TAG mixtures due to the extensive range of different FAs. Bovine milk contains at least 400 FAs including SAFAs from butyric acid (C4:0) to long-chain FAs (60%) and also FAs with an odd number of carbon atoms and a minor amount of branched chain FAs (Christie 1983; Jensen 2002). C18:1*n*-9 is the main unsaturated FA (25%). The amount of PUFAs is relatively low because of biohydrogenation in the rumen. With 400 FAs, theoretical number of TAGs would be 64,000,000, if the FAs were randomly distributed. Because the positional distribution of FAs

in the TAG is genetically controlled, the number of TAGs is not as high as this, but still thousands of TAGs are present, mostly in traces (Jensen 2002). Long-chain SAFAs such as myristic acid (C14:0), C16:0 and stearic acid (C18:0) are mainly distributed at the *sn*-1 and *sn*-2 positions (Christie and Clapperton 1982), whereas position *sn*-3 is dominated by SCFAs and MCFAs such as butyric acid (C4:0), caproic acid (C6:0), caprylic acid (C:8:0) (Christie and Clapperton 1982; Itabashi et al. 1993). C18:1*n*-9 shows preference for positions *sn*-1 and *sn*-3. (Månsson 2008). In ruminant milk TAGs, C16:0 is predominantly located in positions *sn*-1 and *sn*-2 (Blasi et al. 2008). In contrast, human milk TAGs are especially enriched in C16:0 in the position *sn*-2. The FAs used for synthesis of TAGs in human milk are obtained by either the uptake of FAs from plasma or by *de novo* synthesis in the mammary gland (Neville and Picciano 1997). Although the current and long-term maternal diet, genetic factors, duration of pregnancy and stage of lactation influence the FA composition of the milk, the prevalence (20–25% of the milk FAs) and the positional distribution of C16:0 (50% of the FAs in the *sn*-2) are quite constants (Jensen 1995; Fidler and Koletzko 2000; Koletzko et al. 2001; López-López et al. 2002). Unsaturated FAs, especially C18:1*n*-9, typically occupy the positions *sn*-1/3 (Breckenridge et al. 1969; Kallio et al. 2017). Thus, the most abundant TAG regioisomer is O-P-O (Kurvinen et al. 2002a; Kallio et al. 2017; Fabritius et al. 2020). The levels of PUFAs, mainly eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), and *trans*-FAs vary depending on the dietary fat composition (Brenna et al. 2007).

The FA composition of fish and seal oils characterized by long chain *n*-3 FAs (EPA, C20:5*n*-3 and DHA, C22:6*n*-3) reflect their diet (Ruiz-Lopez et al. 2015). The fish species affect the positional distribution of FAs (Beccaria et al. 2015). Herrera and others studied different fish oil samples (tuna, skipjack tuna, commercial fish oil blend), as well as Zhang et al. (anchovy, tuna, salmon) and both studies found that the DHA was preferentially located in the position *sn*-2, while the EPA was either equally distributed in all positions, or located predominantly in the positions *sn*-1 and *sn*-3 in some fish oils (Herrera et al. 2013; Zhang et al. 2018). Other study has compared the positional distributions of FAs in TAGs of seal blubber oil with that of commercially available menhaden oil (Wanasundara and Shahidi 2007). In seal blubber oil, *n*-3 FAs occurred mainly in the positions *sn*-1 and *sn*-2. Whereas, in menhaden oil, the DHA occurred mainly in the position *sn*-2 of the TAG, and the EPA was equally distributed in the positions *sn*-2 and *sn*-3.

2.1.3 Enantiomeric composition of individual TAGs

Enantiomeric ratios are challenging to determine, especially, when the aim is to separate enantiomers and regioisomers in the same analysis. The common challenge is the co-elution of certain TAG species (Lísa and Holčapek 2013). Thus, only a limited amount of knowledge is available concerning the enantiomeric ratio of individual TAGs present in different foods, however, the data covers TAGs of both plant and animal origin (**Table 2**). Enantiomeric ratios of TAGs in different foods are presented in **Table 2**, but in some cases the symmetric TAGs are more abundant than each asymmetric form. For example, the relative ratio of TAG O/O/L isomers in human plasma was O-L-O/O-O-L/L-O-O 43:27:30 and in hazelnut oil 46:15:39 in the study by Lísa and Holčapek (2013). Furthermore, oils from marine mammals contain more symmetric TAGs with two EPAs or two DHAs and one palmitic acid compared to fish oils (Nagai et al. 2013). The relative ratios of TAG P/DHA/DHA isomers in steller sea lion oil were DHA-P-DHA/P-DHA-DHA/DHA-DHA-P 89:11:0 and 71:24:5 in harp seal oil. The relative ratios of TAG P/EPA/EPA isomers were similar; EPA-P-EPA/P-EPA-EPA/EPA-EPA-P 84:16:0 in steller sea lion, and 78:22:0 in harp seal oil. Whereas, in fish oils the corresponding ratios were DHA-P-DHA/P-DHA-DHA/DHA-DHA-P 6:89:5 in TAGs extracted from skipjack tuna and 7:80:13 in sardine oil, and the ratios of TAG P/EPA/EPA isomers were EPA-P-EPA/P-EPA-EPA/EPA-EPA-P 17:78:5 in skipjack tuna, and 18:72:10 in sardine oil.

TAGs consisting of two palmitic acids and one SCFA or MCFA in bovine milk fat were mainly asymmetric containing only one enantiomer with SCFA or MCFA in the *sn*-3 position (Nagai et al. 2015). The positional distribution of these SCFAs and MCFAs in bovine milk is nutritionally essential because lingual and gastric lipases selectively hydrolyze these FAs providing energy for the calf. Similarly, the TAG composition of egg yolk, immature egg yolk and chicken meat changes according to function of the sample material (Nagai et al. 2017), which is illustrated in **Table 2**. Egg yolk has the same role as milk acting as the source of energy for the developing embryo. The findings were similar in regiospecific composition of chicken skin and egg yolk (Kallio et al. 2001).

Table 2. Enantiomeric ratio of selected individual triacylglycerols.

TAG	Enantiomeric ratio				Ref.
	Palm oil				
O-O-P[a,b]	60:40				Nagai et al. 2011a
	Hazel nut oil	Human plasma			
S-O-O	39:61	100:0			Lísa and Holčapek 2013
L-O-O	72:28	53:47			
L-L-O	71:29	43:57			
	Steller sea lion	Harp seal	Skipjack tuna	Sardine	
DHA-P-P	14:86	14:86	5:95	25:75	Nagai et al. 2013
EPA-P-P	0:100	15:85	15:85	35:65	
DHA-DHA-P	0:100	18:82	5:95	14:86	
EPA-EPA-P	0:100	0:100	6:94	12:88	
	Bovine milk (butter)				
P-P-Bu	100:0				Nagai et al. 2015
P-P-Co	100:0				
P-P-Cy	100:0				
P-P-Ca	100:0				
P-P-La	100:0				
	Egg yolk	Immature egg yolk	Chicken meat		
P-P-L	100:0	100:0	71:29		Nagai et al. 2017
P-O-O	100:0	96:4	65:35		
P-P-O	100:0	97:3	72:28		

[a]The first eluting enantiomer of the enantiomeric pair is mentioned.
[b]Fatty acid abbreviations are listed in NOMENCLATURE.

2.1.4 The role of chirality and specific positioning of fatty acids in TAGs

It has been shown that chirality plays a crucial role in the major components of living tissues like nucleic acids, proteins and carbohydrates (Busch and Busch 2006). Correct stereochemistry is vital for the functionality of the chiral biomolecules. It is well-known from chiral drugs that two enantiomers can interact in different ways with dissimilar effects (McConnell et al. 2007). Recently, chiral compounds, among all the compounds found in foods, have increasingly attracted the attention of researchers especially in order to assess their authenticity, origin and safety (D'Orazio et al. 2017; Fanali et al. 2019). Scientists are also interested in interactions between food components and the body environment. To give some examples of the importance of chirality in the food, it is clearly known that chiral compounds may have different sensory properties. Limonene is a cyclic terpene of which the more common D-isomer

has a strong lemon-like aroma, whereas the less common L-isomer has a piney odor. Flavor may be adulterated by addition of synthetic racemic ingredients resulting in a change in the natural enantiomeric ratio (Marchelli et al. 1996). The existence of the other, unnatural enantiomers can also be the result of processing of the natural proteins composed only of L-amino acids. Natural carbohydrates are generally formed from D-saccharides. Consequently, enantiomeric ratios of food components can be regarded as a quality parameter because it may be an indication of a technological process, contamination, adulteration, ageing or extended shelf life (Marchelli et al. 1996; Ebeler 2007).

Chirality of different food components is increasingly studied but the importance of chirality in TAGs is not well established. The type and positional distribution of FAs in TAGs affects the physical properties such as crystal structure, solubility, and viscosity (Itabashi 2005; Foubert et al. 2007) and all these are important to the functional properties of many foods. TAG molecules may pack into fat crystals in different ways, leading to various polymorphic forms with different physicochemical properties. The FA combinations in TAGs impact the liquid-solid phase transitions of the lipids due to the varying melting characteristics of the TAG molecules. (Craven and Lencki 2012a, 2012b). For example, the relationship between the mouthfeel of chocolate and the crystal structures of cacao fat, which mainly contain TAGs with C16:0 and C18:0 in the positions *sn*-1 and *sn*-3 and C18:1*n*-9 in the position *sn*-2, is the basis of chocolate manufacturing. Thus, understanding the structural differences that influence the crystallization and melting characteristics of lipids in foods is essential to the creation of food products with the desired properties.

Another notable example of specific positioning of FAs in TAGs is human milk, in which the stereospecific positioning of C16:0 (*sn*-2) is important for optimal fat and mineral absorption, bone strength, gut microbiota and bowel movement of infants (Carnielli et al. 1996; Lucas et al. 1997; Kennedy et al. 1999; López-López et al. 2001; Litmanovitz et al. 2013; Yaron et al. 2013). The long-chain SAFAs released from the position *sn*-1(3) have an increased tendency to form insoluble FA soaps, which are related to stool hardness (Quinlan et al. 1995). In addition, C16:0 is an essential building block of dipalmitoyl phosphatidylcholine, which in turn is necessary for the functions of lungs (Carta et al. 2017). It was shown, that the phospholipid content in lungs and the amount of C16:0 in the position *sn*-2 was significantly higher in the synthesized TAG group and sow milk group compared to the three other groups, where piglets were fed with a formula containing SAFAs from medium-chain TAGs, coconut, or palm oil (Innis et al. 1996). Significant differences have been detected between the regioisomeric compositions of TAGs in human milk and infant formulas (Kurvinen et al. 2002a; Fabritius et al.

2020), highlighting the fact that the unique composition of human milk is difficult to mimic.

Dietary TAGs undergo enzymatic hydrolysis in the digestive tract to yield free FAs from the positions *sn*-1 and *sn*-3, and *sn*-2 MAG. The stereospecific positions and chain length of FAs influence the postprandial lipid metabolism, which involves multiple steps as digestion, transportation of hydrolysis products to enterocytes, re-synthesis of TAGs, assembly of chylomicrons and transportation in the circulation as well as uptake by tissues (Karupaiah and Sundram 2007; Alfieri et al. 2017). The topic has recently been reviewed and was clearly illustrated by Michalski and others in 2013. Several stereospecific enzymes contribute to the degradation of TAGs with different degrees of efficiency (Mu and Høy 2004). Both gastric and lingual lipases prefer MCFAs and preferentially hydrolyze FAs from the position *sn*-3. It has been suggested, that *sn*-1 and *sn*-3 specific pancreatic lipase prefers the FAs in the position *sn*-1 over FAs in the position *sn*-3. (Rogalska et al. 1990). Pancreatic lipase catalyzes the formation of *sn*-2 MAGs and free FAs, which are in turn absorbed into the enterocytes (Mattson and Volpenhein 1964; Lambert and Parks 2012). The *sn*-2 MAGs are reacylated into new TAGs in the enterocytes before entering the lymph chylomicrons. Consequently, the FA has a higher bioavailability when located in the *sn*-2 position. The metabolic fate of FAs in the positions *sn*-1 and *sn*-3 is affected by the carbon chain length and stereospecific location on the TAG. If the long-chain SAFAs are located in the positions *sn*-1 and *sn*-3, their bioavailability is lower since the melting point of the acid soaps of these LCFAs is very high, and they may precipitate in the intestine and be lost in the feces (Small 1991). LCFAs enter cells both by diffusion and by the action of binding and carrier proteins. SCFAs and MCFAs, originating from the *sn*-1 and *sn*-3 positions, are solubilized in the intestinal environment and are absorbed by enterocyte via diffusion, where they are complexed with albumin, and transported by portal system to the liver for oxidation (Hunter 2001). On the other hand, PUFAs located in the *sn*-1 and *sn*-3 positions influence the hydrolytic activity of pancreatic lipase because of their steric hindrance and thus, slow down the hydrolysis (Michalski et al. 2013). Advancements in methodologies will expand knowledge of the fate of dietary FAs in humans.

Specific FA distribution in TAGs of some natural fats limits their industrial usability. The lipid industry has used different technologies such as interesterification, hydrogenation and fractionation to obtain more desirable physical properties. The aim of interesterification is to modify the melting and crystallization behavior of fats by rearranging the positions of FAs on the glycerol backbone. Unlike hydrogenation producing artificial *trans* FAs, interesterification generates new TAG species, without affecting the *cis/trans*

balance of FAs. There are no legal requirements to specify the FA composition on food labels making it difficult to estimate the consumption of different FAs (Alfieri et al. 2017). Interesterification has largely replaced hydrogenation, and thus more information related to the long-term effects on human consumption of interesterified fats rich in long-chain SAFAs, such as palmitic acid and stearic acid, is needed (Hunter 2001).

It has been shown that many factors contribute to the regioisomeric composition. In plant oils regiospecific TAG composition has been shown to vary within species, subspecies and varieties (Vichi et al. 2007; Leskinen et al. 2008, 2009, 2010b). Furthermore, different environmental factors, especially light and temperature have been found to affect the regioisomerism of TAGs in berry oils. Unfortunately, this has not been widely studied.

In many studies, as also shown in the previous chapters, it has been clearly demonstrated that the TAG composition of fats and oils of different origin seems to be strictly determined in the biosynthesis steps. The characteristic TAG composition is also the key indicator for detecting adulterations. For example, Wei et al. demonstrated that adulterated peanut oil with 5% soybean oil was clearly identified by comparing the qualitative and quantitative results of TAGs obtained by using modern LC-MS techniques combined with principal component analysis (Wei et al. 2015).

In general, it is important to know the specific structural features of the different fats and oils consumed and to study how different molecular structures affect absorption and digestion properties, the effects on fasting and postprandial lipemia, and human health (Berry 2009). Further, the stereospecific distribution of FAs in TAGs is important both technologically and biochemically, as presented in this review. Thus, all random-distribution theories and generalizations concerning the stereospecific composition of TAGs may result in imprecise conclusions highlighting the importance of reliable stereospecific methodologies for analysis of fats and oils. However, from the analytical point of view the chirality of the TAGs still presents a major challenge.

2.2 Methods of studying specific composition of individual TAGs

Enantiomers of TAG have identical chemical properties such as their chromatographic (TLC, HPLC, GC), spectrometric (MS) and spectroscopic (NMR) behavior, as well as their physical properties including melting properties and solubility. However, if two enantiomers are mixed, it results in a change in the melting properties and solubility although the chemical properties

will largely remain unaltered (Mizobe et al. 2013). In order to separate the enantiomers based on their chemical properties it is necessary to introduce an external chiral influence, such as a chiral stationary phase (in HPLC) or a chiral solvent (in NMR). Understanding of the intermolecular forces involved in chiral recognition mechanisms has advanced significantly although there is still much to be done (Scriba 2016). Recently, enantioselective chromatography has also been increasingly used to determine enantiomeric purity.

Several analytical methods including enzymatic and chemical hydrolysis, chromatographic separations including supercritical fluid chromatography (SFC), various mass spectrometric (MS) techniques, and nuclear magnetic resonance (NMR) spectroscopy are available for qualitative and quantitative determination of TAGs as reviewed by several authors over many years (Brockerhoff 1971; Myher and Kuksis 1995; Ruiz-Gutiérrez and Barron 1995; Laakso 1996; Andrikopoulos 2002; Kalo and Kemppinen 2012; Ruiz-Samblás et al. 2015; Indelicato et al. 2017; Řezanka et al. 2017). The analytical methodology has progressed from an overall characterization of TAGs and the FA composition of each stereospecific position towards a molecular level determination of the stereospecific composition of individual TAGs. The development of regio- and stereospecific analyses of TAGs started with enzymatic (Mattson and Lutton 1958; Brockerhoff 1965) and chemical hydrolysis (Brockerhoff 1967), and has now progressed towards more accurate chiral chromatographic methods of individual enantiomeric TAGs (Řezanka et al. 2017). Analytical methods other than chromatography and MS are of negligible use for the resolution and identification of lipid molecules (Myher and Kuksis 1995) as illustrated in **Figure 3**. Since MS is unable to differentiate between enantiomers, chromatographic methods have a particularly important role in the analyses of enantiomeric composition of individual TAGs. SFC as a green analytical method has recently attracted more interest due to significant improvements in instrumentation. NMR is presented as a rapid and non-destructive option. A considerable amount of research has been focused on the regiospecific analysis of TAGs but less attention has been paid to stereospecific studies (Kuksis and Itabashi 2005; Řezanka et al. 2017).

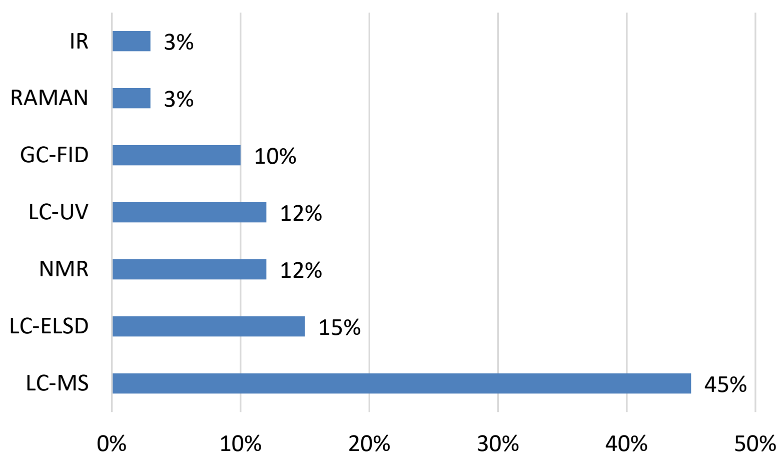


Figure 3. General overview of the analytical techniques used for determination, characterization and quantification of TAGs in edible oils (Indelicato et al. 2017).

2.2.1 Enzymatic and chemical methods

Regio- and stereospecific analysis has its roots in the middle of the last century. The study by Mattson and Lutton was one of the first showing the specific distribution of FAs in TAGs of both animal and plant origin (Mattson and Lutton 1958). Although different even, random, partial random and restricted random theories had been suggested, their results revealed the unique FA composition in the position *sn*-2 in lard compared to other animal fats highlighting the clear evidence of the non-random distribution of FAs.

2.2.1.1 Enzymatic hydrolysis for regiospecific analysis

Porcine pancreatic lipase was first applied for the enzymatic hydrolysis of FAs (Mattson and Lutton 1958; Luddy et al. 1964). In addition, microbial lipases, such as *Rhizopus delemar* lipase, can be applied (Janssen et al. 2006) but reagents and reaction conditions have to be carefully optimized depending on the source of the lipase so as to minimize any undesirable side reactions such as acyl migration. Differences in the positional distribution of FAs in TAGs from natural fats and oils were first systematically studied by Brockerhoff and others (Brockerhoff 1965; Brockerhoff and Yurkowski 1966; Brockerhoff et al. 1966). The basic idea was that TAGs are hydrolyzed by pancreatic lipase to release the FAs from the positions *sn*-1 and *sn*-3. The resulting 2-monoacyl-*sn*-glycerols (*sn*-2 MAGs) are fractionated by thin-layer chromatography (TLC),

chemically further hydrolyzed and analyzed by gas chromatography (GC) to give the FA composition in the position *sn*-2.

Pancreatic lipase hydrolyzes SAFAs and most mono-, di- and trienoic acids from the positions *sn*-1 and *sn*-3 at the same rate. However, PUFAs such as DHA (e.g. from fish oil) are hydrolyzed more slowly due to the steric hindrance, and SCFAs (e.g. from milk) are hydrolyzed more rapidly than the other common FAs. (Christie and Han 2010). The DHA residue is the most resistant to pancreatic lipase hydrolysis due to the high number of *cis* DBs forming a highly bent molecular structure (Bottino et al. 1967; Yang et al. 1990). Because of the FA selectivity of the lipases, the methods using pancreatic or microbial lipases are not suitable for all lipid samples. As an alternative, *Candida antarctica* lipase B (CALB) has been applied to study the regiospecific composition of TAGs from milk fat (Yoshinaga et al. 2016) and sardine oil (Watanabe et al. 2015). CALB is known to be less discriminatory, releasing FAs from all the positions at about an equal rate. The positional selectivity is dependent on the polarity of the reaction environment (Shimada et al. 2003).

2.2.1.2 Chemical hydrolysis for stereospecific analysis

While the regiospecific study with pancreatic lipase hydrolysis is quite simple, the stereospecific methodology is usually more complex including degradation, synthesis, enzymatic hydrolysis, isolation of products, transesterification, and chromatographic separation of the final products. Several authors have modified the basic procedure presented by Brockerhoff in 1965. The first step was to prepare an equimolar mixture of *sn*-1,2- and *sn*-2,3-DAGs by partial hydrolysis of TAGs with pancreatic lipase, which was later replaced by a Grignard reagent. Additionally, *sn*-1,3-DAGs were generated in this reaction. Thus, the *sn*-1,2- and *sn*-2,3-DAGs were separated from the *sn*-1,3-DAGs by TLC and derivatized to phospholipid derivatives (phosphatidylphenols in the original method). They were, in turn, hydrolyzed by the phospholipase A producing a lysophospholipid with FA from the position *sn*-1, free FA from the position *sn*-2 and the unchanged 2,3-diacyl-*sn*-phosphatide. After isolation of these products by TLC and transesterification, the FA composition was determined by GC.

One drawback of the original Brockerhoff method was that the FA composition of the position *sn*-3 was determined indirectly by calculation. The most important step was to produce the representative DAG intermediates, in which the FA composition in each position corresponds to the FA composition of the original TAG. However, both acyl migration and the FA specificities of lipases affect the reliability of the results. It was proposed that using a Grignard

reagent such as methyl magnesium bromide (Brockerhoff 1967) or ethyl magnesium bromide (Christie and Moore 1969) instead of lipase, more representative DAG mixtures could be obtained. In the 1990s, the more reactive allyl magnesium bromide was introduced to minimize errors caused by acyl migration (Becker et al. 1993). In the advanced method, the FA composition in the position *sn*-3 was also determined by isolating *sn*-1,3-DAGs and converting them into 2-phosphatidylphenol, from which phospholipase A cleaved the FA from position *sn*-1. The FA composition of the remaining lysophosphatide corresponds to the FA composition in position *sn*-3 of the original TAG (Brockerhoff 1967). Myher and Kuksis introduced a stereospecific method, which was based on hydrolysis by a Grignard reagent but instead of phosphotidylphenols and phospholipase A, phosphatidylcholines and phospholipase C were applied (Myher and Kuksis 1979).

After different applications of chemical hydrolysis, chiral chromatographic methods were introduced as options to simplify the stereospecific analysis. The more sophisticated methods involved partial chemical deacylation to produce the enantiomeric *sn*-1,2(2,3)-DAGs, which were separated by HPLC, either as diastereoisomers on a silica column (Laakso and Christie 1990; Christie et al. 1991) or, as 3,5-dinitrophenylurethane derivatives on a chiral column (Takagi and Ando 1991; Ando and Takagi 1993; Itabashi et al. 1993). However, conventional methods based on enzymatic and chemical hydrolysis do not give any information about stereochemistry of individual TAG molecules. In addition, in these complex, multistep methods acyl migration is difficult to totally eliminate during the chemical deacylation of TAGs (Turon et al. 2003). Thus, currently different chromatographic and MS methods have largely replaced the conventional methods. The works of Semporé and Bézard as well as of Boukhchina and others were among the pioneering studies of stereospecific structures of individual TAGs. They isolated the main TAG fractions prior to the separate chemical hydrolysis of each fraction, which enabled a more precise determination of the TAG composition of peanut oil and cotton seed oil (Semporé and Bézard 1991) and sunflower seed oil (Boukhchina et al. 2003).

2.2.2 Reversed-phase liquid chromatography

LC methods cover over 70% of the methods used to study TAGs qualitatively and quantitatively (Indelicato et al. 2017, **Figure 3**). Reversed-phase high-performance liquid chromatography (RP-HPLC), commonly known as non-aqueous RP-HPLC (NARP-HPLC) is currently the most widely used method for the separation of the TAG mixtures. Due to the low selectivity for

separation of the TAG regioisomers and TAGs with the same ECN, special LC techniques are used for the structural analysis. The methods include silver-ion LC, chiral-phase LC and on- and offline multidimensional LC methods, in addition to commonly used RP-HPLC (**Table 3**). The clear advantage of HPLC compared to GC is the separation of different TAG molecular species at moderate temperatures. Further, the HPLC methods are not limited by the volatility of the samples.

Table 3. Recent examples of different liquid chromatographic techniques used to study regiospecific and/or stereospecific composition of triacylglycerols over the last decade.

Material	LC method	Stationary phase	Mobile phase	Detection and/or MS for analysis	Ref.
Olive oil	RP-UHPLC	Cortecs C_{18} column (150 × 2.1 mm, 1.6 µm)	MeOH[a]/water and IPA/water (+ ammonium acetate)	ESI-MS/MS (QqQ)	Tarvainen et al. 2019
Reference compound	Chiral-phase HPLC	CHIRALPAK IF-3 (250 × 4.6 mm, 3 µm)	ACN (+ ammonium formate)	ESI-MS/MS (QqQ)	Nagai et al. 2019
Human milk	RP-UHPLC	Waters BEH C_{18} (100 × 2.1 mm, 1.7 µm)	MeOH/IPA + LiCl	ESI-MS/MS (QqQ)	Kallio et al. 2017
Plant oil	RP-HPLC	Nucleodur C_{18} ISIS (50 × 2 mm, 1.8 µm)	ACN/IPA	ELSD	Tamba Sompila et al. 2017
Rat milk	RP-HPLC	2 × Inertsil ODS-P (250 × 4.6 mm, 5 µm) or Sunrise C_{28} (250 × 4.6 mm, 5 µm)	ACN/IPA/hexane (3:2:1) or acetone	UV-APCI-MS/MS (QqQ)	Watanabe et al. 2016
Tuna and algal oil	RP-HPLC	2 × Luna C_{18} (150 × 2.1 mm, 3 µm)	ACN/DCM	APCI-HRMS (hybrid LTQ-Orbitrap)	Baiocchi et al. 2015
Plant oil	Preparative HPLC	Gemini C_{18} (250 × 4.6 mm, 5 µm)	MeOH/IPA	UV and direct-infusion ESI-MS lithiated adducts	Lin et al. 2015
Bovine milk	RP-HPLC	Sunrise C_{28} (250 × 4.6 mm, 5 µm)	Acetone/ACN	APCI-MS (QqQ)	Watanabe et al. 2016
Edible oil	Mixed-mode HPLC	Zorbax Eclipse Plus phenyl-hexyl (250 × 4.6 mm, 5 µm)	IPA/MeOH (+ NH_4OH)	APCI-MS (hybrid QqQ/LIT)	Wei et al. 2015
Plant oil	Mixed-mode HPLC	Zorbax Eclipse Plus phenyl-hexyl (250 × 4.6 mm, 5 µm)	ACN/MeOH	APCI-MS (hybrid QqQ/LIT)	Hu et al. 2014
Fish oil	RP-HPLC	Nova-pak C_{18} (150 × 3.9 mm, 4 µm)	MeOH/IPA	ESI-MS^3 (QqLIT)	Cubero Herrera et al. 2013
Peanut oil	2D: Offline NARP-	Zorbax Eclipse Plus C_{18} (150 × 4.6 mm, 5 µm) +	ACN/IPA (+ NH_4OH) +	APCI-MS (hybrid	Hu et al. 2013

Material	LC method	Stationary phase	Mobile phase	Detection and/or MS for analysis	Ref.
	HPLC + silver ion-HPLC	Varian ChromSpher 5 Lipids (250 × 4.6 mm, 5 µm)	Hexane/ACN	QqQ/LIT)	
Cocoa butter equivalent	2D: Offline NARP-HPLC + silver ion-HPLC	Alltima HP C_{18} HL (150 × 3.0 mm, 3 µm) + ChromSpher 5 Lipids (250 × 4.6 mm)	Acetonitrile/DCM + heptane/acetone	ELSD	Kadivar et al. 2013
Green algal	RP-HPLC	EC-C_{18} (50 × 2.1 mm, 2.7 µm)	MeOH (+ ammonium formate + sodium formate) /IPA+hexane	ESI-MS/MS (LIT/Orbitrap)	Nagy et al. 2013
Milk	RP-HPLC	Sunrise C_{28} (250 × 4.6 mm, 5 µm)	Acetone/ACN	APCI-MS/MS (QqQ)	Yoshinaga et al. 2013
Milk (cow, goat, human)	RP-HPLC	RP-C_{18} Lichrosphere (250 × 4.6 mm, 5 µm)	ACN/DCM	APCI-MS/MS (IT)	Gastaldi et al. 2011
Fish and marine mammal	RP-HPLC	2 × Inertsil ODS-P (250 × 4.6 mm, 5 µm)	ACN/IPA (+hexane)	UV and APCI-MS (quadrupole)	Gotoh et al. 2011a
Animal fat	2D: Offline NARP-HPLC + silver ion-HPLC	2 × Nova-Pak C_{18} (300 + 150 × 3.9 mm, 4 µm) 3 × ChromSpher Lipids (250 × 4.6 mm, 5 µm)	ACN/hexane-IPA	APCI-MS (IT QqTOF)	Lísa et al. 2011
Borago officinals	2D: Online silver ion-HPLC + NARP-HPLC	Customized Nucleosil SA (150 × 1.0 mm, 5 µm) + Ascentis Express C_{18} (50 × 4.6 mm, 2.7 µm)	Hexane/butyronitrile + ACN/IPA	ELSD	Mondello et al. 2011
Reference compound	RP-HPLC	2 × Inertsil ODS-P (250 × 4.6 mm, 5 µm)	ACN/IPA	UV-APCI-MS (QqQ)	Gotoh et al. 2010
Reference compound	Silver ion-HPLC	3 × ChromSpher Lipids (250 × 4.6 mm, 5 µm)	Hexane/IPA/ACN	APCI-MS (5 different mass analyzers)	Holčapek et al. 2010
Rhodococcus erythropolis	RP-HPLC	2 × HIRPB-250AM (C_8/C_{18} multi-alkyl phase) (250 × 2.1 mm, 5 µm)	ACN/IPA	APCI-MS (quadrupole)	Řezanka et al. 2010

[a] MeOH=methanol; IPA=2-propanol; ACN=acetonitrile; LiCl=lithium chloride; DCM=dichloromethane; NH_4OH=ammonium hydroxide

2.2.2.1 Chromatographic separation mechanism

In the RP mode, the chromatographic separation is based on the selective interactions of the analytes with the relatively hydrophobic stationary phase and the relatively hydrophilic (polar) mobile phase. The retention of hydrophobic molecules in the hydrophobic stationary phase is higher, and more hydrophilic molecules are eluted first. The retention of TAGs in RP-HPLC is influenced by a combination of the total length of the acyl chains (acyl carbon number, ACN) and by the degree of unsaturation as well as the *sn*-position of FAs, asymmetry, configuration and position of DBs in the fatty acyl residues (Laakso 1996). It has been demonstrated that the elution time of TAGs increases with the increased length of the acyl chains while the increasing number of DBs reduces the retention time by the equivalent of about two carbon atoms. Thus, the elution order of TAGs can be predicted using the ECN values. TAGs with the same ECN i.e. critical pairs easily co-elute. For example, TAGs P/P/O (ACN:DB 50:1) and P/O/O (ACN:DB 52:2) commonly found in edible oils have both the ECN value 48. In addition to the separation of critical ECN pairs (Holčapek et al. 2005), many studies concentrate on the separation of regioisomers (Leskinen et al. 2007; Kuroda et al. 2008; Gotoh et al. 2010; Nagai et al. 2011b, 2019; Cubero Herrera et al. 2013; Baiocchi et al. 2015; Kallio et al. 2017; Tamba Sompila et al. 2017; Tarvainen et al. 2019). In addition, the positional isomers of TAGs containing FAs that differ only in the position of DBs such as oleic and vaccenic acids or α- and γ-linolenic acids have been studied (Leskinen et al. 2008, 2010b).

2.2.2.2 Chromatographic conditions

Different combinations of stationary phases, column dimensions and mobile phases have been used for separation of complex TAG mixtures to achieve the highest possible resolution (**Table 3**). With the mostly used stationary phases consisting of octadecylsilyl (ODS or C_{18}) groups bonded chemically to silica, the TAGs are separated based on ECN. The ability of RP-HPLC to separate TAG regioisomers was systematically studied for the first time by Momchilova et al. in 2004, and was further optimized by comparing the effects of different stationary phases and mobile phases (Momchilova et al. 2006). The authors tested four C_{18} stationary phases with different amounts of free silanol groups, and mobile phases of acetonitrile mixed with dichloromethane, tetrahydrofuran, 1-propanol, 2-propanol, ethanol, or acetone. Complete separation of five regioisomeric pairs of TAGs (P-P-X/P-X-P, X = L, Ln, A, EPA or DHA, A=arachidic acid, C20:0) using acetonitrile and ethanol (65:35, v/v) on the endcapped Superspher column at 18 °C was achieved. The more polar solvents

were better suited to the stationary phases with higher percentage of free silanol groups. Comparison of the endcapped and non-endcapped columns demonstrated that the residual hydroxyl groups have a clear role in regiospecific separation. Choice of the stationary phase-mobile phase combination depends, for example, on the priority of the analysis time and the resolution (Momchilova et al. 2004a, 2006). However, these studies did not take into account differences between the polymeric and monomeric C_{18} phases.

Kuroda et al. (2008) found that only the non-endcapped polymeric C_{18} column achieved separation of the regioisomeric pair O-O-P/O-P-O at the lowest temperature studied (10 °C). Again, regioisomers P-P-O/P-O-P were separated with a similar column using the same mobile phase acetonitrile/2-propanol/hexane at 25 °C. The optimal temperature was presumably related to the solubility of the analytes in the mobile phase highlighting the importance of column temperature in regioisomeric separation of TAGs. With all chromatographic conditions tested, the isomer with an unsaturated FA in the position *sn*-1(3) was retained more strongly than the symmetric ABA-type isomer (Momchilova et al. 2004a, 2006; Kuroda et al. 2008). According to Kuroda et al., the mechanism of this phenomenon was unclear, and further research with different TAG regioisomers was needed.

In the recent study, six different monomeric and polymeric C_{18}-bonded stationary phases with the mobile phase and temperature conditions described by Momchilova et al. 2004 were tested (Tamba Sompila et al. 2017). A stationary phase with mixed (C_{18} and C_{11}-OH) polymeric bonded silica (Nucleodur C_{18} ISIS) with accessible terminal hydroxyl groups provided good separation of the SSU-type TAG regioisomers containing FAs with 0–6 DBs with a sorter analysis time. This confirmed that the column has an exceptional steric selectivity and that the presence of OH-groups is essential for the regiospecific separation of TAGs.

In theory, a stationary phase similar in chain length to the fatty acyl chains maximizes the interactions, and should offer good resolution. Nagai and others examined the capacity of the C_{28} column for several different regioisomeric pairs with acetone as the mobile phase (Nagai et al. 2011b). TAG regioisomeric pairs containing two C16:0 and one unsaturated FA such as C18:1, EPA or DHA or saturated MCFA C10:0, were almost baseline separated at 10 °C and 15°C with acetone as the mobile phase. However, the regioisomers with two unsaturated FAs and one SAFA were not separated. They also noticed that the retention of regioisomers P-P-O/P-O-P gradually decreased with the increasing column temperature. The resolution of P-P-O/P-O-P was not achieved at 25 °C. Later, the C_{28} column was also applied to separate TAG positional isomers of bovine milk fat, egg yolk, immature egg yolk and chicken meat (Nagai et al.

2015, 2017). The resulted fractions were further analyzed by chiral-phase HPLC to resolve the enantiomers.

Generally, HPLC C_{18} columns with the commonly used dimensions (250 × 4.6 mm, 5 µm) are used in regioisomeric studies of TAGs, despite the lower selectivity for the separation of TAG with the same ECN but different number of DBs (Lísa et al. 2011). Only a few recent studies have been applied with UHPLC columns (Leskinen et al. 2007; Kallio et al. 2017; Tarvainen et al. 2019).

Composition of the mobile phase is crucial to the separation, as the solvent is in interaction with the stationary phase. In the study of Momchilova et al. (2006) different mobile phases for the separation of TAG positional isomers were tested. Binary mixtures of acetonitrile with dichloromethane, tetrahydrofuran or ethanol were found to be the most suitable. For most regiospecific separations of TAGs, acetonitrile or methanol are used as a main component accompanied with a modifier such as acetone or 2-propanol (**Table 3**), mainly in the gradient elution mode. Zeb and Murkovic (2010) tested different organic solvents, acetone, acetonitrile, dichloromethane, 2-propanol, methanol, and propionitrile. They found that 18% 2-propanol in MeOH was the best mobile phase to separate TAGs. They diluted the samples to acetone before adding the mobile phase. Acetone improves the dissolution of oils and the selectivity of critical TAG pairs with the same ECN.

Although the aim of many studies has been the successful separation of isobaric TAGs, there are no universal column-solvent combinations suitable for all sample matrices due to the enormous amount of different TAG species. Usually, the methods have aimed to separate some selected TAG species containing two SAFAs such as C16:0 with one unsaturated FA common in edible oils (Momchilova et al. 2004a, 2006; Šala et al. 2016; Tamba Sompila et al. 2017; Tarvainen et al. 2019) or vaccenic and oleic acids (Leskinen et al. 2010a), or α-linolenic and γ-linolenic acids (Leskinen et al. 2008, 2009). Thus, the analytical conditions have to be carefully considered according to the aim of the study. The chromatographic quantification of TAG regioisomers requires sufficient resolution and is usually based on the relative areas of chromatographic peaks. This kind of approach is better suited for comparison of relative areas than for exact quantification. Although the chromatographic quantification is simple, it neglects the potential differences in the relative responses of TAGs differing in the number of DBs and acyl chain lengths. This approach may lead to systematic errors in the determination of the exact concentration of different TAGs (Holčapek et al. 2005).

2.2.2.3 Detection

As far as detection is concerned, the evaporative light-scattering detector (ELSD) (Février et al. 2001; Momchilova et al. 2004b, 2006; Holčapek et al. 2005; van der Klift et al. 2008), and different mass spectrometric detection methods (Byrdwell and Neff 2002; Leskinen et al. 2007; van der Klift et al. 2008; Holčapek et al. 2009; Řezanka et al. 2010; Gastaldi et al. 2011; Cubero Herrera et al. 2013; Nagai et al. 2013; Baiocchi et al. 2015; Beccaria et al. 2015; Wei et al. 2015; Navarro-Reig et al. 2016; Šala et al. 2016; Watanabe et al. 2016; Kallio et al. 2017; Tarvainen et al. 2019) have been widely employed in LC methods (**Table 3**). The detector available may restrict the choice of the suitable solvent. For example, acetone (UV cut-off 330 λ_c / nm) may cause a drifting baseline if TAGs are detected with an UV detector at 205 nm. Very low wavelengths have to be used, since acylglycerols do not absorb the UV radiation above 220 nm. The UV detector may be the only option if a non-destructive detector is needed. However, large differences between saturated and unsaturated TAGs as well as insufficient sensitivity for saturated TAGs are noticed with UV detection (Holčapek et al. 2005). A refractive index detector does not allow the use of gradient elution.

Consequently, ELSD, although being destructive, seems to be the most suitable for detection of TAGs if MS is excluded. ELSD is sensitive and its clear advantage is the suitability of almost any solvents in complex gradients. The disadvantage of ELSD for quantification is the non-linear response. Holčapek et al. 2005 compared the three typical detection methods used for TAGs; APCI-MS, ELSD and UV. They recommended APCI-MS detection for the analysis and quantification of TAGs due to various advantages. APCI-MS as well as other MS methods widely used for studying regioisomerism of TAGs are reviewed in more detail in chapter "Mass spectrometry".

2.2.3 Normal-phase liquid chromatography

Only a few studies related to normal-phase HPLC (NP-HPLC) of TAG regioisomers have been published (Kalo et al. 2003, 2004, 2006, 2009). Butter fat is among the most complex animal fats, as it contains FAs with a wide range of different chain lengths including odd-chain and unsaturated FAs. Nevertheless, Kalo and others successfully separated TAG regioisomers having short-, medium- and long-chain FAs including TAGs with an odd number of acyl carbons (Kalo et al. 2004, 2009). In NP-HPLC, the stationary phase is more polar than the mobile phase and the less polar analytes elute faster. The retention of TAG molecules is inversely related to the number of carbon atoms. Commonly, the resolution of overlapping chromatographic peaks is a challenge

when the TAGs are analyzed by NP-HPLC. This technique is better suited for separation lipids with different polarities which can be used to separate TAGs from mixtures of MAGs, DAGs and FAs. (Mangos et al. 1999).

2.2.4 Silver-ion chromatography

Silver-ion chromatography was introduced in the 1960s and its applications with TAGs have been reviewed by several authors (Morris 1966; Ruiz-Gutiérrez and Barron 1995; Buchgraber et al. 2004b; Momchilova and Nikolova-Damyanova 2012). Over the decades, silver-ion chromatography has been applied to study TAGs differing in the number (Adlof and List 2004; Lísa et al. 2009, 2011; Holčapek et al. 2010), and position of DBs (Leskinen et al. 2008; Holčapek et al. 2010) as well as in *cis*/*trans* isomers (Macher and Holmqvist 2001). The resolution is based on the weak interactions that are formed between the Ag^+ ions and π-electrons of the DBs. The advantage of this approach is that the exposure to Ag^+ ions does not induce any chemical changes in the TAG structures. Thus, the separated TAG species may be recovered for further analysis, such as RP-HPLC. Moreover, silver-ion chromatography can be accomplished both with TLC systems and HPLC instruments, allowing more versatile implementation in lipid analysis (Momchilova and Nikolova-Damyanova 2012). Subsequently, HPLC columns packed with Ag^+ ions are used for the regiospecific analysis of TAGs.

2.2.4.1 Chromatographic separation mechanism

In silver-ion chromatography, the separation of TAGs occurs mainly according to the total number of DBs in the molecule. In addition, the distribution of DBs among the acyl groups, the configuration and position of the DBs within the acyl groups and the position (a primary or a secondary hydroxyl) at which the unsaturated FA is bound to the glycerol backbone play an important role. The position of unsaturated FA is especially essential in enabling the regiospecific separation. If two TAG species have an equal number of DBs, a molecule in which all or most of those DBs are concentrated into one fatty acyl moiety is retained more firmly by the stationary phase (retention order SAFA-MUFA-MUFA < SAFA-SAFA-PUFA and MUFA-MUFA-PUFA < SAFA-PUFA-PUFA) (Christie 1994). In addition, an unsaturated FA esterified in the position *sn*-2 forms weaker complexes with silver ions than those esterified in the primary positions, because the latter ones are sterically less hindered (Lísa et al. 2011; Řezanka et al. 2017). Retention of TAGs and factors affecting it have been described by Nikolova-Damyanova in 2009.

2.2.4.2 Chromatographic conditions

Considerable effort has been put into developing stable silver loaded columns. These can be prepared according to Christie, who optimized the preparation of a silver-ion HPLC column from a ion-exchange column (Christie 1987, 1988). Based on the same procedure, commercial stationary phases, in which phenylsulfonic acid groups (strong cation exchangers) in the Ag^+ ionic form are chemically bonded to silica, are widely used (Momchilova and Nikolova-Damyanova 2012; Řezanka et al. 2017).

Adlof and List studied the effects of column temperature on the retention of regioisomers (P-P-O/P-O-P, Ln-S-S/S-Ln-S and L-L-O/L-O-L) with ChromSpher Lipids columns (250 × 4.6 mm, 5 μm) using isocratic acetonitrile/hexane as the mobile phase. Regioisomers Ln-S-S/S-Ln-S were separated close to the baseline at 10 °C but the complete baseline separation was achieved at 40 °C (Adlof and List 2004). The resolution was found to depend on the structure of the analytes.

One disadvantage of silver-ion chromatography was that the chlorinated solvents commonly used (Christie 1988) as mobile phases have carcinogenic and mutagenic properties. Nowadays, hexane- and acetonitrile-based solvent mixtures have replaced the chlorinated solvents and have enabled excellent separation of different isomers (Adlof and List 2004; Leskinen et al. 2008; Holčapek et al. 2010). Addition of 2-propanol to the commonly used hexane/acetonitrile mobile phase mixture improve the miscibility, and thus partially solves the problem with the reproducibility in silver-ion systems (Holčapek et al. 2010). For example, Février and coworkers used gradient of hexane/2-propanol/ethyl acetate (820:40:140, v/v/v) and hexane/2-propanol/acetonitrile (956:40:4, v/v/v) for separation of Cy-Cy-L and Cy-L-Cy (Cy, 8:0) regioisomers (Février et al. 2001).

2.2.4.3 Regiospecific analysis of TAGs by silver-ion HPLC

One of the first regiospecific studies dealing with silver-ion HPLC and TAGs of the seed oils rich in α- and/or γ-linolenic acids was published by Laakso and Voutilainen (1996). They described the silver-ion HPLC-APCI-MS analysis of oil from the seeds of cloudberry, evening primrose, borage, alpine currant, and black currant seed oils. Unfortunately, only partial separation within equally unsaturated TAGs was obtained, and identification of regioisomers was based on the ratio of DAG ions. Leskinen et al. applied silver-ion chromatography for identification of the regioisomeric structure of TAGs αLn-L-L and γLn-L-L in black currant seed oils (Leskinen et al. 2008, 2009). They used self-made silver-ion columns and gradient elution using acetone and acetonitrile as

solvents with MS detection. They found that γLn-L-L was retained slightly longer in the column but eluted still in the same chromatographic peak. Thus, αLn-L-L and γLn-L-L were not fully separated but the separation was enough for regiospecific analysis with MS.

Lísa et al. enhanced separation remarkably by using three commercial silver-ion columns in the total length of 75 cm with a silver-ion HPLC-APCI-MS instrumentation (Lísa et al. 2009). The solvent system consisted of hexane/2-propanol/acetonitrile (99.8:0.1:0.1, v/v/v) and hexane/2-propanol/acetonitrile (96:2:2, v/v/v). Partial separation of TAG regioisomers with four to seven DBs was reported, and obvious differences between plant oils and animal fats were demonstrated. For example, in TAGs composed of FAs P, O, and L the ratio of regioisomers O-L-P/L-O-P/O-P-L in sunflower oil was = 63:36:1, but totally opposite in the lard sample i.e. 3:12:85.

Only a limited number of silver-ion HPLC analyses have been performed with complex natural oils such as fish oil (Laakso and Christie 1991). Regioisomers with a high number of DBs present problems (Řezanka et al. 2017). With complex mixtures, RP-HPLC and silver-ion chromatography can be used sequentially. Due to the different separation mechanism of silver-ion chromatography compared to RP-HPLC, these two techniques complement each other. However, they are unable to separate enantiomers of TAGs and silver-ion HPLC does not result in complete separation of TAGs with the same number of DBs or the number of DBs over 5. Several silver-ion columns in series is usually applied to improve resolution of TAG isomers (Holčapek et al. 2010).

2.2.5 Chiral-phase liquid chromatography

The applications of chiral-phase liquid chromatographic separations in stereospecific analysis of TAGs started with HPLC resolution of MAGs (Takagi and Itabashi 1985; Itabashi and Takagi 1986; Turon et al. 2002) and DAGs (Laakso and Christie 1990; Christie et al. 1991; Takagi and Ando 1991; Ando and Takagi 1993; Itabashi et al. 1993). They were separated either using chiral derivatives (Laakso and Christie 1990; Christie et al. 1991) or chiral stationary phases (Takagi and Ando 1991; Ando and Takagi 1993; Itabashi et al. 1993). Both methodologies had some limitations. The chiral derivatizing reagent has to be enantiopure and the presence of the derivatizable groups in the analyte was a prerequisite. Especially *sn*-1,3-DAGs were prone to acyl migration, when DAGs were derivatized after partial hydrolysis (Christie et al. 1991). In that sense, the direct resolution using the chiral column, which is the most widely used in analytical chemistry (D'Orazio et al. 2017), was more

convenient and also applicable for separations on a preparative scale, despite the expensive chiral stationary phases. The third option was to use chiral mobile phases. This approach is a simple and flexible alternative, but is not always applicable and is not used for regioisomeric studies of TAGs according to the literature.

2.2.5.1 Chromatographic separation mechanism

The retention behavior of TAG enantiomers in chiral-phase HPLC is highly complex. Enantiomeric separation depends on the intermolecular forces in specific molecular structures and chromatographic interactions. In 1952, Dalgliesh has already proposed a “three-point interaction model” to explain the enantioselective separation of amino acids on cellulose paper (Dalgliesh 1952). In this model, chiral recognition is based on interactions between the three different substituents attached to the chiral center of compound and a chiral stationary phase as illustrated in **Figure 4** (Pirkle and Pochapsky 1989; Rocco et al. 2013).

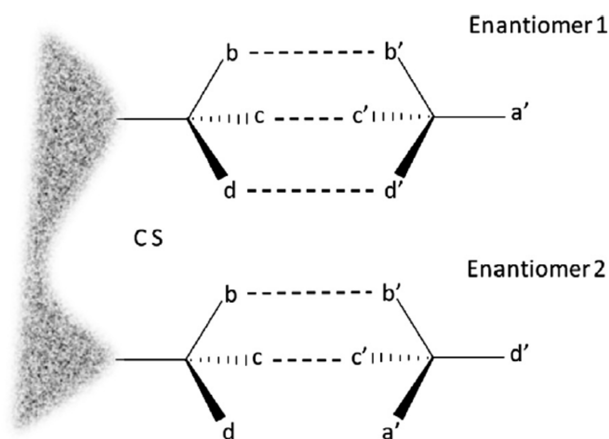


Figure 4. The three-point interaction model. b, c, and d are the interaction sites of the chiral selector (CS). a’, b’, c’, d’ are the interaction sites of the enantiomers 1 and 2 (Rocco et al. 2013).

The enantiomers 1 and 2 bind the chiral selector to its three sites with different stability, due to the spatial configuration of the substituents. Enantiomer 1 joins the chiral selector with three of its substituents, while the enantiomer 2 (mirror image) has only two proper interactions. In a chromatographic analysis, the retention time of enantiomer 1 is longer and, thus, they are separable. (Pirkle and Pochapsky 1989; Rocco et al. 2013;

D'Orazio et al. 2017). In a modern chromatographic system interactions are based on strong Coulomb, hydrogen, π–π, ion-dipole, dipole–dipole, and van der Waals attractive interactions and, also steric hindrance as repulsion is considered as an effect for the enantiomeric separation (Berthod 2006). Although the three-point interaction model is widely accepted, the model has also been challenged, and in the case of TAG enantiomers many aspects such as the stationary phase, mobile phase, physicochemical properties and the multidimensional structure of TAGs have to be considered.

Nagai et al. used asymmetric *sn*-PPO to obtain an idea about the enantioselective mechanism of TAGs. The two palmitic acids bound in the positions *sn*-1 and *sn*-2 might show different hydrophobic interactions with the chiral stationary phase compared to the oleic acid in the position *sn*-3. These three kind interactions may cause chiral separation of *rac*-PPO. This theory also explains the chiral separation of *rac*-OOP and *rac*-PPL. However, the enantiomers of *rac*-OOL and *rac*-PPS were not separated. It is also important to note that *rac*-PPO and *rac*-OOP have the same ECN (48) and very similar chromatographic behavior measured as retention times. Nevertheless, enantiomers of *rac*-PPO were baseline separated, whereas those of *rac*-OOP were not. Evidently differences in the acyl chain length and/or the number, configuration and position of DBs in the asymmetric TAG molecule affect the interactions (Nagai et al. 2011a). This conclusion is supported by a recent study where a deviant elution behavior in the chiral environment of TAGs containing highly unsaturated VLCFAs, was observed (Řezanka et al. 2018). The highly-bent structure of highly unsaturated VLCFAs may also cause more steric hindrances for stereospecific interactions between the chiral selector and stereoisomers. However, in stereoisomeric separation, repulsion and attraction are not opposites. Interactions between chiral selectors and enantiomers depend on the total strength of repulsions and attractions. (Berthod 2006).

In 2015, Nagai and others separated enantiomers of TAGs containing two C16:0 and a SAFA C4:0–C12:0, which was the first report, and according to the literature, the only study, where trisaturated TAGs were enantioseparated. A TAG containing two C16:0 and a short chain SAFA, C4:0, was almost baseline separated in 70 min, whereas TAG enantiomers containing C12:0 was separated as a small shoulder visible after 115 min (Nagai et al. 2015). It was clearly shown that the differences in chain lengths facilitated the enantioseparation with the chromatographic conditions they used. The study of Nagai and others demonstrated that the difference in the length of the saturated acyl chain was also recognized by the chiral stationary phase (Nagai et al. 2015), and the presence of both SAFAs and unsaturated FAs was not always a requirement for enantiomeric separation.

2.2.5.2 Chromatographic conditions

The direct resolution of TAG enantiomers had long remained a challenge. Iwasaki and co-authors achieved the resolution of enantiomers containing C8:0 (Cy) and EPA or DHA, in combinations uncommon in nature on a chiral stationary phase CHIRALCEL OD [cellulose tris-(3,5-dimethylphenylcarbamate)-impregnated silica] (**Table 4**) (Iwasaki et al. 2001). The enantiomer with an unsaturated FA in the position *sn*-1 eluted first. On CHIRALCEL OF [cellulose tris-(4-chlorophenylcarbamate)-impregnated silica] column, the elution order was reversed compared to the OD column and the enantiomeric separation of DHA-Cy-Cy/Cy-Cy-DHA was unsuccessful.

Ten years later, Nagai et al. introduced an enantiomeric resolution of naturally occurring TAGs containing C16:0, C18:1 and C18:2 using chiral-phase HPLC in which the sample was recycled through the same column [CHIRALCEL OD-RH cellulose tris-(3,5-dimethylphenylcarbamate) coated on silica gel] several times (Nagai et al. 2011a). The method was applied to study enantiomeric ratio of P-O-O/O-O-P in palm oil. An enantiomer with unsaturated FA in the position *sn*-1 eluted first when *rac*-POO, *rac*-PPO and *rac*-PPL were studied. Trisaturated (*rac*-PPS) and triunsaturated TAGs (*rac*-OOL) were not enantioseparated. The same stationary phase-mobile phase combination with a recycling system was later applied to resolve enantiomers of TAG extracted from fish oil, marine mammal oil, bovine milk, egg yolk, chicken meat, and different structured TAGs (Nagai et al. 2013, 2015, 2017). The chiral-phase HPLC method was further developed to achieve the resolution of TAG regioisomers and enantiomers at the same time. Lísa and Holčapek (2013) selected non-polar mobile phase due to better solubility of non-polar TAGs. They achieved a resolution of TAG isomers containing 1–8 DBs and different chain lengths using two columns with a rather similar cellulose-based stationary phase as used by Nagai et al. (2011a) – only the manufacturer (**Table 4**) and the particle size were different (3 µm vs. 5 µm). Furthermore, a hexane/2-propanol gradient was applied instead of methanol (Lísa and Holčapek 2013). They were able to determine both regioisomeric and enantiomeric ratio of some TAGs in hazelnut oil and human plasma using a gradient elution in normal-phase mode. Moreover, triunsaturated TAGs such as *rac*-OOL and triacid-TAGs such as A/Ln/O were separated into enantiomers. They observed that better separation of isomers was achieved with hexane/2-propanol than with the hexane/acetonitrile mixture. They also noticed that separation temperature does not show so significant trend in the resolution compared to NARP-HPLC analysis of TAGs. However, specific co-elution problems existed in analysis of TAG enantiomers with a combination of SAFAs and PUFAs in the positions *sn*-1 and *sn*-3 such as P-P-L and P-L-O. In

contrast, in the earlier study of Nagai and others, enantiomers of *rac*-PPL were baseline separated. Due to the co-elutions, several columns in the series or more sophisticated stationary phases are needed. Nevertheless, not all the TAGs are separated using simultaneous separation, and it is impossible to determine the enantiomeric ratio of all enantiomers even when extracted ion chromatograms of each mass-to-charge ratio are used (Nagai et al. 2019) highlighting the complexity of chiral analyses of TAGs. Ultimately, it is also important to notice that the different phases and chromatographic approaches applied so far have their own advantages in the separation of stereoisomers in TAGs. In the current state of art, there is no single method which can be applied for stereospecific separation of all TAGs in natural fats and oils.

Table 4. Examples of chiral-phase HPLC analyses of TAG enantiomers.

Material	Chiral stationary phase	Mobile phase	TAGs enantioseparated[b] (examples)	Enantio-separation unsuccessful[c] (examples)	Ref.
Structured TAGs	CHIRALCEL OF cellulose tris-(4-chlorophenylcarbamate) impregnated silica	Hexane/ IPA[a]	Cy-Cy-EPA	DHA-Cy-Cy with OF column	Iwasaki et al. 2001
	CHIRALCEL OD cellulose tris-(3,5-dimethylphenylcarbamate) impregnated silica		DHA-Cy-Cy, EPA-Cy-Cy		
Structured TAGs, palm oil	CHIRALCEL OD-RH cellulose tris-(3,5-dimethylphenylcarbamate)-coated silica	MeOH	O-O-P, P-P-O, L-P-P	O-O-L, P-P-S	Nagai et al. 2011a
Diatom *Phaeodactylum tricornutum* and structured TAGs	Astec CYCLOBOND™ I 2000 DMP (3,5-dimethylphenyl carbamate-modified β-cyclodextrin)	MeOH:10 mM ammonium acetate	EPA-P-P, EPA-EPA-P		Řezanka et al. 2012
Plasma, hazelnut oil, structured TAGs	2 × Lux Cellulose-1 cellulose tris-(3,5-dimethylphenylcarbamate)-coated silica	Hexane/ IPA gradient	L-O-O, S-O-O, L-L-O	L-P-P, L-L-P, P-P-O, P-O-O, S-S-O	Lísa and Holčapek 2013
Yeast strain	2 × Astec CYCLOBOND™ I 2000 DMP 3,5-dimethylphenyl carbamate modified β-cyclodextrin bonded to silica	Hexane/ IPA gradient	Po-P-P, Po-O-O, P-Po-Po, O-Po-Po, P-P-L, P-L-L	P-P-S	Řezanka et al. 2013
Fish, marine mammal	CHIRALCEL OD-RH cellulose tris-(3,5-dimethylphenylcarbamate)-coated silica	MeOH	DHA-P-P, DHA-DHA-P, EPA-P-P		Nagai et al. 2013
	CHIRALCEL OZ-3R cellulose tris-(3-chloro-4-methylphenyl-carbamate)-coated silica		P-EPA-EPA		

Material	Chiral stationary phase	Mobile phase	TAGs enantioseparated[b] (examples)	Enantio-separation unsuccessful[c] (examples)	Ref.
Bovine milk (butter)	CHIRALCEL OD-3R cellulose tris-(3,5-dimethylphenylcarbamate)-coated silica	MeOH	P-P-Bu, P-P-Co, P-P-Cy, P-P-Ca, P-P-La		Nagai et al. 2015
Protozoan, mold, structured TAGs	Astec CYCLOBOND™ I 2000 DMP 3,5-dimethylphenyl carbamate-modified β-cyclodextrin	Hexane/ IPA gradient	A-L-L, Ln-L-L, EPA-L-L		Řezanka et al. 2015
Egg yolk, immature egg yolk, chicken meat	CHIRALCEL OD-3R cellulose tris-(3,5-dimethylphenylcarbamate)-coated silica	MeOH	L-P-P, O-O-P, O-P-P		Nagai et al. 2017
Ximenia oil, alga, yeast, dinoflagellate, structured TAGs	2 × Astec CYCLOBOND™ I 2000 DMP 3,5-dimethyl-phenyl-carbamate-modified β-cyclodextrin	Hexane/ IPA gradient	TAGs containing VLCFAs such as 24:0- or 24:1- or 30:1-18:1-18:1		Řezanka et al. 2018
Structured TAGs	CHIRALPAK IF-3 amylose tris-(3-chloro-4-methylphenylcarbamate) immobilized silica	ACN	L-L-P, O-O-P, L-P-P, O-P-P, O-O-S, L-S-S, O-S-S, L-L-S, P-S-O, S-O-L	P-O-L, P-P-S, O-O-L	Nagai et al. 2019

[a]MeOH=methanol; IPA=2-propanol; ACN=acetonitrile
[b]The first eluting enantiomer of enantiomeric pair is mentioned
[c]Either another enantiomer or regioisomer co-eluted

2.2.5.3 Chiral selectors and stationary phases

Enantiomeric separation of TAGs has been performed mainly on polysaccharide-based chiral stationary phases (Iwasaki et al. 2001; Gotoh et al. 2011b; Nagai et al. 2011a; Řezanka et al. 2012; Lísa and Holčapek 2013) of which cellulose tris-(3,5-dimethylphenylcarbamate) and cellulose tris-(3-chloro-4-methylphenylcarbamate) have been the most common polysaccharide derivatives. Gotoh and co-authors screened nine polysaccharide-based chiral stationary phases with different chiral selectors to separate the enantiomers of *rac*-PPO. Only CHIRALCEL OD-RH silica column coated with cellulose tris-(3,5-dimethylphenylcarbamate) resulted in enantiomeric separation when methanol was used as the mobile phase. Baseline separation was not achieved without sample recycling (Gotoh et al. 2011b).

In some studies, instead of the derivatized cellulose phases, β-cyclodextrin-based chiral phases have been applied (Řezanka et al. 2012, 2013, 2015). The stationary phase with chiral oligosaccharide β-cyclodextrin modified by tris-(3,5-dimethylphenylcarbamate) enabled the separation of TAG enantiomers P-P-L/L-P-P and L-L-P/P-L-L (Řezanka et al. 2013), which were co-eluted in the previous study (Lísa and Holčapek 2013).

In a recently reported method (Nagai et al. 2019), much faster enantiomeric separation was achieved using amylose-based immobilized chiral stationary phase [CHIRALPAK IF-3 amylose tris-(3-chloro-4-methylphenylcarbamate)]. Another advantage of this type of chiral-phase column was that it enabled the use of wider range of organic solvents, which may be useful in lipid analysis. However, trisaturated or triunsaturated TAGs (e.g. *sn*-PSP/*sn*-PPS/*sn*-SPP and *sn*-OLO/*sn*-OOL/*sn*-LOO) were not separated.

Despite the versatility of the cellulose tris-(3,5-dimethylphenylcarbamate)-coated silica, better resolution of TAG enantiomers EPA-EPA-P/P-EPA-EPA was achieved with the stationary phase cellulose tris-(3-chloro-4-methylphenyl-carbamate)-coated silica (Nagai et al. 2013). The enantiomer with an unsaturated FA in the position *sn*-3 eluted earlier, with the same mobile phase, which was an opposite elution order compared to results obtained with a commonly used stationary phase cellulose tris-(3,5-dimethylphenylcarbamate)-coated silica. The main difference between these two stationary phases was that the functional groups bound in the phenyl group of OD-RH are two methyl groups, whereas in the OZ-3R column these functional groups are a methyl group and a chloride group. The methyl groups have electron-donating characteristics, while the chloride group is electron withdrawing. Therefore, the electron density on the phenyl group may affect the elution order of TAG enantiomers (Nagai et al. 2013).

It has been shown that in the NP-mode of chiral-phase chromatography, TAGs tend to elute in the order of the descending ECN (Lísa and Holčapek 2013; Řezanka et al. 2013, 2013). Furthermore, the TAG retention increases with increasing unsaturation, and also the length of the acyl chains has a crucial impact on retention. It has been demonstrated that TAGs containing more DBs have unusual chromatographic elution behavior. According to Řezanka and others, the main reason was the spatial arrangement of very long carbon chains in the TAG molecules (Řezanka et al. 2018). They noticed that the elution order of TAGs containing at least one VLCFA were different compared to TAGs with MCFAs or LCFAs. Even 20 min difference in the retention times of TAGs with the same ECN was found when TAGs containing at least one VLCFA were chromatographed on two chiral (3,5-dimethylphenyl carbamate modified β-cyclodextrin) columns, using a gradient of hexane and hexane/2-propanol. The *cis*-unsaturated DHA tails in TAGs are very bent compared to TAGs containing mono-, di- or triunsaturated FAs. The retention behavior of TAGs is complicated if there are three different FAs participating in the interaction, and thus a combination of different retention mechanisms. The order of elution must be established by an analysis of the pure enantiomeric reference compounds of a known configuration because there is only a limited amount of knowledge available about the chiral elution behavior of TAGs.

Despite the huge progress in chiral separation of individual TAGs, all the methods applied to separate regioisomers and enantiomers simultaneously have limitations. TAGs of some FA combinations were not separated at all. Therefore, more knowledge about the retention behavior of TAGs in a chiral environment as well as about the development of chiral-phase chromatography requires further study. To date, the choice of the stationary phase-mobile phase combination has been mainly based on trial and error. Deeper knowledge of the chromatographic elution behavior of chiral compounds could aid interpretation of the molecular recognition mechanisms. The clear advantage of chiral-phase HPLC is that it enables the number of identified TAGs to be significantly increased compared to NARP-HPLC.

2.2.6 Techniques to improve resolution of regioisomers and enantiomers

Due to the enormous number of different TAG species in natural samples, there is a common need to improve chromatographic separation. When the separation capacity of a single column is exceeded, the widely-used option is to increase the amount of stationary phase i.e. to use a longer column or more often serially-coupled columns (Holčapek et al. 2003, 2005; Kalo et al. 2009;

Leskinen et al. 2010b; Beccaria et al. 2014; Watanabe et al. 2016). Usually, if the aim is to separate TAGs with the same ECN or TAG regioisomers, a proper resolution is only obtained with multiple column couplings. This increases the backpressure of the system, the total time of analysis, and the solvent consumption.

To overcome challenges with high backpressure, which may limit the number of columns connected in series, recycling HPLC (R-HPLC) can be applied to enhance the separation efficiency and to enable separation of the critical pairs by allowing the small retention time differences to accumulate. By using a recycle valve it is possible to increase the resolution without increasing the length of the single column and the backpressure. The same principle of continuous chromatography is also known as the simulated moving columns technique (Zhang et al. 2007). R-HPLC has been applied to separate TAG isomers, both regioisomers (Kuroda et al. 2008; Gotoh et al. 2010; Watanabe and Yoshinaga 2017) and stereoisomers (Gotoh et al. 2011b; Nagai et al. 2011a, 2015) including the peak purity assessment (Trone et al. 2006). The main restriction of the R-HPLC method is the length of separation time. In theory, recycling can be continued until the desired resolution is achieved but the peak broadening is another limiting factor. According to Liu and others, there is less peak broadening and better separation offered with two identical columns compared to a single-column based method (Liu et al. 2014).

2.2.7 Multidimensional liquid chromatography

Multidimensional liquid chromatography (MD-LC) allows separation of complex TAG mixtures like fish oil by using more columns (usually two) with different stationary phases. The coupled columns allow fractions from the first column (1st dimension) to be selectively transferred to the other column (2nd dimension) for additional separation. This significantly increases the separation power compared to the traditional one-dimensional chromatography. Cacciola et al. (2017) presented different modes used in MD-LC of food analysis, and also their advantages and drawbacks were reviewed. In TAG analyses, the mainly applied combinations are silver-ion/RP-HPLC and RP-/silver-ion HPLC (Laakso and Christie 1991; Dugo et al. 2004; Mondello et al. 2005, 2011; Dugo et al. 2006b; Holčapek et al. 2009; Hu et al. 2013; Beccaria et al. 2015; Navarro-Reig et al. 2016), both in online and offline mode. In the offline technique, the eluent is temporarily fractioned, and then part or all of it is re-injected into the second column. In the online technique, part or all of the eluent is directed into the column via the sample loop.

Pioneering research on MD-LC analysis of TAGs was carried out by Laakso and others (Laakso and Christie 1991; Laakso et al. 1992). An offline MD-LC with complementary techniques made it possible to separate multiple TAG species from herring oil (Laakso and Christie 1991). In addition, Laakso et al. studied the saturation of fractions from butter fat with silver-ion chromatography as a 1st dimension and RP-HPLC as a 2nd dimension but no regiospecific analyses were performed in these studies. The same chromatographic methods in reverse order were applied to analyze TAG regioisomers of rice oil (Dugo et al. 2004). Identification was based on DAG ions derived from APCI-MS detection. Despite the challenges related to reproducibility of retention times with silver-ion columns, the method was applicable, and for example the regioisomeric ratio of P-P-O/P-O-P in rice oil was found to be 14:86.

2.2.7.1 Offline MD-LC

During the last 20 years, several offline MD-LC applications have been reported by using RP and silver-ion couplings (Dugo et al. 2006b; Holčapek et al. 2009; Hu et al. 2013). Dugo et al. (2006b) demonstrated the presence of tallow (5% beef adipose tissue) in lard (pig adipose tissue) through the study of the regioisomeric ratio (P-P-O/P-O-P) by MD-LC-APCI-MS. Identification of the isomers was based on both the elution order of TAGs on the silver-ion column and the observation of the intensities of DAG ions in the MS spectra. Holčapek et al. (2009) demonstrated that the silver-ion LC mode enabled at least partial resolution of TAG species including *cis-/trans*-isomers. RP- (on two columns) and silver-ion HPLC-APCI-MS (on three columns) were combined to determine these isomeric TAGs in the reference samples: black currant oil and beef tallow. Hu et al. (2013) developed an offline MD-LC system coupling the NARP-HPLC and the silver-ion HPLC methods and APCI-MS detection, which was applied to peanut oil samples. The co-eluted TAGs with the same ECN in the 1st dimension obtained better separation in the 2nd dimension due to the improved resolution and peak capacity of MD-LC. Consequently, a total of 48 TAGs including regioisomers were successfully separated and identified. Kadivar and others applied MD-LC to determine undesired asymmetric TAGs during the production of cocoa butter equivalent. Separation of the regioisomers was obtained by combining NARP- and silver-ion HPLC (Kadivar et al. 2013).

2.2.7.2 Online MD-LC

Beccaria and others compared offline MD-LC results achieved with a menhaden oil sample to results obtained with the online stop-flow mode, in terms of peak capacity and analysis time (Beccaria et al. 2015). The use of APCI-MS detection for both LC approaches and FAME analyses by GC supported the TAG determination. The offline mode transpired to be the better option, yielding higher peak capacity and thus, more than 250 TAGs from fish oil were identified. The main drawback was the longer analysis time.

The online connection of RP and silver-ion columns supposedly simplify technically the offline analysis (Mondello et al. 2005; Dugo et al. 2006a; van der Klift et al. 2008). However, a thorough optimization for both dimensions separately and together has to be performed before good separation can be achieved. Mondello (rice oil) and Dugo (soybean and linseed oil) with others applied a micro-bore column (1 mm i.d.) packed with C_{18}, flushed with a 1 M aqueous silver nitrate solution in the 1st dimension and a monolithic C_{18} column in the 2nd dimension (Mondello et al. 2005; Dugo et al. 2006a). The advantage of the use of a micro-bore column in the 1st dimension was that the delivery of incompatible solvents to the 2nd dimension could be avoided (Indelicato et al. 2017). Van der Klift et al. (2008) used a similar approach. The stationary phase in the 1st dimension was a cation exchanger coated with silver ions and C_{18} was the phase in a very short column in the 2nd dimension. Such MD-LC system was successfully employed for the detection of 44 TAGs in corn oil. More recently, the same sample material was investigated in terms of TAG regioisomers by Navarro-Reig et al. (2016) by using a chemometric approach. The combination of MD-LC with the multivariate curve resolution method significantly improved the separation power of the MD-LC technique.

Some advantages of the online MD-LC methods compared with offline ones are the short analysis time, reproducibility and automation. On the other hand, there has to be an interface to transfer the eluate from the first column to the second column, and the two methods have to be compatible. Usually, with MD-LC, compromises have to be made with the 1st dimension as it should enable both an acceptable separation and sufficient sampling of each resolved peak leaving the first column (Mondello et al. 2005). In a gradient elution mode, the column equilibration time has been considered to find the optimal balance between the elution mode and the flow rate. On that basis, the optimization and operation may be complicated. In the case of the offline mode, the time for separation is not so restricted but the total time of analysis is longer due to the collection and the re-injection of the fractions, and the possible solvent exchange increases the solvent consumption.

2.2.7.3 Regiospecific analysis of TAGs by MD-LC

A well optimized MD-LC method is an effective tool for a thorough analysis of the TAG profile of complex food matrices. MD techniques applying RP-HPLC and silver-ion HPLC significantly improve the number of separated compounds, because the separation mechanisms are complementary. So far, MD-LC techniques have been reported as being mainly used for regioisomeric separations of TAGs. However, an achiral RP-HPLC separation prior to the chiral analysis of TAGs is commonly used. Nagai et al. achieved resolution of the regioisomers in natural oils by RP-HPLC on an octacosyl (C_{28}) column followed by chiral-phase HPLC equipped with a recycling system to resolve enantiomers of TAGs of palm oil, fish oil, marine mammal oil, and bovine milk (Nagai et al. 2011a, 2013, 2015). Moreover, in chiral analysis there is a great demand for MD chromatography (Ali et al. 2019). However, the sensitive chiral-phase columns have some restrictions in relation to the mobile phases and the maximum pressure, which makes it challenging to combine it with an online mode.

2.2.7.4 Mixed-mode liquid chromatography

Due to the challenges related to combining two chromatographic separation modes in MD chromatography, mixed-mode chromatography has received attention for characterization of TAGs in complex natural matrixes in recent years (Wei et al. 2013, 2015; Hu et al. 2014). A stationary phase consisting of two or more retention mechanisms, usually reversed-phase and ion-exchange, solves the problems of MD chromatography such as long analysis time and solvent incompatibilities.

Wei and coworkers (2013) illustrated the separation efficiency of mixed-mode chromatography by profiling TAGs. With C_{18} a column, TAGs with the same ECN values were co-eluted, whereas with silver-ion column TAGs with the same number of DBs were co-eluted. For unsaturated γLn-Ln-Ln the retention time was nearly 300 min. The mixed-mode column, packed with octyl and sulfonic co-bonded silica modified with silver-ions (Ag-HiSep OTS) combines the features of a C_8 RP-column and a silver-ion column. It provides hydrophobic interactions as well as π-complexation interactions, and the twelve reference compounds of TAGs with C16–C20 FAs with 0–3 DBs (ECN 36–52) were separated in 20 min. For example, the baseline resolution was achieved for TAGs with the same ECN (P-P-O, O-O-P and S-O-L) but the regioisomers were not determined. The method was applied for the determination and quantification of thirty TAGs in peanut oils, 18 TAGs in corn oils, and 21 TAGs in soybean oils.

In recent studies, promising results were achieved using a phenyl-hexyl column to profile TAGs in edible oils (Hu et al. 2014; Wei et al. 2015). The phenyl-hexyl column combines the features of traditional C_{18} and silver-ion columns that could provide hydrophobic interactions with TAGs under acetonitrile mobile phase conditions and offer π-π interactions with TAGs under methanol conditions. The MD-LC approach using a single column was achieved by altering the mobile phase between acetonitrile and methanol, which exhibited a higher selectivity for the separation of TAGs. The method reported by Wei and others (2015) was improved compared to the earlier research of the same group (Wei et al 2013). TAGs with the same ECN (P-P-O, O-O-P and S-O-L) were well separated and a total of 50 TAGs in peanut oils and 40 TAGs in soybean oils was identified and quantified (Wei et al. 2015). Unfortunately, regioisomers were still neglected, and Hu et al. (2014) reported that co-elutions with phenyl-hexyl column prevent the regioisomeric determination of P-O-O and O-P-O.

A recent study reported good separation of the SSU-type TAG regioisomers containing FAs with 0–6 DBs using a stationary phase of mixed (C_{18} and C_{11}-OH) polymeric bonded silica (Nucleodur C_{18} ISIS) (Tamba Sompila et al. 2017). An offline MD-LC-APCI-MS method with a single mixed-mode column has proven to be a simple and promising technique for analysis of complex TAGs in plant oils. Furthermore, these methodological approaches offer a potential application for complex TAG profiling when the separation of regioisomers is also improved.

2.2.8 Gas chromatography

Until the 1980s, GC and TLC were the principal chromatographic methods in lipid research. After that, the separation power of different LC methods and the structural information offered by MS have revolutionized the structural characterization of lipids and also allowed the studies of individual TAGs in the mixtures. Nevertheless, a gas chromatograph with a flame-ionization detector (GC-FID) is an essential tool to study the fatty acid methyl esters (FAMEs) derived from TAGs. Different acid or base-catalyzed methylation procedures are available (Christie 1997; Seppänen-Laakso et al. 2002). The chromatographic method is quite simple and robust, and offers a linear response over a wide range of chain lengths of typical FAMEs. Analysis of the FA composition is also essential in regio- and stereospecific analyses in order to give an overall picture of the composition.

Originally, high-temperature GC (HTGC) i.e. separation above 300 °C was used in analysis of TAGs (Kuksis and McCarthy 1962; McCarthy et al. 1962).

However, the first challenge of this method is to find a stable column coating material (Mayer and Lorbeer 1997; Aichholz and Lorbeer 1998; Andrikopoulos 2002). Even so, the drifting baseline, the fragmentation of the peaks and even of the analytes at elevated temperatures complicate the data analysis, and have to be taken into consideration in the data handling (Mayer and Lorbeer 1997). Another challenge is to find a proper injection method as TAGs are not easily volatilized and, thus, sample discrimination may occur (Buchgraber et al. 2004a).

Breckenridge and Kuksis determined the molecular weight distributions of the milk fat TAGs of human, cow, goat, horse, guinea pig, dog and sheep (Breckenridge and Kuksis 1967). Řezanka and Mareš separated and identified several TAGs from different dietary plant oils, and also some TAGs with the same ECN were separated (Řezanka and Mareš 1991). Only a limited number of regioisomers have been separated using HTGC (Kemppinen and Kalo 1998), and no studies of enantiomeric separation have been published. In conclusion, current reviews (Andrikopoulos 2002; Buchgraber et al. 2004b; Řezanka et al. 2017) conclude that GC is the most restricted chromatographic separation method for TAGs, and only recommended for analyses of relatively saturated samples (Ruiz-Samblás et al. 2015).

2.2.9 Supercritical fluid chromatography

In the late 1980s and 1990s supercritical fluid chromatography (SFC) was mainly used for TAG characterization because, for example, non-polar stationary phases only separate TAGs into groups according to the ACNs. More polar stationary phases enable the separation of TAGs according to the degree of unsaturation. (Laakso 1996). The methodology and its applicability in lipidomics have been reviewed by Laboureur et al. (2015). Combining SFC with MS has been applied to get regiospecific information in addition to the degree of unsaturation. Kallio et al. (1989) combined SFC with EI-MS for the discrimination between the FAs in the position *sn*-2 and positions *sn*-1/3 in TAG of butter fat on low-polarity GC stationary phase. Manninen and others applied a more polar stationary phase to separate berry oil TAGs containing FAs with differences in the position of the DBs such as α- and γ-linolenic acids using FID (Manninen et al. 1995) and APCI-MS detection (Manninen and Laakso 1997).

Recently, an effective SFC-QqQ-MS method with C_{30} RP stationary phase was developed to profile TAGs, including six regioisomeric TAG pairs (P-P-Ln/P-Ln-P, P-P-L/P-L-P, P-P-O/P-O-P, S-P-Ln/S-Ln-P, S-P-O/S-O-P, S-S-O/S-O-S) in edible oils (Lee et al. 2014). In SFC, the flow rate and the ratio of

modifier have to be optimized carefully as they significantly affect the separation of compounds. The clear advantage of the SFC method is its low-cost and eco-friendly analytical conditions as it uses a pressurized gas, usually CO_2, above its critical temperature and critical pressure as a mobile phase. In the future, there could be more applications of SFC in lipidomics also in the stereospecific analysis of TAGs; this is because the newly established ultra-performance convergence chromatography QTOF-MS system using compressed CO_2 as the primary solvent has been applied to characterize TAGs of cow milk (Zhou et al. 2014) and human milk (Zhang et al. 2019). This method that clearly serves an advanced tool for the determination of complicated TAGs can also be used for chiral separations but it has not yet been applied to study the TAG enantiomers.

2.2.10 Mass spectrometric methods for analysis and detection

LC-MS is the most widespread technique for structural analysis of TAGs, and represents 45% of the recently reviewed literature related to determination, characterization and quantification of TAGs (Indelicato et al. 2017). The use of LC-MS provides clear advantages for analyzing complicated TAG mixtures obtained from natural matrices because of the combination of the separation power of LC and the structural information, high sensitivity and accuracy of MS (Xu et al. 2018). Thus, HPLC-MS has been widely used for both targeted and untargeted analyses. MS generate ions useful for structure elucidation in addition to giving a measure of the amount present (Laakso 2002; Cozzolino and Giulio 2011; Xu et al. 2018; Balgoma et al. 2019). Another advantage is that co-elutions do not limit the identification of TAGs, if the extracted chromatograms can be used. However, the most challenging aspect of the MS methods is the identification of isobaric and isomeric TAGs, whose masses are identical and often produce similar fragmentation patterns.

2.2.10.1 Analysis mechanism

While the analysis mechanisms of chromatographic methods used for regiospecific analysis of TAGs can be different, all MS methods are essentially based on the energetically favored neutral loss of an FA from *sn*-1/3 positions in order to identify the TAG regioisomers. It had already been noticed in 1960 that the cleavage of capric acid (C10:0) from the positions *sn*-1/3 was more favored than the cleavage of lauric acid (C12:0) from the position *sn*-2 (Ryhage and Stenhagen 1960). The ability of MS to distinguish regioisomers with APCI was demonstrated by Mottram and Evershed (1996). The major advantage of MS for regioisomeric studies of TAGs is to generate ions that provide

information on the molecular weight, the fatty acyl residues and their molecular associations, and the positional distribution of FAs between *sn*-1/*sn*-3 and *sn*-2 positions (Laakso 2002). Although MS is unable to separate enantiomers due to their identical mass spectra, after enantiomeric LC separation, for example, MS analysis can be used to determine whether two compounds are enantiomers or not.

2.2.10.2 Direct exposure MS

The first regioisomeric MS analyses of TAGs were carried out without chromatographic separation using direct exposure MS methods (Ryhage and Stenhagen 1960; Currie and Kallio 1993; Kallio and Currie 1993; Mottram and Evershed 1996). In the early 1990s a direct inlet ammonia negative ion chemical ionization (NICI) method was developed (Currie and Kallio 1993; Kallio and Currie 1993) and further optimized (Laakso and Kallio 1996; Kurvinen et al. 2001; Fabritius et al. 2020) to study the TAG regioisomerism. The samples were introduced via a direct exposure probe into the ion source without chromatographic separation. A strong base, ammonia, was capable of deprotonating TAG molecules by NICI. $[M - H]^-$ ions were collided with an inert gas such as argon producing fragments $[M - H - RCOOH - 100]^-$, $[M - H - RCOOH - 74]^-$, and $[M - H - RCOOH - 56]^-$. The masses at *m/z* 100, 74, and 56 represented the fragment ion $[CH_2 - C(O) - (CH_2)_3]^-$, the McLafferty rearrangement product, and its dehydrated form, respectively. The quantification of TAG regioisomers was based on the relative abundances of the unique ketone enolate $[M - H - RCOOH - 100]^-$ product ions. The relative abundance of those product ions was lower for FAs originated from the *sn*-2 position than those originated from the *sn*-1/3 positions. A calculation program and the software to establish the regioisomeric structures of TAGs were developed and applied (Kallio and Rua 1994; Kurvinen et al. 2001). From the MS/MS results, intensities of the $[RCO_2]^-$ ions were used to quantifying the different FA combinations of TAGs, and the intensities of $[M - H - RCOOH - 100]^-$ ions were used to analyze the regioisomeric composition. Furthermore, the program also corrected the spectra for abundances of the naturally occurring ^{13}C isotopes and calculated the relative proportions of the molecular species. The ammonia NICI-MS/MS method was successfully applied to study different food materials (Currie and Kallio 1993; Kallio et al. 2001, 2005; Kurvinen et al. 2002b, 2002a; Linderborg and Kallio 2005; Yang and Kallio 2006; Leskinen et al. 2010a). A recent publication reported an updated and validated ammonia NICI method for analysis of TAG regioisomers with current modern instrumentation improving sensitivity and throughput (Fabritius et al. 2020). The clear advantages of the direct inlet methods are that no

chromatographic separation is required and the analysis time is short. The method is especially practical for quick confirmation of certain TAG structures. However, due to the low sensitivity, the existing studies utilizing this method only show results for the most abundant TAGs.

The direct inlet ammonia NICI-MS/MS method was adapted to an LC-MS/MS instrument. To produce $[M - H]^-$ ions from TAGs eluting from the column an atmospheric pressure chemical ionization (APCI) interface in negative ion mode was used. Pure ammonia was introduced into the system as the nebulizer gas. Although, the $[M - H]^-$ ions were not always recorded in MS/MS mode the current method was suitable for quantification of the TAG regioisomers. Furthermore, some problems related to ammonia gas were encountered. Nevertheless, Leskinen and others identified regioisomers (such as L-L-O/L-O-L, L-O-O/O-L-O and, P-O-O/O-P-O) from lard, rapeseed, sunflower seed, palm, black currant seed, and sea buckthorn pulp oils using ammonia negative ion methods, based on the abundance of the ion $[M - H - RCOOH - 100]^-$ with and without chromatographic separation (Leskinen et al. 2007, 2010a).

2.2.10.3 Ionization techniques

Electrospray ionization (ESI) and APCI are both soft ionization techniques but unlike ESI, APCI usually produces fragmentation to some extent. In addition, APCI ionizes large neutral molecules such as TAGs in a broad range of mobile phases providing valuable information concerning FA structure and position, and is thus a very suitable ionization technique for regioisomeric analysis of TAGs (Lísa and Holčapek 2008). The composition of the mobile phase plays a critical role in ionization, since the analytes should either be ionized in the solution (ESI) or in the gas phase after evaporation of the mobile phase (APCI). Ammonium acetate or sodium acetate can be added to the solvent in order to increase the ionization efficiency, e.g. by post-column addition, because non-polar TAGs are not in ionic form under typical HPLC conditions (Laakso 2002). In addition, silver nitrate can be used as a post-column reagent to produce characteristic $[M + Ag + AgNO_3]^+$ ions with improved MS sensitivity (Lévêque et al. 2010).

HPLC-APCI-MS is now most often applied for characterization of prevailing FAs in the *sn*-2 position in complex natural mixtures such as in soybean oil, peanut oil, corn oil, rapeseed oil, sunflower oil, milk fat, blackcurrant seed oil, fish oil, and different animal fats (Byrdwell 2001; Jakab et al. 2003; Leskinen et al. 2007, 2008; Holčapek et al. 2009; Gastaldi et al. 2011; Gotoh et al. 2011b; Lísa et al. 2011; Hu et al. 2013, 2014; Yoshinaga et al. 2013; Baiocchi et al. 2015; Wei et al. 2015). Byrdwell and Emken (1995)

were the first to report the MS analysis of TAGs using an APCI interface and RP-HPLC. Only DAG ions were observable in the spectra of the trisaturated TAGs. The study of Mottram and Evershed (1996) was also one of the pioneering works in the field of APCI-MS analysis of TAG regioisomers showing that the ratio of $[AA]^+$:$[AB]^+$ is lower for the A-B-A isomer compared to A-A-B, since formation of the 1,2-isomer of the $[AB]^+$ ion requires less energy than that involved in generating the corresponding 1,3-ion from the A-A-B isomer. Mottram & Evershed injected reference compounds directly into the APCI ion source via the loop injector to produce a protonated molecular ion $[M + H]^+$ and DAG fragments for regiospecific structural identification (Mottram and Evershed 1996). They noticed that the relative intensity of the $[M + H]^+$ ion increased with the degree of unsaturation of the TAGs.

The ionization process in the APCI source occurs in the presence of trace amounts of water and nitrogen gas. Protonated molecular ions are formed by gas phase ion-molecule reactions with water cluster ions. (Byrdwell 2001) Thus, the mass spectra of TAGs are quite simple containing protonated molecular ion $[M + H]^+$ as the most common base peak in the positive ion mode. In addition, the degree of diagnostic ions related to the acyl moieties, mainly $[M – RCOO]^+$ is useful for structural characterization. The degree of unsaturation significantly affects the intensities of the molecular ions in the mass spectra of TAGs obtained by APCI-MS, and in particular formation of the DAG type ions $[M + H – RCOOH]^+$ relative to the protonated molecular ion. The more intense protonated molecular ion is observed if the TAG contains more DBs. (Mottram and Evershed 1996; Leskinen et al. 2007). Although identification could be based on the DAG type ions, the low abundancy or absence of $[M + H]^+$ ions of saturated TAGs complicate the identification, and may limit information about the molecular weight distribution.

TAGs are not easily directly ionized by ESI, and formation of adducts is necessary. The mobile phase composition is important, and salts may be introduced to form single charged cations $[M + cation]^+$ with a cation = Li^+, Na^+, K^+, or NH_4^+ (Laakso 1996; Hvattum 2001). The ionization efficiency of TAGs depends on both the number of acyl carbons and DBs (Han and Gross 2001). The interpretation of ESI-MS^n spectra may be complicated because both protonated and sodiated fragment ions are abundant. In recent studies, positional distribution of FAs in TAGs has been analyzed for example in olive oil and other plant oils, fish oil, milk fat, and other animal fats using ESI-MS (Leskinen et al. 2007; Lin and Arcinas 2008; Kalo et al. 2009; Lévêque et al. 2010; Cubero Herrera et al. 2013; Lin et al. 2015; Šala et al. 2016; Kallio et al. 2017; Tarvainen et al. 2019).

The use of ESI-MS and ESI-MS/MS in MAG, DAG, and TAG analyses were first reported by Duffin et al. (1991). They reported that the most

abundant product ions of ammoniated acylglycerols resulted from the loss of FAs in collision-induced dissociation. They found that significantly greater dissociation energy was required for MS/MS fragmentation of sodiated acylglycerols, $[M + Na]^+$, compared to the ammoniated acylglycerols. The product ion spectra of $[M + NH_4]^+$ ions contained $[M – RCOO]^+$ and $[RCO]^+$ ions, which offer valuable information for structural elucidation of the TAGs. Hvattum (2001) was the first to combine RP-HPLC with ESI-MS/MS for regiospecific analysis of TAGs.

ESI is a technique based on ion formation in a solution. The sprayed small droplets form, when the mechanical forces push the solution through the narrow outlet in the ionization chamber. An electric potential is applied between the end of the inlet and the entry into the mass analyzer. Thus, the sprayed droplets can carry net charges, when they are directed into the mass analyzer by the applied electric field. In the positive ion mode, a positive electric potential is applied to the end of the inlet and a negative electric potential at the entrance of the mass analyzer, causing the droplets to carry net positive charges. (Banerjee and Mazumdar 2012; Han 2016). The ESI as a soft ionization technique makes the in-source fragmentation almost non-existent. Typically, ESI of TAGs yields abundant $[M + NH_4]^+$ and $[M + Na]^+$ ions without fragmentation giving easily information about the molecular weight distribution. Thus, the detection sensitivity of ESI-MS in general is higher and Collision-induced dissociation is an essential tool for structural analysis of TAGs. With HPLC-ESI-MS instruments a broad range of flow rates and a wide variety of mobile phases are possible.

Leskinen et al. (2007) were the first researchers who provided comprehensive comparisons of different MS-methods used for TAG analyses. LC-APCI-MS, LC-APCI-MS/MS, LC-ESI-MS/MS and direct inlet ammonia NICI-MS/MS methods using two types of MS instrumentation were applied for identification of regioisomers (such as L-O-L/L-L-O, O-L-O/L-O-O, and O-P-O/P-O-O) from lard, rapeseed, sunflower seed, palm, black currant seed, and sea buckthorn pulp oil. The various regioisomers were determined by MS, because they were not fully separated with LC. Leskinen and others found that the discrimination of regioisomers by observing the different signal intensities of DAG ions and $[M – H – RCOOH – 100]^–$ ions in different regioisomers were obvious with LC-APCI-MS, LC-ESI-MS/MS and direct inlet ammonia NICI-MS/MS methods. The LC-APCI-MS/MS method was not found to be reliable in the quantification of TAG regioisomers because the abundancy of DAG ions did not clearly correspond to the positional distribution of the FAs. In the LC-APCI-MS results the overlapping TAGs, which produced isobaric DAG type fragments produced skewed results. In the LC-ESI-MS/MS method the overlapping TAGs with different molecular weights posed no problem.

Thus, it seemed that ESI-MS/MS of $[M + NH_4]^+$ ions was the easiest and most reliable method for quantifying the regioisomers of TAGs, and ammonia gas was found to be a convenient way to form ammonium adducts. The relative abundances of molecular ions $[M + H]^+$ formed from the saturated TAGs, can be very low or even nonabundant in the APCI mass spectra. In the ESI mass spectra of TAGs, the relative abundances of the $[M + H – RCOOH]^+$ fragments are lower than the cationized TAG molecules but still sufficient for the identification. (Holčapek et al. 2003; Leskinen et al. 2007). $[M + Na]^+$ and $[M + K]^+$ ions are stable making the fragmentation more challenging compared to the APCI technique.

2.2.10.4 Mass analyzers

The mass analyzers applied in the regiospecific studies of TAGs comprise mainly of triple quadrupole (QqQ), ion trap (IT), Q-Trap and hybrid analyzers namely LTQ/Orbitrap and QqQ/LIT. Typically, QqQ is used for targeted analyses of TAGs with selective ion monitoring. Holčapek et al. (2010) studied TAG using five different mass spectrometers (single quadrupole, QqQ, IT, QqTOF and LTQ Orbitrap). They demonstrated that the impact of different mass analyzers on the ratio of DAG fragments in the APCI spectra is lower than the impact of the number or position of DBs in the fatty acyls and the regiospecific distribution of FAs.

2.2.10.5 Quantitative analysis

Various studies have successfully demonstrated the characterization of the regioisomeric composition of TAGs in different fats and oils with different MS methods but the quantitative analysis of TAGs is more complicated. For example, a recent study by Baiocchi et al. (2015) reported efficient analysis of TAG regioisomers of polyunsaturated tuna and algal oils by HPLC-HRMS (MS^2 and MS^3). However, due to the complex sample matrices and the lack of reference compounds of the majority of TAGs, quantitative determination of different regioisomeric TAGs was not possible. Another recent study demonstrated regioisomeric analysis of lithiated adducts of TAGs with UHPLC-ESI-MS/MS together with an automatic data calculation algorithm (Kallio et al. 2017). The method revealed hundreds of different TAG regioisomers from human milk samples. However, the long-term use of lithium salt as an additive in the mobile phase causes corrosion in the instrumentation.

The ratio of regioisomers can be obtained by comparing the less abundant fragmentation products resulted as a loss of FA from the position *sn*-2 $[M + H – R_2COOH]^+$ with fragments resulted as a loss of FA from the positions *sn*-1/3,

$[M + H - R_1COOH]^+$ and $[M + H - R_3COOH]^+$. However, regiopure reference compounds are essential for calibration curves for the reliable quantitative determination due to the differences in fragmentation kinetics of different structures (Jakab et al. 2003; Fauconnot et al. 2004; Leskinen et al. 2007; Wei et al. 2015; Kallio et al. 2017). The intensity of DAG ions is influenced by the structure, mostly the number of DBs and the position of individual FAs (Holčapek et al. 2010). These ratios may also vary with the type of instrument and experimental conditions. However, a similar trend was obtained with five different mass analyzers when the ratios of DAG ions from various TAG reference compounds were compared (Holčapek et al. 2010). Overall, the reliability of the regioisomeric calculations based on DAG ions intensities need to be firmly validated using regiopure TAG reference compounds for each analyte. Unfortunately, commercial reference compounds are expensive and have limited availability. In practice, quantification based on calibration curves for all TAGs is extremely difficult, and usually not reasonable. For A-A-B/A-B-B -type TAGs using the ratio of the two DAG ions to differentiate the two positional isomers have been reported in many studies. Both response factors and calibration curves for each TAG or for saturated and unsaturated TAGs separately have been applied to confirm reliable quantitative results (Jakab et al. 2003; Fauconnot et al. 2004; Malone and Evans 2004; Leskinen et al. 2007; Wei et al. 2015; Watanabe et al. 2016; Kallio et al. 2017) but also direct ratios of the peak areas (Lísa et al. 2011) have been applied. Byrdwell et al. (1996) compared various quantification methods for data obtained by HPLC-APCI-MS. Furthermore, Holčapek et al. (2005) discussed quantification of TAGs carried out by different detection methods. Due to differences between the saturated and unsaturated acyl chains, the use of RFs significantly improves the quantification of TAGs (Holčapek et al. 2005). To date, the calibration curves have been restricted to TAG species with two different FAs. Recently, a UHPLC-ESI-MS/MS method was developed and applied in the analysis of five triacid-TAGs (Tarvainen et al. 2019).

Ion mobility-MS and multidimensional methods which have been more applied in lipidomics are probably also one of the future trends in the MS analyses of TAGs (Hancock et al. 2017). In a recent study, a new differential mobility spectrometry coupled with ESI-MS was developed for determination of the TAG regioisomers containing monounsaturated FAs (MUFA) and SAFAs (Šala et al. 2016). The method enabled the quantitative analysis of TAG regioisomers from porcine adipose tissue in less than one minute, which demonstrated the clear advantage of this optimized method compared to methods where multiple columns are combined. Further development is needed for applicability for TAGs containing PUFAs. Despite significant improvements in quantitative analyses of TAG regioisomers with MS, the

positions *sn*-1 and *sn*-3 in TAG molecule are indistinguishable in an MS spectrum.

2.2.11 NMR spectroscopy

NMR is the basic spectroscopic approach to characterize positional distribution of FAs in TAGs (Lie Ken Jie et al. 1996; Vlahov 1998; Carrasco-Pancorbo et al. 2009; Gouk et al. 2011). In addition to get information on the FAs, NMR can provide information on their positional distribution between the positions *sn*-1/3 and *sn*-2 because of characteristic signals of the alkenyl and carbonyl bonds in the ^{13}C NMR spectrum (Christie and Han 2010).

Kalo and others used ^{1}H NMR for identification of TAG regioisomers such as S-S-Bu/S-Bu-S after chromatographic separation in the 90s (Kalo et al. 1996). Nowadays, the high-resolution ^{13}C NMR spectroscopy has become an important tool for structural analysis of TAGs, if the sample size is not a limiting factor. ^{13}C NMR is an especially promising and reliable tool to replace laborious enzymatic hydrolysis (Tengku-Rozaina and Birch 2014; Ruiz-Lopez et al. 2015). One advantage of this technique is that it may be used to determine the positional distribution of FAs containing DBs close to the carboxyl group, such as TAGs in fish oil and evening primrose oil, where enzymatic methods with lipases are not applicable due to the enzyme specificities (Aursand et al. 1995; Bergana and Lee 1996; Standal et al. 2009).

In the high-resolution ^{13}C NMR, the ^{13}C resonances in TAGs are grouped in four sets of signals: carbonyl (172–174 ppm), unsaturated (124–134 ppm), glycerol (60–70 ppm), and aliphatic carbons (10–35 ppm) of which carbonyl region is mainly used in the regiospecific analysis (Simova et al. 2003; Tengku-Rozaina and Birch 2014). On the ^{13}C NMR spectra, the signals produced by the acyl chains attached to the positions *sn*-1/3 are shifted by 0.4 ppm towards higher frequencies than those of the corresponding chains attached to the position *sn*-2 (Bergana and Lee 1996; Vlahov 1998). Within these two clusters of signals, separate signals for each acid or group of acids must be detected. These signals are easier to distinguish when the acids are unsaturated close to the carboxyl group, for example such as DHA(Δ4,7,10,13,16,19) and EPA(Δ5,8,11,14,17). In fish oil, for example, signals appear in the order of increasing chemical shift: DHA in the position *sn*-2, DHA in the position *sn*-1/3, EPA in the position *sn*-2, and EPA in the position *sn*-1/3 (Aursand et al. 1995; Tengku-Rozaina and Birch 2014; Ruiz-Lopez et al. 2015). Based on the ratio of the integral values, distribution of DHA or EPA between positions *sn*-1/3 and *sn*-2 position can be calculated. However, C18:1(Δ9), C18:2(Δ9,12), C18:3(Δ9,12,15) and C18:3(Δ6,9,12) the

acyl groups are much more challenging to distinguish from each other (Bergana and Lee 1996). Bergana and Lee studied the correlation of the ^{13}C carbonyl chemical shift to the molecular structure of TAG. They found that the ^{13}C carbonyl shift was more dependent on the position than the number of the DBs. The position of the first DB especially affected the chemical shifts. For samples containing unsaturated acids having Δ9 unsaturation, the data acquisition periods of time have to be longer than usual.

^{13}C NMR spectroscopy provides an alternative way to study positional distribution of FAs between the positions *sn*-1/3 and *sn*-2. Especially procedures for obtaining regiospecific information for common vegetable oils (Vlahov 1998, 2006; Vlahov et al. 2010; Gouk et al. 2011) and fish oils (Aursand et al. 1995; Standal et al. 2009; Ruiz-Lopez et al. 2015) have been described. A recent study by Simova et al. (2003) reported a novel method for quantitative determination of the positional distribution of FAs in TAGs with saturated and Δ9-unsaturated acyl chains. In many studies, applicability of ^{13}C NMR spectroscopy has been determined and compared to conventional regiospecific methods based on enzymatic or chemical hydrolysis (Gouk et al. 2011; Tengku-Rozaina and Birch 2014; Ruiz-Lopez et al. 2015). The ^{13}C NMR technique provided data on the regiospecific analysis off plant oils within 44 min with high accuracy and precision - unlike conventional methods (Gouk et al. 2011). Moreover, the long-standing acyl migration problem was avoided. However, various SAFAs were indistinguishable and also distinguishing between the carbonyl carbons of the diene (C18:2) and triene acyl groups (C18:3) in the spectrum was challenging due to only a 0.005 ppm difference in the chemical shifts.

Tengku-Rozaina and Birch (2014) compared pancreatic lipase hydrolysis and ^{13}C NMR spectroscopy to study the positional distribution of FAs in hoki and tuna oils. The methods gave contradictory results for tuna oil. For pancreatic lipase hydrolysis of tuna oil, the results showed that both DHA and EPA were incompletely hydrolyzed from the positions *sn*-1/3. This confirmed that the pancreatic lipase hydrolysis is less reliable than ^{13}C NMR for regiospecific determination in tuna oil and other fish oils with high DHA concentration (>10%).

The high resolution ^{13}C NMR spectroscopy offers a rapid measurement without hydrolysis or derivatization of the sample. The main advantage compared to enzymatic and chemical hydrolysis is that acyl migration during the analysis is eliminated. It is a non-destructive technique, and the TAGs are in the intact form during analysis and, thus, can be used for further investigations. However, the practical applicability is limited by the need for pure compounds in milligram amounts (Standal et al. 2009). Compared with MS, the NMR techniques have only moderate sensitivity. One significant

disadvantage is that if the regioisomers are not separated beforehand, NMR measures the sum of the TAG FAs (Řezanka et al. 2017). Another challenge is that the ^{13}C NMR spectra are complex with hundreds of signals. Consequently, both instrumentation and interpretation of the results requires special skills and knowledge. Enantiomeric composition of TAGs cannot be determined by ^{13}C NMR.

2.3 Summary

TAGs are very complex mixtures of different molecular species depending on the origin of the fat or oil. The TAG composition is determined during the complex biosynthetic pathways catalyzed by stereospecific enzymes resulting in non-random, species-specific FA combinations and their positional distribution in TAGs. The chirality of different food components is being increasingly studied but the role and importance of chirality in TAGs has not yet been fully established. It is evident that the positional distribution of FAs in TAGs is of nutritional (metabolic fate), biochemical (biosynthesis) and technological (physical properties) importance.

Over the years, the analytical methodology (**Table 5**) including enzymatic and chemical hydrolysis, various chromatographic and mass spectrometric techniques, and nuclear magnetic resonance spectroscopy have progressed from an overall characterization of TAGs and the FA composition of each stereospecific position towards a molecular level determination of the stereospecific composition of individual TAGs. Due to the different TAG species with the similar physicochemical properties, the structural analysis of individual TAGs is much more demanding than conventional lipid analysis. Nevertheless, interest in the detailed composition has increased simultaneously with the number of sophisticated methods available. The rapid development of analytical methods has made it possible to analyze highly complex mixtures, and there has been great progress in the stereospecific analysis of individual TAGs. However, lack of enantiopure reference compounds has complicated the stereospecific analysis of individual compounds.

Table 5. Analytical methods mainly used for regio- and stereospecific analyses of triacylglycerols and the structural information obtained, as well as the advantages and disadvantages.

Partial hydrolysis + chromatography	HT-GC	HPLC	MS
Fatty acid composition in positions *sn*-1, *sn*-2 and *sn*-3	Equivalent carbon number value	> High-performance liquid chromatography (HPLC)-Ultra violet/Evaporative light scattering detector	Molecular weight
	Overall fatty acid composition with gas chromatography-flame ionization detector	Equivalent carbon number value	Diacylglycerol fragments for regioisomeric identification
		Regioisomeric separation	
		> Silver-ion HPLC	
+ no expensive instrumentation	+ moderate cost of equipment	Separation according to unsaturation	+ structural information
- multistep	- column stability	> Chiral-phase HPLC	+ high sensitivity and accuracy
- acyl migration	- injection challenging	Stereoisomeric separation	- expensive instruments especially high-resolution mass spectrometry
- no information about individual triacylglycerols		+ moderate temperatures	
		+ lot of information if multidimensional techniques and mass spectrometric detection are used	
		- not possible to separate all triacylglycerols	

NARP-HPLC is the most widely applied separation technique used in the detailed characterization of TAG mixtures. However, it is not the most powerful tool for the separation of regioisomers as the long retention times cause peak broadening (Lísa et al. 2009). Silver-ion HPLC, despite the low reproducibility, together with NARP-HPLC has been the most efficient method to separate the regioisomers of TAGs, while chiral-phase HPLC is needed for

the separation of enantiomers. In addition to the development of more sophisticated stationary phases, the use of MD-LC techniques will, in the future, be solutions to enhance the structural analyses of individual TAGs. Instead of serially-coupled columns, the recycling HPLC seems to be an applicable option when the separation capacity of a single column is exceeded. Usually, pre-separation is needed both before silver-ion and before chiral-phase separations. However, there is lack of use of multidimensional techniques combining NARP-LC and chiral-phase LC online to study the enantiomers of TAGs. For complete analysis of TAGs, especially to reveal the individual TAGs requires: a combination of various separation methods in online or offline mode, careful optimization of the analytical conditions, and usually the use of several detection techniques (Xu et al. 2018). The abundance of different possible TAG molecular species with similar chemical and physical properties has stressed the importance of chromatographic separation, often hyphenated with MS for the characterization and quantification of TAGs. The combination of analytical techniques is evidently time-consuming but it offers the maximum amount of information including regioisomers and even enantiomers of highly complex TAG mixtures. Thus, the analytical approach has to be carefully considered according to the type of information required and the availability of instruments. A number of studies deal with the optimization of the regioisomeric separation but the current data about enantiomeric separations is limited. Therefore, more knowledge about the retention behavior of TAGs in chiral environment as well as development of chiral-phase chromatography is needed in further studies. Deeper knowledge of the chromatographic elution behavior of chiral compounds could aid interpretation of molecular recognition mechanisms. Despite the immense progress in chiral separations of individual TAGs, every method where regioisomers and enantiomers are separated simultaneously has its limitations. For the TAGs of some FA combinations enantiomeric separation has not been detected at all. Usually, either good chromatographic separation, simple instrument configuration or short analysis time is achieved but not all of them at the same time. Thus, development and further improvement of highly sensitive multidimensional chromatographic separation methods together with mass spectrometric structural characterization methods are important. Understanding the nutritional, biological and technological effects of the positional distribution of FAs in TAGs is reliant on accurate analytical data.

3 AIMS OF THE STUDY

The general aim of this PhD research project was to study different methods to analyze the regiospecific structures of individual triacylglycerols in complex mixtures such as human milk and, especially, to develop and apply a method for stereospecific analysis.

The main goal of the thesis was to develop an efficient method of studying the stereospecific composition of individual TAGs in complex mixtures by combining chromatography and mass spectrometry; natural sea buckthorn pulp oil was used as an example.

The specific aims in the individual studies were to:

- apply current methodology to study the TAG composition of human milk and to compare different regiospecific methods as well as to study the impact of maternal body weight and food choice on regioisomeric composition of human milk TAGs (publication I Linderborg et al. 2014)
- establish a reliable and straightforward chiral chromatographic method for stereospecific analysis of TAGs commonly present in fats and oils using synthesized enantiopure TAGs (publication II Kalpio et al. 2015)
- collect analytical data about chromatographic elution behavior of asymmetric TAGs in chiral environment (publication II Kalpio et al. 2015, publication III Kalpio et al. 2020, publication IV Kalpio et al. (submitted)
- study the enantiomeric purity of synthesized TAGs (publication III Kalpio et al. 2020)
- develop a strategy for studying enantiomeric ratios of selected TAGs in natural oil by combining different chromatographic and mass spectrometric methods, using sea buckthorn oil as an example (publication IV Kalpio et al. submitted)

4 MATERIALS AND METHODS

4.1 Sample materials

The samples of this study were enantiostructured TAGs (publications II Kalpio et al. 2015, III Kalpio et al. 2020) and other reference compounds (publications I Linderborg et al. 2014, II Kalpio et al. 2015, III Kalpio et al. 2020), human milk (publication I Linderborg et al. 2014) and sea buckthorn pulp oil (publication IV Kalpio et al. submitted). Chiral elution order was determined using enantioenriched reference mixtures (publications II Kalpio et al. 2015, III Kalpio et al. 2020).

All the 45 reference compounds used in chiral-phase and reversed-phase HPLC analyses (publications II Kalpio et al. 2015, III Kalpio et al. 2020), and MS analyses (publication I Linderborg et al. 2014) are listed in **Table 6**. Altogether 21 diacid-TAG enantiomers (6 UUS-type, 9 USS-type and 6 SSU-type) with C10–C22 FAs and 0–6 DBs were synthesized for the reference compounds (publication III Kalpio et al. 2020, Kristinsson et al. 2014). All racemic TAGs were purchased from Larodan Fine Chemicals (Malmö, Sweden).

Table 6. Reference compounds.

Trivial name	Abbreviation
1,2-dioleoyl-3-decanoyl-*sn*-glycerol[a]	*sn*-18:1-18:1-10:0
1,2-dioleoyl-3-palmitoyl-*sn*-glycerol[a]	*sn*-18:1-18:1-16:0
1,2-dioleoyl-3-arachidoyl-*sn*-glycerol[a]	*sn*-18:1-18:1-20:0
1,2-dioleoyl-3-behenoyl-*sn*-glycerol[a]	*sn*-18:1-18:1-22:0
1,2-dilinoleoyl-3-palmitoyl-*sn*-glycerol[a]	*sn*-18:2-18:2-16:0
1,2-dipalmitoleoyl-3-palmitoyl-*sn*-glycerol[a]	*sn*-16:1-16:1-16:0
1-oleoyl-2,3-dipalmitoyl-*sn*-glycerol[a]	*sn*-18:1-16:0-16:0
1-linoleoyl-2,3-dipalmitoyl-*sn*-glycerol[a]	*sn*-18:2-16:0-16:0
1-oleoyl-2.3-distearoyl-*sn*-glycerol[a]	*sn*-18:1-18:0-18:0
1,2-dipalmitoyl-3-4,7,10,13,16,19-docosahexaenoyl-*sn*-glycerol[a]	*sn*-16:0-16:0-22:6
1-4,7,10,13,16,19-docosahexaenoyl-2,3-dipalmitoyl-*sn*-glycerol[a]	*sn*-22:6-16:0-16:0
1,2-distearoyl-3-4,7,10,13,16,19-docosahexaenoyl-*sn*-glycerol[a]	*sn*-18:0-18:0-22:6
1-4, 7,10,13,16,19-docosahexaenoyl-2,3-distearoyl-*sn*-glycerol[a]	*sn*-22:6-18:0-18:0
1,2-dipalmitoyl-3-5,8,11,14,17-eicosapentaenoyl-*sn*-glycerol[a]	*sn*-16:0-16:0-20:5
1-5, 8,11,14,17-eicosapentaenoyl-2,3-dipalmitoyl-*sn*-glycerol[a]	*sn*-20:5-16:0-16:0

Trivial name	Abbreviation
1,2-distearoyl-3-5,8,11,14,17-eicosapentaenoyl-*sn*-glycerol[a]	*sn*-18:0-18:0-20:5
1-5, 8,11,14,17-eicosapentaenoyl-2,3-distearoyl-*sn*-glycerol[a]	*sn*-20:5-18:0-18:0
1,2-dipalmitoyl-3-9,12,15-octadecatrienoyl-*sn*-glycerol[a]	*sn*-16:0-16:0-18:3
3-9,12,15-octadecatrienoyl-2,3-dipalmitoyl-*sn*-glycerol[a]	*sn*-18:3-16:0-16:0
1,2-distearoyl-3-9,12,15-octadecatrienoyl-*sn*-glycerol[a]	*sn*-18:0-18:0-18:3
1-9,12,15-octadecatrienoyl-2,3-distearoyl-*sn*-glycerol[a]	*sn*-18:3-18:0-18:0
1,2(2,3)-dioleoyl-3(1)-decanoyl-*rac*-glycerol[b]	*rac*-18:1-18:1-10:0
1,2(2,3)-dioleoyl-3(1)-lauroyl-*rac*-glycerol[b]	*rac*-18:1-18:1-12:0
1,2(2,3)-dioleoyl-3(1)-myristoyl-*rac*-glycerol[b]	*rac*-18:1-18:1-14:0
1,2(2,3)-dioleoyl-3(1)-palmitoyl-*rac*-glycerol[b]	*rac*-18:1-18:1-16:0
1,2(2,3)-dioleoyl-3(1)-stearoyl-*rac*-glycerol[b]	*rac*-18:1-18:1-18:0
1,2(2,3)-dioleoyl-3(1)-arachidoyl-*rac*-glycerol[b]	*rac*-18:1-18:1-20:0
1,2(2,3)-dioleoyl-3(1)-behenoyl-*rac*-glycerol[b]	*rac*-18:1-18:1-22:0
1,2(2,3)-dioleoyl-3(1)-linoleoyl-*rac*-glycerol[b]	*rac*-18:1-18:1-18:2
1,2(2,3)-dipalmitoyl-3(1)-oleoyl-*rac*-glycerol[b]	*rac*-16:0-16:0-18:1
1,2(2,3)-dipalmitoyl-3(1)-linoleoyl-*rac*-glycerol[b]	*rac*-16:0-16:0-18:2
1,2(2,3)-dipalmitoyl-3(1)-elaidoyl-*rac*-glycerol[b]	*rac*-16:0-16:0-*tr*18:1
1,2(2,3)-dilinoleoyl-3(1)-palmitoyl-*rac*-glycerol[b]	*rac*-18:2-18:2-16:0
1,2(2,3)-dipalmitoleoyl-3(1)-oleoyl-*rac*-glycerol[b]	*rac*-16:1-16:1-18:1
1,2(2,3)-dipalmitoleoyl-3(1)-palmitoyl-*rac*-glycerol[b]	*rac*-16:1-16:1-16:0
1,2(2,3)-distearoyl-3(1)-oleoyl-*rac*-glycerol[b]	*rac*-18:0-18:0-18:1
1,2(2,3)-dilinoleoyl-3(1)-oleoyl-*rac*-glycerol[b]	*rac*-18:2-18:2-18:1
1(3)-linoleoyl-2-oleoyl-3(1)-palmitoyl-*rac*-glycerol[b]	*rac*-18:2-18:1-16:0
trioleoylglycerol[b]	18:1-18:1-18:1
1,3-dipalmitoleoyl-2-oleoyl-*rac*-glycerol[b]	16:1-18:1-16:1
1,3-dioleoylyl-2-myristoyl-*rac*-glycerol[b]	18:1-14:0-18:1
1(3)-palmitoyl-2-linolenoyl-3(1)-oleoyl-*rac*-glycerol[b]	*rac*-16:0-18:2-18:1
1,3-dioleoylyl-2-linolenoyl-*rac*-glycerol[b]	18:1-18:2-18:1
1,3-distearoyl-2-oleoyl-*rac*-glycerol[b]	18:0-18:1-18:0
triheptadecanoylglycerol[b]	17:0-17:0-17:0

[a]synthesized (publication III Kalpio et al. 2020, Kristinsson et al. 2014)

[b]Larodan Fine Chemicals (Malmö, Sweden)

Regiospecific composition of human milk and its role for the infant are extensively studied. However, the positional distribution of FAs in TAGs of

infant formulas deviates from human milk. Understanding the specific structure of TAGs in human milk is constitutive to the development of infant formulas. The human milk samples were obtained from 40 mothers who participated in the STEPS (Steps to healthy development) study in Finland (Lagström et al. 2012). The Ministry of Social Affairs and Health, and the Ethics Committee of the Hospital District of Southwest Finland approved the STEPS Study (2007-02-27). The mothers were selected from a cohort of 90 overweight (pre-pregnancy BMI ⩾ 25 kg/m^2) women and 73 normal weight (pre-pregnancy BMI < 25 kg/m^2) women based on differences in the food frequency questionnaire (Pietinen et al. 2010) and self-reported pre-pregnancy weight and height. The milk samples were selected to form four groups of ten: 1) milk of normal weight mothers with recommended food choices, 2) milk of overweight mothers with recommended food choices, 3) milk of normal weight mothers with non-recommended food choices, and 4) milk of overweight mothers with non-recommended food choices. The samples were self-collected by the mothers at infant's age of three months according to written instructions. After the collection and during transportation, the samples were kept in +4 – +8 ºC, and then stored at -70 ºC until analysis.

The sea buckthorn pulp oil was selected as a natural source of asymmetric TAGs for developing a strategy for stereospecific analysis of selected TAGs. The FA composition of both seed and berry pulp oils of sea buckthorn is thoroughly studied (Yang and Kallio 2001). The pulp oil contains more saturated FAs than the seed oil, but has still rather high concentration of palmitoleic acid (C16:1). Evidently, pulp oil contains asymmetric TAGs with saturated FAs, mostly C16:0, and the unsaturated C16:1. In addition, C16:1, not common in plant oils, is considered to be a bioactive molecule with potential health effects (Frigolet and Gutiérrez-Aguilar 2017). Three Russian cultivars ('Prozcharachnaya', 'Botanicheskaya' and 'Trofimovskaya') of sea buckthorn (*Hippophaë rhamnoides* L. ssp. *mongolica*) grown in Finland (60° 23' N 22° 09' E, altitude 2 m, Satava, Turku) were used. The berries were picked optimally ripe, frozen and stored at -20 °C immediately after harvesting until analysis.

All solvents were either *pro analysis* quality, HPLC or MS grade and used without further purification.

4.2 Methods

Altogether six MS methods were used with and without chromatographic separation (publications I Linderborg et al. 2014, II Kalpio et al. 2015, IV Kalpio et al. submitted). In addition to reversed-phase chromatography

(publications I Linderborg et al. 2014, IV Kalpio et al. submitted), chiral-phase chromatography and sample recycling system (publications II Kalpio et al. 2015, III Kalpio et al. 2020, IV Kalpio et al. submitted) were applied to enhance the stereospecific separation of the individual TAGs. The methodology of this PhD research is presented in **Figure 5**.

Human milk

Synthesized enantiomers and reference compounds

Sea buckthorn pulp oil

Folch lipid extraction

TAG isolation solid-phase extraction

TAG molecular weight distribution Ammonia NIAPCI-MS

Fatty acid composition

Regioisomeric analysis UHPLC-APCI-MS

TAG fractionation RP-HPLC

Quantification of selected TAGs UHPLC-APCI-MS

Regioisomeric analysis UHPLC-ESI-MS/MS

Regioisomeric analysis Ammonia NIAPCI-MS/MS

Regioisomeric analysis Ammonia NICI-MS and MS/MS

Enantiomeric separations, chiral behavior with chiral R-HPLC

Figure 5. Flow-chart of the analyses.

4.2.1 Sample pretreatment

Whole seeds were picked from the pressed sea buckthorn berries and the remaining berry pulp (fruit flesh and skin) was lyophilized (publication IV Kalpio et al. submitted). Lipids were extracted with chloroform/methanol (2:1, v/v) (Folch et al. 1957) from the thawed milk samples (publication I Linderborg et al. 2014) and from the lyophilized sea buckthorn pulp (publication IV Kalpio et al. submitted). TAGs from the sea buckthorn pulp oil (publication IV Kalpio et al. submitted) were isolated using solid-phase extraction (Vuorinen et al. 2014) while human milk lipids (publication I Linderborg et al. 2014) were analyzed without isolation of TAGs. The enantiostructured TAGs (publications II Kalpio et al. 2015, III Kalpio et al. 2020), the enantioenriched reference mixtures (publications II Kalpio et al. 2015, III Kalpio et al. 2020) and racemic reference compounds (publication I Linderborg et al. 2014) were diluted either in hexane or in hexane/2-propanol (3:2, v/v) prior to analyses. All the synthesized enantiomers were analyzed as such to get the retention times and to automate the switching between the columns (publications II Kalpio et al. 2015, III Kalpio et al. 2020). In addition, enantiopairs consisting of either both pure enantiomers or a racemic mixture and one enantiomer (75%:25%) were analyzed to determine the enantiomeric separation and the elution order. In study III (Kalpio et al. 2020), also enantiomeric mixtures of 99%:1% were prepared to study the enantiomeric purity.

4.2.2 Fatty acids analysis

The total lipids of the milk samples (publication I Linderborg et al. 2014) were transesterified into FAMEs with boron trifluoride (Ågren et al. 1992). For transesterification of TAGs from sea buckthorn pulp oil (publication IV Kalpio et al. submitted), the sodium methoxide method was applied (Christie 1982). All FAMEs were analyzed by GC-FID (GC-2010 Auto Injector / Auto Sampler, Shimadzu, Kyoto, Japan) with a DB-23 (50% cyanopropyl, 50% methylpolysiloxane) column (60 m x 0.25 mm, 0.25 µm film thickness, Agilent Technologies, DE). The peaks of FAMEs were identified by comparing their retention times with those of the known reference mixtures Supelco 37 Component FAME Mix (Supelco, Bellefonte, PA) and GLC reference standard 68D (Nu-Chek Prep, Elysian, MN).

4.2.3 Liquid chromatography

4.2.3.1 Non-aqueous reversed-phase liquid chromatography

Either Kinetex™ C18 RP column (100 × 2.1 mm, 1.7 µm, Phenomenex, Torrance, CA) (publication I Linderborg et al. 2014) or Acquity UPLC BEH C18 column (100 × 2.1 mm, 1.7 µm, Waters Corp., Milford, MA) (publication IV Kalpio et al. submitted) were used with a Waters Acquity UPLC coupled to a Waters Quattro Premier triple quadrupole MS (Waters Corp., Milford, MA) to separate the TAG species. The liquid chromatograph Acquity UPLC acted both as a device for solution delivery to MS (ammonia NIAPCI-MS, -MS/MS) and as an LC pump (publications I Linderborg et al. 2014, II Kalpio et al. 2015, IV Kalpio et al. submitted).

An Ascentis C_{18} column (250 × 4.6 mm, 5 µm, Supelco, Bellefonte, PA) with Shimadzu Prominence preparative HPLC-UV instrumentation consisting of a SIL-20A autosampler, an LC-20AB pump, a CTO-10AC column oven, a DGU-20A5 degasser, and UV/Vis SPD-20A detector (205 nm), and a fraction collector FRC-10A (Shimadzu, Kyoto, Japan) was used to isolate and to further analyze the most prevalent asymmetric TAGs. Acetone and acetonitrile were used as the mobile phases in a gradient mode (publication IV Kalpio et al. submitted).

4.2.3.2 Chiral-phase liquid chromatography

All the enantiostructured TAGs (publications II Kalpio et al. 2015, III Kalpio et al. 2020), the enantiomeric and racemic TAG mixtures (publications II Kalpio et al. 2015, III Kalpio et al. 2020) and the selected TAG fractions from sea buckthorn pulp oil (publication IV Kalpio et al. submitted) were injected to the Shimadzu instrument described in the previous chapter using two identical chiral columns CHIRALCEL OD-RH (150 × 4.6 mm, 5 µm) with cellulose tris-(3,5-dimethylphenylcarbamate) stationary phases and a pre-column (10 × 4.0 mm, 5 µm) (Chiral Technologies Europe, Illkirch, France). The CS3080 Sample Peak Recycler™ (Chiralizer Services, Newtown, PA) including a controlling device and a high-pressure 10-port valve, was installed in the system to improve the efficiency of enantiomeric separation. After the recycling system, another absorbance detector (Waters 486 Tunable Absorbance Detector, Millipore Corp., Milford, MA) was connected in line to ensure that only the peaks of interest were collected. Isocratic methanol at a flow rate of 0.5 mL/min at 25 °C was used as a mobile phase.

4.2.4 Mass spectrometry

In this PhD project, two mass spectrometric instrumentation, either with HPLC (UHPLC-APCI-MS and UHPLC-ESI-MS/MS) or without any chromatographic separation (ammonia NIAPCI-MS and MS/MS, ammonia NICI-MS and MS/MS) were used to study the molecular weight distribution and regioisomeric composition of TAGs.

4.2.4.1 TAG molecular weight distribution

Three different MS methods were used for analyses of the TAG molecular weight distribution. First, in the human milk study (publication I Linderborg et al. 2014), a tandem mass spectrometer Waters Quattro Premier operated with Mass Lynx v4.1 (Waters Corp., Milford, MA) software was used for collection of the full scan mass spectra of the extracted lipids. The negative ion APCI-MS and APCI-MS/MS methods were based on the ammonia NICI-MS and -MS/MS methods and calculation programs published previously (Currie and Kallio 1993; Kallio and Currie 1993; Kallio and Rua 1994; Kurvinen et al. 2001) and later adapted to LC (Leskinen et al. 2010a). To produce $[M - H]^-$ ions, an APCI interface in the negative ion mode was used and ammonia (purity 5:0; Linde AG, Munich, Germany) was introduced into the system as the nebulizer gas (Leskinen et al. 2010a). The instrument settings were optimized for MS and MS/MS operations to generate maximal intensity and stability for $[M - H]^-$ ions of TAGs. The samples were delivered manually to the APCI probe through an injection valve while simultaneously introducing the solvent acetone/acetonitrile (50:50, v/v) at a flow rate 0.4 mL/min. Full scan mass spectra of *m/z* 700–1000 were collected and analyzed. The MS analyses were corrected for the natural occurrence of the ^{13}C isotope.

Additionally, the same Waters Quattro Premier instrumentation using APCI after chromatographic separation with Acquity Ultra Performance LC equipment was used to quantify the selected abundant TAG species from human milk (publication I Linderborg et al. 2014), to obtain the total ion chromatogram of sea buckthorn pulp oil (publication IV Kalpio et al. submitted), and to confirm the molecular weights of the isolated enantiomers (publication II Kalpio et al. 2015). Full scan mass spectra (*m/z* 400–1000) were collected in positive ion mode.

Molecular weights of the stereospecifically separated, isolated TAGs from sea buckthorn pulp oil were determined with direct inlet ammonia NICI-MS method developed in the early 1990s (Currie and Kallio 1993; Kallio and Currie 1993) and previously optimized to the new generation equipment (Fabritius et al. 2020). The direct inlet ammonia NICI-MS instrumentation

consisting of a Thermo Scientific TSQ 8000 EVO mass spectrometer (Thermo Fisher Scientific, Waltham, MA) equipped with a direct exposure probe was used. Instrument settings were according to earlier optimizations (Laakso and Kallio 1996; Fabritius et al. 2020). A calculation algorithm (Kallio and Rua 1994) and later further developed calculation program software MSPECTRA (Kurvinen et al. 2001) were used to analyze the results obtained from the ammonia NICI-MS and ammonia NIAPCI method.

4.2.4.2 Analysis of regioisomers

Four different methods were applied to analyze the selected regioisomers in human milk (publication I Linderborg et al. 2014) and in sea buckthorn pulp oil (publication IV Kalpio et al. submitted).

In the ammonia NIAPCI-MS/MS method the same instrumentation and the parameters were used as with the NIAPCI-MS method (publication I Linderborg et al. 2014). The collision gas (argon) flow was set at 0.23 mL/min and the collision energy at 32 eV. Ammonia gas flow was optimized to generate maximal intensity of negative $[FA]^-$, and $[M – FA – H – 100]^-$ ions of TAGs (Kallio and Rua 1994; Kurvinen et al. 2001). The abundancy of different regioisomers in each sample were determined by using the MSPECTRA calculation program (Kurvinen et al. 2001). The intensities of the $[RCO_2]^-$ ions were used to quantify the different FA combinations of TAGs, and the intensities of $[M – H – RCOOH – 100]^-$ ions were used to analyze the regioisomeric composition. The program also corrected the spectra for abundances of the naturally occurring 13C isotopes. Earlier the ammonia NICI-MS/MS method has been applied to study many foods, for example human milk, human milk substitutes, human plasma lipids, animal fats and plant oils including seed oils (Currie and Kallio 1993; Kallio et al. 2001, 2005; Kurvinen et al. 2002b, 2002a; Yli-Jokipii et al. 2003; Linderborg and Kallio 2005; Yang and Kallio 2006; Leskinen et al. 2010a). Reference compounds 16:1-18:1-16:1, 18:1-14:0-18:1, *rac*-16:0-16:0-18:1, *rac*-16:0-18:2-18:1, *rac*-16:0-18:1-18:1, 18:1-18:2-18:1, *rac*-18:1-18:1-18:0, *rac*-18:0-18:0-18:1 and 18:0-18:1-18:0 (Larodan Fine Chemicals, Malmö, Sweden) were used as external standards to determine the correction factors to correct peak intensity of each fatty acid ($[FA-H]^-$) and discrimination factor, which describes the difference between the CID efficiency for formation of $[M-H-FA-100]^-$ ions between *sn*-1/3 and *sn*-2 (Kallio and Rua 1994; Kurvinen et al. 2001).

In the UHPLC-ESI-MS/MS method the same equipment was used as with the APCI-MS method. Ammonia was introduced to the nebulizer gas flow (nitrogen) to generate positive ammonium adducts $[M + NH_4]^+$. Product ion spectra of adduct ions $[M + NH_4]^+$ from the selected TAGs were scanned.

Reference compounds (TAGs 18:1-18:2-18:1, *rac*-18:1-18:1-18:2, 18:1-16:0-18:1, *rac*-16:0-18:1-18:1, Larodan Fine Chemicals, Malmö, Sweden) were analyzed as pure and as three mixtures (A-B-A/*rac*-A-A-B 25:75, 50:50 and 75:25) of the regioisomeric pairs to compare the ratio of the DAG ion intensities, $[A\text{-}B]^+ / ([A\text{-}B]^+ + [A\text{-}A]^+)$ to the corresponding intensities in the calibration curves (Leskinen et al. 2007).

TAG regioisomers of sea buckthorn pulp oil (publication IV Kalpio et al. submitted) were preliminary characterized with UHPLC-APCI-MS using the same Waters Quattro Premier instrumentation and parameters as were used to obtain the total ion chromatogram of sea buckthorn pulp oil. The TAGs in the oil sample were identified based on their positive-ion APCI mass spectra, including $[M + H]^+$ ions and the DAG fragment ions $[M + H - R_iCOOH]^+$.

The positional distribution of FAs in chirally separated sea buckthorn pulp oil TAG sub-fractions were analyzed with the ammonia NICI-MS/MS using the instrumentation as used in the ammonia NICI-MS analysis. Fragmentation of $[M\text{–}H]^-$ ions was performed using collision-induced dissociation with argon gas. Product ions were scanned between *m/z* 100–650. Calculations of the TAG regioisomer abundances were based on the relative proportions of $[M - H - FA - 100]^-$ and $[RCO_2]^-$ ions, and the results were calculated using the MSPECTRA software.

4.2.5 Statistical analysis

Normal distribution of the data (publication I Linderborg et al. 2014) was tested with the Shapiro-Wilk test. Depending on the normality of the data, either ANOVA or Kruskal-Wallis test was used for multiple comparisons followed by T-test or Mann-Whitney U test, which were Bonferroni -corrected. Statistical significance was indicated by $p<0.05$. Statistical analyses were performed with SPSS 18.0 software (SPSS Inc, Chicago, IL).

The randomized distribution of FAs in TAGs extracted from the sea buckthorn pulp oil was calculated from the analyzed FA composition with the RANDTAGS calculation program (Kallio and Rua 1994; Kallio et al. 2001), (publication IV Kalpio et al. submitted). If in diacid-TAG, the FAs are distributed in a random manner, the regioisomeric ratio of TAGs 1(3)-A-2-A-3(1)-B-*sn*-glycerol and 1-A-2-B-3-A-*sn*-glycerol should be 2:1. Correspondingly, the regioisomeric ratio in triacid-TAG containing *sn*-1(3)-A-2-B-3(1)-C-*sn*-glycerol, 1(3)-B-2-C-3(1)-A-*sn*-glycerol, and 1(3)-C-2-A-3(1)-B-*sn*-glycerol should be 1:1:1.

5 RESULTS AND DISCUSSION

5.1 Characteristics of subjects and dietary evaluation

In the human milk study (publication I Linderborg et al. 2014) there were no between-group differences in the mothers' age (30.01 ± 3.96 years, p=0.472), or infants' age at sampling time (11.55 ± 2.11 weeks, p=0.612). As expected, the mother's pre-pregnancy BMI in the normal weight groups (21.41 ± 2.31 and 20.81 ± 1.69 kg/m^2) deviated from the mother's pre-pregnancy BMI in the overweight groups (31.22 ± 4.25 and 29.79 ± 2.85 kg/m^2, p<0.001). The food choice score deviated between the recommended food choices groups, (4.60 ± 0.16 and 4.4 ± 0.16) and non-recommended food choices groups (1.90 ± 0.10 and 1.8 ± 0.13, p<0.001). Differences between the food choices were seen especially in the consumption of dairy fat.

5.2 Fatty acid composition

The most prevalent FAs in all human milk samples (publication I Linderborg et al. 2014) were C18:1, C16:0 and C18:2. More C18:3*n*-3 and less C18:0 was detected in the milk samples of the normal weight mothers with recommended food choices than in the milk of normal weight mothers with non-recommended food choices.

In sea buckthorn pulp oil (publication IV Kalpio et al. submitted) the most abundant FA was C16:1*n*-7, followed by C16:0 and C18:2. The pulp oil of sea buckthorn, unlike the seed oil, contained a high level of C16:1*n*-7. C16:1*n*-7 is important for human health (Hu et al. 2019), even though it is not one of the essential FAs. Thus, it was important to study the asymmetry and enantiomeric ratio of TAGs with C16:1*n*-7.

5.3 Mass spectrometric characterization

5.3.1 Molecular weight distribution

The ammonia NIAPCI-MS method revealed more than 60 TAG species in the analyzed range of human milk (publication I Linderborg et al. 2014). The major ACN:DB species were 52:2 (ECN 48), 52:3 (ECN 46), 50:2 (ECN 46) and 50:1 (ECN 48), which constituted more than one third of all TAG species detected. The total prevalence of TAGs with less than 1% abundancy was rather large (11.5–14.8%), which indicates the complexity of the TAG composition of the human milk. The amount of different TAG species detected

was lower with UHPLC-APCI-MS method as the chromatographic profile clearly revealed the existence of overlapping TAGs.

A significant difference between the normal weight mothers on the non-recommended food choices and the other groups was seen in ACN:DB 54:6, 54:5, 54:3 and 54:2. The molar percentages of ACN:DB 54:6 and 54:5 were lower, whereas, the molar percentages of ACN:DB 54:3 and 54:2 were higher in the group of normal weight mothers with the non-recommended food choices compared to other groups. The molar percentages of ACN:DB 52:7 and 52:6 were significantly higher in the two recommended food choices -groups compared to the two non-recommended food choices –groups (publication I Linderborg et al. 2014).

The major ACN:DB species of TAGs of sea buckthorn pulp oil were 48:2 (ECN 44), 50:3 (ECN 44), 50:2 (ECN 46) and 48:1 (ECN 46), respectively, which constitute 72.9% of all TAG species detected. The main difference of molecular weight distribution in sea buckthorn pulp oil analyzed with direct inlet ammonia NICI-MS compared to random distribution of FAs was seen in the amount of ACN:DB 48:2 (ECN 44), while the relative abundances were 27% and 16%, respectively. Also molecular weight distributions of TAGs in all the collected fractions of sea buckthorn pulp oil were confirmed with ammonia NICI-MS before MS/MS analysis.

15.3.2 Regioisomeric composition

The selected TAG regioisomers from human milk (publication I Linderborg et al. 2014) were differentiated by UHPLC-ESI-MS/MS and ammonia NIAPCI-MS/MS. Although UHPLC-ESI-MS/MS has been previously applied to rapeseed oil, sunflower seed oil and lard (Leskinen et al. 2007), the method could not give adequate results of the complex human milk TAGs. The chromatographic separation for TAGs of human milk was not sufficient, and overlapping TAGs with isobaric DAG fragments complicated the quantification. For example TAG ACN:DB 52:2, where the DAG fragments $[18:2\text{-}18:0]^+$ and $[18:1\text{-}18:1]^+$ have the same mass, the method cannot distinguish whether the fragments originates from 16:0/18:1/18:1 or 18:2/16:0/18:0 if these are not well separated. Also if the standard curves are only drawn for the most abundant regioisomer pair of each molecular weight fraction, the minor species are either just lost or interfere with the interpretation of the proportions of the major regioisomer pair. Another challenge are the standard curves for TAGs that consist of three different FAs such as ACN:DB 52:3, which contained mainly 18:2/16:0/18:1.

From the data obtained by ammonia NIAPCI-MS/MS, the MSPECTRA calculation program detected ten regioisomers in the group of ACN:DB 50:3,

five regioisomers in ACN:DB 50:1, ten regioisomers in ACN:DB 52:3, five regioisomers in ACN:DB 52:2 and seven regioisomers in ACN:DB 54:4. It was noticed that one isomer in each ACN:DB usually dominates, and traces of minor TAGs exist. TAG O-P-O (ACN:DB 52:2) was the most prevalent regioisomer (13.8 mol-% of all TAGs, publication I Linderborg et al. 2014). Although the milk from normal weight mothers with recommended food choices contained less ACN:DB 50:1 (4.7 mol-%) than the milk from overweight mothers with recommended food choices (6.1 mol-%), there were no differences in the proportions of the different regioisomers within the molecular weight fraction (Table 1 in publication I Linderborg et al. 2014). There were no differences in the proportions of the different regioisomers within the molecular weight fractions ACN:DB 50:3 or ACN:DB 52:3, either, although the largest TAG L-P-O tended to differ (p=0.065). The only between-group difference (p=0.005) in the molecular weight fraction ACN:DB 54:4 was in TAG 16:0-18:1-20:3 (Table 1 in publication I Linderborg et al. 2014), where the normal weight recommended food choices –group (16:0-18:1-20:3 not detected) deviated from the overweight recommended food choices –group (2.1 mol-% within the molecular weight fraction) and the normal weight non-recommended food choices –group (4.0 mol-%) However, the total abundancy of this molecular weight fraction was only about 4 mol-% of all human milk TAGs, and of it less than 5% were 16:0/18:1/20:3. More so, due to the small prevalence, there was a large between-sample deviation. Regioisomers O-O-L/O-L-O dominated this fraction. It may be that the previously detected differences in the FA compositions of the milk between overweight and normal weight mothers (Mäkelä et al. 2013) reflected the regioisomeric compositions obtained in small quantities. Mäkelä et al. have analyzed that the milk of overweight mothers contained higher amount of saturated FAs and lower amount of *n*-3 FAs compared to the milk of normal weight mothers. However, small differences (such as C18:3*n*-3 1.0–4.1% of all FAs in the milk) are difficult to analyze whilst distributed to different regioisomers.

From the four most abundant TAG species of sea buckthorn pulp oil, fraction 2 contained only all unsaturated TAGs (Po/Po/Po, Po/L/Po and L/L/Po), fraction 5 contained mainly Po/Po/P and P/Po/L, fraction 8 composed mostly of P/Po/O and P/O/L, and the main TAG species in fraction 9 were P/P/Po and P/P/L. The three most abundant fractions which contained asymmetric TAGs with saturated and unsaturated FAs (fractions 5, 8 and 9) were selected for chiral analysis. After enantioselective separation, the regiospecific compositions of TAGs of all the sub-fractions were determined with ammonia NICI-MS/MS (publication IV Kalpio et al. submitted).

5.4 Liquid chromatographic separations

5.4.1 Reversed-phase HPLC

Three major TAG fractions from sea buckthorn pulp oil were successfully collected after separation with NARP-HPLC (**Figure 6**) and subjected to chiral separation.

All RP-HPLC methods used in this PhD project separated TAGs according to ECNs. However, the separation of the same ECN but different FA composition was weak. The chromatographic profile and the mass spectral data revealed the existence of overlapping TAGs. The separation efficiency could be improved using two identical columns and sample recycling system also with RP-HPLC.

5.4.2 Chiral retention behavior and enantiomeric separation

The 21 enantiopure TAGs synthesized during this thesis project are valuable reference compounds for chromatographic separation and analysis in the future. The chiral-phase R-HPLC method in a polar-organic phase was demonstrated to enable the enantiomeric resolution of 17 out of 21 enantiomeric pairs of TAGs with C12–C22 FAs with 0–6 DBs. All chromatograms of enantioseparated TAGs was published in the corresponding papers (publications II Kalpio et al. 2015, IV Kalpio et al. submitted). Chiral separation with the two CHIRALCEL OD-RH cellulose tris-(3,5-dimethylphenylcarbamate) columns, methanol as the mobile phase and the conditions examined were effective when the asymmetric TAG molecule contained both saturated and unsaturated FAs. Also TAG enantiomers L-P-P/P-L-L, L-L-P/P-L-L, P-P-O/O-P-P and P-O-O/O-O-P that were not previously separated (Lísa and Holčapek 2013), were clearly separable with the cellulose-based stationary phase with the aid of sample recycling (publication II Kalpio et al. 2015). In an earlier study, they were either co-eluted with another enantiomer or regioisomer when quite similar chiral stationary phases were used but hexane/isopropanol gradient as the mobile phase (Lísa and Holčapek 2013). Nevertheless, Řezanka et al. (2013) succeeded in separating TAG enantiomers P-P-L/L-P-P and L-L-P/P-L-L using the same mobile phase combination but a β-cyclodextrin-based chiral phase, instead of mostly used cellulose-based stationary phase (Řezanka et al. 2012, 2013, 2015).

Only TAG enantiomers containing DHA were separated after the first column without recycling, but not to the baseline. Recycling was needed to separate all the other enantiomeric pairs. Four racemic reference mixtures analyzed did not exhibit any enantioresolution in an applicable time. *Rac*-18:1-

18:1-10:0 contained the shortest fatty acid tested. Triunsaturated TAGs with oleic, linoleic or palmitoleic acids (*rac*-16:1-16:1-18:1 and *rac*-18:1-18:1-18:2) or *rac*-16:0-16:0-*tr*18:1 were not separated. The three-dimensional, straight molecular structure of *rac*-16:0-16:0-*tr*18:1 is in practice similar with trisaturated TAGs due to the linear *trans* DB (publication II Kalpio et al. 2015). The peak shape and area remained constant and the peak broadening did not limit the analyses, despite long elution cycles (32 cycles, running time 9.5 h, *rac*-18:1-18:1-10:0).

Both the length of the acyl chain and the number of DBs of the FAs had a crucial impact on retention of TAGs. Enantiomeric resolution, at the same time with stronger retention of TAGs, increased with increasing FA chain length in the *sn*-1(3) position. A higher degree of unsaturation resulted in a shorter retention time. Based on the elution behavior of different TAG structures, curves to forecast retention behavior of TAG enantiomers under the chromatographic conditions examined were presented (publication II Kalpio et al. 2015). However, as demonstrated, the retention behavior of TAG enantiomers in chiral-phase HPLC is highly complex and depends on the specific molecular structures. Thus, the use of pure enantiomeric reference compounds is essential, and they are never fully replaceable with the curves. For the TAGs that contained maximum one VLCFA with C20 and 5 DBs and two saturated or one saturated and two unsaturated FAs, the retention decreased with increasing unsaturation and with decreasing acyl chain length. Furthermore, TAGs with C12–C22 FAs with 0–5 DBs were eluted in the order of ECN (publications II Kalpio et al. 2015, III Kalpio et al. 2020). However, when the number of DBs increased from 5 (EPA) to 6 (DHA), the elution order of TAG enantiomers did not follow the ECN values. TAGs containing DHA eluted later than TAGs containing EPA, even though the ECNs were the same. Also other authors have noticed abnormal retention behavior of TAGs with at least one VLCFA (Řezanka et al. 2018). The main reason is likely the *cis*-unsaturated FA tails of TAGs containing DHA, due to the highly bent three-dimensional structures. Consequently, ECN values should be used to predict the order of elution in chiral environment only for TAGs having long chain PUFAs with 1–5 DBs under similar chromatographic conditions.

Thus, though some systematic elution behavior can be noticed, it is a challenging task to find any common rule about the elution order of chiral TAGs. This is due to the fact that the chiral recognition mechanisms at molecular level are still not fully understood in the chromatography of lipids.

The study of Nagai and others demonstrated that difference in the length of the saturated acyl chain was recognized by the chiral stationary phase (Nagai et al. 2015), and the presence of both SAFAs and unsaturated FAs was not a requirement for enantiomeric separation. Interestingly, the chiral elution

behavior with the same stationary phase was opposite when TAGs with two C18:1 and one SAFA C10:0–C22:0 were analyzed. In that study, the longer the SAFA, the easier the separation of enantiomers was (publication II Kalpio et al. 2015).

The elution order of enantiomers was determined by co-injection of chemoenzymatically synthesized enantiopure and racemic TAGs or a mixture of two enantiomers. In analyses of all the critical pairs, the enantiomer with an unsaturated FA in the *sn*-1 position eluted faster than the one with the unsaturated FA in the *sn*-3 position, regardless of the carbon chain length and number of DBs. Despite the different retention behavior of TAGs containing DHA compared to other TAGs, there was no difference in elution order between the DHA-TAG enantiomers. The enantiomeric elution order were opposite when non-polar solvents like hexane-based mobile phases were used (Lísa and Holčapek 2013; Řezanka et al. 2018).

5.4.3 Enantiomeric purity

The UV chromatograms of all the 21 structured TAGs analyzed by the chiral-phase R-HPLC showed an absence of impurities, and that no isomerization occurred during synthesis. Performance of the system to detect small enantiomeric impurities was confirmed using samples of TAG enantiomers containing ALA, EPA or DHA spiked with 1% of the opposite enantiomer (publication III Kalpio et al. 2020). When comparing the chromatograms of enantiostructured TAGs with the one spiked with 1% of the less abundant enantiomer, the spiked enantiomer was clearly visible. For the enantiomers with the *n*-3 PUFA that were examined the enantiomeric excess (%) was more than 96%. The small shoulder of the opposite enantiomer i.e. the peak area of impurity was less than 2% of the peak area of the actual target compound. No impurities of the opposite enantiomer were detected from *sn*-ALA-16:0-16:0.

15.5 Stereospecific characterization of natural TAGs

The three major ACN species from sea buckthorn pulp oil were isolated and further analyzed by chiral-phase chromatography using the sample recycling system (publication IV Kalpio et al. submitted, **Figure 6**). They comprised more than 60% of the total TAG pool, and according to the MS analyses these ACN species most probably contained asymmetric TAGs. The chiral-phase R-HPLC method enabled a successful further resolution of all TAGs examined but not to the baseline (**Figure 6**). Progress of the resolution of enantiomers is fully illustrated in the corresponding paper (publication IV Kalpio et al.

submitted). From the three TAG fractions collected in the 1st dimension (RP-HPLC), altogether 10 new sub-fractions were obtained after enantiomeric separations in the 2nd dimension (chiral-phase HPLC). The composition of the sub-fractions was confirmed by ammonia NICI-MS/MS.

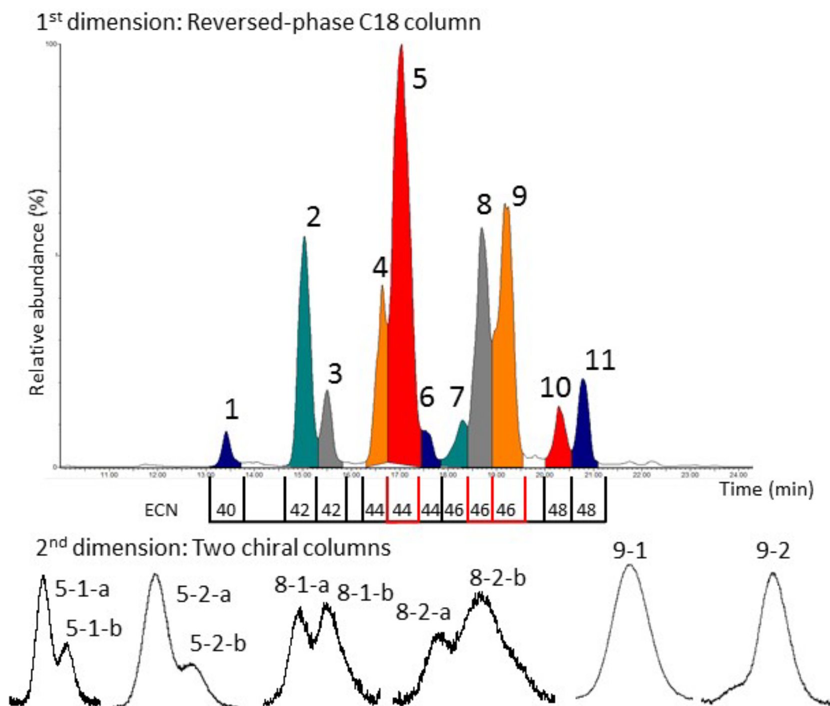


Figure 6. 1st dimension: Separation of TAGs of sea buckthorn pulp oil using RP-UHPLC-APCI-MS (total ion chromatogram). The fractions indicated by red lines were collected on the 1st dimension and re-injected to the enantioselective column on the 2nd dimension.
2nd dimension: Stereospecific separation of the collected fractions using chiral-phase R-HPLC. Sub-fractions are marked with the corresponding numbers and letters.

From fraction 5 altogether four sub-fractions were obtained and collected and analyzed with NICI-MS/MS. Based on the results of NICI-MS/MS, both sub-fraction 5-1-a and 5-1-b composed mostly of 16:1-16:1-16:0 indicating that they contain asymmetric TAGs. In addition, the chromatographic elution behavior in chiral environment and the separation profiles of the sub-fractions 5-1-a and 5-1-b were similar with the reference compound rac-16:1-16:1-16:0. According to our study, sn-16:1-16:1-16:0 elutes before sn-16:0-16:1-16:1 (publication II Kalpio et al. 2015). Thus, the sub-fraction 5-1-a contained

mainly sn-16:1-16:1-16:0 and 5-1-b sn-16:0-16:1-16:1 with enantiomeric ratio 70.5:29.5.

With fraction 8 all four separated fractions were collected in one run. The main $[M - H]^-$ ion in sub-fractions 8-1-a and 8-1-b was *m/z* 829.8 indicating TAGs with 50 acyl carbons and 2 DBs. Again, in sub-fractions 8-2-a and 8-2-b the main $[M - H]^-$ ion was *m/z* 855.8, which indicates TAGs with 52 acyl carbons and 3 DBs. All these fractions contained triacid-TAG species, where unsaturated FAs (either 16:1 or 18:1 or 18:2 or 18:1, respectively) were in the *sn*-2 position. As previously known, in plant oils mainly unsaturated FAs are esterified in the position *sn*-2, whereas in animal fats *sn*-2 position contains mainly saturated FAs (Lísa et al. 2009). Information related to the elution order and the chromatographic retention behavior of triacid-TAGs in chiral environment is very limited (Nagai et al. 2019). In theory, they contain six isomers, which include three regioisomers, each consisting of a pair of enantiomers, thus complicating the enantiomeric separation. Due to the lack of knowledge related to resolution of triacid-TAG enantiomers, and the incomplete separation of the sub-fractions between 8-1-a and 8-1-b as well as 8-2-a and 8-2-b, no stereospecific identification was achieved on the TAG molecular species.

Separation of fraction 9 was effective. After seven columns, two symmetric peaks separated from fraction 9 were clearly seen. The MS/MS analyses of sub-fractions 9-1 and 9-2 were quite straightforward probably due to the symmetric TAGs, which contained only two FAs. The sub-fraction 9-1 contained mainly 16:0-18:2-16:0 and the sub-fraction 9-2 contained 16:0-16:1-16:0, i.e. in both symmetric structures unsaturated FAs were predominantly in the *sn*-2 position and saturated, C16:0, in the positions *sn*-1/3. Only ca 5% had C16:0 in the *sn*-2 position.

These results clearly showed the non-random distribution of natural FAs, and abundance of unsaturated FAs in the position *sn*-2, which is common in plant oils. Other most abundant TAG species included mainly TAGs with three different FAs, which were not possible to analyze with the current methodology.

15.6 Discussion on applicability of the methods

All the MS methods applied were applicable in determining the TAG composition. The abundance of DAG fragments is commonly used for regiospecific structural identification both in positive ion ESI-MS/MS and APCI-MS methods. However, these methods could not give adequate quantitative results of the complex samples such as human milk TAGs,

especially, if only the major regioisomers are taken into account in calculations. In contrast, the MSPECTRA calculation program detected five to ten regioisomers in the ACN:DB groups of 50:3, 50:1, 52:3, 52:2 and 54:4 from human milk. In the positive ion MS methods, the minor TAG species were either lost or interfered with the interpretation of the proportions of the major TAG regioisomers if calibrations curves were drawn only for major TAG species. The standard curves used for accurate determination cannot be established for all possible TAG species. In addition, the interference of isobaric DAG fragments was apparent in the most abundant TAG species of human milk. However, the positive-ion UHPLC-APCI-MS method was very practical for relatively fast but tentative identification of each TAG species of the sea buckthorn pulp oil. It can be applied both for molecular weight characterization and for regioisomeric study as it easily produces diagnostically useful molecular and fragment ions in the same analysis. Alternatively, the direct exposure ammonia NICI-MS/MS method was even faster and very practical for quick confirmation. The amount of sample needed is very low making it a perfect tool to confirm the composition of chirally separated, isolated fractions. In the negative ion analysis (ammonia NIAPCI-MS/MS and ammonia NICI-MS/MS) the use of CI under vacuum was definitely the more practical ionization technique to get more intense $[M - H]^-$ ions. Further developing of the existing methods is needed to get tailored calculation software and a sophisticated method, which can be performed with single equipment, and is applicable to different analyses.

The enantioselective method developed during this thesis project enabled the enantiomeric separation of 17 different SSU- and SUU-type TAGs with C12–C22 FAs with 0–6 DBs in a polar-organic phase. In addition, all TAGs studied of sea buckthorn pulp oil were successfully separated. The number of enantiomers separated from each other at a time is limited due to the peak broadening during chiral chromatographic analysis. The number of these compounds varies according to composition and concentration of TAGs. Thus, all natural mixtures of even hundreds of different TAG species need to be pre-fractionated with sufficient recovery into more simple TAG groups or even single TAGs prior to the enantiomeric analysis.

With the chromatographic conditions examined in this PhD project enantiomeric separation of four TAG reference compounds (*rac*-18:1-18:1-10:0, *rac*-16:1-16:1-18:1, *rac*-18:1-18:1-18:2 or *rac*-16:0-16:0-*tr*18:1) were not successful. It must, however, be noted that, the separation in normal-phase mode was not applied. Generally, the most common natural lipids comprise a mixture of both saturated and unsaturated FAs. Thus, requirement for TAGs with both saturated and unsaturated FAs does often not limit the analysis substantially. The chiral-phase R-HPLC methods developed is applicable for

separation of (SSU- or SUU-type) TAGs with C12–C22 FAs with 0–5 DBs commonly found in nature, if the total time of analysis and the peak broadening cause no restrictions.

Trisaturated TAGs were not examined because of their low UV absorption. The usual disadvantage in detection of TAGs with UV-vis detector is difference in sensitivity between saturated and unsaturated TAGs and the insufficient sensitivity for trisaturated TAGs (Holčapek et al. 2005) which did not hinder the analysis in this study. The routinely used ELSD or MS detectors (Holčapek et al. 2005; Leskinen et al. 2007) are impracticable with recycling system, in which the detection method must be non-destructive. However, after isolation of fractions the enantiomers can be further analyzed with LC-MS, even online, as the mobile phase is LC-MS compatible. R-HPLC was demonstrated to be very practical tool to enhance the separation power. In theory, the sample can be recycled as many cycles as needed for the desired resolution, but in practice peak broadening limits the number of cycles. To overcome challenges with the length of the separation time, the procedure can be fully automated, once timed separately for each mixture.

The stereospecific composition of triacid-TAG is challenging to be determined due to the lack of information related to their behavior in the chiral environment. Chiral-phase chromatographic studies with structured A-B-C-type reference compounds should be provided to obtain more information about the elution order and chromatographic behavior of triacid-TAGs and to improve the applicability of the method.

Another improvement to study enantiomeric ratio of individual TAGs would be the multidimensional chromatography in online mode. However, there may be restrictions for mobile phases to be used in such analysis, because many commonly used LC eluents are not applicable with the sensitive chiral-phase columns. In future research, concentrations of the minor fractions have to be higher in order to obtain sufficient amount of samples for further analytical steps. Also separation of symmetric TAGs from asymmetric TAGs prior to chiral analysis would simplify the resolution of the enantiomers.

The results of the PhD thesis research showed that the strategy combining different chromatographic and mass spectrometric methods is suitable and reliable to determine stereospecific structures of individual TAGs of any biological matrices without derivatization or multiple reaction steps and reagents. The order of elution must be always established by comparison with pure enantiomeric reference compounds of known configuration because there are no commonly known rules about chiral elution behavior of TAGs.

In addition, structural changes in natural products caused by environmental factors or processing can be studied. Further, this study could be continued by studying the effect of place of growth, harvest year, weather conditions, species,

subspecies, or varieties on enantiomeric excess in specific compounds to get more information about TAG biosynthesis and its regulation.

Because the optical activity of TAGs is very low, the chiral-phase chromatography has a crucial role in studies of enantiomeric purity. The chiral R-HPLC method developed during this PhD project proved to be very effective also confirm the purity of the synthesized structured TAG enantiomers being higher than 98% (96% enantiomeric excess). Enantiomeric resolution is obviously essential for the reliable method to study enantiomeric purity due to the inability of UV detector to detect the co-eluting impurities.

6 SUMMARY AND CONCLUSION

The experimental part of the thesis concentrated on the regio- and especially stereospecific analysis of individual TAGs. The aim was to compare current regiospecific methods and to develop a stereospecific method applicable to the study of the positional distribution of FAs in nutritionally important TAGs.

The suitability of two different MS/MS methods in the analysis of TAG regioisomers of human milk was investigated. Overall, the MS/MS methods revealed differences in the fatty acid composition and molecular weight distribution of TAGs between human milk samples from mothers with different pre-pregnancy body mass indices and dietary food choices. Nevertheless, the regioisomerism of TAGs was uniform despite differences in the weight and diet of the mothers. The results highlighted the importance of structure-specific human milk substitutes. All the MS methods proved to be applicable for structural characterization of TAGs. However, the objective of the study and the careful selection of the MS/MS methods for analysis of mixtures of several isobaric TAGs have to be carefully considered prior to analysis. The direct exposure ammonia NICI-MS/MS method proved to be a very simple and fast method for the determination of the regioisomeric composition of major TAGs in natural fats and oils without pre-separation with chromatographic methods. The method was demonstrated to be a useful tool for quick confirmation of pure compounds; for example, the structure of TAGs collected after chiral separation can be confirmed with ammonia NICI-MS and -MS/MS. ESI-MS/MS with calibration curves was a practical method for quantifying the regioisomer composition of selected TAGs but limited by the sufficient chromatographic resolution of analyzed TAG species and the availability of regiopure reference TAGs. Thus, the ammonia NIAPCI-MS method offered the possibility to analyze the more comprehensive regioisomer composition of TAGs. On the other hand, the calculation software has its own limitations. The TAGs with SCFAs or FAs with higher degree of unsaturation are especially complicated to quantitate, and the method requires further development.

Enantiomeric separation is a challenging task in lipid analysis. A straightforward chiral sample recycling method was developed, without enzymatic hydrolysis or derivatization, offering reliable information on the enantiomeric composition of individual TAGs using fewer chemicals and reaction steps than the traditional methods. The length of the stationary phase is multiplied by recycling contrary to traditional column chromatography, where only a maximum of two or three columns in a series are possible due to the high back-pressure. The results of the thesis demonstrate applicability of the

chiral-phase chromatography with combined MS in determining the enantiomeric composition of selected TAGs from sea buckthorn pulp oil.

As there is no universal column-solvent -combination for all analytes, further studies about the chiral-phase chromatographic elution behavior of TAGs are still needed. Chiral-phase chromatographic studies with structured A-B-C-type TAG reference compounds are of especial interest so as to obtain more information about their elution order and chromatographic behavior, as natural oils and fats contain many asymmetric TAGs with three different FAs. Deeper knowledge about the chromatographic elution of chiral compounds could aid the interpretation of molecular recognition mechanisms. Another improvement in the study of the enantiomeric ratio of individual TAGs would be to be able to obtain multidimensional chromatography in online mode with one instrument. However, there may be restrictions for the mobile phase to be used in such an analysis, because many commonly used LC eluents are not applicable with the sensitive chiral-phase columns.

The chiral recognition mechanisms at the molecular level are not fully understood in the chromatography of lipids. Thus, all new information on the chiral retention behavior of TAGs is essential for a deeper understanding of the structure of TAGs and their effects in lipidomic studies. The knowledge obtained about chiral elution behavior and elution profile of the 21 structured TAGs synthesized during this PhD project has provided valuable novel information of chiral-phase chromatography of TAGs for future research. The chiral-phase R-HPLC method enabled resolution of 17 out of 21 TAGs studied, and their elution order and chromatographic elution behavior were firmly established. A TAG with an unsaturated FA in the *sn*-1 position eluted faster than its enantiomer with the unsaturated FA in the *sn*-3 position despite the length of carbon chains or the number of DBs. The TAG enantiomers containing DHA exhibited highly different retention on the chiral-phase column. The enantiomers of DHA-TAGs were separated after the first column, without recycling and their retention was stronger compared to TAGs with LCFAs. Based on these results, it is evident that no common rules can be applied to predict the elution behavior of all the chiral TAGs. The geometrical three-point interaction model can be used to explain the enantioselective separation in theory. However, it was clearly demonstrated that the chiral separation process and mechanisms of asymmetric TAGs are much more complicated, which highlights the importance of further studies in the field.

In addition to the analysis of the stereospecific molecular composition of TAGs, the method developed was demonstrated to provide information about the enantiomeric purity of the products synthesized. The compounds analyzed with the chiral-phase R-HPLC-UV were all of excellent enantiomeric excess (>96%). The knowledge whether a compound is naturally present as a pure

enantiomer or in a specific enantiomeric ratio can lead to further studies on the effects of the growth environment on the processing of the TAG composition of different seed oils, for example.

A targeted strategy was established using mass spectral characterization and achiral-chiral two-dimensional LC in offline mode for analysis of stereospecific structures of individual TAGs in nutritionally important natural oils. It was applied to study the enantiomeric composition of selected TAGs extracted from sea buckthorn pulp oil. The three achirally isolated TAG fractions, representing 60% of the total TAGs, were further separated into ten sub-fractions with chiral-phase R-HPLC-UV instrumentation. Analysis of these sub-fractions with direct inlet NICI-MS/MS enabled determination of regioisomeric and enantiomeric composition of the TAGs. It was shown that the TAGs Po-Po-P/P-Po-Po (enantiomeric ratio 70.5:29.5) and P-Po-P (96% symmetric) in the pulp oil of sea buckthorn berries have different isomeric ratios confirming the stereospecific enzymatic synthesis. Interesting results concerning the stereospecific composition of different TAGs have been obtained previously, however the range of sample materials and the TAG structures studied were limited. Furthermore, this analytical strategy has lead to the detection/discovery of stereospecific structural information about natural TAGs, and a comparison of the enantiomeric ratios, which cannot be resolved by other methods currently available.

In conclusion, this doctoral thesis demonstrates that the developed chiral-phase R-HPLC method can be used to investigate the stereospecific structure of individual TAGs. Application of the method on sea buckthorn pulp oil showed the promising results and demonstrated that by combining different chromatographic methods with mass spectrometry, stereospecific analyses with more complex natural oils and fats such as human milk are also possible. Future research should focus on studying the chiral recognition mechanism of different TAGs in more detail. These findings make the stereospecific analysis with more complex natural samples possible even though development of a single-stage method is essential.

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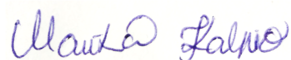

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APPENDIX: ORIGINAL PUBLICATIONS

I. Reprinted from the *Food Chemistry* 2014, 146, 583–590, with permission from Elsevier Ltd.

II. Reprinted from the *Food Chemistry* 2015, 172, 718–724, with permission from Elsevier Ltd.

III. Reprinted from the *Chemistry and Physics of Lipids* 2020, 231, 104937, Elsevier Ltd., an open access article published under the terms of the Creative Commons CC-BY license.

IV. *Submitted*.

DOCTORAL THESES IN FOOD SCIENCES AT THE UNIVERSITY OF TURKU

1. **REINO R. LINKO (1967)** Fatty acids and other components of Baltic herring flesh lipids. (Organic chemistry).
2. **HEIKKI KALLIO (1975)** Identification of volatile aroma compounds in arctic bramble, *Rubus arcticus* L. and their development during ripening of the berry, with special reference to *Rubus stellatus* SM.
3. **JUKKA KAITARANTA (1981)** Fish roe lipids and lipid hydrolysis in processed roe of certain *Salmonidae* fish as studied by novel chromatographic techniques.
4. **TIMO HIRVI (1983)** Aromas of some strawberry and blueberry species and varieties studied by gas liquid chromatographic and selected ion monitoring techniques.
5. **RAINER HUOPALAHTI (1985)** Composition and content of aroma compounds in the dill herb, *Anethum graveolens* L., affected by different factors.
6. **MARKKU HONKAVAARA (1989)** Effect of porcine stress on the development of PSE meat, its characteristics and influence on the economics of meat products manufacture.
7. **PÄIVI LAAKSO (1992)** Triacylglycerols – approaching the molecular composition of natural mixtures.
8. **MERJA LEINO (1993)** Application of the headspace gas chromatography complemented with sensory evaluation to analysis of various foods.
9. **KAISLI KERROLA (1994)** Essential oils from herbs and spices: isolation by carbon dioxide extraction and characterization by gas chromatography and sensory evaluation.
10. **ANJA LAPVETELÄINEN (1994)** Barley and oat protein products from wet processes: food use potential.
11. **RAIJA TAHVONEN (1995)** Contents of lead and cadmium in foods in Finland.
12. **MAIJA SAXELIN (1995)** Development of dietary probiotics: estimation of optimal *Lactobacillus* GG concentrations.
13. **PIRJO-LIISA PENTTILÄ (1995)** Estimation of food additive and pesticide intakes by means of a stepwise method.
14. **SIRKKA PLAAMI (1996)** Contents of dietary fiber and inositol phosphates in some foods consumed in Finland.
15. **SUSANNA EEROLA (1997)** Biologically active amines: analytics, occurrence and formation in dry sausages.
16. **PEKKA MANNINEN (1997)** Utilization of supercritical carbon dioxide in the analysis of triacylglycerols and isolation of berry oils.
17. **TUULA VESA (1997)** Symptoms of lactose intolerance: influence of milk composition, gastric emptying, and irritable bowel syndrome.
18. **EILA JÄRVENPÄÄ (1998)** Strategies for supercritical fluid extraction of analytes in trace amounts from food matrices.
19. **ELINA TUOMOLA (1999)** *In vitro* adhesion of probiotic lactic acid bacteria.
20. **ANU JOHANSSON (1999)** Availability of seed oils from Finnish berries with special reference to compositional, geographical and nutritional aspects.
21. **ANNE PIHLANTO-LEPPÄLÄ (1999)** Isolation and characteristics of milk-derived bioactive peptides.
22. **MIKA TUOMOLA (2000)** New methods for the measurement of androstenone and skatole – compounds associated with boar taint problem. (Biotechnology).
23. **LEEA PELTO (2000)** Milk hypersensitivity in adults: studies on diagnosis, prevalence and nutritional management.
24. **ANNE NYKÄNEN (2001)** Use of nisin and lactic acid/lactate to improve the microbial and sensory quality of rainbow trout products.
25. **BAORU YANG (2001)** Lipophilic components of sea buckthorn (*Hippophaë rhamnoides*) seeds and berries and physiological effects of sea buckthorn oils.
26. **MINNA KAHALA (2001)** Lactobacillar S-layers: Use of *Lactobacillus brevis* S-layer signals for heterologous protein production.
27. **OLLI SJÖVALL (2002)** Chromatographic and mass spectrometric analysis of non-volatile oxidation products of triacylglycerols with emphasis on core aldehydes.
28. **JUHA-PEKKA KURVINEN (2002)** Automatic data processing as an aid to mass spectrometry of dietary triacylglycerols and tissue glycerophospholipids.
29. **MARI HAKALA (2002)** Factors affecting the internal quality of strawberry (*Fragaria x ananassa* Duch.) fruit.
30. **PIRKKA KIRJAVAINEN (2003)** The intestinal microbiota – a target for treatment in infant atopic eczema?
31. **TARJA ARO (2003)** Chemical composition of Baltic herring: effects of processing and storage on fatty acids, mineral elements and volatile compounds.
32. **SAMI NIKOSKELAINEN (2003)** Innate immunity of rainbow trout: effects of opsonins, temperature and probiotics on phagocytic and complement activity as well as on disease resistance.
33. **KAISA YLI-JOKIPII (2004)** Effect of triacylglycerol fatty acid positional distribution on postprandial lipid metabolism.
34. **MARIKA JESTOI (2005)** Emerging *Fusarium*-mycotoxins in Finland.
35. **KATJA TIITINEN (2006)** Factors contributing to sea buckthorn (*Hippophaë rhamnoides* L.) flavour.
36. **SATU VESTERLUND (2006)** Methods to determine the safety and influence of probiotics on the adherence and viability of pathogens.
37. **FANDI FAWAZ ALI IBRAHIM (2006)** Lactic acid bacteria: an approach for heavy metal detoxification.
38. **JUKKA-PEKKA SUOMELA (2006)** Effects of dietary fat oxidation products and flavonols on lipoprotein oxidation.
39. **SAMPO LAHTINEN (2007)** New insights into the viability of probiotic bacteria.
40. **SASKA TUOMASJUKKA (2007)** Strategies for reducing postprandial triacylglycerolemia.

41. **HARRI MÄKIVUOKKO (2007)** Simulating the human colon microbiota: studies on polydextrose, lactose and cocoa mass.
42. **RENATA ADAMI (2007)** Micronization of pharmaceuticals and food ingredients using supercritical fluid techniques.
43. **TEEMU HALTTUNEN (2008)** Removal of cadmium, lead and arsenic from water by lactic acid bacteria.
44. **SUSANNA ROKKA (2008)** Bovine colostral antibodies and selected lactobacilli as means to control gastrointestinal infections.
45. **ANU LÄHTEENMÄKI-UUTELA (2009)** Foodstuffs and medicines as legal categories in the EU and China. Functional foods as a borderline case. (Law).
46. **TARJA SUOMALAINEN (2009)** Characterizing *Propionibacterium freudenreichii* ssp. *shermanii* JS and *Lactobacillus rhamnosus* LC705 as a new probiotic combination: basic properties of JS and pilot *in vivo* assessment of the combination.
47. **HEIDI LESKINEN (2010)** Positional distribution of fatty acids in plant triacylglycerols: contributing factors and chromatographic/mass spectrometric analysis.
48. **TERHI POHJANHEIMO (2010)** Sensory and non-sensory factors behind the liking and choice of healthy food products.
49. **RIIKKA JÄRVINEN (2010)** Cuticular and suberin polymers of edible plants – analysis by gas chromatographic-mass spectrometric and solid state spectroscopic methods.
50. **HENNA-MARIA LEHTONEN (2010)** Berry polyphenol absorption and the effect of northern berries on metabolism, ectopic fat accumulation, and associated diseases.
51. **PASI KANKAANPÄÄ (2010)** Interactions between polyunsaturated fatty acids and probiotics.
52. **PETRA LARMO (2011)** The health effects of sea buckthorn berries and oil.
53. **HENNA RÖYTIÖ (2011)** Identifying and characterizing new ingredients *in vitro* for prebiotic and synbiotic use.
54. **RITVA REPO-CARRASCO-VALENCIA (2011)** Andean indigenous food crops: nutritional value and bioactive compounds.
55. **OSKAR LAAKSONEN (2011)** Astringent food compounds and their interactions with taste properties.
56. **ŁUKASZ MARCIN GRZEŚKOWIAK (2012)** Gut microbiota in early infancy: effect of environment, diet and probiotics.
57. **PENGZHAN LIU (2012)** Composition of hawthorn (*Crataegus* spp.) fruits and leaves and emblic leafflower (*Phyllanthus emblica*) fruits.
58. **HEIKKI ARO (2012)** Fractionation of hen egg and oat lipids with supercritical fluids. Chemical and functional properties of fractions.
59. **SOILI ALANNE (2012)** An infant with food allergy and eczema in the family – the mental and economic burden of caring.
60. **MARKO TARVAINEN (2013)** Analysis of lipid oxidation during digestion by liquid chromatography-mass spectrometric and nuclear magnetic resonance spectroscopic techniques.
61. **JIE ZHENG (2013)** Sugars, acids and phenolic compounds in currants and sea buckthorn in relation to the effects of environmental factors.
62. **SARI MÄKINEN (2014)** Production, isolation and characterization of bioactive peptides with antihypertensive properties from potato and rapeseed proteins.
63. **MIKA KAIMAINEN (2014)** Stability of natural colorants of plant origin.
64. **LOTTA NYLUND (2015)** Early life intestinal microbiota in health and in atopic eczema.
65. **JAAKKO HIIDENHOVI (2015)** Isolation and characterization of ovomucin – a bioactive agent of egg white.
66. **HANNA-LEENA HIETARANTA-LUOMA (2016)** Promoting healthy lifestyles with personalized, *APOE* genotype based health information: The effects on psychological-, health behavioral and clinical factors.
67. **VELI HIETANIEMI (2016)** The *Fusarium* mycotoxins in Finnish cereal grains: How to control and manage the risk.
68. **MAARIA KORTESNIEMI (2016)** NMR metabolomics of foods – Investigating the influence of origin on sea buckthorn berries, *Brassica* oilseeds and honey.
69. **JUHANI AAKKO (2016)** New insights into human gut microbiota development in early infancy: influence of diet, environment and mother's microbiota.
70. **WEI YANG (2017)** Effects of genetic and environmental factors on proanthocyanidins in sea buckthorn (*Hippophaë rhamnoides*) and flavonol glycosides in leaves of currants (*Ribes* spp.).
71. **LEENAMAIJA MÄKILÄ (2017)** Effect of processing technologies on phenolic compounds in berry products.
72. **JUHA-MATTI PIHLAVA (2017)** Selected bioactive compounds in cereals and cereal products – their role and analysis by chromatographic methods.
73. **TOMMI KUMPULAINEN (2018)** The complexity of freshness and locality in a food consumption context
74. **XUEYING MA (2018)** Non-volatile bioactive and sensory compounds in berries and leaves of sea buckthorn (*Hippophaë rhamnoides*)
75. **ANU NUORA (2018)** Postprandial lipid metabolism resulting from heated beef, homogenized milk and interesterified palm oil.
76. **HEIKKI AISALA (2019)** Sensory properties and underlying chemistry of Finnish edible wild mushrooms.
77. **YE TIAN (2019)** Phenolic compounds from Finnish berry species to enhance food safety.
78. **MAIJA PAAKKI (2020)** The importance of natural colors in food for the visual attractiveness of everyday lunch.
79. **SHUXUN LIU (2020**) Fermentation with non-*Saccharomyces* yeasts as a novel biotechnology for berry wine production.
80. **MARIKA KALPIO (2020)** Strategies for analyzing the regio- and stereospecific structures of individual triacylglycerols in natural fats and oils.

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