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Chromatographic and Mass Spectrometric Determination of Molecular Species of Short-Chain Triacylglycerols in Butterfat

Asmo Kemppinen

ACADEMIC DISSERTATION

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Custos

Professor Tapani Alatossava
Department of Food and Nutrition
University of Helsinki

Supervisor

Docent Paavo Kalo
Department of Food and Nutrition
University of Helsinki

Reviewers

Professor Emeritus Heikki Kallio
Food Chemistry and Food Development, Department of Biochemistry
University of Turku

and

Professor Siv Skeie
Faculty of Chemistry, Biochemistry and Food Science
Norwegian University of Life Sciences

Opponent

Associate Professor Kaisa Linderborg
Food Chemistry and Food Development, Department of Biochemistry
University of Turku

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ABSTRACT

Molecules of short-chain triacylglycerols (SC-TAGs) contain at least one acyl group with two to six acyl carbons. A high molar proportion of SC-TAGs in bovine milk fat (MF) is a unique feature among edible fats and oils and results in characteristic biological, chemical, physical, and technological properties, and induces specific requirements for analysis of TAGs. Gas-liquid chromatography (GLC) using polar columns and normal-phase high-performance liquid chromatography (np-HPLC) with mass spectrometric (MS) detection provide efficient methods to analyze slightly polar SC-TAGs and their isomers, but comprehensive quantitative investigations of the regioisomers of SC-TAGs in bovine MF by GC–MS or np-HPLC–MS have not been reported. The main objective of the present study was to identify and quantify regio- and acyl chain isomers of SC-TAG species in model TAG mixtures and in butterfat (BF) by GLC, GC–EI–MS, and np-HPLC–ESI–MS/MS^2 (EI/ESI = electron/electrospray ionization).

In the present study, an improved method was developed to fractionate BF TAGs into saturated, monoene, and polyene TAGs on a solid-phase extraction column in silver ion mode (Ag-SPE) prior to GLC and GC–MS on a polarizable phenyl(65%)methylsilicone column. The method enabled determination of otherwise overlapping regio- and acyl chain isomers of SC-TAG species with different degree of unsaturation. Both Ag-SPE and molecular weight-based SPE fractionation prior to GLC and np-HPLC, respectively, increased the number of quantified TAG species in BF including some minor molecular species of TAGs.

The present study established elution order of the acyl chain isomers of isobaric TAG species and the regioisomers of SC-TAGs on a polarizable phenyl(65%)methylsilicone column using retention indices calculated on the basis of a high number (112) of synthesized TAG species and isomers. Separation power of a phenyl(65%)methylsilicone column proved to be high enough to resolve the regioisomers of monocaproyl TAGs, in addition to those of monobutyryl TAGs. As np-HPLC was used to separate regioisomers of SC-TAGs, all regioisomers of butyryl TAGs resolved to the baseline, monocaproyl TAGs close to the baseline, and dicaproyl TAGs partially. Few isomer pairs of long-chain TAGs containing fatty acyl 20:0 were resolved as well. The present study established that in ESI–MS^2, the cleavage of short-chain acyls (4:0, 6:0) from the *sn*-1(3) positions of ammonium adducts of mono-SC-TAGs was at least 2.3 times higher than that from the *sn*-2 position, which was previously confirmed with long-chain fatty acyls, hence enabling clear-cut differentiation between regioisomers.

Due to intricate TAG composition of BF, specific molar correction factors (MCFs) for TAG classes, TAG species, or TAG isomers were used in all three quantification methods. In two MS methods, extensive variation in MCFs was observed due to variation in molecular size, degree of unsaturation, regio- and acyl chain isomerism of TAGs, but substantial number (> 100) of MCFs enabled extrapolation of missing MCFs for uncommon TAG species. All MS methods provided new information that emphasized the need to use specific MCFs for the *sn*-1(3) and *sn*-2 TAG isomers.

All quantification methods yielded relatively unsurprising and similar general distribution of TAG classes and selection of the most common TAG species. However, 64 minor TAGs of 336 even-numbered TAG species that were determined by np-HPLC–ESI–MS were confirmed for the first time to exist in BF. A high number of them were SC-TAGs including monoacetyl TAGs, rare *sn*-2 isomers of monobutyryl or monocaproyl TAGs, di- and tri-SC-TAGs.

The present study suggested that GLC, GC–EI–MS, and np-HPLC–ESI–MS were reliable choices to determine both most frequently found TAGs and more rare TAGs in BF. All methods enabled quantification of the regioisomers of SC-TAGs in BF. The methods can be adapted to detect adulteration of MF, to elucidate biological or technological function of MF, and to develop effective procedures of MF modification. They can be applied to investigation of structured TAGs with short-chain fatty acyls.

PREFACE

This study was completed in the Department of Food and Nutrition at the University of Helsinki. First of all, I want to express my deepest gratitude to my supervisor Docent Paavo Kalo, whose brilliant expertise in the field of lipid science and unwavering support during these years have quided me through this research project. He has acted as a scientific mentor, invaluable colleague, and trustworthy friend since the first steps of my study. I want to thank Professor Tapani Alatossava for the opportunity to complete my dissertation and especially for his expert advice, patience, and encouragement during the critical final steps of this study. I want to express my gratitude to Professor Arnis Kuksis and Docent Velimatti Ollilainen for their invaluable contribution to this study as cowriters of original publications.

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Helsinki, April 2018

Asmo Kemppinen

CONTENTS

LIST OF PUBLICATIONS

This thesis is based on the following original publications, which are referred to in the text by their Roman numbers **I–VII**.

I Kemppinen A, Kalo P. 1993. Fractionation of the triacylglycerols of lipase-modified butter oil. J Am Oil Chem Soc 70(12):1203-7.

II Kalo P, Kemppinen A. 1993. Mass spectrometric identification of triacylglycerols of enzymatically modified butterfat separated on a polarizable phenylmethylsilicone column. J Am Oil Chem Soc 70(12):1209-17.

III Kemppinen A, Kalo P. 1998. Analysis of *sn*-1(3)- and *sn*-2-short-chain acyl isomers of triacylglycerols in butteroil by gas-liquid chromatography. J Am Oil Chem Soc 75(2):91-100.

IV Kalo P, Kemppinen A, Ollilainen V, Kuksis A. 2003. Analysis of regioisomers of short-chain triacylglycerols by normal phase liquid chromatography–electrospray tandem mass spectrometry. Int J Mass Spectrom 229(3):167-80.

V Kalo P, Kemppinen A, Ollilainen V, Kuksis A. 2004. Regiospecific determination of short-chain triacylglycerols in butterfat by normal-phase HPLC with on-line electrospray–tandem mass spectrometry. Lipids 39(9):915-28.

VI Kemppinen A, Kalo P. 2006. Quantification of triacylglycerols in butterfat by gas chromatography–electron impact mass spectrometry using molar correction factors for $[M–RCOO]^+$ ions. J Chromatogr A 1134(1–2):260-83.

VII Kalo P, Kemppinen A, Ollilainen V. 2009. Determination of triacylglycerols in butterfat by normal-phase HPLC and electrospray–tandem mass spectrometry. Lipids 44(2):169-95.

ABBREVIATIONS

ACN	number of acyl carbons
Ag-/Ag^+-	silver ion
APCI	atmospheric pressure chemical ionization
APPI	atmospheric pressure photoionization
BF/BO	butterfat/butteroil
CF/ECF/MCF	correction factor / empirical CF/molar CF
chir	chiral-phase
CI	chemical ionization
CID	collision induced dissociation
DAG(s)	diacylglycerol(s)
DB	double bond
EI	electron ionization / electron impact ionization
ESI	electrospray ionization
FA/FFA	fatty acid / free fatty acid
FAME	fatty acid methyl ester
FAB	fast-atom bombardment
FID	flame ionization detector
GC/GLC	gas chromatography / gas-liquid chromatography
GPC	gel permeation chromatography
HPLC/UHPLC	high-performance liquid chromatography / ultra HPLC
IBO/IBF	interesterified butteroil/butterfat
IS	internal standard
LC	long-chain with molecules / liquid chromatography with methods
LMBO	lipase-modified butteroil
LSD	light scattering detector
MAG(s)	monoacylglycerol(s)
MC	medium-chain
MF	milk fat
MS	mass spectrometry
MS^2 (MS/MS)	tandem mass spectrometry
NICI/nCI	negative ion chemical ionization
NMR	nuclear magnetic resonance
np	normal-phase
OCI	on-column injector / injection
PICI/pCI	positive ion chemical ionization
rac	racemic
RI	retention index
rp	reversed-phase
SC	short-chain
SGC	silica gel column chromatography
sic	silver ion chromatography
SIM	selected ion monitoring
sn	stereospecific numbering
SPE	solid-phase extraction
TAG(s)	triacylglycerol(s)
TIC	total ion chromatogram
TLC	thin-layer chromatography

Specific abbreviations with TAG/FA species: Ac = acetic (2:0), B/Bu = butyric (4:0), Co = caproic (6:0), Cy = caprylic (8:0), Ci = capric (10:0), La = lauric (12:0), M = myristic (14:0), P = palmitic (16:0), Po = palmitoleic (16:1), S/St = stearic (18:0), O = oleic (18:1), L = linoleic (18:2), Ln = linolenic (18:3); A/B/C/X/Y/Z denotes non-specific fatty acid / fatty acyl in triacylglycerol molecule; S = saturated, M = monoene, D = diene, T = triene, P = polyene

1 INTRODUCTION

Triacylglycerols (TAGs) are esters of glycerol with three fatty acids (FAs) (Nawar 1985), and they compose *ca*. 98 wt% of all lipids in bovine milk fat (MF) (Jensen 2002), which renders them the most influential ingredient in many high-fat dairy products. In spite of simple basic structure of TAG molecule, the composition of bovine MF TAGs is highly complex among edible fats and oils due to the exceptionally high number (> 400) of different FAs in MF (Jensen 2002). On the other hand, the structure of TAGs, *i.e.*, the distribution of FAs within and among TAG molecules, is very specific (Fontecha et al. 2010), which decreases the number of different TAG species in untreated MF compared to randomized MF. In addition, a high content of short-chain (SC) FAs (C2:0–C6:0), and particularly butyric acid in TAGs of bovine MF is a very specific feature among natural fats and oils (Jensen 2002). Consequently, the FA composition of MF TAGs, the distribution of FAs among the *sn*-positions of TAG molecules, and even the content of individual molecular species in MF are considered the main factors that influence on the functional properties of high-fat dairy products (Tzompa-Sosa et al. 2016).

SC-TAGs contain at least one SC-FA in the glycerol backbone. In bovine MF, 36 mol% of all even-numbered TAGs are estimated to be SC-TAGs that have one SC-FA and two long-chain (LC) FAs esterified to the glycerol backbone of TAG molecule (Gresti et al. 1993). The share of di-SC-TAG species is much lower, *ca*. 0.6 mol% (Gresti et al. 1993), and tri-SC-TAGs have been detected only in chemically randomized butteroil (Marai et al. 1994). Further, the structure of mono-SC-TAGs is very specific. Several stereospecific investigations have shown that acetyl acyl groups (*e.g.*, Itabashi et al. 1993) and butyryl acyl groups (*e.g.*, Christie and Clapperton 1982) are located almost exclusively in the *sn*-3 position of TAG molecules, and most caproyl acyl groups are esterified to the *sn*-3 position as well (*e.g.*, Christie and Clapperton 1982). In di-SC-TAGs, butyryl and caproyl acyl groups have been detected both at the secondary position (*sn*-2) and at primary positions (*sn*-1, *sn*-3). Composition and structure of SC-TAGs have major influence on the biological, physical, chemical, and technological properties of MF. SC-TAGs ensure together with medium-chain TAGs and unsaturated TAGs that relatively saturated MF remains liquid at the body temperature, which improves utilization of MF in feed by newborn offspring (Timmen and Putton 1988). A relatively wide melting range of MF (~ from –40 to +40°C) is partly due to the high content of SC-TAGs in MF and the asymmetric structure of mono-SC-TAGs (Walstra et al. 1995). In addition, several natural lipases in gastrointestinal track are *sn*-1,3-specific resulting in quick release and specific digestion of SC-FAs from the TAGs in MF (Bracco 1994, Mu and Høy 2004). In addition, the release of aromatic SC-FAs from the *sn*-3 position by natural and commercial *sn*-1,3-specific lipases can induce off-flavors or desired flavors in fatty dairy products (Urbach and Gordon 1994).

SC-TAGs have a definite effect on the selection of potential chromatographic and mass spectrometric methods which can be used to identify and quantify MF TAGs. Because SC-TAGs are slightly more polar than other MF TAGs, already a polar packed column has shown to separate TAGs with butyryl and caproyl acyl groups from other TAGs with the same number of acyl carbons (ACN) and the same degree of unsaturation (Kuksis et al. 1973). Even better separation has been achieved with long thermostable phenyl(50–65%)-

methylsilicone capillary columns (*e.g.*, Geeraert and Sandra 1985, 1987). Polar normal-phase high-pressure liquid chromatography (np-HPLC) columns have potential to resolve TAGs according to their polarity (Plattner and Payne-Wahl 1979, Rhodes and Netting 1988), but np-HPLC studies on TAGs in MF have not been reported.

Regiospecific analysis, *i.e.*, determination of the *sn*-2 and *sn*-1(3) isomers of TAGs is important, because it aids in elucidation of biological function of edible fats and oils and in development of manufacturing processes and quality control in food industry (Momchilova et al. 2004). Determination of the intact regioisomers of SC-TAGs can be performed by several chromatographic methods. Polarizable phenyl(50–65%)methylsilicone columns have shown to separate the regioisomers of mono- and di-SC-TAGs containing 2:0, 3:0, or 4:0 acyl groups (Myher et al. 1988, Huang et al. 1994, Kalo et al. 1996, Angers and Arul 1999). All kind of regioisomers of mono- and di-SC-TAGs (SC-FA = 2:0, 3:0, 4:0, 6:0) in structured TAGs have shown to be resolved on a np-HPLC column (Mangos et al. 1999, Lee et al. 2002, 2003, 2007, 2008), but corresponding separation of SC-TAGs in MF by np-HPLC has not been reported. Reversed-phase (rp) HPLC is infrequently used for regiospecific analysis of SC-TAGs. However, the regioisomers of monobutyrate TAGs have been resolved on a C18 rp-HPLC column (Kalo et al. 1996) and those of dibutyrate and -caproate TAGs on two C18 columns in series (Lee et al. 2002, 2003). Relatively recently (Nagai et al. 2015), a C28 column with analytes recycling resolved the regioisomers of monobutyryldipalmitoylglycerol. Silver-ion (Ag) HPLC is frequently used for regiospecific analysis of unsaturated TAGs, but the regioisomers of both mono- and di-SC-TAGs resolve on an Ag-HPLC column as well (Adlof 1996). However, regiospecific analysis of SC-TAGs in MF by Ag-HPLC has not been reported. In addition, stereospecific analysis of intact enantiomers of monobutyrate and monocaproate TAGs in MF is possible to perform by chiral HPLC (Nagai et al. 2015).

Regiospecific analysis of intact TAGs by mass spectrometry (MS) is based either *(i)* on the fragment ions that originated almost solely from the *sn*-1(3) positions, *i.e.*, $[M–RCOOCH_2]^+$ in electron ionization (EI) MS (Ryhage and Stenhagen 1960) and $[M–H–RCO_2H–100]^-$ in negative ion chemical ionization tandem MS (NICI–MS^2) (Kallio and Currie 1993) or *(ii)* on the less abundant cleavage of FA from the *sn*-2 position than from the *sn*-1(3) position, *i.e.*, formation of less abundant $[MH–RCOOH]^+$, $[M–RCOO]^+$, and $[(M+NH_4)–NH_3–FA]^+$ ions in CI–MS, atmospheric pressure CI (APCI) MS, and electrospray ionization (ESI) MS^2, respectively (Myher et al. 1984, Mottram and Evershed 1996, Marzilli et al. 2003). Regioisomers of TAGs in MF have been analyzed solely by MS using direct inlet ammonia NICI–MS^2 (Kallio and Rua 1994). However, regiospecific analysis is performed more frequently by combining MS with HPLC, *e.g.,* by rp-HPLC+Ag-HPLC–APCI–MS (Chiofalo et al. 2011), by LC–APCI–MS^2 (Gastaldi et al. 2011, Gotoh et al. 2012, Nagai et al. 2015), and by LC–ESI–MS^2 (Nagy et al. 2013, Linderborg et al. 2014). In addition, quantitative analysis of the regioisomers of TAGs in MF is even more challenging due to the intricate TAG composition and due to the fact that reliable quantification requires highly specific correction factors for each appropriate ions, because intensities and areas of peaks in MS spectra are dependent on the composition and structure of TAGs (*e.g.*, Laakso 1992).

Combining MS detection with chromatographic separation on polar GC or HPLC columns provides theoretically excellent qualitative and quantitative analytical tools to determine regio- and acyl chain isomers of SC-TAGs in bovine MF, which was investigated in the experimental part of this thesis. The literature review gives an overview of the composition and structure of MF TAGs and general principles of separation and identification of TAG species using common chromatographic and mass spectrometric methods. Application of chromatographic and mass spectrometric methods to separate, identify, and quantify MF TAG species and, particularly, the regio- and acyl chain isomers of SC-TAGs in MF is discussed thoroughly in the literature review.

2 OBJECTIVES OF THE STUDY

A highly complex TAG composition of bovine MF, asymmetry in TAG structure, and relatively high molar proportion of short-chain fatty acyls in TAG molecules influence markedly on physical, chemical, technological, and physiological properties of TAGs in practice. The same features in TAG composition and structure render the TAGs in bovine MF a challenging target for chromatographic and mass spectrometric analysis. However, presence of short-chain acyl groups (2:0–6:0) in TAG molecule and their asymmetric distribution increase polarity of TAGs enough to enable resolution of acyl chain isomers and regioisomers of SC-TAGs by appropriate chromatographic methods.

The main aim of the present study was to develop chromatographic (GLC, np-HPLC) and mass spectrometric (EI–MS, ESI–MS/MS^2) methods to separate, identify, and quantify regio- and acyl chain isomers of SC-TAG species in model TAG mixtures made by interesterification and to apply the same methods to determine molecular species of SC-TAGs in BF.

More specific objectives of the present study were:

1) to develop and apply a simple low-pressure column chromatographic method to fractionate BF TAGs according to the degree of unsaturation in order to simplify TAG matrix prior to analytical GLC and GC–MS (**I**, **II**, **III**, **VI**)
2) to investigate to what extent regio- and acyl chain isomers of SC-TAGs can be separated and identified by GLC using phenyl(65%)methylsilicone columns (**II**, **III**) by GC–EI–MS (**II**, **VI**), and by np-HPLC–ESI–MS/MS^2 (**IV**, **V**, **VII**)
3) to assess, how composition and structure of TAG species in model TAG mixtures affect specific correction factors in quantitative analysis by GLC, GC–EI–MS, and np-HPLC–ESI–MS (**II**–**VII**)
4) to determine general distribution of TAG classes, major molecular species of TAGs, and composition and structure of regio- and acyl chain isomers of SC-TAGs in BF by GLC, GC–EI–MS, and np-HPLC–ESI–MS/MS^2 (**III**, **V**, **VI**, **VII**).

3 LITERATURE REVIEW

3.1 Triacylglycerols of bovine milk fat

Triacylglycerols (TAGs) are the main lipid class of bovine milk fat (MF). The proportion of TAGs varies between 97 and 98% (w/w) of all lipids (Padley 1986, Banks 1991, Christie 1995, Jensen 2002). From the biological and nutritional point of view, TAGs of MF are the major source of energy and essential fatty acids (FAs) for newborn calves (Christie 1995). In addition, TAG composition and structure substantially influence on the sensory, physical, and technological properties of milk fat products (Timms 1994, Christie 1995, Jensen 2002). The specific features of the FA composition of MF TAGs, *i.e.*, high proportion of short- and medium chain FAs and existence of hydroxyacids, β-ketoacids, and unsaturated FAs (Urbach and Gordon 1994) contribute to the desired and undesired flavors of milk products. The specific distribution of the FAs among and within the TAG molecules affects digestibility of MF TAGs and flavor release by hydrolytic enzymes (Jensen et al. 1991, Christie 1995, Jensen 2002). Further, the complex FA and TAG composition and especially the high proportion of short-chain FAs in TAGs increase the number of mixed TAG crystals, and hence decrease melting point more than the relatively low degree of unsaturation of MF predicts (Timms 1994).

3.1.1 Molecular structure and nomenclature of triacylglycerols

TAGs are comparatively small biological (macro)molecules and their chemical structure is relatively simple. Essentially TAGs are esters of glycerol with three FAs (Nawar 1985) which may be all alike, two different, or all different (Harwood 1986) resulting in mono-, di-, or triacid TAGs, respectively. An example of the molecular structure of a common triacid TAG in milk fat consisting of triesters of glycerol with butyric- (C4:0), palmitic- (C16:0), and oleic acid (C18:1) is shown in Figure 3.1. Even though a glycerol molecule is symmetrical, the central carbon atom of a TAG molecule ($C_{sn\text{-}2}$ in Fig. 3.1) acquires chirality if two different FAs are esterified at the primary hydroxyl groups on the carbons $C_{sn\text{-}1}$ and $C_{sn\text{-}3}$ (Nawar 1985, Fig. 3.1). In order to describe unambiguously the structure of the TAG enantiomers, the stereospecific numbering (*sn*) system, which was first proposed by Hirschmann (1960), is frequently used. When a Fisher projection (*e.g.*, Cross and Klyne 1976) is depicted for the TAG molecule (Fig. 3.1) so that the esterified FA is shown to the left of the secondary carbon ($C_{sn\text{-}2}$ in Fig. 3.1), the primary carbons are designated *sn*-1 and *sn*-3 from top to bottom, respectively, and consequently, the secondary carbon in the middle is designated *sn*-2 (Fig. 3.1). Accordingly, the TAG in Figure 3.1 can be designated 1-oleoyl-2-palmitoyl-3-butyroyl-*sn*-glycerol, *sn*-glycerol-1-oleate-2-palmitate-3-butyrate, *sn*-18:1-16:0-4:0, or *sn*-OPB/*sn*-OPBu (Nawar 1985). In the case of the racemic mixture of two enantiomers, which is shown by *rac*-prefix, the same FA in the abbreviation is always attached at the *sn*-2 position, but the other two FAs are equally divided between *sn*-1 and *sn*-3 positions (Nawar 1985). For example, if the TAG in Figure 3.1 exists as racemic mixture, the abbreviation *rac*-18:1-16:0-4:0 indicates a mixture of equal amounts of *sn*-18:1-16:0-4:0 and *sn*-4:0-16:0-18:1. Further, the structure of *sn*-triacylglycerol enables

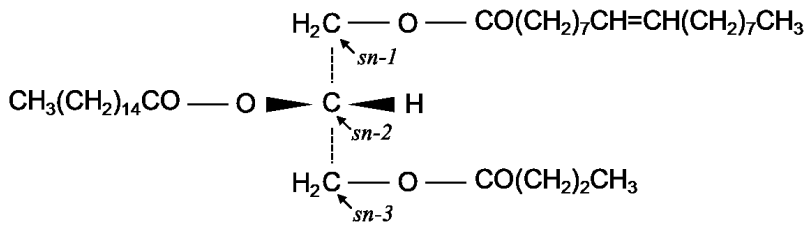

Figure 3.1. A Fischer planar projection of 1-oleoyl-2-palmitoyl-3-butyroyl-*sn*-glycerol.

a specific positional isomerism, *i.e.*, regioisomerism, in which the *sn*-position of the specific FA in the secondary (*sn*-2) or in the primary positions (*sn*-1 or *sn*-3) is indicated. For example, the *sn*-2 isomer of a monobutyrate TAG X-Y-4:0 (X, Y denote FAs other than 4:0) is the mixture of *sn*-X-4:0-Y and *sn*-Y-4:0-X, and the respective *sn*-1(3) isomer is of the mixture of *sn*-X-Y-4:0, *sn*-Y-X-4:0, *sn*-4:0-X-Y and *sn*-4:0-Y-X. Regio- and stereo-isomerism of TAGs in combination with a high number of different FAs result in a great number of possible molecular species of TAGs. As an example, three different FAs (X, Y, Z) can form ten TAG species with different FA composition and altogether 27 different stereo- and regioisomers of those TAGs (Kalo and Kemppinen 2003): *i.e.*, three monoacid TAGs (X-X-X; Y-Y-Y; Z-Z-Z), six diacid TAGs (*sn*-X-X-Y/Y-X-X, *sn*-X-Y-X; *sn*-X-Y-Y/Y-Y-X, *sn*-Y-X-Y; *sn*-X-X-Z/Z-X-X, *sn*-X-Z-X; *sn*-X-Z-Z/Z-Z-X, *sn*-Z-X-Z; *sn*-Y-Y-Z/Z-Y-Y, *sn*-Y-Z-Y; *sn*-Y-Z-Z/Z-Z-Y, *sn*-Z-Y-Z) and one triacid TAG (*sn*-X-Y-Z/Z-Y-X, *sn*-X-Z-Y/Y-Z-X, *sn*-Y-X-Z/Z-X-Y).

3.1.2 Composition and structure of the triacylglycerols in bovine milk fat

The composition of TAGs yields information about the FA content, the number of acyl carbons (ACN), and the number of double bonds (DB) in TAGs (Jensen et al. 1991, Jensen 2002). The structure of TAGs, on the other hand, provides additional information about the distribution of fatty acyls within the TAG molecule and among the TAG molecules, and ultimately, knowledge of the individual molecular species of *sn*-TAGs (Jensen et al. 1991, Jensen 2002). Both composition and structure of the TAGs of bovine MF are undoubtedly highly complex and unique among the TAGs of natural food fats and oils (Christie 1995, Jensen 2002, Fontecha et al. 2010).

Over 400 different FAs have been detected in the lipids of bovine milk as was compiled from several studies by Jensen (2002), who classified them into 1) saturates with normal, monobranched, and multibranched carbon chain, 2) *cis* and *trans* monoenes, 3) dienes, and 4) polyenes (tri–hexa), 5) keto, 6) hydroxy, 7) cyclic, and 8) furan FAs. As Table 3.1 shows, only *ca.* 14 FAs of them exist at high content (> 1%) in the TAGs of MF. Further, the general FA profile of MF is relatively similar in most countries (Table 3.1) showing significant variation only in the content of the major FAs, *i.e.*, palmitic and oleic acid and their ratio. However, it is well-known that several factors can change the FA composition of MF (*e.g.*, Hawke and Taylor 1995, Jensen 2002, Fontecha et al. 2010) due to the origin of FAs of bovine MF from two main sources that are 1) synthesis *de novo* in the mammary gland (4:0–14:0; a part of 16:0) and 2) blood (a part of 16:0; FAs > 16 acyl chain carbons), the FAs of

Table 3.1 Examples of fatty acid (FA) profiles (mol%) of bovine milk fat from assorted European countries, Australia, and USA.

FA	Trivial name[1]	Major FAs in bovine milk fat (mol%)						(wt%)
CN:DB *c/t*[2]	(Abbreviation)	AUS[3]	ESP	FIN	FRA	ITA	SWE[4]	USA
4:0	Butyric (Bu/B)	8.8	10.6	8.5	9.6	8.5	11.1	2–5
6:0	Caproic (Co)	5.0	4.8	4.0	4.9	3.9	4.7	1–5
8:0	Caprylic (Cy)	2.5	2.7	1.8	2.1	2.1	2.2	1–3
10:0	Capric (Ci)	5.3	4.7	3.2	3.9	2.9	4.3	2–4
12:0	Lauric (La)	5.2	4.1	3.2	3.7	3.2	4.3	2–5
14:0	Myristic (M)	13.8	11.2	10.4	11.4	11.7	10.9	8–14
15:0	– (–)	1.0	1.6	1.0		0.5	0.9	1–2
16:0	Palmitic (P)	28	22.6	26.4	24.8	21.9	26.7	22–35
16:1 *9c*	Palmitoleic (Po)	2.3	1.3	1.1	1.5	1.8	2.5	1–3
17:0	Margaric (Ma)	0.6	2.0	0.5		0.5	0.6	0.5–1.5
18:0	Stearic (St/S)	8.3	8.9	11.3	11.4	12.9	8.1	9–14
18:1 *9c*	Oleic (O)	14.1	17.9	19.4	23.0	26.0	19.1	20–30
18:2 *9, 12c*	Linoleic (L)	1.1	1.7	1.4	1.7	1.8	1.5	1–3
18:3 *9,12,15c*	Linolenic (Ln)	0.9	0.5	0.4	0.8	0.5	0.9	0.5–2
Σ		96.9	94.6	92.6	98.8	98.2	97.8	

[1] Commonly used names for major FAs (Gunstone 1986).
[2] ACN:DB *c/t* = Number of acyl carbons:number of double bonds *cis/trans*.
[3] AUS = Australia (Parodi 1982); ESP = Spain (Fraga et al. 1998); FIN = Finland (Laakso et al. 1992); FRA = France (Maniongui et al. 1991); ITA = Italy (Gastaldi et al. 2011); SWE = Sweden (Lund 1988); USA = the United States of America (Jensen 2002).
[4] Composition (mol%) is calculated from wt% by the author.

which originated from diet and from adipose tissue (Hawke and Taylor 1995). In addition, the microbes in the rumen hydrogenate unsaturated FAs modifying the FA composition of digested feed (Padley 1986). According to Jensen (2002), the main factors affecting the FA composition of MF originate from animal itself (genetics of the individual cow and breed, stage of lactation, health and ruminal fermentations) and from feed (energy intake, grain and protein intake, quality and quantity of dietary fat, seasonal and regional variation).

Even though vast majority of the TAGs in milk fat is constructed from the major FAs (n = 14), the possible number of different positional isomers in the hypothetical randomized TAG structure is significant, *i.e.* 2744 (n^3), and correspondingly, the number of TAGs with different FA content is 560 [n * (n + 1) * (n + 2)/6] (Fontecha et al. 2010, Rezanka and Sigler 2014). In practice, merely 50–150 different molecular species of TAGs have been typically identified in bovine MF by chromatographic and spectroscopic methods (Myher et al. 1988, 1993, Fraga et al. 1998, Robinson and MacGibbon 1998b, Mottram and Evershed 2001, Gastaldi et al. 2011, Beccaria et al. 2014) due to the non-random composition of the TAGs of bovine MF and due to the limitations of analytical methods. A

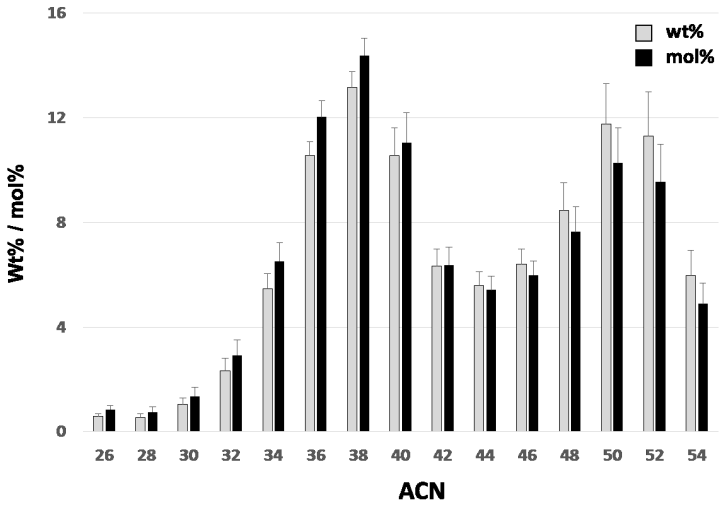

Figure 3.2. A schematic TAG profile (wt%/mol%) of bovine milk fat calculated from the data of Padley 1986 (Table 3.157, page 115). (ACN = number of acyl carbons)

higher number of individual TAG species (*i.e.*, 223) in bovine MF was quantified and 181 minor TAGs more were identified by Maniongui et al. (1991) and Gresti et al. (1993) using reversed-phase liquid chromatography (rp-LC) for fractionation and capillary gas chromatography for quantification. Further, Nagy et al. (2013) determined 565 molecular species (including regioisomers) of TAG in butterfat (BF) by rp-LC–mass spectrometry.

In general, even-numbered TAGs with 24–54 acyl carbons dominate, but odd-numbered TAGs are also present in bovine MF (Myher et al. 1988, Destaillats et al. 2006). The reported proportion of the TAGs with an odd number of acyl carbons varies from *ca*. 5% (*e.g.*, Taylor and Hawke 1975, Kallio et al. 1989) to 15% (*e.g.,* Maniongui et al. 1991, Gresti et al. 1993), and the number of the identified individual odd-numbered TAGs in MF ranges from 1–6 (Fraga et al. 1998, Robinson and MacGibbon 1998b) to 10–17 (Spanos et al. 1995, Mottram and Evershed 2001, Beccaria et al. 2014) depending on the analyzing method. Further, over 20 different odd-numbered molecular species of TAGs have been identified in the most volatile fractions of MF (Myher et al. 1988, 1993). Recently, Nagy et al. (2013) observed a very high number (128) of odd-numbered TAGs that comprised *ca*. 7.6 mol% of all TAGs in MF. The TAG profile of MF (Fig. 3.2) shows two distinct maxima at the ACN range 36–40 and 48–52. Corresponding ACN distribution of the TAGs of bovine MF was reported by Gresti et al. (1993), Marai et al. (1994), Fraga et al. (1998), and Destaillats et al. (2006) and that of anhydrous MF by Shi et al. (2001). The TAG profile with two maxima is due to two different main population of TAGs comprising several of the most abundant molecular species of TAGs in MF. The substantial individual TAGs in the ACN range 36–40 were shown to be 16:0-16:0-4:0 + 18:1-14:0-4:0 (ACN 36), 18:1-16:0-4:0 + 18:0-16:0-4:0 + 16:0-16:0-6:0 (ACN 38), and 18:1-18:1-4:0 + 18:1-16:0-6:0 (ACN 40), all of which consist of one short-chain (4:0/6:0) and two long-chain FAs (Myher et al. 1988, Gresti et al. 1993, Gastaldi et al. 2011). On the other hand, the most abundant TAG species

Table 3.2 Fatty acid (FA) composition (mol%) and stereospecific distribution of some major FAs in the triacylglycerols of bovine milk fat calculated from the data of Parodi (1979a), Christie and Clapperton (1982), and Blasi et al. (2013).

FA	FA composition (mol%)[1]			FA distribution between *sn*-positions, mean ± S.D. (%)[2]					
ACN:DB[3]	Ref 1	Ref 2	Ref 3	*sn*-1		*sn*-2		*sn*-3	
4:0	12.0	12.8	6.7	2.8	± 3.6	0.8	± 1.1	96.4	± 4.7
6:0	5.0	5.0	3.3	10.2	± 15.0	6.6	± 3.8	83.2	± 12.2
8:0	2.3	2.1	2.0	13.4	± 10.0	24.2	± 27.1	62.4	± 27.6
10:0	3.8	4.0	4.1	14.2	± 3.5	31.6	± 23.1	54.3	± 25.2
12:0	3.7	4.2	4.6	28.4	± 11.8	50.7	± 13.4	20.8	± 20.4
14:0	11.0	12.2	15.1	27.9	± 0.8	56.7	± 7.7	15.3	± 7.1
16:0	23.2	26.0	36.1	45.6	± 1.7	44.6	± 1.1	9.8	± 2.0
18:0	13.6	7.6	7.5	51.5	± 2.5	28.5	± 15.0	20.0	± 12.7
18:1	25.3	26.1	20.6	38.0	± 3.4	24.2	± 2.7	37.8	± 5.0

[1] The sum of the values (mol%) of FAs from the Ref 1, 2, and 3, *i.e.*, from Parodi (1979a), Christie and Clapperton (1982), and Blasi et al. (2013), respectively, is normalized to 100 mol%. The values of the FA composition of Ref 1 are the mean of six determinations over a year.
[2] Mean ± S.D. is calculated from the data of the references 1–3.
[3] ACN:DB = Number of acyl carbons:number of double bonds.

with 48–52 acyl carbons were shown to be solely composed of long-chain FAs, *i.e.*, 18:1-16:0-14:0 + 18:1-18:1-12:0 (ACN 48), 18:1-16:0-16:0 + 18:1-18:1-14:0 + 18:1-18:0-14:0 (ACN 50), and 18:1-18:1-16:0 + 18:1-18:0-16:0 (ACN 52) (Gresti et al. 1993, Gastaldi et al. 2011).

The number of double bonds in the TAG molecule of bovine MF ranges usually from zero to five when determined by empirical chromatographic and spectrometric methods (Gresti et al. 1993, Mottram and Evershed 2001, Beccaria et al. 2014). However, trisaturated (SSS; S = saturated FA) and monounsaturated TAGs (SSM; M = monoene FA) are by far the most abundant TAG classes in MF composing 73–81% of all TAGs (Parodi 1981, Gresti et al. 1993, Laakso and Kallio 1993a). Gresti et al. (1993) calculated that there were equal proportion (*ca.* 39 mol%) of SSS and SSM classes in the even-numbered TAGs in MF, but in Parodi's (1981) investigation the proportion of SSS and SSM classes in eight MF samples varied between 32 and 40 mol% and between 34 and 43 mol%, respectively, thus either of them could be the major TAG class.

Further, SMM TAG class composing of one saturated FA and two monoene FAs is another relatively abundant TAG class (13–18 mol%) in MF (Parodi 1981, Gresti et al. 1993, Laakso and Kallio 1993a). In addition to the number of double bonds, variation in the *cis–trans* configuration of double bonds characterizes the TAG molecules of MF, which is uncommon among natural food fats and oils. The proportion of *cis*-isomers in SSM TAG class (SSM^c) has been shown to be from three times (Parodi 1981) to ten times (Laakso and Kallio 1993a) higher than that of *trans* isomers (SSM^t). Similarly, the proportion of SM^cM^c class has shown to be *ca.* 1.5–2 times more frequent than SM^cM^t class in MF (Parodi 1981, Laakso and Kallio 1993a).

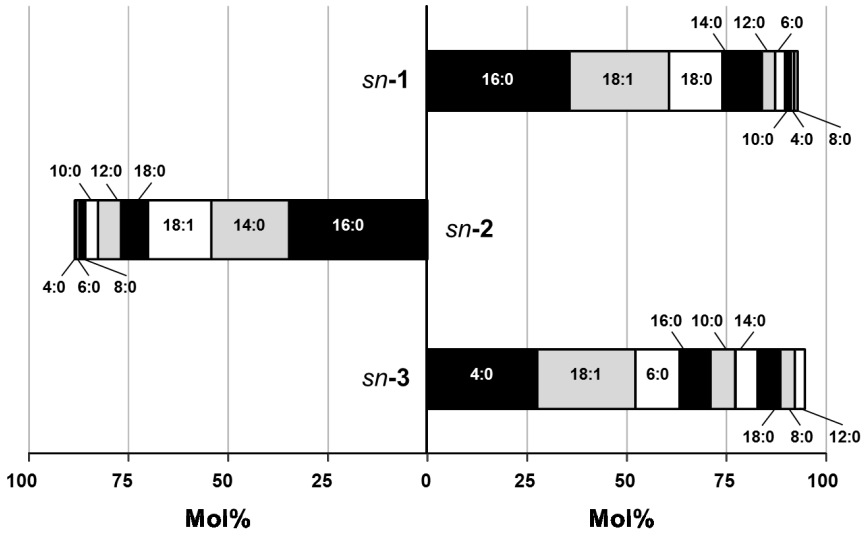

Figure 3.3. An average fatty acid (FA) composition at the *sn*-positions of TAGs in milk fat represented as cumulative mol% from the most abundant to the least abundant FA at each *sn*-position. The values are calculated from the data of three references (Parodi 1979a, Christie and Clapperton 1982, Blasi et al. 2013) as a mean of the respective values (mol% FA at *sn*-i, i = 1,2,3).

The structure of bovine MF TAGs, *i.e.*, the distribution of FAs attached to the *sn*-positions of the glycerol backbone, is non-random. The specific distribution of FAs was observed already in late 1960's (Pitas et al. 1967, Breckenridge and Kuksis 1968a), and was confirmed later in several studies (Bus et al. 1976, Pfeffer et al. 1977, Parodi 1979a, 1982, Christie and Clapperton 1982, Gotoh et al. 2012, Blasi et al. 2013). The general stereospecific distribution of the major FAs of MF is presented in Table 3.2. Some obvious trends of the distribution can be observed: (1) Extremely high proportion of short-chain acyl groups (4:0, 6:0) is in the primary positions (*sn*-1/3) of TAG, especially butyryl groups are almost exclusively in the *sn*-3 position. (2) Major part (> 50%) of the medium-chain acyl groups (8:0, 10:0) are located in the *sn*-3 position. (3) Lauric and myristic acids are most prominent (> 50%) in the secondary position of glycerol backbone. (4) Most of stearic acid is esterified to the *sn*-1 position. (5) Most of palmitic and oleic acids are distributed almost equally between *sn*-1 and *sn*-2, and between *sn*-1 and *sn*-3 positions, respectively. Almost identical regiospecific distribution of FAs, as indicated in Table 3.2, was recently reported by Nagy et al. (2013) when they analyzed regiospecific distribution of FAs in 565 intact BF TAG species.

Due to the uneven distribution of FAs, the average FA composition at *sn*-1, 2, and 3 position is specific as well and the proportion of the two most abundant FAs is over 50 mol% of all FAs at each *sn*-position (Fig. 3.3). The three most abundant FAs esterified to *sn*-1, 2, and 3 position are (16:0, 18:1, 18:0), (16:0, 14:0, 18:1), and (4:0, 18:1, 6:0), respectively (Fig. 3.3). Hence, oleic acid is the sole FA that is among the three most abundant FA at each *sn*-position.

Table 3.3 The most common (> 1 mol%) TAG species of bovine milk fat. The number denotes the order of the relative abundance of the TAGs (most abundant = 1) in each of example studies: Myher et al. (1988), Gresti et al. (1993), and Gastaldi et al. (2011).

ACN:DB[1]	TAG	Myher et al. (1988)[2]	Gresti et al. (1993)	Gastaldi et al. (2011)
36:0	16:0-16:0-4:0	1	2	1
38:1	18:1-16:0-4:0	2	1	7
34:0	16:0-14:0-4:0	3	3	3
38:0	18:0-16:0-4:0	6	6	5
38:0	16:0-16:0-6:0	5	13	2
40:1	18:1-16:0-6:0	7	9	15
36:1	18:1-14:0-4:0	4	10	22
40:0	18:0-16:0-6:0	15	21	6
40:1	18:1-18:0-4:0	13	12	18
40:2	18:1-18:1-4:0	12	14	21
52:2	18:1-18:1-16:0		5	9
52:1	18:1-18:0-16:0		8	8
50:1	18:1-16:0-16:0		7	10
40:0	16:0-16:0-8:0	16		4
36:0	16:0-14:0-6:0	9	16	
48:1	18:1-16:0-14:0		4	
38:1	18:1-14:0-6:0	8		
42:0	16:0-16:0-10:0	10		

[1] ACN:DB = Number of acyl carbons:number of double bonds.
[2] The sample was a highly volatile distillate of butteroil. For details, see Myher et al. (1988).

On the other hand, short- and medium-chain FAs are the least abundant FAs at *sn*-1 and *sn*-2 positions (Fig. 3.3). Recently, Tzompa-Sosa et al. (2014) showed that the three most abundant FAs (mol%) at the *sn*-2 and *sn*-1(3) positions were 16:0 (40.7), 14:0 (16.7), 18:1 (15.1), and 16:0 (21.5), 18:1 (16.3), 4:0 (14.1), respectively, which is in accordance with the average FA composition depicted in Figure 3.3.

A list of the most common (> 1 mol%) TAG species of bovine MF is compiled in Table 3.3 from the example studies (Myher et al. 1988, Gresti et al. 1993, Gastaldi et al. 2011): Each one of the TAG species was among the ten most abundant molecular species at least in one of these studies, or alternatively, among TAG species that were detected in all of these studies. Table 3.3 shows that a high proportion of the most abundant TAG species in bovine MF is mono short-chain TAGs containing one butyric or caproic acid and two long-chain FAs. The three most abundant individual molecular species of TAGs in MF are most probably *sn*-16:0-16:0-4:0, *sn*-18:1-16:0-4:0, and *sn*-16:0-14:0-4:0 (*cf.*, Tables 3.2 and 3.3), which have been shown to be the only TAGs in MF with higher content than 3 mol% (Gresti et al. 1993). Correspondingly, the most common higher molecular weight TAGs consisting solely of long-chain FAs seem to be *sn*-18:1-16:0-18:1, *sn*-18:0-16:0-18:1, *sn*-16:0-16:0-18:1, and *sn*-16:0-14:0-18:1 (Gresti et al. 1993, Gastaldi et al. 2011; *sn*-positions deduced

from Table 3.2), even though Myher et al. (1988) were unable to detect them from highly volatile BF distillates containing mostly low molecular weight TAGs.

3.1.3 Short-chain triacylglycerols of milk fat

Short-chain triacylglycerols (SC-TAGs) can be defined as TAGs that have one to three short-chain FAs (2:0–6:0) esterified to the glycerol backbone of TAG molecule, contrary to medium-chain (MC) and long-chain (LC) TAGs in which the shortest acyl group attached to TAG molecule has, at least, 7–10 acyl carbons and ≥11 acyl carbons, respectively. SC-TAGs containing one short-chain and two long-chain FAs constitute a significant proportion (*ca*. 36 mol%) of all TAGs in bovine MF (Gresti et al. 1993). Several stereospecific determinations have shown that 91–100% of butyryl groups and 70–100% of caproyl groups are located in the *sn*-3 position in bovine MF (Pitas et al. 1967, Breckenridge and Kuksis 1968a, Bus et al. 1976, Pfeffer et al. 1977, Parodi 1979b, 1982, Christie and Clapperton 1982, Blasi et al. 2013). However, due to the fact that small contents of dibutyrates (Gresti et al. 1993, Myher et al. 1993, Gastaldi et al. 2011, Nagy et al. 2013), dicaproates (Gresti et al. 1993, Myher et al. 1993, Nagy et al. 2013), and butyrocaproates (Gresti et al. 1993, Myher et al. 1993, Gastaldi et al. 2011, Nagy et al. 2013, Beccaria et al. 2014) have been detected in bovine MF, at least some SC-FAs are esterified to the *sn*-1 and/or *sn*-2 positions. SC-TAGs containing three short-chain FAs have not been reported to exist in MF, but tri-SC-TAGs with ACN 10–16 (4:0-4:0-2:0, 4:0-4:0-4:0, 4:0-4:0-6:0, and 4:0-6:0-6:0) have been determined in randomized butteroil by LC-MS (Marai et al. 1994). The most common butyrate and caproate TAG species are shown in Table 3.3.

Acetate TAGs are far more infrequently detected in MF. Parodi (1975) observed aceto-diacylglycerols in solvent extracted MF in his plain silica thin-layer chromatography (TLC) and gas chromatograpy (GC) studies. Myher et al. (1988) investigated the most volatile fractions of MF and identified over 20 saturated and unsaturated even- and odd-numbered acetate TAG species. Later, Limb et al. (1999) showed for the first time that acetyl groups can be a natural component in milk synthesizing tissue when they isolated and determined 13 monoacetate TAGs from the bovine udder by ^{1}H- and ^{13}C-nuclear magnetic resonance (NMR) techniques together with collision-induced dissociation fast-atom bombardment (FAB) MS and tandem MS (MS^2). Interestingly, 10 out of 13 molecular species of TAGs in their study were also detected in MF by Myher et al. (1988). In addition, 4–5 monoacetate TAG species in MF were detected by Myher et al. (1993), Itabashi et al. (1993), and Nagy et al. (2013), and one acetate TAG has been reported to exist in buffalo mozzarella cheese (Beccaria et al. 2014). Practically all identified molecular species of acetate TAGs contain one acetyl group and two long-chain (12–18 acyl carbons) acyl groups (Myher et al. 1988, Myher et al. 1993, Itabashi et al. 1993, Limb et al. 1999, Beccaria et al. 2014). In addition, acetyl group had been shown to be in the *sn*-3 position in the glycerol backbone (Itabashi et al. 1993, Limb et al. 1999). Exceptionally, Nagy et al. (2013) detected a regioisomer pair of one di-SC acetate TAG (6:0-2:0-16:0/2:0:16:0-6:0). The most frequently detected molecular species of acetate TAGs were 14:0-16:0-2:0 and 16:0-16:0-2:0 (Myher et al. 1988, Myher et al. 1993, Itabashi et al. 1993, Limb et al. 1999). An uncommon tri-SC acetate TAG (4:0-4:0-2:0) has been identified in randomized butteroil (Marai et al. 1994).

Abundance of SC-TAGs and their highly specific distribution of SC-FAs between *sn*-positions render bovine MF a distinctive food fat and affect physical, digestive, and technological properties of MF. Even though MF is a relatively saturated food fat, it crystallizes totally at quite low temperatures (–40 – –30°C). This is partly due to the high number of mono-SC-TAGs in MF, which increases the number of di- and tri-acid TAG molecules and, on the other hand, due to the specific attachment of SC-FAs to the *sn*-3 position, which increases the asymmetry of TAG molecules (Walstra et al. 1995). Short- and medium-chain TAGs together with long-chain TAGs containing oleic acid have a key role in maintaining MF liquid at the body temperature of lactating cow, which is essential for proper secretion of milk and for utilization of feed by the offspring (Timmen and Patton 1988). It has been shown (Timmen and Patton 1988) that decrease in the incorporation of oleic acid into TAG molecules increases the incorporation of SC-FAs and *vice versa*.

The specific distribution of SC-FAs within TAG molecule influences on the digestion and absorption of MF TAGs, as well as flavor of milk and dairy products. The digestion process starts in the stomach by the lingual and gastric lipases, which both preferentially hydrolyze FAs from the primary positions and especially from the *sn*-3 position (release from *sn*-3 is ~ 2 times faster than from *sn*-1), thus releasing substantial amount of SC-FAs and *sn*-1,2-DAGs from TAGs (Mu and Høy 2004). Most of the released short- and medium-chain FAs are readily absorbed into the portal vein and transported to the liver (Bracco 1994) resulting in quick digestion, absorption and utilization of SC-FAs of MF. In addition, hydrolytic activity of industrial and native lipases on the primary *sn*-positions of SC-TAGs contributes to the release of desired and undesired flavors in dairy products. Free SC-FAs, if present in concentration higher than 100 ppm, can induce strong, unpleasant flavors, *i.e.*, lipolytic rancidity in fresh milk (Urbach and Gordon 1994). Further, the flavor threshold of free SC-FAs is 3–10 times lower than that of LC-FAs in high-fat dairy products (Urbach and Gordon 1994). SC-TAGs are essential substrate for proper flavor development of Provolone and Romano type cheeses (Urbach and Gordon 1994, Jensen 2002), characteristic flavor of which is partly derived from free SC-FAs released by (commercial) lipases from the *sn*-3 position of SC-TAGs.

3.2 Chromatographic and mass spectrometric determination of triacylglycerols in milk fat

3.2.1 Prefractionation of the triacylglycerols in milk fat

Chromatographic and mass spectrometric analyses of the TAGs of bovine MF are challenging due to small differences in size, chemical structure, and polarity of TAG molecules, and a vast number of various isomers. In order to separate, identify and, ultimately, quantify relatively similar TAG species as small subgroups of TAGs or as individual TAG species, chromatographic prefractionation is often an essential step prior to analytical chromatography and mass spectrometry. Tables 3.4–3.6 summarize the most common prefractionation methods used in the analysis of MF TAGs.

Overview of the prefractionation methods of MF TAGs on normal-phase thin-layer chromatography (np-TLC) is given in Table 3.4. In general, plain silica TLC of MF TAGs yields two or three distinct TAG fractions according to the polarity of TAGs. Table 3.4 indicates that fractionation results in a low molecular weight (LMW) TAG fraction containing slightly polar SC-TAGs and one or two apolar fractions with high molecular weight (HMW) TAGs. In early investigations (Blank and Privett 1964), MF was fractionated into one fraction containing TAGs with SC- and MC-FAs and the other fraction containing TAGs with MC- and LC-FAs. More recently, Mottram and Evershed (2001) used similar prefractionation system prior to HPLC–atmospheric pressure chemical ionization (APCI)–MS analysis of bovine MF TAGs. However, a more common way to prefractionate MF TAGs is to separate them into HMW, medium molecular weight (MMW), and LMW fraction (Kuksis and Breckenridge 1968, Breckenridge and Kuksis 1968b, Myher et al. 1993). The studies of Banks et al. (1989) and Steele and Banks (1994) showed that the 3rd/LMW fraction consisted mostly of butyrate TAG species and, thus, more asymmetric molecular species. Steele and Banks (1994) assumed that separation of 1st/HMW and 2nd/MMW fraction was partly due to the differences in stereospecific distribution of acyl groups. Parodi (1975) showed that acetate TAGs in MF could be separated from other TAGs on np-TLC plates. This finding was later confirmed by Myher et al. (1988) in fractionation of a highly volatile MF distillate with concentrated amount of short-chain TAGs on a plain silica TLC plate. They showed that the most polar fraction consisted of acetate TAG species. Solvent systems used to develop np-TLC plates were not highly uniform (*cf.*, Table 3.4) in MF prefractionation. In general, three-component solvent systems were used (Kuksis and Breckenridge 1968, Breckenridge and Kuksis 1968b, Banks et al. 1989, Myher et al. 1993, Steele and Banks 1994, Mottram and Evershed 2001) consisting of non-polar alkane (hexane, heptane), more polar ether (diethyl or isopropyl ether), and the most polar acid component (acetic or formic acid) in varying volumetric proportions. However, Myher et al. (1988) used successfully a two-component system (hexane–ethyl acetate, 88:1, v/v) in np-TLC of volatile MF fraction with high concentration of short-chain TAGs.

Silver ion thin-layer chromatography (Ag-TLC) is another common prefractionation method of MF TAGs (Table 3.5). The separation of TAGs using silver ion chromatography is based on the different mobility of TAG molecules due to the number of double bonds, double bond position in the acyl groups, and *cis–trans* isomerism (Christie 1988, Nikolova-Damyanova et al. 1990) and, partly, due to the size and asymmetry of TAG molecules (Jeffrey 1991). Interactions of π-electrons of double bond(s) and silver ions of the stationary phase of Ag-TLC plate enable formation of reversible complexes, which retard progression of unsaturated TAGs (*e.g.*, Ruiz-Gutiérrez and Barron 1995, Buchgraber et al. 2004b). The general rule is that TAGs with higher number of double bonds are retarded more than less unsaturated TAGs due to the stronger complex formation. Already Gunstone and Padley (1965) showed, however, that TAGs with two double bonds in one linoleic acyl were retarded more than TAGs with two double bonds in two oleic acyls. In addition, they showed that TAGs with three double bonds in one linolenic acyl were retarded more than TAGs

Table 3.4 Examples of prefractionation of the triacylglycerols (TAGs) of milk fat (MF) based samples by normal-phase thin-layer chromatography (np-TLC).

Method	**Sample** => Fractions (F)	Reference
Silica gel G plate (0.25 mm)	**Bovine MF =>** F 1: TAGs with MC/LC-FAs F 2: TAGs with SC/MC-FAs	Blank and Privett (1964)
Silica gel plate (0.5 mm) developed in heptane–isopropyl ether–AcCOOH (60:40:4, v/v/v)	**Bovine MF =>** HMW, MMW, LMW fractions ***A highly volatile MF distillate*** **=>** *HMW, MMW, LMW fractions*	Kuksis and Breckenridge (1968), Breckenridge and Kuksis (1968b) *Myher et al. (1993)*
Silica TLC plate (0.25 mm) developed in HEX–ethyl acetate (88:1, v/v)	**A highly volatile MF distillate =>** F 1–3 F 3: Acetate TAGs	Myher et al. (1988)
Silica TLC plate (1.25 mm) developed in HEX–DEE–formic acid (40:10:1, v/v/v)	**Hydrogenated MF =>** F 1–3 F 3: Mostly butyrate TAGs	Banks et al. (1989), Steele and Banks (1994)
Silica TLC plate (0.25 mm, particle size 60 Å); developed in HEX–DEE–AcCOOH (94:4:2, v/v/v)	**Bovine MF =>** F 1: Majority of TAGs F 2: Mostly butyrate TAGs	Mottram and Evershed (2001)

Abbreviations: HMW/MMW/LMW = high/medium/low molecular weight; AcCOOH = acetic acid; HEX = hexane; DEE = diethyl ether

with four double bonds in two linoleic acyls. As a rule, the elution order (according to increasing retention) is SSS>SSM>SMM>SSD>MMM>SMD>MMD>SDD≥SST>SMT≥MDD>MMT>SDT≥DDD>MDT≥STT>DDT>MTT>DTT>TTT, in which S, M, D, and T denote fatty acyl groups with 0, 1, 2, and 3 double bonds, respectively; however, the position of S/M/D/T do not refer to the *sn*-positions in TAG molecule (*e.g.*, Nikolova-Damyanova 2009). Further, *cis* monoene TAGs were shown to be retarded more than *trans* monoene TAGs, and *cis*-*cis* diene TAGs more than *cis*-*trans* diene TAGs on Ag-TLC plates (Parodi 1980).

In early analysis of MF TAGs (Blank and Brivett 1964, Kuksis and Breckenridge 1968, Parodi 1980), Ag-TLC was primarily used to fractionate HMW, MMW, and LMW fractions into 5–7 TAG fractions with different degree of unsaturation and content of *cis–trans* isomers. According to above principle, Parodi (1980) fractionated MF into six distinct fractions containing (1) saturated, (2) *trans* monoene, (3) *cis* monoene, (4) *cis*-*trans* diene, (5) *cis*-*cis* diene, and (6) polyene TAGs. Later, MF samples were more frequently separated by Ag-TLC without preceding fractionation by np-TLC resulting in 3–9 Ag-TLC bands (Lund 1988, Myher et al. 1988, Marai et al. 1994, Fraga et al. 1998, Robinson and MacGibbon 1998a,b, 2000, Fontecha et al. 2000). Lund (1988) used unfractionated MF as a TAG sample to produce saturated, monoene, and polyene MF fractions for further capillary GLC analysis of TAGs. Myher et al. (1988) showed that Ag-TLC was an excellent

Table 3.5 Examples of prefractionation of the triacylglycerols (TAGs) of milk fat (MF) based samples by silver ion thin-layer chromatography (Ag-TLC).

Method	**Sample** => Fractions (F)	Reference
Silica gel G plate, impregnated with $AgNO_3$; developed in 25% DEE in petroleum ether (30–60°C)	**HMW, LMW F from bovine MF =>** 7 fractions from HMW TAGs 5 fractions from LMW TAGs	Blank and Privett (1964)
Silica gel plate, impregnated with 10% $AgNO_3$; developed in chloroform (0.65% methanol)	**HMW, MMW, LMW F from MF =>** Saturated, monoene, diene, triene, and polyene fractions (F)	Kuksis and Breckenridge (1968)
Silica gel G plate, impregnated with 25% $AgNO_3$; developed in chloroform–benzene–etOH (70:30:0.25, v/v/v)	**HMW, MMW, LMW F from MF =>** F 1: Saturated TAGs F 2: Monoene TAGs, *trans* F 3: Monoene TAGs, *cis* F 4: Diene TAGs, *cis-trans* F 5: Diene TAGs, *cis-cis* F 6: Polyene TAGs	Parodi (1980)
Silica gel G plate, impregnated with 10% $AgNO_3$; developed in chloroform (0.6–1.0% etOH)	**Butterfat =>** F 1: Saturated TAGs F 2: Monoene TAGs F 3: Polyene TAGs	Lund (1988)
Silica gel G/H plate, impregnated with 15% $AgNO_3$; developed in chloroform (0.75% etOH)	**A highly volatile MF distillate =>** F 1: Saturated TAGs, caproate TAGs and higher F 2: Saturated TAGs, butyrate TAGs and higher F 3: Monoene TAGs, 72% *trans*, 28% *cis* F 4: Monoene TAGs, 100% *cis* F 5: Diene TAGs, two monoenoic FAs F 6: Other diene and triene TAGs	Myher et al. (1988)
Silica gel G plate (0.25 mm) impregnated with 15% $AgNO_3$ developed in chloroform (0.75% etOH)	**Randomized butteroil =>** Saturated, monoene, diene, triene, and polyene fractions	Marai et al. (1994)
Silica gel plate (0.25 mm), impregnated with 20% $AgNO_3$ developed in chloroform	**Bovine MF (from butter) =>** F 1–2: Saturated butyrate TAGs + other saturated TAGs F 3–4: Monoene butyrate TAGs + other monoene TAGs F 5 and 6: Diene and triene TAGs, respectively	Fraga et al. (1998)

Method	**Sample** => Fractions (F)	Reference
Silica gel plate (0.25 mm), impregnated with 20% $AgNO_3$ developed in chloroform–toluene (1:1, v/v)	**Anhydrous bovine MF =>** F 1: Saturated TAGs (ACN > 36) F 2: Saturated TAGs, caproate TAGs and higher F 3: Saturated TAGs + *trans* monoene TAGs, butyrate TAGs and higher F 4: Monoene TAGs F 5: Monoene TAGs, caproate TAGs and higher F 6: Monoene TAGs, butyrate/caproate TAGs F 7: Monoene TAGs + *cis, trans* diene TAGs F 8: Diene TAGs, *cis-cis* F 9: Polyene TAGs	Robinson and MacGibbon (1998a,b), Robinson and MacGibbon (2000)
Silica gel plate (0.25 mm), impregnated with 20% $AgNO_3$ developed in chloroform (0.5–0.8% etOH)	**Goat MF =>** F 1: Saturated TAGs, caproate TAGs and higher F 2: Saturated TAGs, butyrate TAGs and higher F 3: Monoene TAGs, F 4: Polyene TAGs	Fontecha et al. (2000)

Abbreviations: HMW/MMW/LMW = high/medium/low molecular weight; ACN = number of acyl carbons; DEE = diethyl ether; etOH = ethanol

alternative for simple fractionation of MF which was naturally relatively saturated fat with high proportion of SC-TAGs and naturally occurring *trans* isomers. They separated highly volatile MF distillate into two saturated fractions, two monoene fractions and two diene/polyene fractions on the basis of acyl chain length of TAGs (butyrate TAGs *vs*. other TAGs), *cis–trans* isomerism (*cis-trans vs*. all-*cis*), and degree of unsaturation (diene TAGs with two oleic acids *vs.* other di- and polyene TAGs), respectively. Robinson and McGibbon (1998a) developed an improved Ag-TLC method for anhydrous MF. They separated TAGs into nine different fractions which consisted of two fractions of pure saturated TAGs, one fraction of combined saturated and monoene TAGs, three fractions of pure monoene TAGs, one fraction of combined monoene and diene TAGs, one fraction of pure diene TAGs, and one fraction of polyene TAGs (for details, see Robinson and MacGibbon 1998a and Table 3.5). Fontecha et al. (2000) showed applicability of Ag-TLC for fractionation of goat MF and separated TAGs into two saturated, one monoene, and one polyene Ag-TLC bands.

Robinson and MacGibbon (1998a) stressed that impregnation of silica gel TLC-plates with silver ions and choice of the solvents for mobile phase are the critical steps in Ag-TLC. Table 3.5 indicates that impregnation step was carried out relatively similarly using 10–25% $AgNO_3$ (Kuksis and Breckenridge 1968, Parodi 1980) in Ag-TLC analysis of MF TAGs. Chloroform was most frequently used solvent as mobile phase when total MF TAGs were analyzed (Table 3.5). Content of ethanol in chloroform ranged from 0% (Fraga et al. 1998)

Table 3.6 Examples of prefractionation of the triacylglycerols (TAGs) of milk fat (MF) based samples by low- and high-pressure column chromatography.

Method	**Sample** => Fractions (F)	References
SGC		
Silicic acid (100 mesh) column, 400 x 18 mm, eluted with HEX + DEE (0%–10%), flow rate 2 mL/min	**Bovine MF (from butter) =>** HMW F: TAGs > ACN 38 MMW F: TAGs ACN 32–38 LMW F: TAGs < ACN 32	Parodi (1980)
rp-HPLC		
C18 Hypersil rp-column (4 mm x 250 mm, 5 μm particle size); elution with propionitrile-AcN (6-7:4-3, v/v)	**MF, Human MF =>** 49 fractions for MF 39 fractions for human MF	Weber et al. (1988a,b)
LiChrospher 100 RP-18 + precolumn (4 mm x 250 mm, 4 μm particle size); isocratic elution with ACE-AcN (55:45, v/v) 1 mL/min ACE-AcN (50:50, v/v) 1 mL/min (2nd step for the first 27 fractions)	**Bovine MF (from butter) =>** 47 fractions	Maniongui et al. (1991), Gresti et al. (1993)
2 x Discovery HS C18 rp-columns in series (4.6 mm x 250 mm, 5 μm particle size); gradient elution with 2-propanol-AcN (70%=>30%)	**Donkey MF =>** > 50 fractions	Dugo et al. (2005)
Ag-HPLC		
Nucleosil (4.6 mm i.d. x 250 mm) cation exchange column loaded with silver ions ($AgNO_3$); binary gradient: (A) DCM + 1,2-di-chloroethane (4:1, v/v) and (B) ACE; flow rate 1.0 mL/min	**Liquid and solid fractions of BF =>** **Anhydrous BF =>** F 1: Saturated TAGs F 2: Di-saturated *trans*-monoene TAGs F 3: Di-saturated *cis*-monoene TAGs F 4: Saturated *cis, trans*-dimonoene TAGs F 5: Saturated *cis, cis*-dimonoene TAGs F 6: Polyene TAGs	Laakso et al. (1992), Laakso and Kallio (1993a,b), Kallio et al. (2006)
Nucleosil (1.0 mm i.d. x 250 mm) column loaded with silver ions ($AgNO_3$); linear gradient: (A) DCM, (B) DCM/ACE (1:1, v/v), (C) ACE/AcN (4:1, v/v), flow rate 6.0 mL/min	**Human MF =>** 13 fractions	Brühl et al. (1993, 1994)
Nucleosil (1.0 mm i.d. x 150 mm) microbore column loaded with silver ions ($AgNO_3$); binary gradient:	**Donkey MF =>** Volume (20 mL) based fractions injected onto rp-HPLC column	Dugo et al. (2006)

Method	**Sample** => Fractions (F)	References
(A) 0.7% AcN in HEX (v/v) and (B) 0.9% AcN in HEX (v/v), flow rate 11 μL/min		
GPC		
PL Gel column (polystyrene–divinylbenzene, 60 cm x 10 mm, 10 μm particle size, 50 Å pore size); elution with DCM-meOH (1:2, v/v), 3 mL/min	**Bovine MF =>** 16 fractions	Mottram and Evershed (2001)

Abbreviations: SGC = silica gel column chromatography; rp-HPLC = reversed-phase high-performance liquid chromatography; Ag-HPLC = silver ion high-performance liquid chromatography; GPC = gel permeation chromatography; HMW/MMW/LMW = high/medium/ low molecular weight; ACN = number of acyl carbons; HEX = hexane; DEE = diethyl ether; AcN = acetonitrile; ACE = acetone; DCM = dichloromethane; meOH = methanol

to 1% (Lund 1988). Interestingly, Robinson and MacGibbon (1998a,b, 2000) modified successfully the developing solvent and used chloroform:toluene-mixture (1:1, v/v) in fractionation of total MF.

Several low and high pressure column chromatographic methods have been used to pre-fractionate MF TAGs prior to qualitative and quantitative determination by chromato-graphic and spectrometric methods (Table 3.6). Parodi (1980) utilized a low-pressure column chromatography on silica columns to produce low (ACN < 32), medium (ACN 32–38), and high molecular weight (ACN > 38) TAG fractions of bovine MF TAGs. More recently, high pressure column chromatography in reversed-phase mode (Weber et al. 1988a,b, Maniongui et al. 1991, Gresti et al. 1993, Dugo et al. 2005) or in silver-ion mode (Laakso et al. 1992, Laakso and Kallio 1993a,b, Brühl et al. 1993, 1994, Dugo et al. 2006, Kallio et al. 2006,) has been applied to the fractionation of MF TAGs.

Weber et al. (1988a,b) used rp-HPLC to separate TAGs of both bovine and human MF on a octadecylsilyl (ODS/C18) column resulting in 49 and 39 TAG fractions, respectively. Similarly, Maniongui et al. (1991) and Gresti et al. (1993) used rp-HPLC to yield 47 major TAG fractions of bovine MF for further analysis by GC. Dugo et al. (2005) studied donkey MF showing that the use of two C18 rp-columns in series increased the number of separated TAG fractions over 50, which were further analyzed by Ag-HPLC–APCI–MS. Due to the high separation power, rp-HPLC is more generally used as a sole chromatographic method than as a prefractionation step in the analysis of MF TAGs. Thus, the separation of MF TAGs on rp-HPLC columns will be elaborated in Chapter 3.2.2.

Prefractionation of MF TAGs by Ag-HPLC (Table 3.6) is based on the same principles as that by Ag-TLC. Altogether, six different TAG fractions were separated according to the number and *cis–trans* configuration of double bonds when liquid and solid fraction of MF (Laakso et al. 1992) and anhydrous MF (Laakso and Kallio 1993a,b, Kallio et al. 2006) were prefractionated by Ag-HPLC. Further, Brühl et al. (1993, 1994) used Ag-HPLC to fractionate TAGs of human MF into 13 TAG fractions as follows: SSS, SSE, SSM, SEM, SMM, SSD, (MMM + SED), SMD, EMD, MMD, (SDD + SST), (MDD + SMT), and (DDD

and higher unsaturated TAGs), in which S, E, M, D, and T denote saturated, *trans*-monoene, *cis*-monoene, diene, and triene fatty acyl groups, respectively.

In addition, Mottram and Evershed (2001) studied prefractionation of bovine MF by gel permeation chromatography using a polystyrene–divinylbenzene gel. They separated TAGs into 16 fractions, after which most fractions were analyzed by rp-HPLC–APCI–MS and GC–MS. The results showed that TAG species of MF separated on a gel column according to polarity rather than molecular size, which was slightly unexpected.

3.2.2 Separation of the triacylglycerols in milk fat on GLC and HPLC columns

Gas-liquid chromatography. Examples of separation of TAGs in MF are given in Table 3.7 in chronological order. For the first time, TAGs of MF were sufficiently well separated and quantified on GLC columns by Kuksis and McCarthy (1962) and Kuksis et al. (1963), after which the method has been an important analytical tool in the analysis of MF TAGs. In the early investigations (Kuksis and Breckenridge 1965, 1966, Murata and Takahashi 1973, Murata 1977, Parodi 1972, 1979b, Morrison and Hawke 1977), apolar packed columns were used to separate TAGs solely according to the number of acyl carbons. Later, GLC with similar packed columns together with mathematical and statistical modelling had been used mainly to investigate adulteration of dairy product (*e.g.*, Precht 1991, Molkentin and Precht 1994, 2000, Lipp 1996, Molkentin 2007). TAG analysis using short capillary columns (length < 5 m) yielded similar separation of TAGs, but with shorter analysis time (Molkentin and Precht 1994, Lozada et al. 1995, Molkentin and Precht 2000, Gutiérrez et al. 2009). More recently, development of ultrafast GC enabled the separation of MF TAGs on apolar columns according to the number of acyl carbons within four minutes with excellent resolution of TAGs with ACN 24–54 (Destaillats et al. 2006, Povolo et al. 2008). Further, odd-numbered TAGs separated partially between the peaks of even-numbered TAGs on short packed columns (Molkentin and Precht 1994), but practically baseline resolution was achieved when MF TAGs were analyzed by GC on a high-quality short apolar capillary column (Destaillats et al. 2006). Introduction of long (> 15 m) thermostable apolar capillary columns (Grob et al. 1980) resulted in an improved resolution within the same ACN of TAGs due to separation of acyl chain isomers, especially those containing short-chain acyls (Grob et al. 1980, Geeraert et al. 1983). Further, Geeraert and De Schepper (1982) showed that the improved resolution of TAGs was partly based on the number of unsaturated fatty acyls in TAG molecules. In general, unsaturated TAGs eluted prior to saturated TAGs on long apolar capillary columns (Grob et al. 1980, Geeraert and Sandra 1987, Ruiz-Samblás et al. 2015). However, regioisomers of TAGs did not resolve on apolar columns as was noted by Geeraert et al. (1983).

Even though thermostable apolar GC columns were excellent choices for separation and quantification of TAGs (see above), polar GC columns were needed for a more comprehensive analysis. Kuksis et al. (1973) showed that a polar packed column (ethylene glycol succinate) resolved acylglycerols with butyryl, caproyl, and capryloyl acyls within the same ACN. Later, Geeraert and Sandra (1985, 1987) introduced a thermostable phenyl(50%)methylsilicone fused-silica capillary column with excellent resolving power,

which was demonstrated in the analysis of MF TAGs. Geeraert and Sandra (1985), Myher et al. (1988), and Kalo et al. (1990) showed that phenyl(50%)methylsilicone columns separated TAGs with the same ACN according to the level of unsaturation and the different carbon chain length combinations of fatty acyls due to different polarity of TAGs. Unlike apolar capillary columns, polar phenylmethylsilicone columns have shown to separate regioisomers of SC-TAGs, which is discussed in Chapter 3.3.2 in detail. Accordingly, separation capacity of long, thermostable polar or polarizable phenyl(50–65%)-methylsilicone capillary columns enabled detailed analysis of TAG composition, *i.e.*, analysis of individual molecular species of TAGs (including some positional isomers) or, at least, relatively restricted groups of TAG species by GLC. Even though, GC analysis of TAG composition of bovine MF (Geeraert and Sandra 1985, 1987, Hinshaw and Seferovic 1986b, Lozada et al. 1995, Evershed 1996, Fraga et al. 1998, Mottram and Evershed 2001, Naviglio and Raia 2003) and modified bovine MFs (Geeraert and Sandra 1985, Myher et al. 1988, 1993, Fraga et al. 1998, Mottram and Evershed 2001) has been the main interest, several studies concerning TAG composition in MF of other mammals have been published lately (Table 3.7). TAG composition of goat (Fontecha et al. 2000), ovine (Fontecha et al. 2005), buffalo (Romano et al. 2011), camel (Haddad et al. 2011a), equine (Haddad et al. 2012), and human MF (Haddad et al. 2012) has been investigated using long (30-m) polarizable phenyl(65%)methylsilicone columns (Rtx-65 TG), split injection, FID (or MS) as detector, and helium as carrier gas.

Injection of TAG samples is another critical point of TAG analysis by GC due to the high evaporation temperature of intact TAGs. Split-splitless, programmable temperature vaporization (PTV) and (cold) on-column injectors (OCI) have been the most common choices in the analysis of MF TAGs by GC (Table 3.7). Grob (1979) and Grob et al. (1980) regarded OCI superior to split injection in GC analysis of TAGs due to better recovery, accuracy, and precision. Similarly, Hinshaw and Seferovic (1986a,b) showed that PTV injection was as good choice as OCI in MF TAG analysis, and much better than split injection, which could discriminate HMW TAGs in injector. Molketin and Precht (1994) compared repeatability of TAG analysis by capillary GC with cold (80°C) and high-oven temperature (190°C) OCI, PTV, and split injection. They observed that cold OCI was the best choice and PTV relatively close to it, but high-oven temperature OCI and split injections were unsuitable for TAG analysis. On the contrary, Buchgraber et al. (2004a) showed in their extensive international collaborative GC studies that all injectors (OCI, PTV, and split-splitless) yielded reliable quantitative results in TAG analysis by capillary GC of cocoa butter. However, Table 3.7 suggests that OCI is the most commonly used injector in capillary GC of MF TAGs. Lozada et al. (1995) summarized that cold OCI was the best choice for injection when short or long capillary columns were used, particularly, in quantitative analysis. In addition, Table 3.7 shows that FID is the most frequently used detector in the TAG analysis of MF, but EI–MS (Murata and Takahashi 1973, Wakeham and Frew 1982, Myher et al. 1988, Fontecha et al. 2000, Mottram and Evershed 2001, Fontecha et al. 2005) and CI–MS (Murata 1977, Myher et al. 1988, Evershed 1996) were used as well. Hydrogen (H_2) was preferred carrier gas in TAG analysis due to column efficiency as reviewed by Kaal and Janssen (2008), but both nitrogen (N_2) and helium (He) were frequently used in TAG separation on both packed and capillary columns (Table 3.7).

Table 3.7 Examples of the analysis of triacylglycerols (TAGs) in milk fat (MF) by gas-liquid chromatography (GLC).

Sample	Column/Pol.[1]; L(m) x id(mm)[2]	Gas[3]	Inj./°C[4]	Det./°C[5]	References
BO distil-late	Packed (5% SE-30)/aP; 0.5/0.9/1.5/2.5 x 3	N_2		FID	Kuksis and Breckenridge 1965
MF	Packed (5% SE-30)/aP; 0.5 x 3 Packed (3% JXR)/aP; 0.5 x 3	N_2		FID/325	Kuksis and Breckenridge 1966
MF	Packed (1% OV-1)/aP; 0.35 x 3	He	/350	MS/EI	Murata and Takahashi 1973
MF	Packed (3% JXR)/aP; 0.61 x 2	N_2	OCI/330	FID/360	Parodi 1972
IBF	Packed (1% OV-1)/aP; 0.50 x 2 Packed (1% EGSS-X)/P; 1.8 x 2	N_2	OCI	FID	Kuksis et al. 1973
MF	Packed (1% OV-1)/aP; 0.35 x 3	He	/350	MS/CI	Murata 1977
MF	Packed (3% JXR)/aP; 0.58 x 2.5	N_2	/325	FID/350	Morrison and Hawke 1977
MF	Packed (3% OV-101)/aP; 0.6 x 2	N_2	OCI/330	FID/360	Parodi 1979b
BF, IBF	OV-1/aP; 15 x 0.30	H_2	Cold OCI	FID	Grob et al. 1980
BF	SE-52/aP; 26 x 0.3 SE-30/aP; 15 x 0.3	H_2 He	Cold OCI/ 100	FID/350 MS/EI	Wakeham and Frew 1982
BO HydroBF	Phe(50%)metsilicone/P*; 25 x 0.25	H_2	Cold OCI	FID	Geeraert and Sandra 1985, 1987
MF fractions	SE-54/aP; 10 x 0.32 and 10 x 0.20	H_2	OCI/160	FID	Kalo et al. 1986b
BF	Methylsilicone/aP; 15 x 0.25 Phe(65%)Met/P; 25 x 0.25	H_2	Cold OCI PTV	FID/425	Hinshaw and Seferovic 1986b
BO distil-late	RSL300/P*; 25 x 0.25 SE-54/aP; 8 x 0.32	H_2 H_2	Cold OCI/40 Cold OCI/40	FID/360 FID/360 MS/EI+CI	Myher et al. 1988
BF & frac.	CPSil 5CB/aP; 10 x 0.32	He	OCI	FID/350	Lund 1988
IBF	TAP/P; 25 x 0.25	H_2	Cold OCI	FID/360	Kalo et al. 1990
BO distil-late	RSL300/P*; 25 x 0.25 (Met(65%)Phe) SP-2380/P; 15 x 0.32	H_2 H_2	OCI OCI	FID/360 FID/360	Myher et al. 1993
BF	Packed (3% OV-1)/aP; 0.5 x 2	N_2	Splitless/210	FID/370	Molkentin and Precht 1994
	Capillary: dimethylpolysiloxane/ nonP; 5 x 0.5	N_2	Cold OCI/80 OCI/190 PTV/70	FID/370	Molkentin and Precht 2000

Sample	Column/Pol.[1]; L(m) x id(mm)[2]	Gas[3]	Inj./°C[4]	Det./°C[5]	References
BF	Rtx-65 TG/P*; 2.5 x 0.22 Rtx-65 TG/P*; 25 x 0.22 DiPhe(65%)DiMet	He	Cold OCI PTV/280	FID/370	Lozada et al. 1995
BF	Packed (3% OV-1)/aP; 0.3 x 2	He	/370	FID/370	Lipp 1996
BF	Phe(65%)Met/P*; 25 x 0.25 Quadrex Al-clad	He	OCI	MS/NICI (ammonia)	Evershed 1996
BF & frac. *Goat MF* Ovine MF	Rtx-65 TG/P*; 30 x 0.22 DiPhe(65%)DiMet	He	Split 1:4/355	FID/370 *MS/EI* MS/EI	Fraga et al. 1998 *Fontecha et al. 2000* Fontecha et al. 2005
MF, GPC fractions	Rtx-65 TG/P*; 30 x 0.32 DiPhe(65%)DiMet		OCI	MS/EI	Mottram and Evershed 2001
BF	Rtx-65 TG/P*; 30 x 0.25 DiPhe(65%)DiMet	H_2	Split 1:80/ 60 > 370	FID/370	Naviglio and Raia 2003
BF	Met(95%)Phe/aP; 4 x 0.25 (Made from DB-5HT; 15 x 0.25)	H_2	Split 50:1	FID/380	Destaillats et al. 2006
AMF	Packed (3% OV-1)/aP; 0.3 x 2	N_2	Splitless	FID/370	Molkentin 2007
MF Sheep MF Goat MF Buffalo MF	Dimethylpolysiloxane/aP; HP-1, 4 x 0.32 Dimethylpolysiloxane/aP; Ultra-fast module, 2.4 x 0.32	H_2 H_2	Cold OCI PTV/60 Split 1:30	FID/350 FastFID/ 350	Povolo et al. 2008
MF	Met(95%)Phe/aP; 2 x 0.25	He	Split/340	FID/350	Gutiérrez et al. 2009
Buffalo MF (Mozzarella)	Rtx-65 TG/P*; 30 x 0.25	He	PTV/50 Split 1:60	FID/360	Romano et al. 2011
Camel MF *Equine MF* *Human MF*	Rtx-65 TG/P*; 30 x 0.25 DiPhe(65%)DiMet	He	Split 50:1	FID/360	Haddad et al. 2011a *Haddad et al. 2012*

[1] Pol = polarity (aP = apolar, P = polar, P* = polarizable)
[2] L x id = length x internal diameter
[3] Carrier gas
[4] Injector/temperature (OCI = on-column injector; PTV = programmable temperature vaporization injector)
[5] Detector/temperature (FID = flame ionization detector; MS/EI, CI = mass spectrometry/ electron ionization, chemical ionization)
Abbreviations: BO = butteroil; BF = butterfat; IBF = interesterified butterfat; HydroBF = hydrogenated BF; AMF = anhydrous MF; frac. = fraction; GPC = gel permeation chromatography

Reversed-phase HPLC. Table 3.8 demonstrates the established employment of rp-HPLC in the analysis of TAG composition of MF and summarizes the trends of this chromatographic method. Recent reviews concerning TAG analysis by rp-HPLC agreed the significance and versatility of the method (*e.g.*, Buchgraber et al. 2004b, Kalo and Kemppinen 2012, Rezanka et al. 2017). In the late 1990's and in early 2000's, most TAG analysis of MF was carried out using a single conventional ODS column (250 x 4.6 mm, 5 μm) or using two ODS columns in series (Table 3.8). Recently, the contact of stationary phase with the TAG analytes has been extended by connecting up to three columns in series (Beccaria et al. 2014), and by recycling analytes through one analytical column several times (Gotoh et al. 2012, Nagai et al. 2015). Recycling has enabled separation of the regioisomers of intact trisaturated SC-, MC-, and even LC-TAGs (Gotoh et al. 2012, Nagai et al. 2015). New rp-column technologies in MF analysis have improved resolution of TAG species. Gotoh et al. (2012) used polymeric ODS column and Gotoh et al. (2012) and Nagai et al. (2015) used octacocyl silylation (C28) column in the analysis of regioisomers of MF TAGs. Beccaria et al. (2014) and Linderborg et al. (2014) were the first to apply fused-core C18 columns in the analysis of ruminant and human MFs under ultra-high-performance liquid chromatography (UHPLC) conditions. Recently, Kallio et al. (2017) quantified numerous regioisomers of TAGs in Finnish and Chinese human MF using UHPLC–ESI–MS^2 in combination with a two-phase optimization algorithm to estimate their concentration. Beccaria et al. (2014) improved identification and resolution of TAG species using HPLC–APCI–MS/MS with three rp-columns in series (Ascentis Express Fused-core C18, 150 mm x 4.6 mm i.d., 2.7 μm) resulting in identification of 243 different molecular species of TAGs in bovine, goat, buffalo, and human MFs. Ten-Doménech et al. (2015) determined TAGs of bovine, ovine, goat, and human MFs using a slightly shorter fused-core column (KinetexTM C18, 150 mm x 4.6 mm i.d., 2.6 μm) under conventional operating pressure resulting in similar resolution of TAGs and shorter analysis time than those reported by Beccaria et al. (2014).

In early investigations (Robinson and Macrae 1984, Frede 1986, Frede and Thiele 1987, Weber et al. 1988a,b, Maniongui et al. 1991, Gresti et al. 1993), refractive index (RI) detectors were used in rp-HPLC with isocratic elution. However, isocratic elution was not the best choice for MF TAGs with a wide range of ACN, hence other detectors were applied in gradient elution (Robinson and Macrae 1984, Stolyhwo et al. 1985). Light scattering detectors (LSDs) were common choices when gradient elution was applied to TAG analysis (Table 3.8). First studies of MF TAGs using LSD detection were carried out by Stolyhwo et al. (1983, 1984, 1985) and Robinson and Macrae (1984). For MF TAG separation, several modifying solvents have been successfully used in combination with acetonitrile, *e.g.*, acetone (Stolyhwo et al. 1983, 1984, 1985, Ruiz-Sala et al. 1996), ethanol (Robinson and Macrae 1984, Herslöf and Kindmark 1985), hexane (Herslöf and Kindmark 1985), dichloromethane (Laakso et al. 1992, Laakso and Kallio 1993a,b, Robinson and MacGibbon 1998b, 2000), 1,2-dichloroethane (Laakso et al. 1992, Laakso and Kallio 1993a,b), chloroform (Kermasha et al. 1993), isopropylalcohol (Myher et al. 1993, Marai et al. 1994), propionitrile (Brühl et al. 1993, 1994, Spanos et al. 1995), and n-pentanol (Ten-Doménech et al. 2015). UV detectors were used frequently in MF TAG analysis as a sole detector and in combination with LSD or mass spectrometry (Table 3.8), even though they were assessed less sensitive and incompatible with some solvents in gradient elution (Stolyhwo et al.

1985). Common binary mobile phases in UV–rp-HPLC were acetonitrile (AcN) based solvents modified by acetone (Haddad et al. 2012), ethanol (Robinson and Macrae 1984), isopropanol (Gilkison 1988, Haddad et al. 2011a), methyl tert-butyl ether (Barron et al. 1990), dichloromethane (Robinson and MacGibbon 1998b, 2000), and n-pentanol (Ten-Doménech et al. 2015). Nurmela and Satama (1988) showed that flame ionization detector (FID) was a good alternative to LSD and UV in the analysis of MF TAGs. Lately, increasing need of unambiguous identification of coeluting TAG species of MF has increased mass spectrometric detection of TAGs (Table 3.8). In recent years, the most common rp-HPLC–MS methods in MF analysis have been atmospheric pressure chemical ionization (APCI) MS (*e.g.*, Mottram and Evershed 2001, Dugo et al. 2005, 2006, Chiofalo et al. 2011, Gastaldi et al. 2011, Gotoh et al. 2012, Beccaria et al. 2014, Nagai et al. 2015) and electrospray ionization (ESI) MS (*e.g.*, Haddad et al. 2012, Nagy et al. 2013, Linderborg et al. 2014, Kallio et al. 2017). However, already Kuksis et al. (1986) investigated TAGs in goat MF by rp-HPLC and chemical ionization (CI) MS, and later, several rp-HPLC–MS studies were performed by negative (ion) CI–MS (Kuksis et al. 1991, Kallio et al. 2006), positive ion CI–MS (Kuksis et al. 1991, Myher et al. 1993, Marai et al. 1994), and desorption CI–MS (Spanos et al. 1995).

In general, TAGs are separated on rp-HPLC columns due to different number of acyl carbons and double bonds in TAG molecules. Plattner et al. (1977) showed that TAGs eluted in order of ascending ACN and descending number of double bonds. In addition, they noted that the effect of one DB on retention volume/time was equal to that of two methylene groups in isocratic elution. Hence, TAGs eluted in an ascending equivalent carbon number (ECN), which was defined as follows: ECN = ACN – (*a* x DB), in which ACN = number of acyl carbons, DB = number of double bonds, and *a* = 2. The influence of DBs (*i.e.*, *a*) on the retention time of TAGs was confirmed to be close to two in several other studies (*e.g.,* Maniongui et al. 1991, Gresti et al. 1993, Ruiz-Sala et al. 1996). However, existence of numerous different TAG isomers in MF induces further variation in retention times of TAG species and separation of critical pairs of TAG molecules. For example, MF TAGs with the same ECNs have been shown to resolve due to acyl chain isomerism, *e.g.,* TAG molecules containing 1–2 short-chain acyl groups have shown to elute later than those containing long-chain acyls (Nurmela and Satama 1988, Maniongui et al. 1991, Laakso and Kallio 1993a). Kuksis et al. (1991) determined the elution order of short- and medium-chain TAGs in a volatile fraction of MF using rp-HPLC for separation of TAGs and positive ion CI–MS and negative ion CI–MS with chloride attachment for identification of TAG species. They showed that mono caprylates preceded mono caproates, after which eluted mono butyrates on a conventional C18 column (300 mm x 4.6 mm i.d., 5 μm) with linear gradient elution (10–90% PCN in AcN). Laakso and Kallio (1993a) reported that *cis* isomer of SSM type MF TAGs eluted prior to corresponding *trans* isomer on rp-columns. Earlier, El-Hamdy and Perkins (1981b) observed similar resolution due to *cis–trans* isomerism in the separation of triolein (*cis*) and trielaidin (*trans*). Even the isomerism due to the double bond position in the fatty acyl moieties of TAG molecule has shown to induce resolution of positional isomers as was shown by Phillips et al. (1984) in the separation of triolein (54:3Δ9) and tripetroselinin (54:3Δ6), in that order, on a ODS column (Zorbax C18, 250 x 4.6 mm, 5 μm) using linear gradient elution. In addition, separation of regioisomers of synthesized

Table 3.8 Examples of the analysis of triacylglycerols (TAGs) in milk fat (MF) by high-performance liquid chromatography (HPLC).

HPLC[1]	Column; L(mm) x id(mm)/°C[2]	Mobile phase[3]	Detector[4]	References
rp	LiChrosorb™ RP-18 (5 µm); 200 x 2.0	ACE, AcN linear gradient	LSD	Stolyhwo et al. 1983
rp	LiChrospher™ 1000CH-18 (5 µm); 1–2 x (250 x 4.0) in series	ACE, AcN linear gradient	LSD	Stolyhwo et al. 1984 Stolyhwo et al. 1985
rp	Spherisorb™ ODS2 (5 µm); 50 x 4.6 and 250 x 4.6 in series	ACE, AcN isocratic	LSD, RI	Robinson and Macrae 1984
		etOH, AcN linear gradient	LSD, UV	
rp	LiChrospher™ RP100; 250 x 4.6	AcN, HEX, etOH linear gradient	LSD	Herslöf and Kindmark 1985
rp	Supelcosil™ LC-18/30	AcN, PCN linear gradient	MS/CI	Kuksis et al. 1986
rp	Hibar RP18 (5 µm); 2 x (250 x 4.6) in series/10 => 60	PCN, ether isocratic	RI	Frede 1986
rp	Nucleosil™ C18 (5 µm); 150 x 4.0 and Microspher™ C18 (3 µm); 100 x 4.6 in series/30, 35	ACE, AcN isocratic	RI	Frede and Thiele 1987
rp	Hypersil™ ODS (5 µm); 250 x 4.0/10–60	AcN, PCN isocratic	RI	Weber et al. 1988a,b
rp	LiChrospher™ 100CH-18/2 (5 µm); 2 x (250 x 4.0) in series/40	ACE, AcN non-linear gradient	FID	Nurmela and Satama 1988
rp	Ultrasphere™ ODS-IP (5 µm); 250 x 4.6/25	AcN, IPA, HEX; linear gradient	UV/220	Gilkison 1988
rp	LiChrospher™ 100RP-18 (4 µm); 250 x 4.0/30, 40	ACE, AcN isocratic	RI	Maniongui et al. 1991, Gresti et al. 1993
rp	Supelco C18 (5 µm); 300 x 4.6	AcN, PCN linear gradient	MS/pCI	Kuksis et al. 1991
		ACE, PCN, DCM	MS/nCI	
rp	Spherisorb™ ODS (5 µm); 2 x (200 x 4.6) in series/30	MTBE, AcN linear gradient	UV/220	Barron et al. 1990
rp	Zorbax™ ODS (5 µm); 250 x 4.6 and Spheri™ ODS (5 µm); 250 x 4.6 in series	DCM, 1,2-dichloro-ethane, AcN linear gradient	LSD	Laakso et al. 1992, Laakso and Kallio 1993a,b
rp	Supelcosil™ LC-18 (5 µm) 2 x (150 x 4.6) in series/32	ACE, AcN isocratic	RI	Bornaz et al. 1992
rp	Supelcosil™ LC-18 (5 µm) 250 x 4.6	IPA, AcN linear gradient	LSD	Myher et al. 1993, Marai et al. 1994
		PCN, AcN linear gradient	MS/pCI	
rp	Spherisorb™ ODS-2 (5 µm); 2 x (150 x 4.6) in series/25	Chloroform, AcN linear gradient	LSD	Kermasha et al. 1993
rp	Hypersil™ ODS (5 µm); 250 x 4.6	PCN, AcN; isocratic ACE. AcM linear gradient	LSD	Brühl et al. 1993, Brühl et al. 1994
rp	Spherisorb™ ODS-2 (5 µm); 250 x 4.6 and Zorbax™ C18; 250 x 4.6 in series	AcN, PCN, methylene chloride	LSD MS/DCI	Spanos et al. 1995
rp	Spherisorb™ ODS-2 (3 µm); 250 x 4.6 and 150 x 4.6 in series	ACE, AcN linear gradient and isocratic	LSD	Ruiz-Sala et al. 1996
rp	Nova-Pak™ C18 (4 µm);	AcN, DCM	LSD	Robinson and MacGibbon 1998b,

HPLC[1]	Column; L(mm) x id(mm)/°C[2]	Mobile phase[3]	Detector[4]	References
	150 x 3.9/20	linear gradient	UV/233	Robinson and MacGibbon 2000
rp	Supelcosil™ LC-18 (5 µm) 2 x (250 x 10) in series	PCN, isocratic	MS/APCI	Mottram and Evershed 2001
rp	Discovery™ HS C-18 (5 µm); 2 x (250 x 4.6) in series	IPA, AcN linear gradient	MS/APCI	Dugo et al. 2005, Chiofalo et al. 2011
rp	Chromolith™ RP-18; 100 x 4.6	IPA, AcN linear gradient	MS/APCI	Dugo et al. 2006
rp	Discovery™ HS C-18 (5 µm); 250 x 4.6	IPA, meOH linear gradient	LSD MS/NICI	Kallio et al. 2006
rp	LiChrospher™ RP-18 (5 µm); 250 x 4.5	AcN, DCM gradient	MS/APCI	Gastaldi et al. 2011
rp	Restek Ultra C18 (5 µm); 2 x (250 x 4.6) in series/25	AcN, IPA, HEX; isocratic	UV/210 MS/ESI	Haddad et al. 2011a
rp	Inertsil™ ODS-P (5 µm); polymeric 2 x (250 x 4.6)/10	AcN, IPA, HEX; isocratic	MS/APCI UV	Gotoh et al. 2012
	Sunrise™ C28 (5 µm); 250 x 4.6/15	ACE; isocratic		
rp	Restek Ultra C18 (5 µm); 2 x (250 x 4.6) in series/30	ACE, AcN isocratic	UV/210 MS/ESI	Haddad et al. 2012
rp	Poroshell™ 120 EC-C18 (2.7 µm) 250 x 2.1	NH_4-, Na-formate, meOH, IPA, HEX gradient	MS/ESI	Nagy et al. 2013
rp	Ascentis Express Fused-core C18 (2.7 µm); 1–3 x (150 x 4.6) in series	AcN, IPA gradient	MS/APCI	Beccaria et al. 2014
rp	Kinetex™ C18 (1.7 µm); 100 x 2.1	ACE, AcN gradient	MS/MS/ ESI	Linderborg et al. 2014
rp	Kinetex™ C18 (2.6 µm); fused-core 150 x 4.6/10	AcN, n-pentanol gradient	UV/205 LSD	Ten-Doménech et al. 2015
rp	Sunrise™ C28 (5 µm); 250 x 4.6/15	ACE, AcN linear gradient	MS/APCI	Nagai et al. 2015
rp	BEH C18 (1.7 µm); 100 x 2.1	meOH (+LiCl), IPA gradient	MS/MS/ ESI	Kallio et al. 2017
sic	Nucleosil™ 5SA in Ag+-mode 250 x 4.6	1,2-dichloroethane, DCM, ACE;gradient	LSD	Laakso et al. 1992, Laakso and Kallio 1993a,b Kallio et al. 2006
sic	Nucleosil™ 10 SA (10 µm) in Ag+-mode, 250 x 10	DCM, ACE, AcN linear gradient	LSD	Brühl et al. 1993, 1994
sic	Nucleosil™ 5SA (5 µm) in Ag+-mode; 250 x 4.6	DCM, ACE, AcN gradient	LSD	Winter et al. 1993
sic	Chromspher™ 5 Lipids (5 µm); in Ag+-mode, 250 x 4.6	HEX, AcN isocratic	MS/APCI	Dugo et al. 2005
sic	HotSep™ (5 µm) in Ag+-mode; 150 x 1.0	HEX, AcN gradient	LSD	Dugo et al. 2006
chir	ChiralGel™ OD-3R (3 µm); 150 x 4.6 (recycle)/25	meOH	MS/APCI	Nagai et al. 2015

[1] High-performance liquid chromatography: rp = reversed-phase HPLC; sic = silver ion HPLC; chir = Chiral HPLC

[2] L x id = length x internal diameter

[3] ACE = acetone; AcN = acetonitrile; DCM = dichloromethane; etOH = ethanol; HEX = hexane; IPA = isopropanol; meOH = methanol; MTBE = methyl tert-butyl ether: PCN = propionitrile

[4] FID = flame ionization detector; LSD = (evaporative) light scattering detector; MS = mass spectrometry; CI = chemical ionization; APCI = atmospheric pressure CI; DCI = desorption CI; nCI = negative CI; pCI = positive CI; ESI = electrospray ionization; RI = refractive index detector; UV = ultra violet detector

SC-TAGs (Kalo et al. 1996, Lee et al. 2002, 2003) and intact LC-TAGs (Momchilova et al. 2004, 2006) on ODS columns was reported for the first time. However, the separation of regioisomers of TAGs will be discussed in more detail in Chapter 3.3.2.

Silver ion HPLC. Silver ion high-performance liquid chromatography (Ag-HPLC) is frequently used in the analysis of polyunsaturated oils, but the method is less common in the analysis of more saturated MF (Table 3.8). Applications of Ag-HPLC to TAG analysis were recently reviewed by Nikolova-Damyanova (2009), Momchilova and Nikolova-Damyanova (2012), and Kalo and Kemppinen (2012). Although, TLC in silver ion mode was successfully used to fractionate MF TAGs for decades (*cf.*, Table 3.5), utilization of HPLC in silver ion mode is a more modern method due to the fact that preparation of stable HPLC columns with similar principles than that of Ag-TLC plates proved to be more complicated. In 1987, Christie reported on preparation of a stable silver-ion loaded HPLC column (Nucleosil^{TM} 5SA) which separated TAGs of palm oil into six fractions according to the degree of unsaturation: SSS, SSM, SMM, SSD, MMM, SMD (S, M, and D denote saturated, monoene, and diene acyl moieties, respectively). In addition, he noted that similar resolution was obtained with the MF TAGs and cocoa butter. Later, he successfully applied a corresponding method to analysis of several other natural fats and oils (Christie 1988). Later, Christie's (1987, 1988) method was utilized in TAG analysis of different MFs: Laakso et al. (1992) applied Ag-HPLC to the TAGs of liquid and solid BF fractions resulting in separation of MF TAGs into six fractions. Similar separation was observed for the TAGs of winter butterfat (Laakso and Kallio 1993a,b) and those of unsalted butterfat (Kallio et al. 2006). Linear gradient elution together with a binary gradient of (A) dichloroethane/DCM and (B) acetone was used in all analysis of Laakso, Kallio, and their coworkers. Brühl et al. (1993, 1994) and Winter et al. (1993) fractionated TAGs of human MF into 13–14 fractions using gradient program including the following eluents: DCM/(DCM,ACE)/(ACE,AcN). Later, Dugo et al. (2005, 2006) applied Ag-HPLC separation to the TAGs of donkey (Dugo et al. 2005, 2006) and human milk (Dugo et al. 2005). Both isocratic (Dugo et al. 2005) and gradient (Dugo et al. 2006) elution with 2-component mixtures of n-hexane and acetonitrile were used in separation. In general, light scattering detector was well fitted for determination of TAGs (Table 3.8), but APCI–MS was chosen for the identification of TAG species of MF in the study of Dugo et al. (2005).

In Nikolova-Damyanova's review article (2009), the elution order of TAG classes and *cis–trans* isomers on Ag-HPLC columns was assessed to depend mainly on the number of double bonds in fatty acyl moieties and the interaction of DBs with silver ions of the stationary phase resulting in resolution which resembled that of Ag-TLC (*cf.,* Chapter 3.2.1). However, more complex interactions, hence resolution patterns were observed when double bonds positional isomers, regioisomers and acyl chain isomers of TAGs (TAG classes) were resolved on Ag-HPLC columns. For example, Nikolova-Damyanova et al. (1990) demonstrated that both double bond position in a monoenoic fatty acyl group and the carbon chain length of a fatty acyl group induced distinctive resolution in the MMM TAG class (M denotes monoene acyl moiety) as follows: 20:1(11) > 20:1(5) > 22:1(13) > 18:1(5) > 18:1(9), double bond position is in parenthesis. Effect of short- and medium-chain acyl groups and regioisomerism on the elution pattern of TAGs will be dicussed more thoroughly in Chapter 3.3.2.

Normal-phase HPLC. Normal-phase HPLC (np-HPLC) is more frequently used in separation of lipid classes than in separation of TAGs as reviewed by Lin (2007). However, already Plattner and Payne-Wahl (1979) and Rhodes and Netting (1988) demonstrated the resolving power of np-columns according to the descending number of acyl carbons and the ascending number of double bonds of TAG molecules. Later, Mangos et al. (1999) showed that the method enabled separation of the regioisomers of mono- and di-short-chain TAGs. Regioisomeric analysis of TAGs by np-HPLC will be discussed in detail in Chapter 3.3.2.

Chiral-phase HPLC. A relatively short history of the applications of chiral-phase HPLC to enantiomeric analysis of intact TAGs was reviewed recently by Rezanka et al. (2017). First successful resolution of enantiomers was performed by Iwasaki et al. (2001), who separated *sn*-ECyCy/*sn*-CyCyE and *sn*-DCyCy/*sn*-CyCyD enantiomers (Cy = 8:0, E =20:5, D = 22:6) on chiral HPLC columns (Chiralcel^{TM} OF and/or Chiralcel^{TM} OD). They observed that *sn*-SSU enantiomer eluted after *sn*-USS enantiomer (S, U = saturated, unsaturated acyl) on an "OD" column but the retention order of the *sn*-CyCyE/*sn*-ECyCy pair was reversed on an "OF" column. Kalpio et al. (2015) performed successfully separation of enantiomers of 11 synthetic racemic TAGs containing acyl groups with 12–22 acyl carbons and 0–2 double bonds by a recycle HPLC-UV system with two Chiralcel^{TM} OD-RH chiral columns using isocratic elution (methanol). They reported that enantiomers with unsaturated fatty acyl attached to the *sn*-1 position eluted prior to enantiomers with unsaturated acyl group in the *sn*-3 position. In addition, Rezanka et al. (2017) noted that isomers with unsaturated fatty acyl in the primary positions elute normally later than respective isomer with unsaturated fatty acyl in the secondary position (*sn*-2). Nagai et al. (2015) focused on the enantiomers of trisaturated TAGs and used a chiral column (Chiralcel^{TM} OD-3R) with sample recycling HPLC system in the analysis of *sn*-PPS$_n$/*sn*-S$_n$PP type TAGs (P = palmitic acid; S$_n$ = saturated fatty acid with n acyl carbons; n = 4, 6, 8, 10, or 12). They observed that enantiomers separated almost to the baseline when n = 4–10, and partially when n = 12. The elution order was as follows: first *sn*-S$_n$PP followed by *sn*-PPS$_n$ in all cases. However, enantiomers of the TAGs with three unsaturated long-chain fatty acyls (Nagai et al. 2011b, Kalpio et al. 2015) and those with three saturated long-chain fatty acyls (Nagai et al. 2011b) did not resolved on chiral columns. Recently, chiral-phase HPLC method was successfully applied to separation of enantiomeric TAG species of natural fats and oils such as *rac*-OOP in palm oil (Nagai et al. 2011b), TAGs of hazelnut oil and those of human plasma (Lisa and Holzapek 2013), and TAGs of microbial oils (several studies of Rezanka and his coworker as was reviewed by Rezanka et al. 2017). In the analysis of MF TAGs (Table 3.8), the chiral HPLC method was applied only once by Nagai et al. (2015), who studied enantiomeric ratio of asymmetric saturated TAGs containing two palmitoyl acyls and one short- or medium/long-chain acyl (*rac*-PPS$_n$, where n = 4, 6, 8, 10, and 12). They confirmed the existence of only *sn*-3 enantiomers (*sn*-PPS$_n$) in SC- and MC-TAGs in MF.

3.2.3 Detection and identification of triacylglycerols by mass spectrometric methods

Mass spectrometry (MS), as a sole analytical method or as a detector in chromatographic analysis, has been applied to characterize TAGs since 1960's (*e.g.*, Ryhage and Stenhagen 1960, Barber et al. 1964, Hites 1970, Murata and Takahashi 1973). MS provides information on molecular weight of TAGs on the basis of molecular ion M^+, quasi-molecular ion $QM^{+/-}$ ($[M+H]^+/[M-H]^-$), or other adduct ions ($[M+X]^{+/-}$), and in addition, that on the structure of TAGs on the basis of the fragment ions yielded by primary ionization or by collision induced dissociation in tandem MS (Duffin et al. 1991).

Electron ionization mass spectrometry (EI–MS). In EI–MS, identification of TAGs is based on extensive fragmentation of molecular ions resulting in a characteristic fragmentation pattern under relatively harsh EI condition. Ryhage and Stenhagen (1960) were the first to describe characteristic fragmentation of TAGs in EI under low pressure. The most abundant fragment ions were acyl ions $[RCO]^+$ and ions formed by cleavage of acyloxy groups from the molecular ion $[M-RCOO]^+$. In addition, they showed that EI resulted in loss of acyloxymethylene from M^+ ($[M-RCOOCH_2]^+$). Barber et al. (1964) named other less intense peaks in the mass spectra of TAGs due to ions of $[M-RCOOH]^+$, $[M-18]^+$, $[RCO+74]^+$, and $[RCO+128]^+$. Later, Lauer et al. (1970) and Aasen et al. (1970) explained the structures of fragment ions using specific deuterium labeling of TAGs and observed other characteristic fragment ions, *i.e.*, $[RCO+115]^+$, and homologous series of ions $[RCO+128+14n]^+$ (n = 0, 1, 2,…). In addition, Lauer et al. (1970) stressed that the structure of esterified fatty acids influenced on the relative yields of fragmented ions as well. For example, the ratio of $[M-RCOO]^+/[M-RCOOCH_2]^+$ increased with increasing carbon chain length of fatty acyls in saturated monoacid TAGs. Further, Lauer et al. (1970) showed that unsaturation of fatty acyls induced increase in the amount of $[RCO-1]^+$ ions in the spectra and interrupted the homology in the $[RCO+128+14n]^+$ series. Detection of the regioisomers of TAGs by EI–MS will be considered in Chapter 3.3.3.

Chemical ionization mass spectrometry (CI–MS). Lauer et al. (1970) noted that practically no molecular ions (M^+) were observed in the mass spectra of short-chain monoacid TAGs when EI was used, which complicated elucidation of molecular weight of TAGs. Even though, the abundancy of M^+ in EI slightly increased with increasing chain length of esterified FAs (Lauer et al. 1970), utilization of CI yielded relatively intense QM^+ ions with less fragmentation and provided an easier determination of molecular weight of TAGs, thus ACN and number of DBs of TAG molecule (Murata 1977, Murata and Takahashi 1977). The type and abundancy of adduct ions depend on the reagent gas used in CI. Murata (1977), and Murata and Takahashi (1977) noted that the use of ammonia as the reagent gas yielded $[M+NH_4]^+$ adduct ion as the base peak in mass spectra of TAGs, and $[MH-CHOOH]^+$ ion as a sole fragment, on the contrary to the use of isobutene as the reagent gas, which resulted in $[MH-CHOOH]^+$ fragment ion as the base peak, and no adduct ions at all. On the other hand, Spanos et al. (1995) reported that positive DCI–MS with isobutene as the reagent gas produced relatively intense $[M+H]^+$ ions without any remarkable fragmentation. Further, a high energy collision induced dissociation (CID) of $[M+H]^+$ yields information on the esterified FAs and that on the location of DBs in unsaturated fatty acyl groups (Spanos et al. 1995). Myher et al. (1988) used methane as the reagent gas and identified MF TAGs by

mass chromatograms of $[M–RCOOH]^+$ ions in GC–CI–MS. Kuksis et al. (1983, 1986) and Myher et al. (1984) applied HPLC solvents (acetone-acetonitrile, acetonitrile-propionitrile) to the reagent gas in CI–MS to yield protonated molecular ions ($[M+H]^+$) and $[MH–CHOOH]^+$ fragment ions, together with low abundances of adduct ions of acetonitrile and propionitrile. Myher et al. (1984) observed that the relative abundancy of $[M+H]^+$ ion was much higher for polyunsaturated than saturated TAGs. In addition, they reported that the rate of release of $[MH–CHOOH]^+$ fragment ions from TAGs varied with the fatty acyl composition of TAGs, being lower for unsaturated fatty acyls. Further, Myher et al. (1984) stated that cleavage of fatty acyls from the primary positions of TAG molecule was *ca*. four times higher than that from the secondary position showing the significance of regioisomerism for the ease of cleavage of FAs in soft ionization techniques. Instead of positive ion CI–MS (PICI), negative ion CI (NICI) has been applied to the analysis of TAGs resulting in different characteristic ions. Kuksis et al. (1991) used 1% dichloromethane in acetonitrile-propionitrile mobile phase to yield negative chloride adduct ions ($[M+35]^–$) in determination of MF TAGs with rp-HPLC–NICI–MS. The observed $[M+35]^–$ ions with no fragment ions, together with characteristic fragmentation induced by PICI provided a reliable basis for identification of TAGs (Kuksis et al. 1991). Instead of any prominent quasimolecular ions, ammonia NICI GC–MS yielded characteristic negative $[RCOO]^–$, $[RCOO–H_2O]^–$, and $[RCOO–H_2O–H]^–$ fragment ions, which enabled elucidation of TAG composition of BF TAGs by GC–NICI–MS (Evershed et al. 1990). On the contrary, relatively abundant quasimolecular ions, *i.e.*, negative deprotonated molecular ions ($[M–H]^–$) were produced by NICI with ammonia in other TAG studies (Kallio and Currie 1993, Laakso and Kallio 1993a,b). Further, employing tandem MS for $[M–H]^–$ ions provided structural information of TAGs, *i.e.*, composition of esterified fatty acids and their regiospecific positions, on the basis of negative fragment ions, $[M–H–RCO_2H]^–$, $[M–H–RCO_2H–74]^–$, and especially, abundant $[M–H–RCO_2H–100]^–$ ions, the cleavage of which from the *sn*-2 position was very low compared to that of the *sn*-1(3) positions (Kallio and Currie 1993).

Atmospheric pressure chemical ionization mass spectrometry (APCI–MS). In recent years, other soft ionization techniques than conventional PICI or NICI have been successfully applied to TAG analysis of MF, mainly due to the increased utilization of LC–MS for separation of TAGs. In general, applicability of APCI in TAG analysis by HPLC–MS was demonstrated by Byrdwell and Emken (1995), who observed that the APCI mass spectra of monoacid TAG standards yielded protonated molecular ions $[M+1]^+$ and "diacylglycerol" fragment ions (DG^+/$[M–RCOO]^+$/$[M+1–RCOOH]^+$), which enabled determination of both molecular weight of TAGs and their FA composition. However, no $[M+1]^+$ ions were observed in the APCI–MS spectra of saturated TAG molecules (Byrdwell and Emken 1995), but the relative proportion of these ions increased with increasing degree of unsaturation (Byrdwell and Emken 1995, Mottram and Evershed 1996). In addition, Mottram and Evershed (1996) noted that the peaks corresponding to the cleaved acyls ($[RCO]^+$) could be detected in the spectra of some TAG, as well as low intensity peaks of $[M–RCOO+18]^+$ ions, and in some cases, adduct ions $[M+55]^+$ formed with propionitrile (Mottram et al. 1997). Laakso and Manninen (1997) used supercritical fluid chromatography (SFC) in combination with APCI–MS to separate and identify the TAGs of MF. For the ionization, vapor of ammonia (0.5%) in methanol was introduced in ionization chamber resulting in the

mass spectra containing prominent $[M–RCOO]^+$ ions and $[M+18]^+$ ions, the latter of which enabled reliable elucidation of ACN and the number of double bonds in both saturated and unsaturated TAG molecules present in MF (Laakso and Manninen 1997). Further, already Mottram and Evershed (1996) showed that APCI–MS enabled differentiation of the regioisomers of TAGs. The regiospecific analysis of TAGs using APCI–MS and APCI–MS^2 will be reviewed in more detail in Chapter 3.3.3.

The same principles for detection and identification of TAGs by LC–APCI–MS(/MS) as mentioned above have been employed for the analysis of natural MF (Gastaldi et al. 2011, Gotoh et al. 2012, Zou et al. 2013, Beccaria et al. 2014, Nagai et al. 2015) and prefractionated MF (Mottram and Evershed 2001, Dugo et al. 2005, Dugo et al. 2006) extracted from several animal species. Mottram and Evershed (2001) identified *ca.* 120 TAGs of bovine MF by APCI–MS and by GC–EI–MS from TAG fractions produced by silica TLC and by gel permeation chromatography. Dugo et al. (2005) investigated TAGs present in donkey milk by off-line two-dimensional HPLC–APCI–MS approach using characteristic quasimolecular ($[M+1]^+$) and fragment ions ($[M–RCOO]^+$) for identification together with partition number on rp-HPLC column. At the first dimension, they separated TAGs on two rp-columns (Discovery^{TM} HS C18) in series into 50 fractions, which were analyzed further by Ag-HPLC–APCI–MS resulting in 55 identified and quantified (area-%) TAG species (without regioisomers). Later, based on the similar identification principle, Dugo et al. (2006) reported separation and identification of close to 60 TAGs in donkey MF by on-line 2D LC–APCI–MS using micro-Ag-HPLC as the first chromatographic dimension and fast rp-HPLC (Chromolith^{TM} RP-18) as the second dimension. Gastaldi et al. (2011) used $[M+1]^+$ and DG^+ ions to identify TAGs in bovine, goat and human milks. Their method enabled to determine principal regioisomers of MF TAGs and acyl chain isomers containing rare polyunsaturated TAGs with very long-chain polyunsaturated FAs. Regioisomers of selected TAGs (consisting of palmitic, capric, oleic, and docosahexaenoyl acids) in human, bovine, and rat milks and those in cheeses made from buffalo, sheep, and goat milks were investigated by Gotoh et al. (2012) with HPLC–APCI–MS/MS using two polymeric ODS columns (Inertsil^{TM} ODS-P) in series or a C28 column with or without sample recycling. Selected ion monitoring was used to analyze TAG species, *i.e.*, characteristic $[M+NH_4]^+$ (ammonium derived from acetonitrile in mobile phase) and $[M–RCOO]^+$ ions for OOP/OPO regioisomers, and $[M+1]^+$ and $[M–RCOO]^+$ ions for isomers of other TAGs (Gotoh et al. 2012). Later, Nagai et al. (2015) employed a recycle HPLC–APCI–MS/MS equipped with a C28 column (Sunrise^{TM} C28) to separate regioisomers of butterfat TAGs consisting of two palmitic acid and one other even-numbered saturated FAs (4:0–12:0). They used $[M+NH_4]^+$ as precursor ions and DG^+ as product ions in APCI tandem MS for identification of regioisomers. Zou et al. (2013) carried out an extensive study concerning MF TAGs from several mammalian species (human, cow, buffalo, sheep, donkey, camel). They identified 19–28 TAGs or TAG groups from all MFs by rp-HPLC–APCI–MS using a single rp-column (Lichrospher^{TM} C18) for separation. Beccaria et al. (2014) improved the resolution and increased the number of identified TAG species by combining APCI-ion trap-time of flight mass spectrometry (IT-TOF-MS) with UHPLC using three core-shell C18 columns (Ascentis Express Fused-core C18) in series. They determined 51, 141, 165, and 136 TAGs from human, bovine, and goat milks, and Mozzarella cheese, respectively. Identification was based on retention times, protonated

molecular ions, and diacylglycerol ions of TAGs (Beccaria et al. 2014). Recently, Nagai et al. (2015) reported a successful identification of the enantiomers of *sn*-X-16:0-16:0/ *sn*-16:0-16:0-X TAGs (X = 4:0, 6:0,…, 12:0) in TAG standards and BF TAGs on the basis of positive protonated molecular ions and diacylglycerol ions by a recycle HPLC–APCI–MS/MS system equipped with a chiral column (Chiralcel^{R} OD-3R).

Electrospray ionization mass spectrometry (ESI–MS). ESI is another atmospheric pressure soft ionization method, which is commonly used in the analysis of polar organic compounds in general, but it is more infrequently applied to the analysis of non-polar TAGs. However, Duffin et al. (1991) introduced a practical way to analyze acylglycerols, including TAGs, by ESI–MS using non-polar solvent (chloroform:methanol, 70:30, v/v) for dissolution of TAGs and ionic modifiers (ammonium and sodium acetate) to increase ionization. The method yielded abundant adduct ions $[M+NH_4]^+$ or $[M+Na]^+$ depending on the modifier. Several other ionic modifiers than those used by Duffin et al. (1991) have been applied to adduct formation in ESI–MS of TAGs. For example, ammonium formate (Hvattum 2001, Byrdwell and Neff 2002, Malone and Evans 2004, Herrera et al. 2010, Nagy et al. 2013), ammonium hydroxide (Leskinen et al. 2007), lithium acetate dehydrate (Herrera et al. 2010), lithium chloride (Kallio et al. 2017), lithium iodide (Cheng et al. 1998), cesium iodide (Cheng et al. 1998), silver trifluoromethane sulfonate (Herrera et al. 2010), and silver nitrate (Lévêque et al. 2010) have been used as a source for ionic modifier to yield respective adduct ions, *i.e.*, $[M+NH_4]^+$, $[M+Li]^+$, [M+Cs]+, $[M+Ag]^+$/$[M+Ag+AgNO_3]^+$.

Fragmentation of ammonium adducts by low energy collision-induced dissociation (CID) yielded characteristic product ions in ESI–MS^2, *i.e.*, diacyl product ions due to cleavage of fatty acid (and ammonia) from ammoniated TAGs ($[DAG]^+$/$[(M+NH_4)–NH_3–FA]^+$), acyl ions ($[RCO]^+$), and acyl + 74 ions ($[RCO+74]^+$) (Duffin et al. 1991). In the early investigation of Duffin et al. (1991), the *sn*-position of FA in the glycerol backbone of TAG molecule or location of DBs in unsaturated FAs could not be distinguished. Later, several investigators (*e.g.*, Hsu and Turk 1999, Hvattum 2001, Dorschel 2002, Byrdwell and Neff 2002, Marzilli et al. 2003, Malone and Evans 2004, Lévênque et al. 2010, Kallio et al. 2017) have demonstrated unquestionable differences between the *sn*-1(3) and *sn*-2 regioisomers of TAGs in the fragmentation of TAG molecules in their ESI–MS^2 studies, which will be discussed more thoroughly in Chapter 3.3.3. In addition, ESI–MS^2 results in additional information concerning the location of double bonds in unsaturated acyl groups. Already, the study of Cheng et al. (1998) showed that the positions of DBs in unsaturated fatty acyl chains were possible to detect by tandem sector MS using high-energy CID of ESI-produced $[M+NH_4]^+$ ions. Later, respective information was confirmed in other investigations, for example, by ESI–MS^2 with source CID of dilithiated ions $[R_nCO_2Li_2]^+$ derived from fatty acyl moieties of TAG molecules (Hsu and Turk 1999), and by ESI multi-state (MS^n, n = 2, 3, 5) linear ion-trap MS with CID (MS^3) of $[M+Li–RCOOH]^+$ ions (Hsu and Turk 2010).

Even though, the product ions formed by CID of ammonium adducts are generally agreed, several fragmentation mechanisms of $[M+NH_4]^+$ ions are suggested (Duffin et al. 1991, Cheng et al. 1998, Marzilli et al. 2003, McAnoy et al. 2005, Kalo et al. 2006). According to Duffin et al. (1991) all fragment ions formed directly from $[M+NH_4]^+$, but Cheng et al. (1998) suggested that $[RCO+74]^+$ ions originated from $[DAG]^+$ ions, instead. Marzilli et al.

(2003) observed another fragment ion, which resulted from $[(M+NH_4)–NH_3–FA]^+$ ion of unsaturated TAGs after cleavage of fragment 74. McAnoy et al. (2005) stated that CID of ammonium adducts of TAGs resulted in a neutral loss of ammonia (NH_3) and that of acyl chain as a carboxylic acid producing diacyl product ions, $[(M+NH_4)–NH_3–FA]^+$. Kalo et al. (2006) suggested a three-stage fragmentation mechanism, in which ammonia was released from ammonium ion of $[M+NH_4]^+$ at the first stage, after which free fatty acid was formed from protonated TAGs yielding $[DAG]^+$ ions. At the third stage, $[RCO]^+$, $[RCO+74]^+$, and $[DAG–74]^+$ ions are fragmented from $[DAG]^+$.

Relatively few studies have been published concerning the analysis of MF TAGs by ESI–MS and ESI–MS^n techniques. Haddad et al. (2011a) used ESI–MS and ESI–MS^3 together with rp-HPLC and high-resolution GC to identify 135 molecular species of TAGs in camel milk. Soon after the previous study, Haddad et al. (2012) published a comparative study of the TAGs in human and equine milks using similar technique as previously (Haddad et al. 2011a), and identified 180 and 98 molecular species of TAGs in equine and human milks, respectively. Identification of TAGs based on fragmentation of $[DAG]^+$ ions in ESI–MS^3 experiments resulting in characteristic $[RCO]^+$, $[RCO–H_2O]^+$, and $[RCO+74]^+$ ions in each rp-HPLC fractions (Haddad et al. 2011a, 2012). Linderborg et al. (2014) investigated human milk, as well, by UHPLC–ESI–MS^2 yielding valuable information on the composition and structure of TAGs even though the separation of TAG species on UHPLC column was not optimized. Later, use of a novel optimization algorithm together with UHPLC–ESI–MS^2 enabled quantification of hundreds of regioisomeric TAGs in human MF by Kallio et al. (2017). Nagy et al. (2013) used a high-resolution LTQ-Orbitrap XL hybrid MS and positive electrospray ionization to determine regioisomers of TAGs in several edible fats and oils, including BF resulting in identification and quantification of 565 different TAGs (including regioisomers) and determination of regioisomeric balance of the main FAs in MF.

Other mass spectrometric methods. A recent review of Holčapek et al. (2012) showed clearly the dominant role of atmospheric pressure ionization methods (APCI, ESI) in the LC–MS analysis of organic compounds, including TAG analysis. In the early MS analysis of TAGs, EI and CI were the most common ionization methods in MS (Laakso 1992). Alternatively, other ionization methods were tested in early MS studies of TAGs, *e.g.*, field ionization and field desorption (Fales et al. 1975), which utilized powerful electric field to remove electrons from molecules resulting in relatively abundant $[M]^+/[M+1]^+$, $[M–RCO]^+$, $[RCO]^+$ ions in mass spectra of TAGs (Games 1978). A more frequently used method is desorption chemical ionization, which enables separation of TAGs according to the ACN and the degree of unsaturation on the basis of $[M+NH_4]^+$ ions. The method was employed, for example, in fingerprinting of TAGs in animal fats including MF (Schulte et al. 1981), and in the analysis of TAGs in human plasma (Mares et al. 1991). Similarly, matrix-assisted laser desorption and ionization mass spectrometric (MALDI–MS) applications suit well to profile TAG composition of natural fats and oils on the basis of sodium adducts and CID of $[M+Na]^+$ ions (Fuchs et al. 2010). For example, Picariello et al. (2007) used positive ion MALDI–time-of-flight (TOF)–MS to comparative characterization of TAGs in main animal fats (MF, lard, tallow). A modified laser desorption and ionization method was applied to fingerprinting of TAGs in standard solutions and those in vegetable oils (soy, sunflower, and olive oils) without any matrix, hence without any interference peaks from matrix using

sodium and potassium adduct ions for identification of TAG species and improved shot-to-shot reproducibility (Calvano et al. 2005). Evans et al. (1991) analyzed TAGs in alcohol solution containing sodium salt (NaI) by fast atom bombardment (FAB) ionization producing $[M+Na]^+$ adduct ions and $[M–RCOO]^+$ ions. Later, Kim et al. (2000) used FAB–MS^2 with high-energy CID of sodium adducted TAGs ($[M+Na]^+$) to determine regioisomers of di-short-chain TAGs in bovine udder. In addition to APCI and ESI, a third atmospheric pressure ionization method, *i.e.*, atmospheric pressure photoionization (APPI) has been applied to TAG analysis. In APPI, ionization of TAG molecules is induced by photons, which ionize TAGs directly or indirectly with the assistance of ionized dopant, *e.g.*, toluene or acetone (Holčapek et al. 2012). APPI–MS of TAGs (with dopant) showed several characteristic ions in full mass spectra, *i.e.*, protonated molecular ions $[M+H]^+$, molecular radical ions $[M]^{\bullet+}$, acyl ions $[RCO]^+$ and $[RCO–H_2O]^+$ ions, which have been employed in the analysis of molecular weight, degree of unsaturation, and structure of TAG molecules in olive oil (Gómez-Ariza et al. 2006a,b).

3.2.4 Quantification of triacylglycerols using GC–MS and LC–MS

Table 3.9 lists, in chronological order, investigations in which MF TAGs have been quantified by mass spectrometric methods. In most reported studies (Table 3.9), mass spectrometric detection was used after chromatographic separation by GC, HPLC, or SFC, but in few cases quantification was performed solely by mass spectrometer using direct inlet injection as reported by Laakso and Kallio (1993a,b), Spanos et al. (1995), Kallio et al. (2006), Haddad et al. (2011a, 2012), and Linderborg et al. (2014). In the early MS studies, quantification rested on the use of EI, PICI, and NICI techniques with full mass spectra recording (Table 3.9). Later, the atmospheric pressure ionization techniques (ESI, APCI) have been used almost without exception, in combination with full mass spectra monitoring or, increasingly, with selective ion monitoring (Table 3.9). Several strategies have been applied to quantification of MF TAGs. In GC–MS studies, the actual quantification was carried out by GC–FID with (Fontecha et al. 2000, 2005) or without (Myher et al. 1988) correction factors (CFs), and mass spectrometry was used merely to determine relative abundances of TAG species on the basis of specific ions. The CFs in GC–FID were calculated from the known BO composition (Fontecha et al. 2000, 2005). In 1990's, no CFs were applied to quantification of MF TAGs by HPLC–CI–MS studies (Kuksis et al. 1991, Myher et al. 1993, Marai et al. 1994), and TAGs were quantified using uncorrected peak areas of specific fragment ions (Table 3.9), which, in some cases, was accompanied by GC–FID determination (Kuksis et al. 1991). In APCI–MS studies of MF TAGs, quantification has been carried out with or without CFs. Dugo et al. (2005) and Chiofalo et al. (2011) determined the TAG from uncorrected peak areas in APCI–MS spectra. Later, Gastaldi et al. (2011) quantified MF TAGs on the basis of DAG^+ ions using CFs calculated from four TAG standards. Gotoh et al. (2012) and Nagai et al. (2015) used area based CFs for selected ions of eight and ten positional isomers of standard TAGs, respectively, in APCI–MS/MS^2. Nagy et al. (2013) used 25 TAG standards including mainly LC-TAGs to determine area based CFs for ammonium adducts in HPLC–ESI–MS. Similarly, Linderborg et al. (2014) used molar CFs of $[M+NH_4]^+$ derived from TAG standards in their UHPLC–ESI–MS investigation. Kallio et al. (2017) determined regioisomeric TAGs in human MF by a three-

Table 3.9 Examples of quantification of TAGs in milk fat using mass spectrometric methods.

Sample[1]	MS type[2]	Inlet	Ioni-zation	*m/z*[3]	CF/A,I[4]	References
BO distill.	Q	GC	EI+CI	200–850	–/FID (+ Rel. abund. by $[MH–RCOOH]^+$)	Myher et al. 1988
BF	DF	SFC	EI	200–1100 + SIM	–/I(M^+)	Kallio et al. 1989
BO distill.	Q	HPLC	NICI	200–1000	–/A($[M+Cl]^-$)	Kuksis et al. 1991
BO distill.	Q	HPLC	PICI	200–1000	–/($[MH]^+$/$[MH–RCOOH]^+$)	
BO/BO distill.	Q	HPLC	PICI	200–900	–/A($[MH–RCOOH]^+$)	Myher et al. 1993
IBO	Q	HPLC	PICI	200–900	–/A($[MH–RCOOH]^+$)	Marai et al. 1994
BF	QqQ	Direct	NICI, NH_4	n.a.	–/I($[M–H]^-$)	Laakso and Kallio 1993a,b
BO	DF	Direct	DCI, IBu	100–1000	–/I(Rel. abund. of $[MH–RCOOH]^+$)	Spanos et al. 1995
Goat MF	DF	GC	EI	50–1000	MCF/FID (known BO) + Rel. abund. by GC–MS	Fontecha et al. 2000
Ovine MF	DF	GC	EI	50–1000	MCF/FID (known BO) + Rel. abund. by GC–MS	Fontecha et al. 2005
Donkey MF	QqQ	HPLC	APCI	215–1100	–/A(APCI–MS spectra)	Dugo et al. 2005
BF	QqQ	Direct	NICI	200–900	MCF/I($[RCOO]^-$)	Kallio et al. 2006
Human, Donkey MF	QqQ	HPLC	APCI	215–1100	–/(APCI–MS spectra)	Chiofalo et al. 2011
Cow, Goat Human MF	IT	HPLC	APCI	n.a.	RF (4 TAG std)/DG^+	Gastaldi et al. 2011
Camel MF	LCQ	Direct	ESI	n.a.	MCF (TAG STD)/GC + Rel. abund. of $[DAG]^+$	Haddad et al. 2011a
Human, Cow Rat, Buffalo, Sheep, Goat MF/Cheese	Q	HPLC	APCI	SIM	CF (8 TAG STD)/A of selected ion chromatograms in APCI–MS/MS^2	Gotoh et al. 2012
Human, Equine MF	LCQ	Direct	ESI	n.a.	MCF (TAG STD)/GC + Rel. abund. of $[DAG]^+$, $[RCO]^+$	Haddad et al. 2012
BF	LTQ-orbitrap	HPLC	ESI	100–2000	CF (25 TAG STD)/A of $[M+NH_4]^+$	Nagy et al. 2013

Sample[1]	MS type[2]	Inlet	Ioni-zation	*m/z*[3]	CF/A,I[4]	References
Human MF	QQ	UHPLC Direct	ESI APCI	SIM SIM	MCF (TAG STD)/A of selected $[M+NH_4]^+$	Linderborg et al. 2014
BF	Q	HPLC	APCI	SIM	CF (10 TAG STD)/A of selected ion chromatograms in APCI–MS/MS^2	Nagai et al. 2015
Human MF	Q	UHPLC	ESI	SIM	–/I (TAGs & their *sn*-2/*sn*-1(3) ratio calculated by 2-phase optimization algorithm	Kallio et al. 2017

[1] BO = butteroil; BO distill. = butteroil distillate; IBO = interesterified butteroil, BF = butterfat; MF = milk fat

[2] Q = quadrupole; QQ = tandem quadrupole; QqQ = triple quadrupole; LCQ = liquid chromatography quadrupole; LTQ = linear trap quadrupole; DF = double focusing MS; IT = ion trap MS

[3] Scanning range (*m/z*) of full mass spectra; SIM = selected ion monitoring; n.a. = not available

[4] CF/A,I = type of correction factor (CF) / based on area (A) or intensity (I) of peaks; MCF = molar correction factor

step method consisting of (1) resolution of lithiated TAGs by UHPLC, (2) qualitative analysis of TAG species by ESI–MS^2, and (3) quantitative estimation of the regioisomers by optimization algorithm which based on the fragmentation pattern of the reference TAGs (*i.e.*, six regioisomer TAG pairs) in ESI–MS^2. In the early CI–MS studies using direct inlet of samples, MF TAGs were quantified using uncorrected peak intensities of $[M–H]^–$ (Laakso and Kallio 1993a,b) and $[MH–RCOOH]^+$ ions (Spanos et al. 1995). Later, Kallio et al. (2006) used more specific peak intensity based molar CFs for $[RCOO]^–$ ions to determine molar composition of MF TAG of ACN:DB 48:1–52:1 using direct inlet NICI–MS. Haddad et al. (2011a, 2012) combined direct inlet with ESI–MS in order to quantify TAGs in camel MF (Haddad et al. 2011a), in human MF and in equine MF (Haddad et al. 2012). In their studies, actual quantification was done by GC utilizing molar CFs, but relative abundancies of TAG species were determined by ESI–MS on the basis of specific fragment ions ($[DAG]^+$, $[RCO]^+$).

Correction factors are crucial in achieving reliable results in the quantification of TAGs with MS methods. It is generally known that use of CFs in MS measurements is challenging due to the fact that peak intensities and areas are dependable on the composition and structure of TAG species and on the conditions of MS measurements (*e.g.*, Laakso 1992). Already Hites (1970) quantified TAGs in ten vegetable oils on the basis of the intensities of $[M]^+$ and $[M–18]^+$ ions produced by EI–MS after direct introduction of samples into the ion source. Due to the direct inlet system, molecular distillation of different TAG molecules varied according to the ACN of TAGs, which was corrected using fractionation correction factors and, further, the effect of unsaturation on response was corrected systematically (Hites 1970). Later, Myher et al. (1984) showed the necessity of the use of appropriate CFs in their quantitative rp-HPLC–MS studies of TAGs. They showed that increase of unsaturation of TAGs decreased the response when total ion current was used for quantification of resolved peaks. When characteristic fragment ion was used for

quantification of unresolved TAGs, regioisomerism was shown to affect the response, and hence accurate quantification, because the yield of $[MH–RCOOH]^+$ ions was *ca.* four times higher from the *sn*-1/3 positions than from the *sn*-2 position (Myher et al. 1984). Duffin et al. (1991) connected the ion current response in ESI–MS to polarity of TAGs and reported that relatively more polar TAGs consisting of unsaturated FAs and/or short-chain FAs yielded more adduct ions ($[M+Na]^+$/$[M+NH_4]^+$) in electrospray ionization than less polar long-chain saturated TAGs.

Even though early studies (*e.g.*, Hites 1970, Myher et al. 1984, Duffin et al. 1991, Kallio and Currie 1993) unquestionably showed that ACN of TAGs, the chain length of esterified FAs of isobaric TAGs (isobaric = the same ACN:DB), the degree of unsaturation, and regioisomerism influenced on the abundance of molecular ions, quasi-molecular ions, and fragment ions in different ionization methods, and hence correction factors for TAGs in quantification, surprisingly few investigators have applied CFs to quantification of MF TAGs (*cf.*, Table 3.9). A natural reason for the scarce use of response factors (RFs) in the analysis of TAGs by MS was the huge number of different TAG species in natural fats and oils, which has complicated the search for relevant TAG standards for the determination of CFs (Holčapek et al. 2005). Correction factors have been calculated on the basis of limited number of commercial monoacid TAG standards (Kallio et al. 1991, Holčapek et al. 2005, Gastaldi et al. 2011, Nagy et al. 2013), mixed-acid TAG standards (Kallio and Currie 1993, Holčapek et al. 2005, Nagy et al. 2013, Linderborg et al. 2014), and regio- or enantiomeric TAG standards (Fauconnot et al. 2004, Nagy et al. 2013, Linderborg et al. 2014, Nagai et al. 2015). A higher number of TAG species has been synthesized for CF calculations from commercial TAGs by chemical interesterification of monoacid TAG standards (Byrdwell et al. 1996, Lisa et al. 2009), which enabled to produce several regioisomers of TAGs as well (Lisa et al. 2009). In addition, CFs derived from calculated composition (Myher et al. 1984, Byrdwell et al. 1996), or from known composition of natural fats and oils (Myher et al. 1984, Byrdwell et al. 1996, Fontecha et al. 2000, 2005) have been successfully used for determination of TAGs.

Byrdwell et al. (1996) assessed reliability and accuracy of four different LC–APCI–MS quantitative analysis of synthetic TAG mixtures and that of TAGs in natural and randomized soybean oil and lard. They calculated correction factors which based (*i*) on monoacid TAG standards, (*ii*) on randomized 35-component TAG standard mixture of known composition, (*iii*) on comparison of randomized samples to their calculated composition, and (*iv*) on comparison of FA compositions derived from calculated uncorrected TAG composition to that of GC–FID analysis (Byrdwell et al. 1996). The most accurate quantitative results for the known (*i.e.*, calculated) composition of randomized soybean oil and that of randomized lard were obtained with the use of GC–FID derived CFs with average relative error of 8.6 and 10.1%, respectively (Byrdwell et al. 1996). Further, the use of the CFs calculated on the basis of randomized soybean oil or lard resulted in almost similar TAG composition of natural soybean oil and natural lard than the use of GC–FID based CFs (Byrdwell et al. 1996), but CFs based on randomized TAG mixture were not applicable to the natural samples in their study. The last finding was contradictory to Myher et al. (1984), who stated that CFs derived from synthetic TAG standards would yield most reliable results. Holčapek et al. (2005) used 133 CFs (relative to triolein) calculated on the basis of commercial mono-

and mixed-acid TAG standards in quantification of TAGs of vegetable oils with HPLC using APCI–MS, LSD, and UV detectors. When FA compositions of seven oils were determined by GC–FID and compared to that calculated from the TAG composition analyzed by APCI–MS using CFs, average relative errors between GC and MS determinations varied from 7.8 to 32.0% (Holčapek et al. 2005), which were rather similar to those reported by Byrdwell et al. (1996). Due to the fact that commercial TAG standards, which are relevant to the determination of CFs for TAGs in edible fats and oils, are expensive and/or not available at all (Holčapek et al. 2005, Lisa et al. 2009), chemical interesterification (*i.e.*, randomization) of the mixtures of common and relatively inexpensive monoacid TAGs offers a reasonable alternative to produce more uncommon TAG species at well-defined molar ratios for the determination of CFs. Byrdwell et al. (1996) used randomization of the mixture of five monoacid TAGs to synthesize 35 different TAGs (regioisomers not included) for CFs determination. In their investigation (Byrdwell et al. 1996), the relatively large number of different TAG species in one mixture undoubtedly complicated exact integration of all chromatographic peaks of TAG species due to overlapping of TAGs, hence made the determination of CFs more difficult. Lisa et al. (2009) interesterified TAG mixtures containing equal amounts of only two or three monoacid TAGs enabling a baseline separation of even the regioisomer pairs of several TAG species in the randomized mixtures on three silver-ion HPLC columns in series, which facilitated reliable CF calculation. In addition, they noted that chemical interesterification had to be optimized so that the experimental distribution of TAG species in randomized mixtures was equivalent to theoretical calculations of TAG composition. Further, it is worth noting that isotope effects may have a significant influence on quantification of TAGs, especially when direct MS was applied, hence corrections on mass spectra, mainly due to the effects of naturally occurring (~1.11%) ^{13}C isotope have to be made prior to final calculation (Han and Gross 2001, Linderborg et al. 2014).

Tables 3.8 and 3.9 suggest that APCI- and ESI–MS/MS^n combined with HPLC are the most common analyzing techniques to identify and quantify TAGs in MF at present, as well as they are in the analysis of other biological samples (Holčapek et al. 2012). Generally, ESI–MS yields less fragmentation of adduct ions, hence producing more simple mass spectra to interpret and more intense peaks to quantify (Nagy et al. 2013). On the other hand, APCI–MS yields also some fragment ions, hence structural information of TAGs without additional CID of adduct ions. Depending on the analytical conditions, ESI–MS can produce other adduct ions (*e.g.*, $[M+Na]^+$) than solely $[M+NH_4]^+$ adduct, enabling additional and alternative way to identify TAGs of natural fats and oils, even if it results in slight decrease in sensitivity in quantification due to less intense (ammonium) adduct ions (Nagy et al. 2013).

3.3 Analysis of the regioisomers of triacylglycerols

Stereospecific analysis of TAGs yields information on the positional distribution of acyl groups between the *sn*-1, *sn*-2, and *sn*-3 positions of a TAG molecule or, alternatively, that on the general distribution of acyl groups among the three *sn*-positions of all TAGs in a mixture of TAG species, for example, in natural fats and oils, as was already stated by

Brockerhoff (1971). Respectively, regiospecific analysis describes the distribution of esterified FAs between the primary *sn*-positions (*sn*-1,3) and the secondary *sn*-position (*sn*-2) of a TAG molecule or between those of all TAGs in synthesized or natural TAG mixtures (Kalo and Kemppinen 2003). Further, the regiospecific distribution of FAs can be calculated from the stereospecific distribution. In lipid literature, regio- and stereospecific analysis of TAGs have been assessed from different viewpoints by several researchers (*e.g.*, Brockerhoff 1971, Kuksis et al. 1985, Laakso 1992, Ruiz-Gutiérrez and Barron 1995, Kuksis and Itabashi 2005, Kalo and Kemppinen 2012). Applicable methods for regiospecific analysis of TAGs can be roughly divided into five main categories: enzymatic methods (Brockerhoff 1971, Kalo et al. 1996, Lisa et al. 2009, Baiocchi et al. 2015), chemical methods (Brockerhoff 1971, Kalo et al. 1996, Baiocchi et al. 2015), chromatographic (GLC, HPLC) methods (Kalo et al. 1996, Lisa et al. 2009, Kalo and Kemppinen 2012, Baiocchi et al. 2015), spectrometric (MS, NMR) methods (Kalo et al. 1996, Lisa et al. 2009, Kalo and Kemppinen 2012, Baiocchi et al. 2015), and combined methods (*e.g.*, GC–MS or HPLC–MS) (Kuksis and Itabashi 2005, Lisa et al. 2009, Kalo and Kemppinen 2012, Baiocchi et al. 2015).

Regiospecific analysis of TAGs is an important field of lipid reseach due to the fact that the distribution of FAs between the *sn*-2 and *sn*-1(3) positions is related to the functionality of TAGs in fatty foods, as well as to their physical and physiological properties (Hunter 2001, Kalo and Kemppinen 2003, Watanabe et al. 2014). For example, unique melting properties of cocoa butter with very narrow melting range slightly below body temperature are due to specific, symmetrical distribution of fatty acyl moieties (16:0, 18:0, and 18:1) among primary and secondary *sn*-positions of TAG molecules (Hunter 2001, Kalo and Kemppinen 2003). Several technologically important hydrolytic enzymes used in food industry are regiospecific and release FAs primarily from the *sn*-1(3) positions, which was shown to intensify desirable or undesirable flavors of food products, *e.g.,* in milk and milk products with potentially aromatic short-chain FAs in the *sn*-3 position (Urbach and Gordon 1994, Jensen 2002). In animal metabolism (humans included), digestion and absorption of dietary TAGs are controlled by *sn*-1(3)-specific enzymes resulting in digestion of TAGs as *sn*-2 MAGs and FFAs released from the primary positions (*e.g.*, Bracco 1994, Hunter 2001, Kalo and Kemppinen 2003, Mu and Høy 2004). It is generally known that palmitic acid and other long-chain saturated FAs esterified to the *sn*-2 position had an increased absorption as 2-MAGs than as respective FFAs due to their high melting temperature and ability to form unsoluble calcium soaps (Hunter 2001, Innis 2011) resulting in an increased risk of cardiovascular diseases in animal studies, but not unambiguously in human studies, as was reviewed (Hunter 2001, Karupaiah and Sundram 2007). Further, in order to develope tailor-made edible fats and oils with desired properties by chemical or enzymatic modification of natural oils and fats or by synthesis of specific structured lipids (*e.g.*, infant milk substitutes), regiospecific distribution of esterified FAs is essential to know during the production and in final products (Février et al. 2001, Mu et al. 2001, Kalo and Kemppinen 2003, Rønne et al. 2005). In addition, regio- and stereospecific analysis have used to elucidate biosynthesis of TAGs (Ruiz-Gutiérrez and Barron 1995) and to assess regio/stereospecificity of lipases (Iwasaki et al. 2001).

3.3.1 Chemical and enzymatic methods

Enzymatic methods. Initially, most chemical and enzymatic methods of structural analysis were applied to reveal the whole stereospecific structure of TAGs or solely FA composition in the *sn*-2 position (Luddy et al. 1964, Brockerhoff 1965, 1971), hence they were not used for regiospecific analysis *per se*. Nevertheless, early enzymatic regiospecific analyses were based on partial hydrolysis of TAGs with 1,3-specific pancreatic lipase (EC 3.1.1.3) resulting in a multi-component mixture consisting non-hydrolyzed TAGs, 1,2(2,3)-DAGs, 2-MAGs, and FFAs (Hendrikse and Harwood 1986, Kalo and Kemppinen 2003). After hydrolysis step, FA composition of the *sn*-2 position ($FA_{2\text{-}MAG}$, mol%) was determined by GC using FAME analysis of isolated (by TLC) 2-MAG fraction (Hendrikse and Harwood 1986). Though, use of longer carbon chain derivatives (*i.e.*, ethyl, propyl, butyl esters) than methyl esters has been recommended for FA analysis of MF containing plenty of short-chain FAs (Ulberth et al. 1999) in order to avoid underestimation of the share of SC-FAMEs due to their higher volatility and water solubility compared to MC- and LC-FAMEs (Yoshinaga et al. 2016). The regiospecific analysis was completed by calculating the mean composition of FAs in the primary positions ($n(FA_{sn\text{-}1(3)})$ as follows (Fontecha et al. 2010): $n(FA_{sn\text{-}1(3)})$, mol% = [3 x $n(FA_{TAG})$ – $n(FA_{2\text{-}MAG})$] / 2; $n(FA_{TAG})$ = FA composition (mol%) of native TAGs in fat/oil. On the other hand, regioisomeric distribution of FAs has been successfully determined directly on the basis of the released FAs from the *sn*-1(3) positions (Arcos et al. 2000, Dourtoglou et al. 2001).

Obviously, strict 1,3-specificity and non-discriminatory FA specificity of pancreatic lipase together with a low migration rate of fatty acyl moieties between primary and secondary positions during analysis are essential for reliable results (Turon et al. 2003). In general, pancreatic lipase was shown to be strictly regiospecific in practice (*e.g.*, Sampugna et al. 1967, Franzke et al. 1973). However, already in some early studies (Bottino et al. 1967, Franzke et al. 1973), definite specificity problems were noticed. Bottino et al. (1967) showed that pancreatic lipase released long-chain polyunsaturated FAs (20:5:n-3, 22:6n-3) more slowly than long-chain saturated FAs, which complicated analysis of marine oils. Brockerhoff (1971) summarized the same observation more widely. On the other hand, regio- and stereospecific analysis of TAGs in ruminant MFs are challenging due to a high number of short-chain TAGs. Sampugna et al. (1967) showed that short-chain TAGs containing butyryl acyls were hydrolyzed more rapidly than higher molecular weight TAGs.

In addition to the use of pancreatic lipase, some other 1,3-specific lipases originated from fungi have been used for regioisomeric analysis and/or determination of FA composition in the *sn*-2 position, *i.e.*, *Rhizomucor miehei* (Dourtoglou et al. 2001), *Rhizopus arrhizus* (Arcos et al. 2000), and *Rhizopus oryzae* (*delemar*) (Kosugi et al. 2002, Pérignon et al. 2012). For example, Kosugi et al. (2002) successfully determined FA composition of the *sn*-2 position in TAGs of beef tallow by *R. oryzae*. Further, Perignon et al. (2012) confirmed that *R. oryzae* lipase was strictly regiospecific and hydrolyzed medium- and long-chain FAs without FA selectivity. Unfortunately, *R. oryzae* lipase has shown to hydrolyze polyunsaturated FAs more slowly (< 35%) than oleic acid (Shimada et al. 1997), which restricts its suitability for structural analysis of highly unsaturated TAGs in edible fats and oils. *Pseudozyma* (prev. *Candida*) *antarctica* lipase (B) is a promising alternative to

pancreatic lipase to carry out a regiospecific analysis of TAGs (Irimescu et al. 2001, 2002, Shimada et al. 2003, Watanabe et al. 2009, 2014, 2015, Yoshinaga et al. 2015, 2016). Even though, *P. antarctica* was generally regarded as unspecific lipase (Irimescu et al. 2001, 2002), the enzyme was shown to be highly 1,3-specific when immobilized and applied to transesterification of TAGs using a high excess (TAGs:ethanol ~1:3–1:57) of ethanol (Irimescu et al. 2001, 2002, Shimada et al. 2003, Watanabe et al. 2009), and no excessive acyl migration was observed (Watanabe et al. 2009). Importantly, the enzyme has shown to be suitable for regiospecific analysis of TAGs containing saturated and unsaturated FAs with 4–24 acyl carbons without any fatty acid specificity (Watanabe et al. 2009). On the other hand, Shen and Wijesundera (2006) questioned equal hydrolysis rate of long-chain polyunsaturated fatty acids by *P. antarctica* despite the substrate:ethanol ratio. Further, the yield of representative 2-MAGs from TAGs by *P. antarctica* lipase was observed to be *ca.* 30 mol% (after 4 h of reaction at 30°C), which was higher than the theoretical yield of 2-MAG (4.8 mol%) in complete chemical hydrolysis and in conventional hydrolysis with pancreatic lipase due to above mentioned fatty acid specificity and due to only partial hydrolysis (*ca*. 10%), which is essential in order to avoid excessive acyl migration as was assessed by Watanabe et al. (2009).

Enzymatic regio- and stereospecific analysis of TAGs in MF were initially performed with 1,3-specific hydrolysis of TAGs with pancreatic lipase in order to figure out the general distribution of FAs in TAGs (Pitas et al. 1967, Breckenridge and Kuksis 1968a, Parodi 1979a, Christie and Clapperton 1982, Haddad et al. 2010). Later, hydrolysis with pancreatic lipase had an important role in monitoring synthesis and final composition of enzymatically modified MF products (Kalo et al. 1989a, 1990, Christensen and Holmer 1993). In addition, regiospecific analysis yielded valuable information, for example, how FA composition in the nutritionally significant *sn*-2 position of TAGs was affected by feeding of cows (DePeters et al. 2001, Mistry et al. 2002). Recently, strict regiospecificity of *P. antarctica* lipase B was utilized in regiospecific analysis of *trans*-FAs in MF (Yoshinaga et al. 2015) showing that *trans*-11-C18:1 was mainly esterified to the primary positions. In addition, the same enzyme was applied successfully to a challenging analysis of the regioisomers of short- and medium-chain TAGs in MF showing that mainly *sn*-1(3) isomers of C4:0–C8:0 containing TAGs existed in MF (Yoshinage et al. 2016). In this study (Yoshinaga et al. 2016), TAGs were transesterified in excess of ethanol with immobilized *P. antarctica* lipase B and the 2-MAG fraction was separated from the reaction mixture with silica gel chromatography (Sep-Pak silica cartridge), after which FA composition of 2-MAGs and that of total FAs were determined as FA propyl esters (area%) by GC.

Chemical analysis: In chemical regio- and stereospecific analysis, determination involves usually partial deacylation of TAGs with Grignard reagent (methyl, ethyl, or allyl magnesium bromide) resulting in a reaction mixture of intact TAGs, *sn*-1,2(2,3) and *sn*-1,3 DAGs, *sn*-1, *sn*-2, and *sn*-3 MAGs, and tertiary alcohols derived from acyl groups (*e.g.*, Yurkowski and Brockerhoff 1966, Laakso 1992, Kalo and Kemppinen 2003). Grignard degradation is frequently used especially for stereospecific analysis of TAGs due to equal deacylation of ester bonds at *sn*-1, 2, and 3 positions and due to lack of FA specificity (Yurkowski and Brockerhoff 1966, Laakso 1992), which enables relatively reliable determination of average FA composition in all *sn*-positions of TAG on the basis of

representative partial acylglycerols, especially diacylglycerols (Brockerhoff 1971, Laakso 1992, Turon et al. 2003). However, some limitations of the method have resulted in unreliable results, which were related to the spontaneous migration between *sn*-1,2(2,3) and *sn*-1,3 DAGs (Yurkowski and Brockerhoff 1966, Kalo and Kemppinen 2003, Baiocchi et al. 2015), and swift isomerization of MAGs (Yurkowski and Brockerhoff 1966, Becker et al. 1993), together with long and laborious separation of partial acylglycerols for further analysis (Shen and Wijesundera 2006). In addition, some studies suggested that ethyl (Turon et al. 2002) and allyl magnesium bromide (Becker et al. 1993) had a slightly higher selectivity to the *sn*-1(3) positions and to the *sn*-2 position, respectively, which could yield an unbalanced ratio of *sn*-1(3) and *sn*-2 MAGs.

In early investigations, stereospecific analyses of TAGs using Grignard degradation were performed on the basis of *sn*-1,2(2,3) DAGs, which were assessed to reflect most accurately the original FA distribution in the primary positions of TAGs (Yurkowski and Brockerhoff 1966, Brockerhoff 1971, Laakso 1992, Turon et al. 2003). After partial deacylation of TAGs, *sn*-1,2(2,3) DAGs were separated by TLC and converted to phosphatidylphenols, which were hydrolyzed with phospholipase A_2 (EC 3.1.1.4) in order to produce specifically FFAs released form the *sn*-2 position and lysophosphatides with unhydrolyzed FA at the *sn*-1 position, from which the FA composition of the *sn*-1 position could be determined with FAME analysis by GC (Yurkowski and Brockerhoff 1966, Brockerhoff 1971, Laakso 1992, Turon et al. 2003, Kalo and Kemppinen 2003). Regiospecifically important FA composition of the *sn*-2 position could be determined by GC from the above mentioned FFAs (Blasi et al. 2008, 2013, Cossignani et al. 2011), or more commonly from 2-MAGs produced by pancreatic lipase from the original TAGs (Yurkowski and Brockerhoff 1966, Brockerhoff 1971, Laakso 1992). In addition to phospholipase A_2 method (see above), *sn*-1,2 and *sn*-2,3 DAGs derived from Grignard degradation have been separated and analyzed as their 3,5-dinitrophenyl urethane (DNPU) derivatives on a chiral HPLC columns (Itabashi and Takagi 1987, Itabashi et al. 1990, Itabashi 1993) or as their diastereomeric derivatives on a silica gel HPLC column (Laakso and Christie 1990, Christie et al. 1991). Takagi and Ando (1991) represented a stereospecific method based on the analysis of DNPU derivatives of *sn*-1(3) MAGs and underivatized *sn*-2 MAGs with relatively low acyl migration rate in MAGs (2.5–3%). After partial Grignard degradation of TAGs with ethyl magnesium bromide, *sn*-1(3) and *sn*-2 MAGs were separated by TLC on boric acid-impregnated silica gel plates (Takagi and Ando 1991). From the isolated MAG fractions, *sn*-2 MAGs were determined directly by GC, but *sn*-1 and sn-3 MAGs were first converted to representative 3,5-DNPUs, after which they were separated on a chiral HPLC column prior to FAME analysis (Takagi and Ando 1991).

Chemical methods have been used more infrequently for direct regiospecific analysis than for stereospecific analysis, but some MAG-based methods have been developed (Becker et al. 1993, Angers et al. 1998, Angers and Arul 1999, Turon et al. 2002, Tzompa-Sosa et al. 2014). Even though quick acyl migration in MAGs has been reported to be a serious problem and to result in unpresentative MAG population (Yurkowski and Brockerhoff 1966), Takagi and Ando (1991) observed relatively low (2.5–3%) acyl migration rate in MAGs in their investigation. Becker et al. (1993) used a high amount of allyl magnesium bromide for a quick partial deacylation of TAG standards (*sn*-1,3-distearoyl-2-

oleoylglycerol, *sn*-1,3-didecanoyl-2-palmitoylglycerol) to produce representative *sn*-2 MAGs, which were separated by silica TLC plates impregnated with boric acid. FA composition of the *sn*-2 position ($FA_{2\text{-}MAG}$) and that of the intact TAGs (FA_{TAG}) were determined by GC–FID on the basis of peak areas of FAMEs, after which FA composition of the *sn*-1,3 positions ($FA_{sn\text{-}1(3)}$) was calculated as follows: ($FA_{sn\text{-}1(3)}$) = 1.5 x FA_{TAG} – 0.5 x $FA_{2\text{-}MAG}$ (Becker et al. 1993). Corresponding methods using allyl magnesium bromide as a catalyst have been utilized to quantify acyl migration during the synthesis of structured lipids (Mu et al. 2001) and to assess the regiospecificity of lipases in the enzyme-catalyzed interesterification of butterfat–rapeseed oil mixtures (Rønne et al. 2005). In addition, ethyl magnesium bromide was used to deacylate TAGs instead of allyl magnesium bromide in order to produce representative 2-MAGs for regiospecific analysis of TAGs in MF fractions separated by rp-HPLC (Angers et al. 1998) and those in MFs of individual cows (Tzompa-Sosa et al. 2014). On the other hand, Turon et al. (2002) chose to produce representative *sn*-1(3) MAGs by quick Grignard degradation using an excess amount of ethyl magnesium bromide as a catalyst to deacylate the TAG substrates (*i.e.*, *sn*-1,3-dipalmitoyl-2-oleoylglycerol TAG standard and TAGs of borage oil, milk fat, and tuna oil). MAGs were swiftly separated by boric acid TLC and analyzed by GLC after methyl ester preparation followed by calculation of the FA composition in the secondary position on the basis of FA compositions in the *sn*-1(3) MAGs and in intact TAGs ($FA_{2\text{-}MAG}$ = 3 x FA_{TAG} –2 x $FA_{1(3)\ MAG}$) (Turon et al. 2002). Angers and Arul (1999) deacylated TAGs with ethyl magnesium bromide and prepared dibutyrate derivatives of both 1(3)- and 2-MAGs with n-butyryl chloride without any separation of MAGs by TLC after Grignard degradation. The regiospecific distribution of FAs in TAG standards containing FA with 12–20 acyl carbons and that in TAGs of three edible oils and fats (beef tallow, grapeseed and cotton oils) were determined by GC using a phenyl(65%)methylsilicone column for separation of the regioisomers of the dibutyrate derivatives (Angers and Arul 1999).

Preliminary studies concerning regio- and stereospecific analysis of TAGs in MF by Grignard degradation with methyl magnesium bromide encountered problems in separation of DAGs on silica TLC plates probably due to overlapping of *sn*-1,3 DAGs containing a butyryl group with *sn*-1,2(2,3) DAGs containing solely long-chain fatty acyl groups (Yurkowski and Brockerhoff 1966). Later, Parodi (1982) used ethyl magnesium bromide to produce DAGs for structural analysis of MF TAGs of nine different mammals, but prefered pancreatic lipase hydrolysis to obtain DAGs from TAGs in bovine MF. Itabashi et al. (1993) analyzed fully hydrogenated acetyl- and butyryl-enriched TAGs of np-TLC isolated fractions of a butteroil redistillate and produced DAGs by partial Grignard degradation. TLC resolved DAGs from acetyl-TAG fraction showed three well-separated bands containing *sn*-1,2(2,3) DAGs with two long-chain acyl groups, *sn*-1,3 DAGs with one acetyl and one long-chain acyl group, and *sn*-1,2(2,3) DAGs with one acetyl and one long-chain acyl group (Itabashi et al. 1993). However, *sn*-1,3 DAGs with one butyryl and one long-chain acyl group overlapped with *sn*-1,2(2,3) DAGs containing medium- and long-chain acyl groups when separated on TLC plates, which was previously noted by Yurkowski and Brockerhoff (1966). Further, Itabashi et al. (1993) showed an alternative for GLC determination of partial acylglycerols derived from Grignard degradation, and separated both enantiomers (*sn*-1,2 *vs.* *sn*-2,3 DAGs) and regioisomers (*sn*-1,2(2,3) *vs.* *sn*-1,3 DAGs) of short-chain DAGs as their 3,5-DNPU derivatives on a chiral HPLC column using (R)-(+)-l-

(1-naphthyl)ethylamine polymer as the stationary phase and hexane/l,2-dichloroethane/ethanol (40:10:1) as the mobile phase. In the past decade, stereospecific structure of TAGs in MFs of several ruminant species, *i.e.*, cow, ewe, goat, and buffalo (Blasi et al. 2008) and camel (Haddad et al. 2010), and that of non-ruminant species, *i.e.*, human (Haddad et al. 2012), horse (Haddad et al. 2011b), and donkey (Blasi et al. 2008, Cossignani et al. 2011) has been studied by chemical methods in order to find out their usability in nutritional applications, especially as infant formulas, and in order to prevent adulteration of rare MFs with bovine MF (Blasi et al. 2013). Blasi et al. (2008, 2013) and Cossignani et al. (2011) used partial Grignard degradation with ethyl magnesium bromide to yield *sn*-1,2(2,3) DAGs and phospholipase A_2 method to produce *sn*-1-lysophosphatidylcholines and FFAs from chemically synthesized *sn*-1,2(2,3)-phosphatidylcholines. FA composition in the *sn*-1 and *sn*-2 position were determined by GC analysis of FAMEs (corrected area-%) of the *sn*-1-lysophosphatidylcholines and that of FFAs from the *sn*-2 position, respectively, and FA composition in the *sn*-3 position was calculated. Haddad et al. (2010, 2011b, 2012) applied relatively similar phospholipase A_2 procedure except that FA composition in the *sn*-3 position was determined on the basis of *sn*-1,3 DAGs and synthesized *sn*-1,3-phosphatidylcolines, from which *sn*-3-lysophosphatidylcholine were released by phospholipase A_2 for FA analysis (*sn*-3) by GC, hence FA composition in the *sn*-2 position was calculated.

Most reported direct regiospecific analysis of TAGs in MF by chemical methods comprised rather similar procedures (Angers et al. 1998, Turon et al. 2002, Tzompa-Sosa et al. 2014): partial deacylation of TAGs with ethyl magnesium bromide, isolation of the *sn*-2 and *sn*-1(3) MAGs on a silica gel TLC plate impregnated with boric acid, preparation of fatty acid methyl esters from the desired MAG fraction, analysis of FAMEs by GC, and calculation of the FA composition in the *sn*-1(3) positions on the basis of determined FA composition in the *sn*-2 position and in intact TAGs. Angers et al. (1998) and Tzompa-Sosa et al. (2014) rested their determination on representative 2-MAGs, but Turon et al. (2002) used 1(3)-MAGs instead of 2-MAGs. All methods enabled determination of general regiospecific distribution of FAs in MF TAGs. In addition, they provided profound information about biosynthesis (Angers et al. 1998, Tzompa-Sosa et al. 2014) and nutritional potential of TAGs (Tzompa-Sosa et al. 2014). According to Angers et al. (1998), size of TAG molecules influenced on regiospecific distribution of oleic acid and FAs with 10–14 acyl carbons, but did not affected that of FAs with 4–8, 16, and 18 acyl carbons. Tzompa-Sosa et al. (2014) showed that high amount of palmitic acid in MF TAGs decreased the proportion of long-chain saturated FAs at the *sn*-2 position and increased that of oleic acid at the same position, which resulted in negative changes in the nutritional value of bovine MF-based infant formulas. Rønne et al. (2005) chose analysis of 2-MAGs produced by Grignard degradation with allyl magnesium bromide to monitor interesterification reaction of the blends containing butterfat and rapeseed oil (70/30, w/w) and vegetable oils and fats (sunflower oil, fully hydrogenated cottonseed oil) using six commercial lipases (both 1,3-specific and non-specific). They observed no significant differences in the action of the lipases on butterfat blends containing an abundant amount of short-chain FAs compared to that on vegetable fats and oils containing longer chain FAs (Rønne et al. 2005).

In summary, regiospecific analysis of bovine MF by chemical and enzymatic methods is challenging partly due to high proportion of short-chain FAs in MF TAGs. Hydrolysis with 1,3-specific lipases provides a relatively easy way to determine regiospecific distribution of FAs in the *sn*-2 position assuming that the procedure yields representative 2-MAGs. Unfortunately, the conventional enzymatic method with pancreatic lipase has been reported to hydrolyze low molecular weight butyrate TAGs more rapidly than high molecular weight TAGs without short-chain acyls (*e.g.*, Sampugna et al. 1967). Alternatively, *Pseudozyma (Candida) antarctica* lipase B has been shown to hydrolyze TAGs containing short-chain acyls without any special selectivity to them (Watanabe et al. 2009), hence Yoshinaga et al. (2015, 2016) applied successfully hydrolysis with *P. antarctica* lipase to the regiospecific analysis of TAGs in MF, recently. On the other hand, chemical methods using Grignard degradation result in practically random deacylation of MF TAGs, and hence, yield representative 1,2(2,3)-DAGs for further structural analysis of TAGs (*e.g.*, Turon et al. 2003). However, partial acylglycerols, *i.e.*, 1(3)-MAGs, 2-MAGs, and 1,3-DAGs, which are suitable for direct regiospecific analysis, are particularly prone to rapid acyl migration (Yurkowski and Brockerhoff 1966). Slight adjustments of parameters concerning reaction time and quality and quantity of Grignard reagent have enabled production of representative MAGs, on the basis of which reliable regiospecific analysis of MF TAGs has been carried out (Angers et al. 1998, Turon et al. 2002, Tzompa-Sosa et al. 2014). In general, chemical and enzymatic methods provide knowledge of the regiospecific distribution of FAs in all TAGs of MF. If more specific regiospecific information of individual TAG species and specific TAG groups are essential, chromatographic separation of TAGs prior to regiospecific analysis (*e.g.*, Angers et al. 1998) or that of MAGs/DAGs after partial hydrolysis or deacylation are needed (*e.g.*, Christie et al. 1991, Itabashi et al. 1993, Angers and Arul 1999).

3.3.2 Chromatographic methods

Chromatographic methods are essential parts of practically every chemical or enzymatic structural analysis of TAGs, because they are applied to the separation of partial acylglycerols by TLC after hydrolysis or deacylation step, and quantification of partial acylglycerols by GC or HPLC, as was discussed above. For example, separation of molecular species of MAGs or DAGs has been performed by GC on a polar capillary column (Angers and Arul 1999) and by HPLC on a chiral column (Takagi and Ando 1991, Itabashi et al. 1993) and on a silica gel column (Christie et al. 1991) after preparation of respective dibutyrate derivatives from MAGs, 3,5- DNPU derivatives from 1(3)-MAGs or DAGs, and diastereomeric derivatives from DAGs. However, separation power of current chromatographic columns enables regiospecific analysis without any chemical or enzymatic procedure prior to GC or HPLC analysis. Separation of the regioisomers and enantiomers by polar high-resolution GC (HRGC), np-HPLC, and chiral HPLC after derivatisation of partial acylglycerols is discussed shortly in the previous chapter (3.3.1). In addition, separation of the regioisomers of TAGs has been conducted on two rp-HPLC column in series after conversion of unsaturated TAGs to their brominated derivatives (Neff et al. 2001). However, this chapter (3.3.2) focuses on the discrimination of *sn*-2 and *sn*-1(3) isomers of intact TAGs by chromatographic methods without any derivatization step.

Gas chromatography (GC). Due to the fact that apolar GC columns do not differentiate between *sn*-2 and *sn*-1(3) isomers of TAGs (Geeraert et al. 1983), polar columns are needed for regiospecific analysis of TAGs. Further, in order to estimate reliability of the quantification of TAG species including several regioisomers by GC–FID, Yoshinaga et al. (2017) synthesized 47 TAG species (including several regioisomers) containing 30–54 acyl carbons and 0–9 double bonds and analyzed them with Ultra ALLOY^{+}-65 column (30 m × 0.25 mm i.d., 0.10 μm film thickness, He as carrier gas). They (Yoshinaga et al. 2017) observed that correction factors (CFs) of different TAG species varied markedly, but no marked differences between the CFs of regioisomers were observed. In general, polar or polarizable phenyl(50–65%)methylsilicone columns have shown to separate regioisomers of TAGs containing short-chain (2:0–4:0) acyl groups (Myher et al. 1988, Huang et al. 1994, Klemann et al. 1994, Kalo et al. 1996, Angers and Arul 1999), but separation of regioisomers of TAGs containing medium- and long-chain FA residues (> 6:0) has not been reported. Myher et al. (1988) investigated regioisomers of monoacetate and -butyrate TAGs in MF and showed that of *rac*-SXX eluted prior to *rac*-XSX (S = 2:0 or 4:0, X = other acyl group) on a polar flexible quartz column (RSL-300, 25 m x 0.25 mm i.d., H_2 as carrier gas). Huang et al. (1994) reported similar resolution of monoacetate and -propionate TAGs on a more polar phenyl(65%)methylsilicone column (Quadrex^{TM}, 25 m x 25 mm i.d., 0.1 μm film thickness, H_2 as carrier gas). In addition, they (Huang et al. 1994) demonstrated that *rac*-SSX eluted later than *rac*-SXS regioisomer of diacetate and -propionate TAGs (S = 2:0 or 3:0, X = long-chain acyl group). Later, Angers and Arul (1999) reported that *sn*-BXB and *rac*-BBX type regioisomers of dibutyrate TAGs (B = butyryl acyl group, X = long-chain acyl group with CN:DB = 12:0–20:0, 17:0, 18:1, 18:2, or 18:3) resolved near baseline resolution (1.0–1.2) from each other on a phenyl(65%)methylsilicone column (Quadrex^{TM}, 30 m x 25 mm i.d., 0.1 μm film thickness, H_2 as carrier gas). In all cases, *sn*-BXB regioisomer eluted prior to *rac*-BBX isomer (Angers and Arul 1999). Further, Kalo et al. (1996) showed separation of the regioisomers of synthetic monobutyrate TAGs containing two long-chain acyls (12:0 and/or 18:0) on a highly polar phenyl(65%)methylsilicone column (Quadrex^{TM}, 25 m x 25 mm i.d., 0.1 μm film thickness, H_2 as carrier gas). In conclusion, gas chromatography with polarizable thermostable phenylmethyl columns enables determination of the regioisomers of TAGs containing 1–2 short-chain FAs (2:0–4:0) and 1–2 long-chain FAs. GC methods have been used for separation of regioisomers of SC-TAGs in a simple mixture of synthesized TAGs (Kalo et al. 1996), in more complex mixtures of structured TAGs (Huang et al. 1994, Klemann et al. 1994), and in highly complex TAG fractions of natural fats and oils (Myher et al. 1988, Angers and Arul 1999).

Normal-phase HPLC (np-HPLC): Even though, np-HPLC is relatively seldom applied to TAG analysis, some investigations concerning separation of regioisomers of SC-TAGs have been carried out (Mangos et al. 1999, Lee et al. 2002, 2003, 2007, 2008). Separation of the regioisomers of SC-TAGs on a silica gel column is based on a slightly higher polarity of TAG molecules in which a short-chain acyl group(s) is (are) located in the primary position(s) than those in which short-chain acyl is in the secondary position (Lee et al. 2002). Accordingly, when Mangos et al. (1999) separated the regioisomers of mono- and diacetate and -propionate TAGs on a silica column (Chromegasphere^{TM} Si60, 3 μm, 250 mm x 4.6 mm i.d.) using isocratic elution, *rac*-LSS regioisomers eluted prior to *rac*-SLS and *rac*-LSL isomers prior to *rac*-LLS (L = 16:0 or 18:0, and S = 2:0 or 3:0). Similar elution pattern

was shown for the regioisomers of mono- and dibutyrates (Lee et al. 2002, 2003, 2007, 2008) and for those of partially separated mono- and dicaproates (Lee et al. 2002) on a Chromegasphere^{TM} Si60 column (3 μm, 250 mm x 4.6 mm i.d.) (Lee et al. 2002, 2003) or on a Chrompac^{TM} Si60 column (5 μm, 300 mm x 3.0 mm i.d.) (Lee et al. 2007, 2008) using an isocratic binary solvent system (15% MTBE, 85% hexane, v/v, + 0.4% acetic acid). Short-chain TAG isomers have been synthesized by interesterification of triacetin or tripropionin with hydrogenated soybean oil (Mangos et al. 1999), tributyrin with hydrogenated soybean oil (Lee et al. 2002), macadamia oil (Lee et al. 2003), palm oil (Lee et al. 2008), olive and soybean oils (Lee et al. 2007), and tricaproin with hydrogenated soybean oil (Lee et al. 2002). In all studies above (Mangos et al. 1999, Lee et al. 2002, 2003, 2007, 2008), np-HPLC method has been used to monitor the molecular species of TAGs including regioisomers in the final products, *i.e.*, in the structured TAGs.

Reversed-phase HPLC (rp-HPLC): First reported separations of the regioisomers of underivatized TAGs by rp-HPLC were related to synthetic short-chain TAGs. Kalo et al. (1996) showed that regioisomers of TAGs containing one butyryl acyl group (B) and two long-chain (L) acyl groups (L/L = 18:0/18:0 or 18:0/12:0) separated on a non-endcapped rp-HPLC column (Spherisorb^{TM} S5ODS1, 5 μm, 250 mm x 4.6 mm i.d.) using a linear gradient of 1–70% isopropanol in acetonitrile as a mobile phase. The elution order of the regioisomers, *i.e.*, *rac*-LBL prior to *rac*-BLL, was confirmed with ^{1}H NMR spectra of the corresponding isolated TAG fractions (Kalo et al. 1996). Later, Lee et al. (2002, 2003) reported to separate regioisomers of dibutyrate and -caproate TAGs by rp-HPLC using two Chrompack^{TM} Inertsil ODS columns (3 μm, 100 mm x 3.0 mm i.d.) in series and a binary (Acetonitrile/Acetone) mobile phase gradient (Lee et al. 2002) or using a Beckman-Altex Ultrasphere^{TM} ODS column (5 μm, 250 mm x 4.6 mm i.d.) with a binary (acetonitrile/ethylene chloride) mobile phase gradient (Lee et al. 2003). Analogous resolution of the regioisomers of dicaproates was achieved by the same method (Lee et al. 2002). Further, separation of the regioisomers of long-chain intact TAGs on ODS columns using non-aqueous mobile phases was reported by Momchilova et al. (2004, 2006) for the first time. They demonstrated separation of a limited number of *rac*-PPU/PUP type regioisomers (P = palmitoyl acyl group, U = unsaturated acyl group: 18:1, 18:2, 18:3, 20:4, 20:5, and 22:6) on endcapped and non-endcapped ODS columns using binary mobile phases of acetonitrile modified by eight different solvent (methanol, ethanol, 2-propanol, 1-propanol, 1-butanol, acetone, dichloromethane, and tetrahydrofuran) in isocratic elution. The studies (Momchilova et al. 2004, 2006) showed that *rac*-PUP isomers eluted prior to *rac*-PPU isomers in all cases. Later, Kuroda et al. (2008) managed to separate respective *rac*-PPU/PUP type regioisomers (U = 18:1) on a non-endcapped polymeric ODS column (Inertsil^{TM} ODS-P, 5 μm, 250 mm x 4.6 mm i.d.) with a recycle HPLC system using a mobile phase of acetonitrile, 2-propanol, and hexane (3:2:1, v/v/v), but three other ODS columns (*i.e.*, a monomeric non-endcapped, a monomeric endcapped, and a intermediate endcapped ODS) failed to separate the regioisomers. In addition, Kuroda et al. (2008) observed that *rac*-UUP type regioisomer (U = 18:1) eluted prior to *rac*-UPU on the same non-endcapped monomeric column. The best column temperature in separation was different for *rac*-PPU/PUP and *rac*-UUP/UPU regioisomer pairs (25°C and 10°C, respectively), probably due to the different solubility of TAG species in mobile phase (Kuroda et al. 2008).

The structure of the stationary phase has a definite effect on the separation of TAGs. Already, El-Hamdy and Perkins (1981a) showed that increasing the chain length (from 1 to 8, and further to 18) of hydrocarbons bonded to silica in rp-columns improved the separation of TAG species. A recent proof of this finding was introduction of octacocyl silylation (C28) columns (Nagai et al. 2011a), which enabled separation of regioisomers of TAGs containing two palmitic acids (16:0) and one other (structurally different) fatty acid (*i.e.*, 8:0, 18:1, 20:5, and 22:6). Gotoh et al. (2012) applied a recycle rp-HPLC–UV–APCI–MS/MS to separate regioisomers of *rac*-PCiP/PPCi type (Ci = 10:0, P = 16:0) medium-chain TAGs in MFs of several ruminants, human, and rat milk. The *rac*-PPCi isomer eluted after the *rac*-PCiP isomer and they separated close to the baseline on a C28 column (Sunrise C28, 250 mm x 4.6 mm i.d., 5 μm) without using recycling. Earlier, Gotoh et al. (2010) showed that regioisomers of MC-TAGs containing two C8:0 acyls (Cy) and one saturated long-chain acyl (L) were separated on two non-endcapped polymeric ODS columns (Inertsil^{TM} ODS-P, 250 mm x 4.6 mm i.d., 5 μm;) in series without recycling, if the length of the L was 14 or higher. In all cases, *rac*-CyCyL retained more strongly than *sn*-CyLCy (Gotoh et al. 2010). Later, Nagai et al. (2015) showed that a recycle LC–APCI–MS method using a C28 column (Sunrise C28, 250 mm x 4.6 mm i.d., 5 μm) with a linear gradient elution was an excellent choice to separate regioisomers of mono short-chain TAGs containing two palmitoyl acyls. Baseline separation of *rac*-PBP/*rac*-PPB and *rac*-PCoP/*rac*-PPCo regioisomers was observed and the elution order was identical to that of mono medium-chain TAGs on a C28 column (Gotoh et al. 2012). Further, Nagai et al. (2015) confirmed excellent resolution of the regioisomers of trisaturated mono medium-chain TAGs (*i.e.*, *rac*-PSnP/*rac*-PPSn, where n = 8 and 10) and one trisaturated long-chain TAG (*i.e., rac*-PLaP/*rac*-PPLa, where La = 12:0).

Silver-ion HPLC (Ag-HPLC): Since early 1990's, laboratory-made (Jeffrey 1991) and commercial (Adlof 1995) silica gel columns in silver ion mode were shown to separate regioisomers of unsaturated LC-TAG, which are common components of commercial vegetable oils. When regioisomers of SSU- and SUU-type TAGs (S, U = saturated, unsaturated acyl group, respectively) were analyzed, the retention order of the isomers was shown to be as follows: *rac*-S_LUS_L>*rac*-S_LS_LU, and *rac*-S_LUU>*rac*-US_LU for TAGs containing long-chain fatty acyls (S_L) (Jeffrey 1991, Adlof 1995), and correspondingly *rac*-S_MUS_M>*rac*-S_MS_MU for TAGs containing medium-chain fatty acyls (S_M) (Fevrier et al. 2001). However, *rac*-S_MUU and *rac*-US_MU isomers were not separated (Fevrier et al. 2001). Holčapek et al. (2010) studied resolution of 43 synthetic regioisomer pairs of LC-TAGs on three silver-ion columns (ChromSpher Lipids, 250 x 4.6 mm, 5 μm) in series using APCI–MS and observed a general rule for separation, *i.e.*, those regioisomers of unsaturated TAGs with more double bonds in primary positions of TAG molecule had a slightly higher retention. This observation is in agreement with the above mentioned findings in previous studies concerning unsaturated TAGs (Jeffrey 1991, Adlof 1995, Fevrier et al. 2001). Additional reaction sites in TAG molecule and those in stationary phase, *i.e.*, ester moieties of TAG molecules and free silanol groups of stationary phase were the most probable reason for the observed separation of regioisomers (Adlof 1997, Nikolova-Damyanova 2009). Further, Holčapek et al. (2010) reckoned that double bonds in the primary positions of TAG molecule interacted more strongly with stationary phase due to more favorable steric availability. Interestingly, Holčapek et al. (2010) showed that different positions of double

bonds in fatty acyls, *e.g.*, in linolenic (Ln) and γ-linolenic acyl (γLn) induced separation of positional isomers (*e.g.*, *rac*-OOLn *vs*. *rac*-OOγLn), if Ln and γLn acyl were in the primary positions, but no resolution was observed, if Ln and γLn were in the secondary position (*e.g.*, *rac*-OLnO *vs*. *rac*-OγLnO). Unsaturated TAGs containing SC fatty acyls showed a different elution pattern than TAGs with 1–2 MC or LC acyl groups. When SC acyl group was an acetyl group (Ac), the elution order on an Ag-HPLC column (ChromSpherTM) was UUU>*rac*-UAcU>*rac*-UUAc>*rac*-UAcAc>*rac*-AcUAc>AcAcAc (Adlof 1996) suggesting a strong influence of SC acyl carbons on resolution due to their interactions with polar sites of stationary phase. The elution order was identical even if palmitoyl group (P) was substituted for U, *e.g.*, *rac*-PAcAc>*rac*-AcPAc (Adlof 1996). Further, already Smith et al. (1980) showed that the decrease of column temperature increased the resolution of TAGs and their regioisomers. In the study of Adlof and List (2004), decrease of column temperature decreased significantly the retention time, but resolution of regioisomers was not improved. Smith et al. (1980) showed that an optimum loading-% (5%) of silver ions could be detected as well. In addition, Jeffrey (1991) showed that column conditioning prior to analysis was essential for good separation and shape of peaks as well as unchanged retention times and elution order of TAGs on an Ag-HPLC column. Interestingly, no regiospecific analysis of MF TAGs solely by Ag-HPLC has been reported, which is probably due to relatively saturated nature of TAGs in bovine MF.

In summary, regiospecific analysis of TAGs in bovine MF solely by chromatographic methods is mainly applied to short- and medium-chain TAGs using polar high-resolution GC (*e.g.,* Myher et al. 1988) or using sophisticated rp-HPLC methods (*e.g.*, Gotoh et al. 2012, Nagai et al. 2015). However, np-HPLC in plain or silver ion mode has shown to separate regioisomers of SC-TAGs in other TAG mixtures as was discussed above, accordingly enabling regiospecific analysis of MF TAGs as well. Regiospecific analysis of TAGs by chromatographic methods enables determination of regiospecific distribution of FAs in individual TAG species or that in a specific group of TAG, which is practically impossible to achieve with chemical and enzymatic methods. On the other hand, general regioisomeric analysis of TAGs in whole fat or oil is easier to complete with chemical and enzymatical methods. Chromatographic analysis of regioisomers provides information of intact TAGs in natural fats and oils, which is more difficult to accomplish after partial hydrolysis or deacylation of TAGs, in consequence of acyl migration. Altogether, regiospecific analysis of TAG species in edible fats and oils is challenging even with chromatographic methods due to numerous different molecular species of TAGs, hence coelution of several TAG species on chromatographic columns. In recent decades, chromatographic resolution is combined with mass spectrometric detection of the regioisomers of TAGs, which has markedly improved identification and quantification of otherwise inseparable regioisomers of TAGs.

3.3.3 Mass spectrometric methods

Differentiation of regioisomers of TAGs by MS is based on the specific fragment ions which originated from the *sn*-1(3) positions of TAG molecules (Ryhage and Stenhage 1960, Kallio and Currie 1993), or on the different fragmentation efficiency of FAs from the *sn*-2 and *sn*-1(3) positions (Myher et al. 1984, Mottram and Evershed 1996, Hvattum 2001, Marzilli et al. 2003). MS analysis of regioisomers can be performed solely by MS or by chromatography-MS as was recently reviewed by Kalo and Kemppinen (2012). General principles of regiospecific analysis of TAGs by different MS and tandem MS methods are discussed below.

Electron ionization MS (EI–MS). Already in 1960's, EI–MS was used to detect regioisomers of TAGs on the basis of characteristic fragment ions (Ryhage and Stenhagen 1960, Barber et al. 1964, Lauer et al. 1970). Population of acyloxymethylene ions ($[M–RCOOCH_2]^+$) was shown to be much less abundant if the ions originated from the *sn*-2 position than from the *sn*-1(3) positions of TAG molecule (Ryhage and Stenhagen 1960, Barber et al. 1964, Lauer et al. 1970), which enabled structural elucidation of TAG molecule. Later, this observation was demonstrated by Kallio et al. (1989), who applied SFC–EI–MS with selected ion monitoring to a regioisomer pair of *rac*-16:0-16:0-18:0/*sn*-16:0-18:0-16:0 and detected only one $[M–RCOOCH_2]^+$ ion (derived solely from 16:0) when *sn*-16:0-18:0-16:0 isomer was analyzed. In addition, Barber et al. (1964) suggested that the regioisomer pair of *rac*-18:0-18:0-18:1/18:0-18:1-18:0 could be distinguished on the basis of the abundances of $[M–RCOO]^+$ ions originated from *sn*-2 and *sn*-1(3) positions. However, Lauer et al. (1970) stressed that the structure of fatty acid residues, *i.e.*, carbon chain length of acyl groups and the degree of unsaturation probably influenced more on the abundances of $[M–RCOO]^+$ ions than did the *sn*-position of acyl groups in mixed TAGs.

Chemical ionization MS (CI–MS). In most CI–MS methods, ionization produces relatively abundant amount of positive or negative quasimolecular ions (QM^+/QM^-) from TAGs, which facilitates a reliable identification (ACN:DB) of TAG species (Murata 1977, Murata and Takahashi 1977), but does not yield information on regioisomers of TAGs *per se*. Hence, fragment ions yielded by CI–MS or alternatively by CI–MS^2 are essential for regiospecific analysis. Myher et al. (1984) conducted positive ion CI–MS using the components of mobile phase (acetonitrile, propionitrile) in rp-HPLC as a reagent gas to produce QM^+ and fragment ions ($[MH–RCOOH]^+$) in CI–MS. They observed (Myher et al. 1984) that the abundance of $[MH–RCOOH]^+$ ions cleaved from the *sn*-1(3) positions was *ca.* four times higher than that from the *sn*-2 position. Further, the difference of abundances of $[MH–RCOOH]^+$ ions between the primary and secondary positions was higher than the difference resulting from the variation (*ca.* 1.25-fold) of CN:DB in fatty acyl groups (Myher et al. 1984). This finding was in contrast to the observation of Lauer et al. (1970) concerning $[M–RCOO]^+$ ions in EI–MS and was probably due to more gentle ionization (Myher et al. 1984). Kallio and Currie (1993) developed a direct inlet ammonia negative CI–MS^2 method for regiospecific analysis of TAGs based on abundant $[M–H–RCO_2H–100]^-$ ions cleaved from $[M–H]^-$ ions, and the method was later applied to the regioisomeric analysis of TAGs in human MF (Kallio and Rua 1994). Further, the method was improved with a software algorithm used for automatic interpretation of mass spectra (Kurvinen et al. 2001b). Correspondingly to the

study of Myher et al. (1984), Kallio and Currie (1993) showed that the secondary position of TAG molecule was less prone to the cleavage of fatty acyl group than the primary positions in negative ion tandem MS regardless of the esterified (long-chain) fatty acyl groups. When *rac*-AAB and *rac*-ABA-type TAGs were analyzed with the NICI–MS^2 method, the ratio of the intensities $[M–H–B–100]^–$/ $[M–H–A–100]^–$ were 0.145 and 0.764 for *rac*-ABA and *rac*-AAB, respectively (Kallio and Currie 1993). In addition, the ratio was observed to increase steadily when the proportion of *rac*-AAB isomers in the mixture of (AAB+ABA) increased from 0% to 100% (Kallio and Currie 1993), which enabled regiospecific selectivity and determination of the ratio of AAB/ABA-type regioisomers. However, when Kurvinen et al. (2001a) studied TAG mixtures of AAB/ABA-type regioisomers of mono medium-chain TAGs (A = 18:2; B = 8:0), they observed that the ratio $[M–H–8:0–100]^–$/$[M–H–18:2–100]^–$ remained constant until the proportion of *rac*-8:0-18:2-18:2 isomer exceeded 50% in the mixture resulting in an impossible generic quantification of regiosiomers. In addition, the direct inlet NICI–MS^2 method was difficult to apply to TAGs with the same molecular weight with varying FA content, *e.g.,* containing short-chain and/or polyunsaturated FAs together with more common LC-FAs (Kallio and Currie 1993, Leskinen et al. 2007, 2010). However, Leskinen et al. (2010) suggested an improved method in which direct inlet ammonia NICI–MS^2 was adapted to UHPLC, which enabled better separation of TAG isomers prior to MS determination. In general, combination of chromatographic methods with APCI–MS/MS^2 and ESI–MS^2 are nowadays very practical in regioisomeric analysis of TAGs (see below).

Atmospheric pressure chemical ionization MS (APCI–MS). Mottram and Evershed (1996) showed that APCI ionization of TAG molecules yielded information concerning the *sn*-position of esterified long-chain FAs in ABC- and AAB/ABA-type TAGs (A, B, and C denote different FAs). They showed that the ratio of $[M–RCOO]^+$ ions of AAB/ABA-type TAGs (*i.e.*, $[AA]^+$/$[AB]^+$) was *ca*. 4–5 higher for *rac*-AAB than ABA TAGs due to energetically less favorable cleavage of FA from the *sn*-2 position than from the *sn*-1(3) positions of TAG molecule. Later, principles of this method were applied successfully to the analysis of the regioisomers of TAGs present in eight different vegetable oils (Mottram et al. 1997). However, they (Mottram et al. 1997) noted that identification of regioisomers was not reliable, if two (or more) co-eluting TAGs yielded the same DG^+ ion in APCI, *e.g.,* $[16:0-20:0]^+$ and $[18:0-18:0]^+$ (*m/z* 607) from TAGs *rac*-16:0-20:0-18:2 and 18:0-18:2-18:0, respectively. Employing ESI–MS^2 in identification and quantification of regioisomers of TAGs has shown to be more challenging due to the fact that formation of $[M+H]^+$ precursor ions from saturated TAGs was shown to be very low (Byrdwell and Emken 1995, Mottram and Evershed 1996). In addition, Leskinen et al. (2007) and Herrera et al. (2010) reported that the yield of DG^+ ions from the protonated molecular ions in CID was independent from the regioisomeric position of fragmented FA. However, APCI–MS^2 has been applied to the regioisomeric analysis of TAGs in combination with excellent chromatographic resolution of regioisomers prior to MS detection (*e.g.*, Gotoh et al. 2012, Nagai et al. 2015). Further, Leskinen et al. (2010) demonstrated that linear relationship in calibration curves was obtained between the ratio of production ion (100 x $[M–H–A–100]^–$/($[M–H–A–100]^–$ + $[M–H–B–100]^–$) intensities (y-axis) and the regioisomer proportions (100 x *sn*-ABA/(*sn*-ABA + *rac*-AAB) in binary mixtures (x-axis) when ammonia negative ion APCI–MS^2 applied to the analysis of the regioisomers of unsaturated AAB/ABA-type TAGs in TAG standards

and those in five edible oils. Jakab et al. (2003) were the first, who applied linear calibration plots in positive APCI–MS to determine the ratio of the regioisomers of *rac*-18:2-18:2-18:1 (*rac*-LLO) and *sn*-18:2-18:1-18:2 (*sn*-LOL) in five vegetable oils on the basis of $[LL]^+$ and $[LO]^+$ diacylglycerol fragment ions. They (Jakab et al. 2003) analyzed the regioisomeric ratio using a linear calibration curve in which the ratio $[LL]^+/[LO]^+$ (y-axis) was plotted against the concentration of *sn*-LOL regioisomer (%, v/v; 100**sn*-LOL/(*sn*-LOL + *rac*-LLO). Corresponding linearity of calibration plots for the regioisomers of long-chain TAGs was observed in other positive APCI–MS studies as well (Fauconnot et al. 2004, Leskinen et al. 2007). In some recent studies (Gotoh et al. 2012, Nagai et al. 2015), in which the regioisomers of short- and medium-chain TAGs were sufficiently separated from each other prior to APCI–MS^2, quantification of the regioisomers has been performed using specific correction factors for the diacylglycerol fragment ions of each regioisomer which were calculated on the basis of linear calibration and internal standard methods. Use of multidimensional chromatographic separation of the regioisomers of long-chain TAGs prior to APCI–MS determination enhanced the applicability of APCI–MS for regiospecific analysis. For example, Dugo et al. (2004) used the off-line two-dimensional chromatographic system containing rp-HPLC in combination with Ag-HPLC–APCI–MS to determine relative abundances of regioisomers in nine AAB-type TAGs and two ABC-type TAGs of rice oil. Further, corresponding 2D-system was used to determine several regioisomers in a very complex blackcurrant oil (Holčapek et al. 2009).

In the regioisomeric analysis of TAGs in complex MFs, identification and quantification of the isomers by APCI–MS/MS^2 are carried out after efficient separation of TAG species by reversed- or chiral-phase HPLC (Gastaldi et al. 2011, Gotoh et al. 2012, Nagai et al. 2015) or by two-dimensional chromatography (Chiofalo et al. 2011). Gastaldi et al. (2011) determined principal regioisomer of the most abundant TAG species ($C_{TAG} > 1\%$) in bovine, goat, and human MF using non-aqueous (na) rp-HPLC–APCI–MS and APCI–MS^2 methods. Further, Gotoh et al. (2012) used APCI–MS^2 in combination with high-resolution HPLC to determine ratios of selected regioisomers of TAGs containing palmitic (P) acid (*i.e.*, *rac*-PPX/PXP, in which X = 10:0, 18:1, or 22:5, and *rac*-PXX/XPX, in which X = 18:0) in cow, human, and rat MF, and in cow, buffalo, goat, and sheep cheese fat. A more comprehensive study of the TAG isomers in bovine MF by APCI–MS^2 was performed by Nagai et al. (2015), who determined several regioisomers and enantiomers of dipalmitate TAGs (*i.e.*, even-numbered *sn*-PXP, *sn*-XPP, *sn*-PPX TAGs; X = 4:0–12:0). Chiofalo et al. (2011) determined the regioisomeric ratio of six LC-TAG species in human MF and that of nineteen MC- and LC-TAG species in donkey MF with similar 2D-HPLC–APCI–MS system as was used in the study of Dugo et al. (2004). Further, Yoshinaga et al. (2013) showed that LC–APCI–MS^2 methods can be used to determine the proportion of MF and randomized MF in foods using regioisomers of *sn*-16:0-16:0-4:0 and *sn*-16:0-4:0-16:0 as an indicator of the content of MF and modified MF, respectively.

Electrospray ionization MS (ESI–MS). Since the first ESI–MS studies (Duffin et al. 1991), ESI–MS^2 has been assessed as one possibility for regiospecific analysis of TAGs. However, FA composition of TAGs (Hsu and Turk 1999), type of adduct ions (Hvattum 2001, Herrera et al. 2010), and level of collision energy in tandem MS (Hvattum 2001) were observed to influence on the relative abundances of fragment ions, hence complicating the analysis. In

the early TAG studies with ESI–MS^2, no regioisomeric information was achieved when low-energy CID (Duffin et al. 1991) and high-energy CID of ammonium adducts of TAGs (Cheng et al. 1998) were applied. Similarly, Han and Gross (2001) could not detect any difference in the loss of long-chain FA from primary and secondary positions when lithium adducts of TAGs were analyzed by ESI–MS–MS under low-energy CID conditions. Neither did Segall et al. (2004) found any difference in the preferential loss of fatty acyls from the secondary and primary *sn*-positions of medium-chain TAGs. On the other hand, Hsu and Turk (1999) noted that the *sn*-position of esterified FA influenced more on the relative abundances of fragment ions than did the identity of FAs when ESI–MS^2 with low-energy CID of lithium adducts of TAGs (from lithium acetate) were used for analysis. Accordingly, a slightly greater cleavage of long-chain acyl groups from the primary positions than from the secondary position of $[M+NH_4]^+$ ions was observed when low-energy CID was used (Hvattum 2001, Dorschel 2002, Byrdwell and Neff 2002). Later, Marzilli et al. (2003) and Malone and Evans (2004) analyzed ammonium adducts of AAB/ABA and ABC-type long-chain TAGs by ESI–MS^2 ion trap instrument and found unquestionably that the loss of a acyl group from the *sn*-2 position was energetically more unfavorable than that from the *sn*-1(3) positions. In addition, the above mentioned difference in the CID mass spectra derived from all kind of adduct ions (*i.e.*, $[M+NH_4]^+$, $[M+Li]^+$, $[M+Ag]^+$, $[M+Na]^+$) was substantial enough to allow identification and quantification of the regioisomers with linear calibration curve (Herrera et al. 2010). On the other hand, Cheng et al. (1998) could not differentiate regioisomers of TAG by CID of $[M+NH_4]^+$ ions, but high energy CID of other adduct ions ($[M+Na]^+$, $[M+Li]^+$, $[M+Cs]^+$) allowed unambiguous detection of regioisomers of long-chain TAGs. Similarly, Lévêque et al. (2010) received respective results after CID of $[M+Ag+AgNO_3]^+$ adduct ions. Consequently, different cleavage of FAs from the *sn*-2 and *sn*-1(3) positions in CID enabled to model a linear relationship between intensities of selected product ions in CID spectra and relative abundance of the regioisomers in binary mixtures of known regioisomeric ratios in order to plot calibration curves for quantification of unknown regioisomeric mixtures in natural fats and oils (Malone and Evans 2004, Leskinen et al. 2007).

In spite of an undeniable ability of ESI–MS/MS^2 to separate and quantify *sn*-1(3) and *sn*-2 isomers of TAGs in edible oils and fats, regioisomers of MF TAGs have been investigated very rarely by this powerful method. Linderborg et al. (2014) analyzed the regioisomers of TAGs in human MF by UHPLC–ESI–MS^2, but they were unable to differentiate unambiguously the isomers of several unsaturated LC-TAGs due to coelution of more than one regioisomer pair of the same ACN:DB-class on a UHPLC-column. Nagy et al. (2013) analyzed TAGs in BF by narp-HPLC combined with a high-resolution LTQ-Orbitrap XL hybrid MS using positive ESI-mode in ionization. Automatically calculated identification of TAGs and that of the regioisomers of TAGs were based (*i*) on the list of specific ammonium adducts with highly accurate theoretical masses, (*ii*) on the (positive) mass tag (*m/z* 4.955395) between ammonium and sodium adducts, (*iii*) product ion spectra from ammonium adducts, and (*iv*) theoretical fragmentation patterns of regioisomers consisting of fatty acyls with different carbon chain length and degree of unsaturation (Nagy et al. 2013). Quantification of TAGs was performed on the basis of response factors calculated from the analysis of 25 commercially available TAG standards using linear calibration and internal standard methods (Nagy et al. 2013). The applied method enabled to determine the

regiospecific distribution of the main FAs in BF TAGs (Nagy et al. 2013). Recently, Kallio et al. (2017) developed a UHPLC–ESI–MS^2 method that automatically identify all potential TAG species of specific *m/z* value on the basis of 21 different fragment ions from lithium adducts yielded by tandem MS. In addition, the method automatically quantify the regioisomers of detected TAG species using calculation algorithm, which was optimized by relative intensities of eight fragment ions (ESI–MS^2) of six regioisomeric pairs of diacid TAG standards. In this particular case, no CFs were needed for reliable quantification of hundreds of regioisomeric TAGs in human MF.

In summary, determination of the regiospecific distribution of esterified fatty acyl groups among the primary and secondary positions in intact individual TAG species of MF can be performed more reliably by MS than by any other methods of regiospecific analysis, especially in the case of coeluting molecular species. However, separation of TAG groups, individual TAG species, or regioisomers of MF TAGs by chromatographic methods is practically an essential step prior to MS detection due to a large number of different molecular species of TAGs in bovine MF. Accordingly, regiospecific analysis of MF TAGs using direct inlet MS is challenging due to a large number of similar (fragment) ions derived from several TAG species, even though characteristic fragment ions, which are relatively specific to the *sn*-1(3) position, have been detected in EI–MS (Ryhage and Stenhagen 1960), and in NICI–MS of TAGs (Kallio and Currie 1993). In addition, MS or tandem MS methods using PICI (Myher et al. 1984), APCI (Mottram and Evershed 1996), and ESI ionization (Marzilli et al. 2003, Malone and Evans 2004) enabled differentiation of the regioisomers of TAGs due to the less abundant cleavage of FAs from the *sn*-2 than *sn*-1(3) positions. Regioisomers of TAGs in human MF have been analyzed solely by direct inlet ammonia NICI–MS^2 (Kallio and Rua 1994), but more common are methods, which combine atmospheric pressure soft ionization MS or tandem MS with liquid chromatography. Chiofalo et al. (2011) separated MC- and LC-TAGs in human and donkey MF by 2-dimensional HPLC (rp-HPLC + Ag-HPLC) and determined regioisomers by APCI–MS. Further, LC–APCI–MS^2 has provided a powerful alternative to determine principal regioisomer of the most abundant TAGs (Gastaldi et al. 2011) or to perform highly specific regioisomeric analysis of selected individual molecular species of TAGs in MFs (Gotoh et al. 2012, Nagai et al. 2015). Similarly, ESI–MS^2 combined with high-resolution LC separation was shown to yield information on the regioiomeric distribution of FAs in TAGs of human (Linderborg et al. 2014) and bovine MFs (Nagy et al. 2013).

4 EXPERIMENTAL

4.1 Materials

4.1.1 Butterfat

Butterfat (BF) was supplied by Valio Ltd. (Helsinki, Finland). Prior to further analysis, fractionation, or interesterification, anhydrous BF was prepared as follows (**I–III**, **V–VII**): BF was dried under vacuum for 100 min at 95°C to a water content of 55–85 ppm, and bleached for 30 min at 95°C with 2% of bleaching earth, which was activated at 130°C for 2 h in advance and filtered. All analyses were carried out using the same batch of unhydrous BF. If not used directly, BF was stored at –18°C under argon in the dark. The fatty acid (FA) composition of BF (**II**, **VI**) was [mol% (FA)]: 12.8 (4:0), 5.1 (6:0), 2.2 (8:0), 3.8 (10:0), 3.5 (12:0), 11.0 (14:0), 0.9 (14:1), 0.9 (15:0), 24.2 (16:0), 1.3 (16:1), 0.4 (17:0), 0.3 (17:1), 10.0 (18:0), 18.8 (18:1), 1.7 (18:2), 0.5 (18:3), 0.2 (20:0), and 2.4 (other).

4.1.2 Acylglycerol and fatty acid standards

Monoacid triacylglycerols (TAGs): Monoacid TAG standards of 99% purity were purchased from Fluka (Buchs, Switzerland) (**III–VI**), Merck (Darmstadt, Germany) (**III**, **VI**), Nu-Check-Prep (Elysian, MN, USA) (**I–II**), and Sigma (St. Louis, MO, USA) (**IV–VI**). The following monoacid TAGs were used in the present study: tritetranoylglycerol (tributyrin, BBB), trihexanoylglycerol (tricaproin, CoCoCo), triheptanoylglycerol (trienanthin, EEE), trioctanoylglycerol (tricaprylin, CyCyCy), tridecanoylglycerol (tricaprin, CiCiCi), tridodecanoylglycerol (trilaurin, LaLaLa), tritetradecanoylglycerol (trimyristin, MMM), trihexadecanoylglycerol (tripalmitin, PPP), trioctadecanoylglycerol (tristearin, SSS), *cis*-9-trihexadecenoylglycerol (tripalmitolein, PoPoPo), and *cis*-9-trioctadecenoylglycerol (triolein, OOO). Trinonanoylglycerol (trinonanoin) was used as internal standard (IS).

Monoacylglycerols (MAGs): 1-monotetradecanoylglycerol (1-monomyristin), 1-monohexadecanoylglycerol (1-monopalmitin), and 1-mono-*cis*-9-octadecenoylglycerol (1-monoolein) were purchased from Larodan Fine Chemicals (Malmö, Sweden) (**IV–V**).

Fatty acid methyl esters (FAMEs): The standard mixture (GLC74) of FAMEs was purchased from Nu-Chek-Prep (Elysian, MN, USA) (**I–II, VI–VII**).

4.1.3 Solvents

All solvents were purchased from Rathburn Chemicals Ltd. (Walkerburn, Scotland, United Kindom) and Merck (Darmstadt, Germany) and they were HPLC and pro analysis grade, respectively (**I–VII**).

Table 4.1 Interesterified equimolar standard mixtures of three or two monoacid TAGs used in **III–VI**. (Table adapted from Table 1 in **III** and **VI**, and from data in **IV** and **V**.)

Name[1]	Interesterified mixture of	Isolation method	Name	Interesterified mixture of	Isolation method
Saturated			**Unsaturated**		
BM	BBB+MMM	SPE[2]	BO	BBB+OOO	SPE
BP	BBB+PPP	SPE			
BCoE	BBB+CoCoCo+EEE	SPE	BLaPo	BBB+LaLaLa+PoPoPo	SPE
BLaM	BBB+LaLaLa+MMM	SPE	BLaO	BBB+LaLaLa+OOO	TLC[3]
BLaP	BBB+LaLaLa+PPP	SPE	BMPo	BBB+MMM+PoPoPo	SPE
BLaS	BBB+LaLaLa+SSS	TLC	BMO	BBB+MMM+OOO	TLC
BMP	BBB+MMM+PPP	TLC	BPO	BBB+PPP+OOO	TLC
BMS	BBB+MMM+SSS	TLC	BSO	BBB+SSS+OOO	TLC
BPS	BBB+PPP+SSS	TLC			
CoLaP	CoCoCo+LaLaLa+PPP	SPE	CoLaO	CoCoCo+LaLaLa+OOO	SPE
CoMP	CoCoCo+MMM+PPP	TLC	CoMPo	CoCoCo+MMM+PoPoPo	SPE
CoMS	CoCoCo+MMM+SSS	TLC	CoMO	CoCoCo+MMM+OOO	TLC
CoPS	CoCoCo+PPP+SSS	TLC/SPE	CoPO	CoCoCo+PPP+OOO	TLC/SPE
			CoSO	CoCoCo+SSS+OOO	TLC
CyLaM	CyCyCy+LaLaLa+MMM	TLC	CyLaO	CyCyCy+LaLaLa+OOO	TLC
CyLaS	CyCyCy+LaLaLa+SSS	TLC	CyMO	CyCyCy+MMM+OOO	TLC
CyMP	CyCyCy+MMM+PPP	TLC			
CyMS	CyCyCy+MMM+SSS	TLC			
CiLaP	CiCiCi+LaLaLa+PPP	TLC/SPE	CiLaO	CiCiCi+LaLaLa+OOO	TLC/SPE
CiLaS	CiCiCi+LaLaLa+SSS	TLC			
CiMP	CiCiCi+MMM+PPP	TLC			

[1] Abbreviations for acyl groups (the number of carbons: the number of double bonds): B = 4:0, Co = 6:0, E = 7:0, Cy = 8:0, Ci = 10:0, La = 12:0, M = 14:0, P = 16:0, S = 18:0, Po = 16:1, O = 18:1
[2] SPE = Solid phase extraction column chromatography
[3] TLC = Thin-layer chromatography

4.2 Synthesis of molecular species of triacylglycerols

4.2.1 Synthesis of randomized TAG mixtures by chemical interesterification

Altogether, 32 mixtures of randomized TAGs (Table 4.1) were prepared by chemical interesterification of equimolar amounts of three monoacid TAGs (**III–VI**). In addition, three randomized TAG mixtures (Table 4.1) were synthesized by chemical interesterification of equimolar amounts of two monoacid TAGs (**IV**). Prior to interesterification, TAG mixtures were dried under reduced pressure (3.3 kPa) for 100 min at 95°C and bleached for 30 min at 95°C with 2% bleaching earth in order to remove the impurities, which may retard interesterification. The interesterification was performed using 1% sodium methoxide as catalyst at 85–90°C (1 h) under argon atmosphere. The isolation of TAGs from reaction mixture is described below (Chapter 4.4) and in Table 4.1. All synthesized TAGs were stored at –18°C in solvent under argon in the dark before further use.

4.2.2 Synthesis of 1-long acyl chain-2,3-dibutyroyl-*rac*-glycerols by acylation

Three TAGs, *i.e.*, 1-myristoyl-2,3-dibutyroyl-*rac*-glycerol, 1-palmitoyl-2,3-dibutyroyl-*rac*-glycerol, and 1-oleoyl-2,3-dibutyroyl-*rac*-glycerol, were synthesized from 1-monomyristin, 1-monopalmitin, 1-monoolein, and butyric acid by acylation as described in detail elsewhere (Kodali et al. 1987).

4.3 Modification of butterfat

4.3.1 Chemically catalyzed interesterification of BF

A part of dried and bleached BF was interesterified for 1 h using 1% sodium methoxide as catalyst at 85–90°C under argon atmosphere resulting in randomized TAG composition in BF (**III, VI–VII**). TAGs were isolated from reaction mixtures by TLC (**III, VI**) or by solid phase extraction (SPE) column chromatography (**VI–VII**) as described in details in Chapter 4.4. All TAG samples were dissolved in isooctane or chloroform and stored at -18°C under argon in the dark.

4.3.2 Lipase catalyzed modification of BF

Lipase (EC 3.1.1.3) from *Pseudomonas fluorescens* was immobilized by adsorption on Celite Hyflo Supercel (Johns-Mansville Co, Ltd., Richmond, Surrey, United Kingdom) and used as catalyst for interesterification of dried and bleached BF (**I–II**). The interesterification reactions were carried out in microaqueous reaction conditions and in two reaction time/reaction temperature combinations: 50°C/8–10 d (Kalo et al. 1990) and 60°C/17 h (**II**). After isolation of TAGs by TLC (see Chapter 4.4), the lipase-modified BF samples stored at –18°C under argon in the dark.

4.4 Isolation of triacylglycerols

The TAGs of BF, modified BF, and synthesized TAGs were isolated principally with two different methods. (i) In **I–VI**, TAGs were isolated by thin-layer chromatography (TLC) on 20 x 20 cm silica gel plates (Kieselgel 60; Merck, Darmstadt, Germany) with 0.25 mm layer thickness. After developing with hexane/diethyl ether/formic acid (80:20:2, v/v/v), the TLC plates were sprayed with 0.2% 2,7-dichlorofluorescein in ethanol and TAG bands were separated off from the plates. TAGs were eluted from silica gel matrix by chloroform/methanol (98:2, v/v). (ii) In **IV–VI**, the isolation of TAGs was performed by flash chromatography grade silica gel column chromatography (Silica gel 60; Merck, Darmstadt, Germany; particle size range 40–63 μm). The chromatographic columns (5.4 x 1.5 cm) were prepared in a 15-mL Isolute filtration tube with micropore filters (International Sorbent Technology, Hengoed, United Kindom) using a dry packing method. The following stepwise elution schemes were used in the isolation of TAGs:

(i) 40 mL of dichloromethane (DCM)/hexane (HEX) 22:78 (v/v); (2) 60 mL of DCM/HEX 60:40 (v/v); (3) 40 mL of DCM/HEX 65:35 (v/v); (4) 40 mL of DCM/HEX 85:15 (v/v); and (5) 40 mL of DCM; TAGs in fractions (2)–(5) (**IV–V**)
(ii) 40 mL of diethylether (DEE)/HEX 1:99 (v/v); (2) 40 mL of DEE/HEX 3:97 (v/v); (3) 80 mL of DEE/HEX 5:95 (v/v); and (4) 40 mL of DEE/HEX 8:92 (v/v); TAGs in fractions (3)–(4) (**VI**)

In **VII**, TAGs were isolated using SPE column STRATA^{TM}-SI-1 (Phenomenex). The following elution scheme (iii) was used: (1) Introduction in 0.1 mL of DCM/HEX 22:78 (v/v); (2) 4 mL of DCM/HEX 22:78 (v/v); (3) 4 mL of DCM; (4) 4 mL of acetone (ACE)/DCM 40:60 (v/v). TAGs were in fraction (3). A more detailed description of the SPE column chromatography is in **IV–VII**.

4.5 Fractionation of butterfat by silica gel solid phase extraction

In **V** and **VII**, silica gel SPE chromatography was used in the fractionation and concentration of short-chain TAGs of BF. The chromatographic columns (5.4 x 1.5 cm) was prepared in a 15-mL Isolute filtration tube with micropore filters (International Sorbent Technology, Hengoed, United Kindom) using a dry packing method. The following method was used for fractionation of TAGs: prior to elution of TAG fractions, cholesterol esters were eluted with 40 mL of DCM/HEX 22:78 (v/v). The four TAG fractions were eluted using the following stepwise elution scheme: (1) 60 mL of DCM/HEX 60:40 (v/v); (2) 40 mL of DCM/HEX 65:35 (v/v); (3) 40 mL of DCM/HEX 85:15 (v/v) and (4) 40 mL of DCM. A more detailed description of the method is in **V** and **VII**.

4.6 Silver ion column chromatography of triacylglycerols

BF was separated into TAG fractions differing in degree of unsaturation on SPE columns loaded with 0.5 g of SCX (*p*-propylbenzene sulfonic acid) sorbent (BondElut™ SCX, Analytichem International, Harbor City, CA, USA) (**I–II**) or 2.0 g of Bulk Isolute™ SCX sorbent (International Sorbent Technology Ltd., Hengoed, United Kingdom) (**III, VI**). Prior to fractionation, the columns were impregnated with silver ions and washed according to the principles described by Christie (1989b). A total amount of 1600 (8 x 200) μg lipase-modified BF was fractionated into saturated (S), monoene (M), diene (D) and triene (T) TAG fractions using the following stepwise elution scheme: 6 mL of pentane/DCM 50:50 (v/v), S; 5 mL of ACE/DCM 1:99 (v/v), M; 7.5 mL ACE/DCM 5:95 (v/v), D; 5 mL of ACE (100%), T (**I–II**). In **III** and **VI**, a total amount of 2400 (3 x 800) μg of BF and chemically interesterified BF were fractionated into S, M, and polyene (P) fractions with a different stepwise elution scheme: 35 mL of pentane/DCM 25:75 (v/v), S; 45 mL of ACE/DCM 1:99 (v/v), M; 40 mL of ACE (100%), P. After fractionation, the TAGs were dissolved in isooctane (**I–II**) or DCM (**III**, **VI**) and stored at –18°C under argon in the dark.

4.7 Gas-liquid chromatography

The molar composition of the TAGs in BF and in standard mixtures was determined by a Carlo Erba gas chromatograph (Milano, Italy) using a 25 m x 0.25 mm i.d. polarizable phenyl(65%)methylsilicone capillary column (Quadrex, New Haven, CT, USA) with 0.1 μm film thickness (**I–VI**). Hydrogen was used as carrier gas, and the GC was equipped with a flame-ionization detector and constant pressure (cp)/constant flow (cf) control module. Cold on-column injection was made with constant hydrogen pressure (cp mode) at high-oven temperature (170–200°C) after which the cp–cf module was changed to cf mode (linear velocity 61–62 cm/s). The temperature program in **I–II** was: after an isothermal period of 1 min at 200°C, the column temperature was raised 15°C/min to 320°C, hold 1 min, and finally raised at a rate of 7°C/min (or 2°C/min) to 360°C (hold 14 min). In **III–VI**, the following temperature program was used: after an isothermal period of 1 min at 170°C, the temperature was raised 10°C/min to 310°C (hold 1 min), after which, 0.5°C/min to 315°C (hold 1 min), and finally, the column temperature was raised 8°C/min to 360°C (hold 12 min).

The fatty acid composition of TAG samples and FAME standards was determined by a MicroMat Model HRGC 412 gas chromatograph (HNU^{R}-Nordion Ltd., Helsinki, Finland) using a 25 m x 0.32 mm i.d. (NB-351, HNU^{R}-Nordion) capillary column with 0.50 μm film thickness (**I–II, VI**). Helium was used as carrier gas (inlet pressure 0.7 bar). The gas chromatograph was equipped with a flame-ionization detector (FID, 225°C) and a split injector (split 1:20, 250°C). The following temperature program was used: after 1 min at 60°C, the temperature was increased at 10°C/min to 250°C (hold 7 min). In addition, *cis-trans* isomers of FAMEs were analyzed by a Carlo Erba gas chromatograph (Milano, Italy) using a 100 m x 0.25 mm i.d. (Supelco, Inc. Bellefonte, PA, USA) capillary column with 0.2 μm film thickness (**I**). For more details, see the original paper (**I**). Acylglycerols (TAGs, MAGs) were transesterified to FAMEs (**I–II, VII**) according to the principles described by Badings and De Jong (1983).

4.8 Gas chromatography–electron ionization mass spectrometry

The TAGs in BF and in standard mixtures were analyzed (**II**, **VI**) with a Finnigan MAT INCOS 50 quadrupole mass spectrometry (San Jose, CA, USA) coupled to a Varian 3400 gas chromatograph (Palo Alto, CA, USA) using a 25 m x 0.25 mm i.d. polarizable phenyl(65%)methylsilicone column with 0.1 μm film thickness (Quadrex, New Haven, CT, USA) with helium as carrier gas. Cold on-column injections were made by using a SGE OCI-3 injector (Ringwood, Australia) (**VI**), or by piercing the septum of the septum-equipped programmable injector with a 40 x 0.7 mm syringe needle and by introducing a *ca*. 15 cm long, 0.23 mm O.D. silica capillary needle of a 10 μL syringe through that needle into the analytical column (**II**). Two different temperature programs were selected to separate analytes: (i) One program started at 50°C and followed 50–320°C at the rate of 20°C/min, hold 1 min, 320–360°C at the rate of 4°C/min with the final isothermal period of 5 min at 360°C (**II**), and (ii) the other started with an isothermal period of 0.5 min at 50ºC

Table 4.2 Parameters for multistep binary gradient system in np-HPLC. (Table adapted from Table 1 in **IV**, and from data in **V** and **VII**.)

Time (min)	%B (v/v) Two columns	Three columns	Flow (mL/min)
0	0	0	0.1
2	15	5	0.1
16	15		0.1
18		18	0.1
22	18		0.1
30	18	18	0.1
31	90	90	0.1
49	90	90	0.1
50	99	99	0.5/0.1[1]
64	99	99	0.5
65	0	0	0.5
75		0	0.5

Solvent A: hexane; solvent B: hexane/methyl-*tert*-butyl ether/acetic acid (60:40:1, v/v/v).
[1] In **VII**

and followed 50–310ºC at a rate of 18ºC/min, hold 1 min, 310–315ºC at a rate of 0.5ºC/min, hold 1 min, and finally to 360ºC at a rate of 8ºC/min with a final isothermal period of 10 min (**VI**). Full mass spectra (*m/z* 100–610, every 2 s) (**II**) or selected ions ($[RCO]^+$, $[M–RCOO]^+$, and $[M–RCOO+1]^+$) (**VI**) were collected in electron ionization (EI) mode (70 eV ionization energy). In selected ion monitoring, scan time was 1 s/scan and 0.05 s/each ion. In addition, several different descriptors (*i.e.*, a group of monitored ions) of 12–19 ions were used as described in detail in the original publication (**VI**). The temperature of ion source was 190°C (**II**) or 175ºC (**VI**). The temperature of transferline between GC and MS was 350ºC. The mass analyzer was cleaned daily and calibrated after each cleaning procedure.

4.9 Liquid chromatography–electrospray ionization mass spectrometry

An ion-trap Bruker Esquire LC–ESI–MS (Bruker Daltonic, Bremen, Germany) was used in the LC–MS analysis of TAGs in BF and in standard mixtures. Prior to ESI–MS detection, the analytes were resolved by a normal-phase HPLC using two or three Phenomenex Luna 3-μm silica columns (100 mm x 2.0 mm) and a guard column (4 mm x 2.0 mm), in series (**IV, V, VII**). For elution of TAGs, a multistep binary gradient of hexane (A) and hexane/methyl-*tert*-butyl ether/acetic acid (60:40:1, v/v/v) (B) was applied in the analysis (Table 4.2).

An ion-trap Bruker Esquire LC–ESI–MS (Bruker Daltonic, Bremen, Germany) was operated in positive ESI mode using the following parameters: capillary voltage was 3000 V, capillary exit offset was 60 V, skimmer potential was 20 V, and ion-trap drive value for ion trap was 55. Conventional ESI mass spectra of the ammonium adducts of TAGs were recorded using a scan range of 50–1000 *m/z* and summation of 15 spectra. Nebulizer

(nitrogen) pressure was 275 kPa (~40 psi), drying gas (nitrogen) flow 8 L/min and drying temperature 300°C. Auto-MS/MS spectra for two most intense ions eluting concurrently were recorded using helium (99.996%) as the collision gas. The reagent solvent, chloroform/methanol/ammonia water (25%) 20:10:3 (v/v/v), was pumped with a flow rate of 6.0 mL/min via a 1:100 split device to effluent flow.

4.10 Other methods

4.10.1 Calculation of retention indices

In order to identify major TAG species of BF by GC, retention indices for 112 different molecular species of TAGs were calculated with Micman program (Sunicom Ltd., Helsinki, Finland) using cubic spline curve-fitting method (**I–III**). Calculation of unknown indices based on the index values for eight monoacid TAGs (12:0–54:0) that were 100 times the number of acyl carbons (*i.e.*, 1200, 1800,…, 5400). Monoacid TAGs were added as index compounds to the standard TAG mixtures and analytical TAG samples, if they did not contain them naturally.

4.10.2 Determination of empirical correction factors

Linear calibration was used in the determination of empirical molar correction factors (MCF) for correction of ion yield in GC–EI–MS and HPLC–ESI–MS studies of TAGs (**IV–VI**). Trinonanoylglycerol (trinonanoin) was used as internal standard (IS) in all calibrations. Three solutions of selected interesterified TAG standard mixtures with different analyte and IS ratios were prepared. The ion chromatograms of $[M–RCOO]^+$ ions or ammonium adducts $[M + NH_4]^+$ of TAGs were recorded by EI–MS (**VI**) or ESI–MS (**IV,V**), respectively, and extracted from total ion chromatograms and the areas of the selected ions were integrated. The plots of the molar ratio of each TAG species (i) to IS [n(i)/n(IS)] *vs*. the area ratio of specific ion to IS [A(i)/A(IS)] were produced for calculation of MCF by linear regression. The slope (a) of the linear regression curve (y = ax) was used as MCF. Further, linear calibration was applied to the determination of MCFs in GLC analysis of TAG (**III**) with slight modifications to MS analysis. For determination of MCFs, five different concentrations of TAG standard mixture of nine monoacid TAGs (12:0–54:0, 54:3) were used. The integrated GC chromatograms (FID) were used as the basis for the areas of analytes (i) and IS [A(i)/A(IS)].

In **VII**, empirical MCFs for correction of ion yield of ammonium adducts produced by np-HPLC–ESI–MS were determined on the basis of calculated molar composition of TAG species in the randomized BF (RBF) and uncorrected mol% (*i.e.*, area mol%) of each ACN:DB class. In calculation, uncorrected molar amounts in all four SPE fractions of RBF were reckoned in by using a known molar amount of IS In addition, acyl chain and regiospecific MCFs for butyrate and caproate TAGs and acyl chain specific for medium-chain TAGs were calculated by dividing the mol% of the TAG species in the RBF by the experimentally determined respective uncorrected mol%. The randomness of chemically interesterified BF was confirmed by hydrolysis with 1,3-specific pancreatic lipase and

determination of the FAME composition of *sn*-2 MAG yielded by hydrolysis (*cf.*, Chapter 4.10.4). Altogether, 105 different ion chromatograms for TAG ammonium adducts were extracted from the MS data recorded in duplicate for SPE fractions (1)–(3) and in triplicate for SPE fraction (4) and integrated. Trinonanoin was used as IS.

In addition, for determination of empirical molar and mass correction factors for the quantification of FA and TAG composition of BF by GLC, calibration mixtures of known FAME and TAG compositions were analyzed (**I–II**). Analysis of a FAME standard (GLC-74; Nu-Check-Prep, Elysian, MN, USA) containing even-numbered saturated FAs 4:0–22:0 and selected unsatured FAs (18:1, 18:2, and 18:3) was repeated six times and MCFs were calculated. For the calculation of MCFs of TAGs, the composition of a calibration mixture containing 7 monoacid TAGs (24:0, 30:0, 36:0, 42:0, 48:0, 54:0, and 54:3) was analyzed six times (**I**).

4.10.3 Data processing

Gas chromatographic data was acquired and integrated with a Microsoft Windows-based SC-Chromatographic Workstation 1.2B program (Sunicom Ltd., Helsinki, Finland) and calculated with Micman Software (Nordion Ltd., Helsinki, Finland) (**I–III, VI**). Data processing of GC–EI–MS analysis was performed by Mass Spectrometer Data System program (MSDS) supplied by Finnigan MAT Inc. (San Jose, CA, USA) (**II, VI**). Statistical calculations were carried out using the software of Microsoft Excel (Microsoft Corp., Redmond, WA, USA) and that of Microcal Origin (Microcal Software Inc., Northampton, MA, USA) (**I–VII**).

4.10.4 Confirmation of the random distribution of regioisomers in chemically interesterifed TAG mixtures

Random distribution of short-chain acyls between primary and secondary *sn*-positions in the chemically randomized TAGs was confirmed using ^{1}H nuclear magnetic resonance (NMR) spectroscopy (**IV**) or the determination of FAs in the *sn*-2 position (**VII**). ^{1}H NMR spectra of randomized TAG mixtures were recorded by Varian Unity 600 spectrometer (Palo Alto, CA, USA) and the signals were referenced to internal tetramethylsilane as described by Kalo et al. (1996). The FA composition of the *sn*-2 position of randomized TAGs was determined by hydrolysis with 1,3-specific porcine pancreatic lipase, type II (Sigma, St. Louis, MO, USA) according to the principles described by Hendrikse and Harwood (1986). After hydrolysis, the *sn*-2 MAGs were isolated by TLC, transesterified to FAME and FA composition was determined by GC. A more detailed description of the method is in **VII**.

5 RESULTS

Milk fat (MF) has a highly complex fatty acid (FA) and triacylglycerol (TAG) composition, which complicates resolution of TAG species and TAG classes on various chromatographic columns. Prefractionation of the TAGs of MF or its derivatives prior to chromatographic and mass spectrometric determination is essential to achieve reliable identification and quantification of the molecular species of TAGs. Further, use of highly specific TAG models and standards increases accuracy of chromatographic and spectrometric analysis of MF TAGs.

5.1 Synthesis of randomized model mixtures of triacylglycerols

5.1.1 Randomized model mixtures of TAG standards

In order to produce sufficient amounts of TAG standards with known structure and composition, 35 model TAG mixtures were prepared by chemical interesterification of equimolar amounts of three (or two) monoacid TAGs (Table 4.1). In theory, chemical interesterification of three equimolar monoacid TAGs (~33.33 mol% each) will result in a randomized mixture of three monoacid (á 3.70 mol%), six diacid (á 11.11 mol%), and one triacid (22.22 mol%) TAG species as shown in Table 5.1. In addition, the ratio of *sn*-1(3) and *sn*-2 isomers of the synthesized TAG species will be 2:1. Table 5.1 shows that experimental composition of an interesterified mixture of 10:0/10:0/10:0, 14:0/14:0/14:0, and 16:0/16:0/16:0 and its theoretical random composition were rather similar. The theoretical random composition and experimental composition of 29 standard mixtures, which were prepared by interesterification from one short- or medium-chain monoacid TAG and two long-chain monoacid TAGs, are listed in Appendix 1. Theoretical content (mol%) was calculated from the baseline composition of monoacid TAGs according to the principles described by Kalo et al. (1986a), and experimental composition was calculated from GC data. The average deviation (%) of experimental value from calculated value varied from 2.17 to 18.73 (avg. 8.22 ± 4.38) for di- and triacid TAGs of model mixtures (Appendix 1). The ratio of *sn*-1(3) and *sn*-2 isomers of mono-SC-TAGs was on the average 1.89:1 (S.D. = 0.20, n = 51), which was close to the expected 2:1 ratio of the regioisomers in a randomized mixture of TAG (Appendix 1). In addition, the random composition of selected interesterified TAG mixture was confirmed previously by ^{1}H NMR measurement of the ratios of primary and secondary butyrate methyl signals at *sn*-1(3) (δ 0.957) and those at *sn*-2 (δ 0.963), which were shown to be 1.96:1 and 1.77:1 for interesterified mixtures of BLaS and BSO, respectively (Kalo et al. 1996). In the present study (**IV**), the ratio of butyroyl methyl signals at *sn*-1(3) to those at *sn*-2 which originated from the dibutyrates synthesized by interesterification and those synthesized from long-chain-1-monoacylglycerols were compared. The ratio was 1.7:1, 2.1:1, 1.9:1 for regioisomeric mixtures of dibutyroylmyristoyl-, dibutyroylpalmitoyl-, and dibutyroyloleoylglycerol, respectively, in the randomized standards, and 1.1:1.0, 1.1:1.0, and 1.0:1.0 for 1-myristoyl-2,3-dibutyroyl-, 1-palmitoyl-2,3-dibutyroyl-, and 1-oleoyl-2,3-dibutyroyl-*rac*-glycerols, respectively, which were in accordance with the respective theoretical ratios 2:1 and 1:1.

Table 5.1 **(A)** An example of the theoretical composition of model TAG mixture prepared from three equimolar mixtures of monoacid TAGs (A/A/A + B/B/B + C/C/C á 33.33 mol%) by chemical interesterification (~randomization). **(B)** An example of theoretical and determined composition of an interesterified model TAG mixture (= CiMP).

(A) Randomized mixture of A/A/A (33.33 mol%) + B/B/B (33.33 mol%) + C/C/C (33.33 mol%) [1]		**(B)** Randomized mixture of 10:0/10:0/10:0 (31.88 mol%) + 14:0/14:0/14:0 (33.95 mol%) + 16:0/16:0/16:0 (34.17 mol%)		
TAG species	Calc.[2] mol%	TAG species	Calc. mol%	GC [3] mol%
A/A/A	3.70	10:0/10:0/10:0	3.24	3.16
A/A/B + A/B/A	11.11	10:0/10:0/14:0 + 10:0/14:0/10:0	10.35	10.01
A/A/C + A/C/A	11.11	10:0/10:0/16:0 + 10:0/16:0/10:0	10.42	10.93
A/B/B + B/A/B	11.11	10:0/14:0/14:0 + 14:0/10:0/14:0	11.02	10.58
A/B/C + A/C/B + B/A/C	22.22	10:0/14:0/16:0 + 10:0/16:0/14:0 + 14:0/10:0/16:0	22.19	21.43
A/C/C + C/A/C	11.11	10:0/16:0/16:0 + 16:0/10:0/16:0	11.17	12.25
B/B/B	3.70	14:0/14:0/14:0	3.91	3.56
B/B/C + B/C/B	11.11	14:0/14:0/16:0 + 14:0/16:0/14:0	11.82	11.96
B/C/C + C/B/C	11.11	14:0/16:0/16:0 + 16:0/14:0/16:0	11.89	11.96
C/C/C	3.70	16:0/16:0/16:0	3.99	4.17
Sum [4]	99.98		100.00	100.01

[1] A/A/A, B/B/B, and C/C/C = monoacid TAGs containing three similar FAs (A, B, or C)

[2] Random TAG composition (Calc.) was calculated according to the equations (a)–(c): (a) AAA = $A^3/10000$, (b) AAB = $3A^2B/10000$, (c) ABC = 6ABC/10000, where A, B, And C are mol% of (different) FAs and A/A/A, A/A/B, and A/B/C are examples of monoacid, diacid, and triacid TAGs, respectively (Kalo et al. 1986a).

[3] Composition was determined by gas-chromatography (GC)

[4] Difference from 100 mol% is due to rounding

5.1.2 Randomized butterfat synthesized by chemical interesterification

TAG composition of chemically interesterified BF was estimated according to the same principles as that of the randomized mixture of equimolar TAG standard (*cf.*, Kalo et al. 1986a; Table 5.1), which enabled utilization of the data of calculated composition in qualitative and quantitative chromatographic and mass spectrometric analysis of other TAG samples. Hence, the randomness of the distribution of FAs in the TAG molecules of chemically interesterified BF was confirmed by analysing the FA composition at the *sn*-2 position of the TAGs of randomized butterfat (RBF). A small TAG sample of RBF was hydrolysed by 1,3-specific pancreatic lipase, after which the *sn*-2 MAGs were isolated by TLC and FA composition of *sn*-2 MAG and that of TAGs of RBF were analyzed by GC. The average proportion of 14 FAs (*i.e.*, even-numbered SAFA 6:0–18:0 and MUFA 14:1–18:1, 18:2, and three odd-numbered FAs 15:0, 17:0, and 17:1) at the *sn*-2 position was 32.7% (S.D. 5.2), which was close to the expected 33.3%. The average deviation (%) of determined

Table 5.2 Fatty acid (FA) composition (mol%) of lipase-modified butter oil (LMBO) and its saturated (S), monoene (M), diene (D), and triene (T) fractions determined by GLC (n = 3) using NB-351 capillary column, split injection (split 1:20, 250°C), flame ionization detection (FID 225°C), and He as carrier gas. (Table adapted from Table 4 in **I**.)

FA	S		M		D		T		LMBO		CALC[2]
CN:DB	mol%	%RSD[1]	mol%	%RSD	mol%	%RSD	mol%	%RSD	mol%	%RSD	mol%
4:0	**15.1**	3.84	**15.0**	7.00	**5.1**	10.59	**5.1**	1.18	**12.8**	2.66	**13.2**
6:0	**6.1**	3.61	**5.6**	4.82	**2.3**	10.87	**1.9**	1.58	**4.8**	1.67	**5.2**
8:0	**2.7**	2.96	**2.3**	3.04	**1.1**	5.45	**0.8**	2.50	**2.1**	1.90	**2.3**
10:0	**4.9**	3.88	**3.6**	1.39	**1.9**	4.74	**1.3**	1.54	**3.6**	1.94	**3.8**
12:0	**4.6**	2.61	**3.3**	1.21	**2.0**	3.00	**1.3**	5.38	**3.4**	1.76	**3.6**
14:0	**14.6**	2.40	**10.0**	1.20	**6.7**	2.09	**4.5**	4.89	**11.0**	1.27	**11.4**
16:0	**32.2**	1.02	**21.4**	3.79	**15.4**	2.86	**11.6**	15.43	**24.5**	0.61	**25.0**
18:0	**13.0**	4.08	**8.2**	6.22	**5.8**	4.14	**3.6**	11.11	**10.2**	0.20	**9.8**
20:0	**0.2**	5.00	**0.1**	20.00	**0.1**	10.00	**0.1**	50.00	**0.2**	0.00	**0.2**
15:0	**1.2**	3.33	**0.9**	3.33	**0.7**	5.71	**0.5**	20.00	**0.9**	24.44	**1.0**
17:0	**0.5**	4.00	**0.4**	5.00	**0.3**	3.33	**0.2**	10.00	**0.4**	25.00	**0.4**
Σ(Saturated)	*95.1*		*70.8*		*41.4*		*30.9*		*73.9*		*75.9*
14:1			**1.2**	6.67	**2.0**	6.00	**1.8**	2.78	**0.9**	24.44	**0.8**
16:1	**0.1**	30.00	**1.9**	5.26	**4.3**	9.07	**4.7**	7.45	**1.3**	26.15	**1.6**
17:1			**0.4**	2.50	**0.8**	7.50	**0.8**	12.50	**0.3**	23.33	**0.3**
18:1	**1.2**	20.83	**22.5**	3.33	**43.8**	2.72	**38.8**	5.82	**18.8**	0.43	**16.2**
Σ(Monoene)	*1.3*		*26.0*		*50.9*		*46.1*		*21.3*		*18.9*
18:2	**0.1**	20.00	**0.2**	45.00	**2.7**	8.52	**11.5**	1.57	**1.0**	4.00	**1.2**
18:3	**0.2**	50.00					**4.8**	13.75	**0.5**	2.00	**0.4**
other	**3.3**	24.85	**3.0**	0.33	**4.9**	10.00	**6.7**	13.28	**3.3**	1.82	**3.6**

[1] %RSD = Relative standard deviation (%): %RSD = (100 * S.D.)/mean

[2] CALC = Calculated FA composition (mol%): CALCi = $[(n_{iS} * \%Frac_S) + (n_{iM} * \%Frac_M) + (n_{iD} * \%Frac_D) + (n_{iT} * \%Frac_T)] / 100$, where n_i = content (mol%) of FA_i in fraction S, M, D, or T; $\%Frac_X$ = relative proportion (%) of fraction X in LMBO (X = S, M, D, or T).

percentage from the theoretical random value was 13.2 ± 8.2% (n = 14), 10.0 ± 3.7% (n = 9), and 18.9 ± 11.3% (n = 5) for all FAs, saturated FAs and unsaturated FAs, respectively, indicating random distribution of FAs among the three *sn*-positions of glycerol backbone. Due to a small sample of RBF subjected to hydrolysis of pancreatic lipase, main proportion of *sn*-2 butyrate MAGs remained on the TLC plate, hence butyric acid was not included in the calculations, in order to avoid disturbance on the percentages of other FAs.

5.2 Prefractionation of triacylglycerols of milk fat

An improved method for fractionation of the molecular species of TAGs into saturated, monoene, and polyene TAGs was developed based on the use of p-propylbenzene sulfonic

acid solid-phase extraction (SPE) columns loaded with silver ions. The applicability of the method was tested on the fractionation of lipase-modified butteroil (LMBO). The content (mol%) of major FAs in LMBO and that of saturated (S), monoene (M), diene (D), and triene (T) TAG fractions of LMBO are listed in Table 5.2. Unspecified minor FAs (*e.g.*, branched and some odd-carbon numbered FAs) are presented in the FA-group 'other'. The relative proportion of each fraction was assessed using internal standard method and determined FA composition. The proportions of the four fractions [mean ± S.D. (fraction)] were 45.8 ± 3.52 (S), 36.2 ± 3.34 (M), 11.0 ± 0.33 (D), and 7.0 ± 0.04% (T). The difference between calculated and analyzed content of FAs varied from 0.0 to 23.1% and was on the average 8.3% indicating that the recovery of TAGs from SPE columns was adequate.

Table 5.3 Proportion (mol%) of the class of triacylglycerols (TAGs) in lipase-modified butter oil (LMBO) and its saturated (S), monoene (M), diene (D), and triene (T) fractions determined by GLC using phenyl(65%)methylsilicone column, cold on-column injection and flame ionization detection. Carrier gas was hydrogen. (Table adapted from Table 3 in **II**.)

TAG	S mol%	M mol%	D mol%	T mol%	LMBO	CALC.[1]
TAG ACN 24–35						
* Saturated	18.07	1.11			9.30	8.68
* Monoene	1.71	10.23			4.56	4.49
* Diene			1.21			
* Triene						
TAG ACN 36–43						
* Saturated	29.09	6.04	0.64	1.97	14.16	16.09
* Monoene	4.12	24.30	3.65	1.34	10.27	11.18
* Diene	0.23	2.28	13.83		3.34	2.45
* Triene		0.24		7.25	0.65	0.59
TAG ACN 44–54						
* Saturated	35.31	1.24	1.67	4.19	17.80	17.10
* Monoene	2.52	44.72	4.50	4.51	22.65	18.15
* Diene	1.46	0.62	53.32	1.96	10.81	6.90
* Triene	0.83	0.91	0.40	55.76	3.50	4.66
Σ						
* Saturated	82.47	8.39	2.31	6.13	41.26	
* Monoene	8.35	79.25	8.15	5.85	37.48	
* Diene	1.69	2.90	68.36	1.96	14.15	
* Triene	0.83	1.15	0.40	63.01	4.15	
Not identified TAGs	0.97	2.17	11.43	5.34	2.91	
Not identified minor peaks	5.69	6.14	9.35	17.71	0.05	

[1] CALC = Calculated TAG class composition (mol%): $CALCi = [(n_{iS} * \%Frac_S) + (n_{iM} * \%Frac_M) + (n_{iD} * \%Frac_D) + (n_{iT} * \%Frac_T)] / 100$, where n_i = content (mol%) of $(TAG\ class)_i$ in fraction S, M, D, or T; $\%Frac_X$ = relative proportion (%) of fraction X in LMBO (X = S, M, D, or T) determined on the basis of FA composition.

In theory, the ratio of saturated FAs (SAFA) to monounsaturated FAs (MUFA) should be 100:0 and 67:33 in S and M fractions, respectively. On the basis of the excess of MUFA in S fraction and SAFA in M fraction, the degree of overlapping of two fractions can be estimated to be 3.9–9.3% (Table 5.2). Accordingly, overlapping of D and T fractions with S fraction was 0.3 and 0.6%, respectively. FA composition of D and T fractions suggested a higher proportion of SAFA than was expected. In addition, unknown degree of unsaturation of the FAs in group 'other' increased uncertainty to the assessment of overlapping.

In lipase-modified BO (LMBO), the FA distribution in TAGs was shown to be nearly random (Kalo et al. 1990), hence the ratio of individual FA to the total amount of FAs was expected to be almost constant in all fractions. However, the relative amount of butyric acid (4:0) to the total amount of all identified saturated acids was 15.8 and 21.1% in the S and M fractions, respectively (Table 5.2). The difference between the similar ratios of other SAFA of the two fractions was at most 3.5 percentage points. When the difference of the ratio of each SAFA in S and M fractions was proportionated to the total content of SAFA in LMBO, the average was 17.4%. Short-chain SAFAs (4:0, 6:0) and C20:0 had the highest (> 30%) proportional differences, suggesting untypical migration of short-chain and very long-chain saturated TAGs during elution. Further, the proportion of *cis–trans* isomers of MUFA was tentatively analyzed. The ratio of *cis*-18:1 to *trans*-18:1 was shown to be similar (ratio ~ 10:1) in the whole fat (LMBO) and in the M fraction, but a relative abundance of *trans* isomer (ratio ~ 1:1) was observed in S fraction (**I**).

Determination of TAG class composition of LMBO fractions suggested almost identical overlapping of fractions as FA analysis (Table 5.3). The content of saturated TAGs in M fraction was 8.4 mol% and, *vice versa*, that of monoene TAGs in S fraction was 8.4 mol%, which agreed with the observations based on the FA composition. Some overlapping (2–6%) of S fraction with D and T fractions was detected. Overlapping of unsaturated fractions (M, D, T) was also evident, *e.g.*, monoene TAGs were detected in D and T fractions 8.2 and 5.9 mol%, respectively. The difference between calculated and analyzed content of TAG classes showed slightly higher variation (1.6–36.2%; 15.9% on the average) than FA analysis suggested. However, in the high resolution area of gas chromatograms (ACN 24–43) calculated content differed from analyzed composition on the average 11.0% suggesting relatively even recovery of various TAG classes in fractionation process.

Effect of fractionation is illustrated in Figure 5.1, which shows a gas chromatogram of LMBO and those of saturated (S), monoene (M), diene (D), and triene (T) fractions. Influence of fractionation was most evident in the high ACN range (RI > 4400), in which fewer acyl chain isomers existed and the difference in RIs of major peaks of each fraction was distinct. The effect of fractionation was less evident in the lower ACN range due to the more substantial number of the TAG isomers with the same ACN and degree of unsaturation. In addition, the chromatograms showed clearly that each fraction contained, in addition to major peaks, small peaks with the same retention index (RI) as a major peak in another fraction.

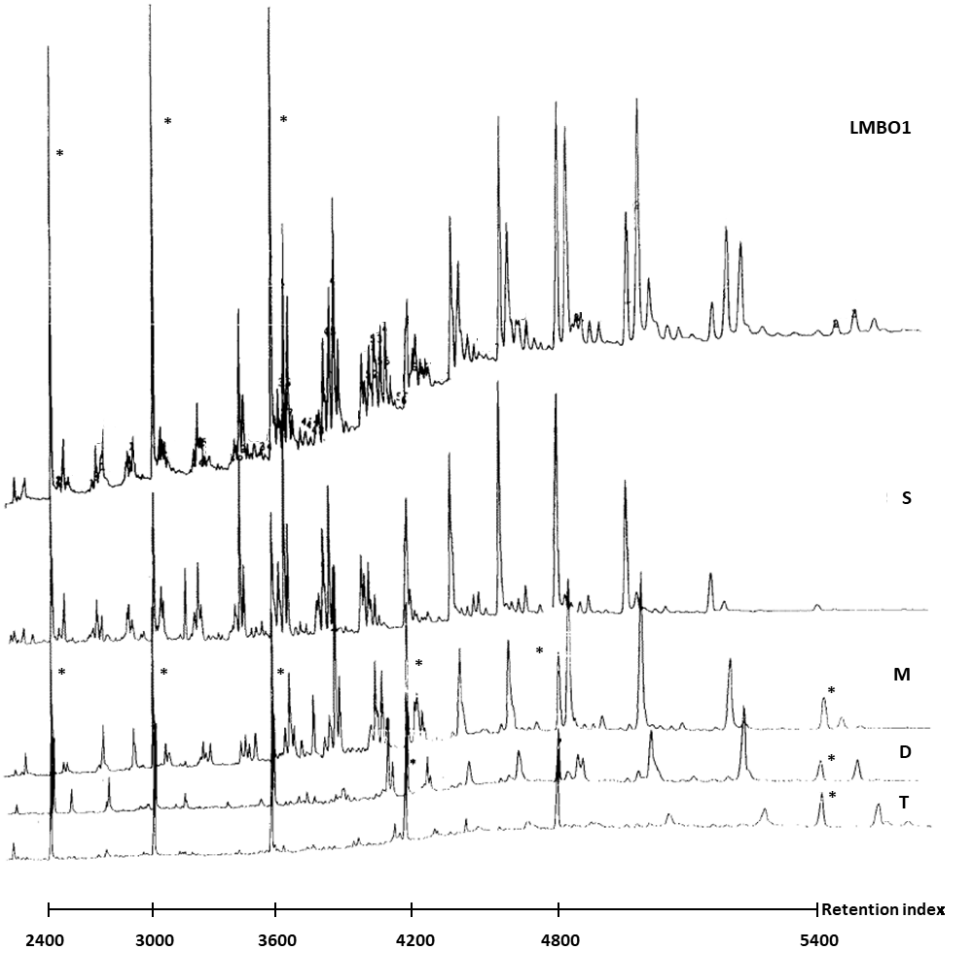

Figure 5.1. Gas chromatogram of lipase-modified butter oil (LMBO) and those of saturated (S), monoene (M), diene (D), and triene (T) fractions of LMBO. * = peaks for monoacid TAGs, which are added as index compounds (see text for details). (Figure adapted from Fig. 1 in **I**.)

5.3 Identification of the molecular species of triacylglycerols

Principal methods that were used in the identification of various TAG classes, molecular species of TAGs, and isomers of TAGs in standard mixtures and milk fat samples are summarized in Table 5.4. In GC and CG–EI–MS, the main sources of identification were RIs or relative retention times (RRT) and specific fragment ions in EI mass spectra. In HPLC–MS studies, TAG species were identified according to elution order (RRT) and ammonium adducts (*m/z* $[M+NH_4]^+$) in ESI–MS or specific fragment ions together with the relative abundance of the ions (*m/z* $[(M+NH_4)–NH_3–FA]^+$) in ESI–MS^2. In all chromatographic and mass spectrometric methods combining information from elution order of acyl chain isomers and regioisomers of TAG species with that of mass spectra of TAG isomers increased the number of identified molecular species of TAGs.

Table 5.4 Chromatographic and mass spectrometric methods used to identify acyl chain isomers of TAGs and regioisomers of short-chain TAGs.

Method[1]	Source of identification	Target of identification	Original publication
GLC	RIs[2]	ACN:DB[3] class of TAG species	**I, II, III**
		Elution order of acyl chain isomers of TAG species	
	RIs for known TAG species (including regioisomers of short-chain TAGs)	Elution order of acyl chain isomers and regioisomers of short-chain TAGs	**III**
GC–EI–MS	TIC[4] *m/z* 100–610, specific fragment ions: $[M–RCOO]^+$, $[RCO]^+$, $[RCO+74]^+$, $[RCO+128]^+$	ACN:DB class of TAG species	**I, II**
	RIC[5] of specific fragment ions *m/z* $[M–RCOO]^+$, $[RCO]^+$	ACN:DB class of TAG species	**VI**
	RIs	Elution order of acyl chain isomers of TAGs	**II**
	RRT[6] in RIC of selected fragment ions	Elution order of acyl chain isomers of TAGs and that of regioisomers of short-chain TAGs	**VI**
np-HPLC–ESI–MS	*m/z* $[M+NH_4]^+$	ACN:DB class of TAG species	**IV, V, VII**
	RRT in TIC/EIC[7] of $[M+NH_4]^+$	Elution order of TAG isomers	**IV, V, VII**
np-HPLC–ESI–MS^2	*m/z* $[(M+NH_4)–NH_3–FA]^+$	Acyl chains of TAG species ➔ Acyl chain isomers of TAG species	**IV, V, VII**
	Relative abundance of *m/z* $[(M+NH_4)–NH_3–FA]^+$	Regioisomer of separated short-chain TAGs; predominant (regio)isomer of medium- and long-chain TAGs	**IV, V, VII**

[1] GLC = Gas-liquid chromatography; GC–EI–MS = Gas chromatography–electron ionization mass spectrometry; np-HPLC–ESI–MS(MS^2) = normal phase high-performance liquid chromatography–electrospray ionization mass spectrometry (tandem mass spectrometry)
[2] RIs = Retention indices
[3] ACN:DB = Number of acyl carbons:number of double bonds
[4] TIC = Total ion chromatogram
[5] RIC = Reconstructed ion chromatogram
[6] RRT = Relative retention time
[7] EIC = Extracted ion chromatogram

5.3.1 Identification of TAG species by high-resolution gas–liquid chromatography

In order to define the elution order of TAG species and resolution of isomers on phenyl(65%)methylsilicone columns, 112 TAG species, including 22 pairs of the regioisomers of SC-TAGs, were synthesized and their RIs were determined (Tables 5.5 and 5.6). Separation and elution order of the principal ACN:DB classes of even-numbered TAGs were evident as indicated in Table 5.5. The higher the ACN of TAG species, the higher was their RI. Retention indices of even-numbered TAGs showed that most polar molecular species of saturated SC-TAGs overlapped with unsaturated TAGs with the same ACN. However, saturated TAGs did not overlapp with any of the most polar monoene and diene TAG isomers of preceding ACN class (Tables 5.5 and 5.6). Difference between the RI of monostearate (XX18:0) and monooleate (XX18:1) with the same ACN and that of distearate (X18:0-18:0) and dioleate (X18:1–18:1) with the same ACN was on the average 16.3 ± 8.0 (n = 17) and 38.5 ± 17.3 (n = 8), respectively (Table 5.5), suggesting adequate resolution on phenylmethylsilicone columns.

RIs showed that even-numbered saturated acyl chain isomers of TAG eluted on phenyl(65%)methylsilicone columns in the following order: XXX >> XX8:0 >> XX6:0 >> XX4:0, where X = medium or long-chain acyl (Table 5.5). In general, RIs increased (*i.e.*, elution time increased) when the difference between the carbon chain length of the shortest and longest acyl increased. Only few exceptions were observed with most symmetric TAGs (see Table 5.5; TAG 38:0, 40:0, 44:0, and 46:0). In most cases, the resolution on a phenylmethylsilicone column was high enough to resolve butyrates from caproates from caprylates from other acyl chain isomers. Difference between the mean of RIs of monobutyrates and monocaproates with the same ACN:DB was (mean ± S.D.) 28.9 ± 5.0 (n = 7), that of monocaproates and monocaprylates 14.9 ± 2.5 (n = 8), and that of monocaprylates and monocapriates 15.9 ± 5.2 (n = 9) (Table 5.5).

RI data showed that *sn*-1(3) and *sn*-2 isomers of monobutyrates and -caproates containing two long-chain acyl groups (12:0, 14:0, 16:0, 18:0, and 18:1) eluted in two separate peaks on a phenyl(65%)methylsilicone column (Table 5.6). Regioisomers of dibutyrates separated from each other only partially, shown as shouldering in peaks. In general, regioisomers were not separated from each other, if the number of acyl carbons of the shortest acyl was eight or higher. Even so, in the case of caprylyldioleoylglycerol partial separation of positional isomers was detected as a slight shouldering in chromatogram.

The difference between RIs of regioisomers of saturated monobutyrates and monocaproates ranged from 14 to 18 (avg. 16.1 ± 1.5, n = 8) and from 9 to 11 (avg. 9.5 ± 0.8, n = 6), respectively (Table 5.6). The corresponding average difference for unsaturated monobutyrates and monocaproates was slightly higher, *i.e.,* 18.0 ± 1.4 (n = 4) and 12.0 ± 3.6 (n = 4), respectively. The average difference between the carbon chain length of the shortest and longest acyl group was 12.8 ± 2.0 (n = 12) and 11.2 ± 1.4 (n = 10) for butyrates and caproates, respectively, suggesting that the chain length of esterified short-chain acyl affected more strongly on the difference between RI of *sn*-1(3) and *sn*-2 isomers than that of the longest acyl group.

Table 5.5 Retention indices (RIs) of the molecular species of synthesized TAGs and selected differences of RI between TAG species determined by gas-liquid chromatography on a phenyl(65%)methylsilicone column. For chromatographic conditions see text. (Table adapted from Table 3 in **III**.)

ACN:DB[1]	TAG	ΔLength[2]	Mean	Min	Max	n[3]	Δ_{RI}(B–Co)[4]	Δ_{RI}(Co–Cy)	Δ_{RI}(Cy–Ci)	Δ_{RI}(18:1)[5]
12:0	**BBB**	**0**	**1200**	**1200**	**1200**	**14**				
18:0	**CoCoCo**	**0**	**1800**	**1800**	**1800**	**12**				
20:0	**BBLa**	**8**	**2037**	**2036**	**2037**	**2**				
22:0	**BBM**	**10**	**2252**	**2251**	**2253**	**6**				
24:0	**CyCyCy**	**0**	**2400**	**2400**	**2400**	**12**				
	BBP	12	2466	2465	2467	4				
26:0	**CoCoM**	**8**	**2633**	**2627**	**2638**	**5**				
	BBS	14	2680	2678	2683	7	47[6]			
26:1	BBO	14	2683	2681	2686	6				3
28:0	**CyCyLa**	**4**	**2813**	**2807**	**2816**	**6**				
	BLaLa[7]	8	2838	2838	2838	2				
	CoCoP	10	2842	2839	2845	5		29		
30:0	**CiCiCi**	**0**	**3000**	**3000**	**3000**	**8**				
	CyCyM	6	3014	3012	3017	8				
	CoCoS	12	3048	3045	3050	4		34		
30:1	CoCoO	12	3056	3054	3059	4				8
32:0	**CiCiLa**	**2**	**3189**	**3187**	**3190**	**6**				
	CyLaLa	4	3194	3190	3200	6				
	CyCyP	8	3207	3206	3208	2			18	
	BMM	10	3237	3234	3239	6				
34:0	**CiLaLa**	**2**	**3389**	**3387**	**3391**	**6**				
	CiCiM	4	3394	3392	3395	2				
	CyLaM	6	3400	3399	3400	2			11	
	CoMM	8	3412	3408	3415	5		12		
	CyCyS	10	3416	3413	3419	4			22	
34:1	CyCyO	10	3429	3428	3429	4				13
34:0	BMP	12	3444	3444	3444	2	32			
	BLaS	14	3448	3447	3449	2	36			
36:0	**LaLaLa**	**0**	**3600**	**3600**	**3600**	**14**				
	CiCiP	6	3613	3612	3614	4				
	CyMM	6	3616	3613	3619	8				
	CoMP	10	3632	3631	3632	2		16		
	BPP	12	3658	3656	3660	6	26			
	BMS	14	3664	3662	3666	2	32			
36:1	BMO	14	3678	3676	3680	2				14
38:0	**CiMM**	**4**	**3810**	**3803**	**3816**	**2**			22	
	LaLaM	2	3814	3813	3814	2				
	CiLaP	6	3818	3817	3818	2			13	
	CiCiS	8	3824	3822	3825	2				
	CyMP	8	3831	3828	3833	2		16	21	
	CyLaS	10	3832	3832	3832	2		13	14	

ACN:DB[1]	TAG	ΔLength[2]	Mean	Min	Max	n[3]	Δ_{RI}(B–Co)[4]	Δ_{RI}(Co–Cy)	Δ_{RI}(Cy–Ci)	Δ_{RI}(18:1)[5]
38:1	CiCiO	10	3836	3831	3840	2				12
38:0	CoPP	10	3845	3841	3848	6	24	14		
	CoMS	12	3847	3845	3848	2		15		
38:1	CyLaO	10	3848	3847	3849	2				16
38:1	CoMO	12	3853	3848	3858	2				6
	BPS	14	3869	3868	3870	2	22			
38:1	BPO	14	3889	3887	3891	2				20
40:0	**LaLaP**	**4**	**3997**	**3996**	**3997**	**2**				
	CiMP	6	4002	3992	4012	2			23	
	LaMM	2	4005	4004	4006	2				
	CiLaS	8	4010	4009	4011	2			8	
	CyPP	8	4018	4016	4019	2		20	16	
40:1	CiLaO	8	4023	4010	4035	2				13
40:0	CyMS	10	4025	4024	4025	2			15	
	CoPS	12	4038	4033	4043	2		13		
40:1	CyMO	10	4049	4046	4051	2				24
	CoPO	12	4060	4058	4061	2				22
40:0	BSS	14	4068	4057	4078	6	30			
40:1	BSO	14	4091	4087	4094	2				23
40:2	BOO	14	4101	4100	4104	4				*33*[8]
42:0	**MMM**	**0**	**4200**	**4200**	**4200**	**22**				
	LaLsS	6	4200	4200	4200	6				
	CiPP	6	4215	4214	4216	2				
42:1	LaLaO	6	4225	4220	4231	4				25
42:0	CoSS	12	4251	4243	4260	6				
42:1	CoSO	12	4273	7270	4276	2				22
42:2	CoOO	12	4282	4275	4321	6				*31*
44:0	**LaPP**	**4**	**4449**	**4448**	**4450**	**2**				
	MMP	2	4454	4449	4458	3				
	CySS	10	4475	4468	4482	4				
44:2	CyOO	10	4489	4474	4499	4				*14*
46:0	**MMS**	**4**	**4642**	**4627**	**4651**	**6**				
	MPP	2	4646	4641	4651	4				
	CiSS	8	4648	4648	4648	2				
46:1	MMO	4	4648	4640	4658	6				6
46:2	CiOO	8	4681	4678	4683	2				*33*
48:0	**PPP**	**0**	**4800**	**4800**	**4800**	**18**				
	LaSS	6	4800	4800	4800	6				
48:2	LaOO	6	4836	4834	4840	4				*36*
50:0	**PPS**	**2**	**4966**	**4964**	**4966**	**4**				
	MSS	4	4971	4966	4978	6				
50:1	PPO	2	4984	4975	4995	4				18
50:2	MOO	4	5009	4998	5021	6				*38*
52:0	**PSS**	**2**	**5159**	**5158**	**5160**	**4**				
52:2	POO	2	5208	5200	5217	4				*49*

ACN:DB[1]	TAG	ΔLength[2]	Mean	Min	Max	n[3]	Δ_{RI}(B–Co)[4]	Δ_{RI}(Co–Cy)	Δ_{RI}(Cy–Ci)	Δ_{RI}(18:1)[5]
54:0	**SSS**	**0**	**5400**	**5400**	**5400**	**20**				
54:1	SSO	0	5432	5422	5436	4				32
54:2	SOO	0	5474	5469	5482	4				*74*
54:3	OOO	0	5498	5487	5514	18				

[1] ACN:DB = Number of acyl carbons:number of double bonds
[2] ΔLength = Difference between the length of the longest and that of the shortest acyl chain in the TAG molecule
[3] n = Number of determinations
[4] Δ_{RI}(B–Co) = Difference between the mean of RIs of a monobutyrate (or dibutyrate) TAG and a monocaproate (or dicaproate) TAG with the same ACN:DB
[5] Δ_{RI}(18:1) = Difference between the mean of RIs of a monostearate (or distearate) TAG and a monooleate (or dioleate) TAG with the same ACN:DB
[6] Underlined numbers refer to Δ_{RI} of di-short or di-medium-chain TAG species
[7] RIs and the differences of RIs have been calculated solely using the RI of *sn*-1(3) isomers of short-chain TAGs
[8] *Underlined italics* numbers refer to Δ_{RI} between distearate and dioleate TAGs

5.3.2 Identification of TAG species by GC–EI–MS

In gas chromatography–EI–mass spectrometry, retention indices (RI) or relative retention times (RRT), full mass spectra (*m/z* 100–610), and specific fragment ions in EI mass spectra ($[RCO]^+$, $[RCO + 74]^+$, $[RCO + 128]^+$, and $[M–RCOO]^+$) were the principal sources for identification. In addition, due to the difference in the resolution between the chromatograms of high-resolution (HR) GC and GC–EI–MS, peak size and shape and elution order of TAG species in partly resolved peaks or unresolved peaks of mass chromatograms were taken into consideration in identification process.

In order to identify common TAG species containing 24–54 acyl carbons, interpretation table of *m/z* values for specific fragment ions were calculated (*cf.*, Table 5.7, part a). Mass chromatograms with full mass spectra (**I, II**) or selected ion chromatograms (**VI**) were recorded for TAG samples (BOs, TAG standards). When full mass spectra were used, the data from mass chromatograms with a scan number interval of 1–4 were collected to interpretation tables resembling Table 5.7 (part b) with an example of TAG 38 of LMBO. In general, identification of TAG species was regarded as positive when at least three of the four specific fragment ions were detected at the same or close to the same scan number (Table 5.7, part c). $[M–RCOO]^+$ ion was assessed necessary for interpretation. Further, detection of both $[RCO + 74]^+$ and $[RCO + 128]^+$ ion of TAG species containing 4:0 and 6:0 acyls was essential because *m/z* 71 and 99 were not recorded due to their abundance in every mass spectra of TAG. In some cases interpretation was challenging, because of low and varying intensity of key fragments and the same *m/z* of some $[RCO + 74]^+$ and $[RCO + 128]^+$, ions, *i.e.*, the $[RCO + 74]^+$ fragments for 16:1 and 18:1 and the $[RCO + 128]^+$ fragments for 12:0 and 14:0 had the same mass, *m/z* 311 and *m/z* 339, respectively. Use of selected ion monitoring in GC–EI–MS (**VI**), increased intensity of the specific fragment ions and improved identification of major TAG species, but concurrently, mass information of uncommon TAG species was lost. Due to their identical fragment ions in EI–MS, differentiation of the regioisomers of TAG species was solely based on their distinct retention (RI/RRT).

Table 5.6 Retention indices (RIs) of *sn*-1(3) and *sn*-2 isomers of synthesized short-chain TAGs (SC-TAGs) determined by gas-liquid chromatography on a phenyl(65%)methyl-silicone column. For chromatographic conditions see text. (Table adapted from Table 3 in **III**.)

ACN:DB[1]	TAG	ΔLength[2]	Mean	Min	Max	n[3]	ΔRI[*sn*(2)–*sn*-1(3)][4]
Saturated SC-TAG							
28:0	B/La/La[5]	8	2838	2838	2838	2	14
	La/B/La[5]	8	2852	2852	2852	2	
32:0	B/M/M	10	3237	3234	3239	6	14
	M/B/M	10	3251	3249	3253	6	
34:0	Co/M/M	8	3412	3408	3415	5	9
	M/Co/M	8	3421	3420	3422	3	
	B/M/P	12	3444	3444	3444	2	16
	M/B/P	12	3460	3460	3460	2	
	B/La/S	14	3448	3447	3449	2	17
	La/B/S	14	3465	3464	3466	2	
36:0	Co/M/P	10	3632	3631	3632	2	9
	M/Co/P	10	3641	3640	3641	2	
	B/P/P	12	3658	3656	3660	6	17
	P/B/P	12	3675	3673	3677	6	
	B/M/S	14	3664	3662	3666	2	18
	M/B/S	14	3682	3679	3685	2	
38:0	Co/P/P	10	3845	3841	3848	6	10
	P/Co/P	10	3855	3852	3858	6	
	Co/M/S	12	3847	3845	3848	2	9
	M/Co/S	12	3856	3856	3856	2	
	B/P/S	14	3869	3868	3870	2	16
	P/B/S	14	3885	3884	3885	2	
40:0	Co/P/S	12	4038	4033	4043	2	9
	P/Co/S	12	4047	4042	4051	2	
	B/S/S	14	4068	4057	4078	6	17
40:0	S/B/S	14	4085	4074	4094	6	
42:0	Co/S/S	12	4251	4243	4260	6	11
	S/Co/S	12	4262	4254	4271	6	
Monoene SC-TAG							
36:1	B/M/O	14	3678	3676	3680	2	19
	M/B/O	14	3697	3694	3700	2	
38:1	Co/M/O	12	3853	3848	3858	2	9
	M/Co/O	12	3862	3857	3867	2	
	B/P/O	14	3889	3887	3891	2	18
	P/B/O	14	3907	3906	3908	2	
40:1	Co/P/O	12	4060	4058	4061	2	9
	P/Co/O	12	4069	4067	4070	2	
	B/S/O	14	4091	4087	4094	2	16
	S/B/O	14	4107	4103	4110	2	
42:1	Co/S/O	12	4273	7270	4276	2	14

ACN:DB[1]	TAG	ΔLength[2]	Mean	Min	Max	n[3]	ΔRI[*sn*(2)–*sn*-1(3)][4]
42:1	S/Co/O	12	4287	4284	4290	2	
Diene SC-TAG							
40:2	B/O/O	14	4101	4100	4104	4	19
	O/B/O	14	4120	4117	4123	6	
42:2	Co/O/O	12	4282	4275	4321	6	16
	O/Co/O	12	4298	4275	4321	6	

[1] ACN:DB = Number of acyl carbons:number of double bonds

[2] ΔLength = Difference between the length of the longest and that of the shortest acyl chain in TAG molecule

[3] n = Number of determinations

[4] Δ_{RI}(B–Co) = Difference between the mean of RIs of a monobutyrate (or dibutyrate) TAG and a monocaproate (or dicaproate) TAG

[5] An example: B/La/La = 3-butyroyl-1,2-dilauroyl-*rac*-glycerol; La/B/La = 2-butyroyl-1,3-dilauroylglycerol

5.3.3 Identification of TAG species by np-HPLC–ESI–MS and ESI–MS^2

Identification of TAGs by HPLC–ESI–MS. In HPLC–ESI–MS analysis, ACN:DB classes of TAG species were identified according to the mass-to-charge ratio of ammonium adducts (*m/z* $[M+NH_4]^+$) and elution order (RRT) on a normal-phase column. Figure 5.2 shows an example of a total ion chromatogram (TIC) of interesterified equimolar mixture of three monoacid TAGs (4:0/4:0/4:0, 12:0/12:0/12:0, and 18:0/18:0/18:0) and extracted ion chromatograms (EIC) of ammonium adducts of mono- and di-short-chain TAG species (A–E) of the model mixture. TIC and EIC of ammonium adducts showed a good chromatographic resolution of ACN:DB classes of TAG species and *sn*-1(3) and *sn*-2 positional isomers of short-chain TAGs (Fig. 5.2). Under the experimental conditions, mass spectra recorded at the top of the peaks were relatively clean as shown in Figure 5.2. In general, ESI mass spectra showed only ammonium adduct ions, and no sodium or potassium adducts were detected. In addition, $[M+NH_4]^+ + 1$ and $[M+NH_4]^+ + 2$ isotope peaks were observed (Fig. 5.2, F). Fragment ions were not frequently observed with an exception of low intensity fragments in the spectra of low molecular weight (*m/z* < 500) $[M+NH_4]^+$ ions (*e.g.*, Fig. 5.2, E). Overlapping with some closely eluting TAG species could be recognized in some mass spectra (Fig. 5.2, A–C) as a presence of other $[M+NH_4]^+$ ions.

Interpretation of mass spectra of ESI–MS^2. Figure 5.3 shows an extracted ion chromatogram of ammonium adduct of 514.7 from a fraction of BF sample, and two product ion tandem mass spectra. The two tandem mass spectra show two intense fragment ions *m/z* 409.5 and 215.2 which were formed by the loss of ammonia together with butyric acid and oleic acid, respectively, from the ammonium adduct (marked M + 18). On the basis of $[M + NH_4]^+$ and the fragment ions, both of the molecular species could be identified as dibutyroyloleoylglycerol. However, there is a distinct difference between the relative intensity of the fragment ions of the two spectra and the retention times of the two peaks. The regioisomers of 4:0-4:0-18:1 were shown to be eluted in the order: 1,2-butyroyl-3-oleoyl-*rac*-glycerol >> 1,3-butyroyl-2-oleoylglycerol. Hence, the lower relative abundance of *m/z* 215.2 in the right hand side spectrum suggested a markedly lower cleavage of oleic acid from the secondary *sn*-position than from the primary positions in ESI–MS^2.

Table 5.7 An example of interpretation of mass spectral data (*m/z*) for TAG species with 38 acyl carbons (ACN 38). a) Calculated mass values for $[RCO]^+$, $[RCO+74]^+$ and $[RCO+128]^+$ ions and respective $[M–RCOO]^+$ ions; b) Data from mass chromatograms (EI–MS) of the fragment ions; c) Identification of possible TAG species with ACN 38. (Table adapted from Table 1 in **II**.)

a) Interpretation table (*m/z*, EI–MS) for ACN 38

Fragment ion	FA										
	18:0	16:0	14:0	12:0	10:0	8:0	6:0	4:0	18:1	16:1	18:2
$[RCO]^+$	267	239	211	183	155	127	99	71	265	237	263
$[RCO+74]^+$	341	313	285	257	229	201	173	145	339	311	337
$[RCO+128]^+$	395	367	339	311	283	255	227	199	393	365	391
$[M–RCOO]^+_{38:0}$	383	411	439	467	496	524	552	580			
$[M–RCOO]^+_{38:1}$	381	409	437	465	494	522	550	578	383	411	
$[M–RCOO]^+_{38:2}$	379	407	435	463	492	520	548	576	381	409	383
$[M–RCOO]^+_{38:3}$	377	405	433	461	490	518	546	574	379	407	381

b) Detected fragment ions (*m/z*)					**c) Interpretation**	
Scan no. / RI	$[RCO]^+$	$[RCO+74]^+$	$[RCO+128]^+$	$[M–RCOO]^+$	Acyl	TAG species
478 / 3785	127	201	255	524	8:0	
	155	229	283	496	10:0	
	183	257	311	467	12:0	14:0-14:0-10:0
	211	285	339	439	14:0	16:0-12:0-10:0
	239	313	367	411	16:0	16:0-14:0-8:0
481 / 3807		173	227	552	6:0	
	239	313	367	411	16:0	16:0-16:0-6:0
485 / 3807		145	199	580	4:0	
	239	313	367	411	16:0	
	267	341	395	383	18:0	18:0-16:0-4:0
488 / 3859		145	199	578	4:0	
		173	227	550	6:0	
	211	285	339	437	14:0	
	239	313	367	409	16:0	18:1-16:0-4:0
	265	339	393	383	18:1	18:1-14:0-6:0
490 / 3874		145	199	578	4:0	
	237	311	365	411	16:1	
	267	341	395	381	18:0	16:1-18:0-4:0

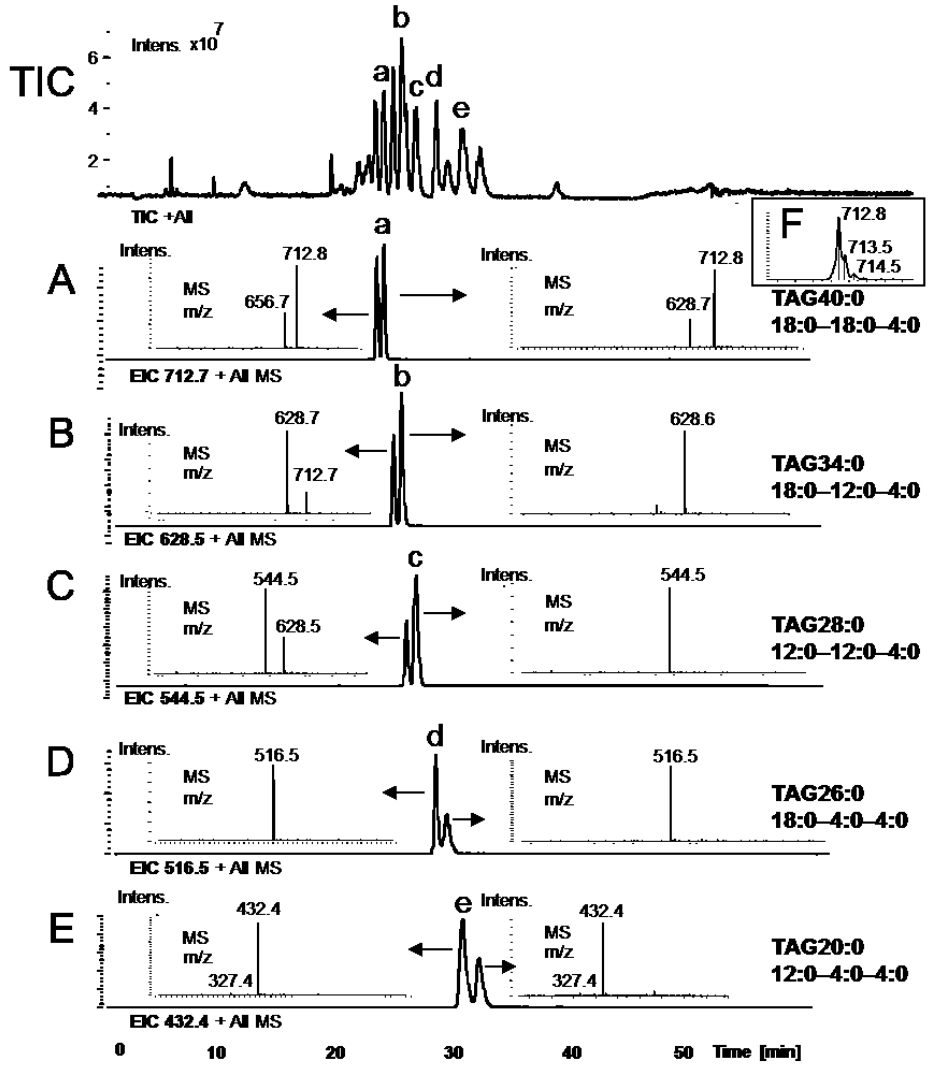

Figure 5.2. Total ion chromatogram (TIC) of interesterified equimolar mixture of TAGs 4:0/4:0/4:0, 12:0/12:0/12:0, and 18:0/18:0/18:0 and extracted ion chromatograms (EIC) of ammonium adducts of short-chain TAGs (A–E) together with mass spectra recorded at the top of the peaks of regioisomers (a–e) and an example of isotope peaks (F) of ammonium adducts. (Figure adapted from Fig. 1 in **IV**.)

Relative abundance of the fragment ions formed by cleavage of FA from the secondary and primary positions of short-chain triacylglycerols in ESI–MS^2 was investigated with di- and triacid TAGs originated from 20 interesterified equimolar TAG mixtures. For the resolution of TAG species, two or three microbore silica gel columns in series were applied (**IV, V**). The following parameters were calculated for the regioisomers of SC-TAG species: RA = Relative abundance (%) of $[(M+NH_4) - NH_3 - \text{SC-FA}]^+$ normalized to $\Sigma\,[(M+NH_4) - NH_3 - FA_i]^+ = 100\%$ (i = FA *sn*-1–3); RAR = Relative abundance ratio = $[(M+NH_4) - NH_3 - \text{SC-FA}]^+ / [(M+NH_4) - NH_3 - \text{LC-FA}]^+$, and RRAR = Ratio of relative abundance ratio of $[(M+NH_4) - NH_3 - \text{SC-FA}]^+ / [(M+NH_4) - NH_3 - \text{LC-FA}]^+$ of the regioisomer pairs

sn-1(3) *vs. sn*-2. RRAR describes how much higher the cleavage of fatty acyls is from *sn*-1(3) position than from *sn*-2 position. Table 5.8 summarizes the RRARs for the regioisomers of SC-TAG classes. Without exception, the cleavage of FAs from primary positions was on the average 3.74–16.68 times higher than from secondary position in all SC-TAG classes and in both np-HPLC–ESI–MS^2 analytical systems (Table 5.8). The average cleavage varied from 4.60 to 12.29 and from 3.74 to 9.33 for monobutyrate and monocaproate TAGs, respectively, and the average RRAR was invariably higher for monobutyrate TAG classes than respective monocaproate TAG classes. The same trend was observed in di-SC-TAG classes. Further, the relative ease of cleavage of the SC-FA and LC-FA from *sn*-1(3) position of mono and di-SC-TAGs, respectively, was higher in the HPLC–MS system with improved resolution, *i.e.*, when three silica gel HPLC-columns were used in series. Coincidentally, the variation of RRAR was higher in three than two column HPLC system.

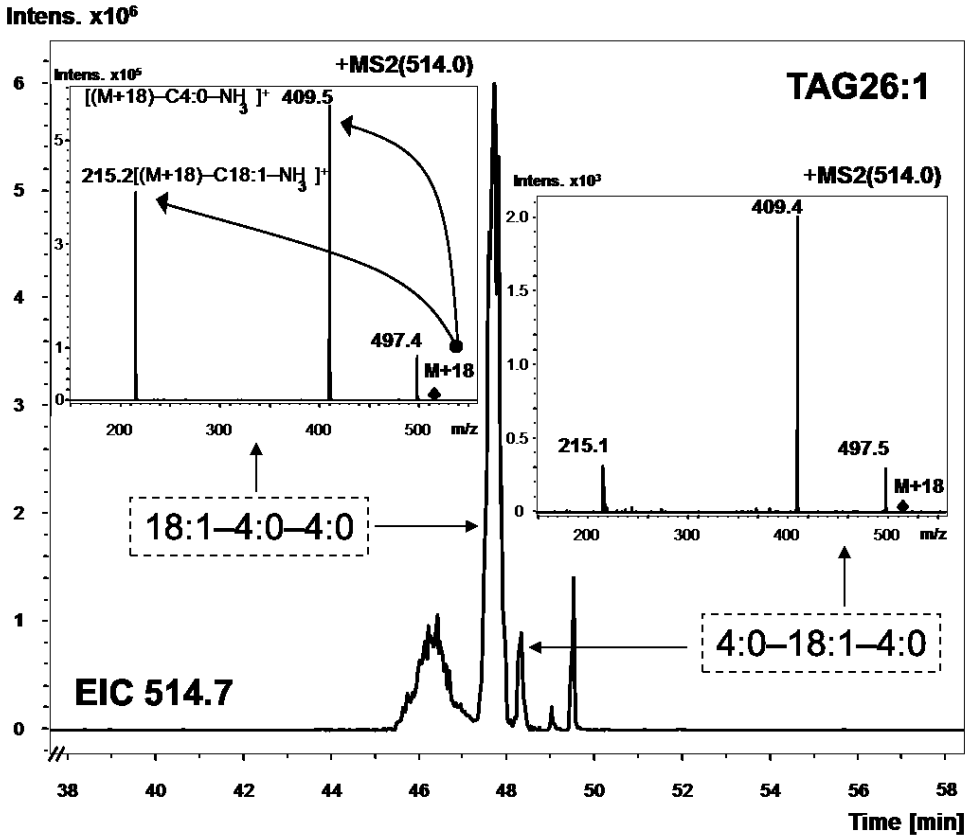

Figure 5.3. Extracted ion chromatogram (EIC) of ammonium adduct TAG 26:1, *m/z* 514.7, determined from a sample of butterfat by np-HPLC–ESI–MS and identification of the regioisomers of dibutyroyloleoylglycerol by np-HPLC–ESI–MS^2.

The product ion tandem mass spectra showed that RA of $[(M + NH_4)–NH_3–4:0]^+$ for *sn*-1(3) isomers in the diacid monobutyrate TAG class was in the range 34.5–53.9% (n = 12) and 36.7–68.2% (n = 13) in the 2 column (**IV**) and 3 column system (**V**), respectively. RAR for the same TAG class varied respectively 0.527–1.127 and 0.580–2.140. The relative abundance for *sn*-2 isomers of butyrate TAGs of the same TAG class was markedly lower and ranged from 4.1 to 13.6% (n = 9) and 4.9–12.7% (n = 13) in the studies **IV** and **V**, respectively. The RAR in L/4:0/L class varied between 0.043 and 0.157 in (**IV**) and between 0.051 and 0.146 in (**V**). The RA of $[(M+NH_4)–NH_3–6:0]^+$ and RAR varied from 40.4 to 49.5% and 0.678–0.980 (n = 6) for the *sn*-1(3) isomers in the diacid monocaproate TAG class (**IV**). For *sn*-2 isomers (L/6:0/L), the variation of RA / RAR was 11.1–21.1% / 0.125–0.267 (n = 4) when two HPLC columns are in series (**IV**). Similar trends were observed in the RA and RAR of regioisomers of monocaproate TAGs in three HPLC column system: The RA / RAR values for *sn*-1(3) isomers (n = 4) were 43.6–60.5% / 0.776–1.534 and those for *sn*-2 isomers (n= 4) 9.5–14.5% / 0.105–0.169 (**V**). In the tandem mass spectra of the regioisomers of diacid TAGs containing two short-chain acyl groups, the RAR $[(M + NH_4)–NH_3–LC\text{-}FA]^+$ / $[(M + NH_4)–NH_3–SC\text{-}FA]^+$ varied between 0.262–0.873, 0.0267–0.0941, 0.216–0.715, and 0.0604–0.220 for L/4:0/4:0, 4:0/L/4:0, L/6:0/6:0, and 6:0/L/6:0 TAG classes, respectively, when two microbore silica gel columns in series were used (**IV**). A slightly higher variation in the RAR ranges was observed when three columns in series were used (**V**): The respective RAR ranges were 0.511–2.034, 0.021–0.120, 0.283–1.636, and 0.043–0.286.

In accordance with diacid TAG classes, CID spectra of ammonium adducts for triacid mono SC-TAGs revealed lower RA and RAR for *sn*-2 than *sn*-1(3) isomers regardless of the np-HPLC–ESI–MS^2 system (**IV, V**). The relative abundance of $[(M + NH_4)–NH_3–4:0]^+$ for *sn*-2 [*sn*-1(3)] isomers was in the range 11.4–19.4% [36.4–48.5%] and 9.0–13.8% [39.9–79.7%] when 2 and 3 columns in series, respectively, were used for separation of TAG species (**IV, V**). The RAR of *sn*-2 [*sn*-1(3)] isomers ranged from 0.129 to 0.241 [from 0.572 to 0.942] in the study (**IV**), and from 0.087 to 0.171 [from 0.794 to 3.931] in the (**V**) study. A close examination of the product ion tandem mass spectra of the three isomers of enanthoylcaproylbutyroyl-*rac*-glycerol revealed that the poor cleavage of fatty acid from the *sn*-2 position was actually independent of the chain length of acyl group, even though the absolute value of RA was varying. The RAs for the $[(M+NH_4)–NH_3–FA_i]^+$ of the isomers *rac*-6:0–7:0–4:0, *rac*-7:0–6:0–4:0, and *rac*-7:0–4:0–6:0 were 44.4/18.2/37.4%, 39.3/11.7/49.0%, and 22.6/4.5/72.8% [RA(%) is in the order shown in the formula], respectively (**IV**). Based on the observation, reverse isomers (*e.g.*, *rac*-6:0–7:0–4:0 and *rac*-7:0–6:0–4:0) could be differentiated from each other if the resolving power of a chromatographic column is high enough or, at least, the main reverse isomer could be detected, even if the isomers co-eluted in the same peak.

Elution order and resolution of the regioisomers of SC-TAGs on np-HPLC columns. The resolving power of microbore silica gel columns in series was high enough to resolve regioisomers of SC-TAGs. Mono-SC-TAGs with the shortest acyl chain in the *sn*-2 position were shown to elute in the first peak followed by the two regioisomers which have the shortest acyl group in the *sn*-1(3) position of TAG molecule (*e.g.*, Fig. 5.4). Elution pattern

Table 5.8 Ratio of relative abundance ratios (RRAR) of the ions formed by cleavage of fatty acid from the primary positions *vs*. secondary position of short-chain triacylglycerols (SC-TAGs) in the ESI–MS[2]. (Table adapted from Table 2 in **V**, and from data in Tables 3–5 in **IV**.)

Class of SC-TAGs	RRAR[1]				Publ.[2]
	Mean	Range	S.D.	n	
L/L/4:0 *vs*. L/4:0/L	7.78	4.60–20.74	5.44	8	**IV**
	12.29	4.92–41.63	9.65	13	**V**
L/L'/4:0[3] *vs*. L/4:0/L'	4.60	2.38–6.28	1.82	4	**IV**
	12.27	5.35–24.53	6.08	7	**V**
L/L/6:0 *vs*. L/6:0/L	3.74	2.66–6.27	1.71	4	**IV**
	8.86	5.70–13.03	3.15	4	**V**
L/L'/6:0[4] *vs*. L/6:0/L'	4.05	2.31–6.06	1.90	3	**IV**
	9.33	6.79–12.75	3.08	3	**V**
L/4:0 /4:0 *vs*. 4:0/L/4:0	12.53	7.37–20.98	4.01	20	**IV**
	16.68	7.02–35.89	8.21	8	**V**
L/6:0/6:0 *vs*. 6:0/L/6:0	4.37	2.36–7.75	1.70	10	**IV**
	7.32	4.15–12.70	2.93	6	**V**

[1] Ratio of $[(M+NH_4)–NH_3–SC\text{-}FA]^+$/ $[(M+NH_4)–NH_3–long\text{-}chain\ (LC)\text{-}FA]^+$ of the regioisomer pairs L/L/4:0 *vs*. L/4:0/L, L/L'/4:0 *vs.* L/4:0/L', L/L/6:0 *vs*. L/6:0/L, and L/L'/6:0 *vs*. L/6:0/L'. Ratio of $[(M+NH_4)–NH_3–LC\text{-}FA]^+$/ $[(M+NH_4)–NH_3–SC\text{-}FA]^+$ of the regioisomer pairs L/4:0/4:0 *vs*. 4:0/L/4:0/L, and L/6:0/6:0 *vs*. 6:0/L/6:0.

[2] Original publication

[3] Mixture of reverse isomers L/L'/4:0 + L'/L/4:0

[4] Mixture of reverse isomers L/L'/6:0 + L'/L/6:0

of TAG species containing two or three short-chain acyls was similar. For example, the regioisomers of butyroylcaproylenanthoyl glycerols were eluted in the order (i) 1-enanthoyl-2-butyroyl-3-caproyl-*rac*-glycerol, (ii) 1-enanthoyl-2-caproyl-3-butyroyl-*rac*-glycerol, and (iii) 1-caproyl-2-enanthoyl-3-butyroyl-*rac*-glycerol (**IV**). The last two reverse isomers eluted in the leading edge and in the tailing end of the second peak, respectively.

Figure 5.4 indicates that the resolving power of three np-HPLC columns in series was much higher than two np-columns in series for regioisomers of SC-TAGs. In general, all *sn*-1(3) and *sn*-2 isomers of monobutyrates were separated with baseline resolution and dibutyrates close to it when two columns were connected in series (Fig. 5.4; A, C). However, in Figure 5.4 (B, D) it can be clearly seen that all regioisomers of butyrate TAGs were separated to the baseline on three np-columns in series. The resolution (RS) values varied between 1.6 and 2.9 and between 1.5 and 2.0 for monobutyrate and dibutyrate TAGs, respectively. In the two np-columns system, *sn*-1(3) and *sn*-2 isomers of mono- and dicaproate TAGs were separated only partially or eluted in the same peak (Fig. 5.4; E, G). However, resolution of caproates on three microbore silica gel columns in series was definitely higher: The RS value for the regioisomers of monocaproate TAGs was in the range 1.0–1.5 indicating close to the baseline resolution (Fig. 5.4; F). Regioisomers of dicaproate TAGs resolved partially on three np-columns in series – RS value varied between 0.5 and 1.3 (Fig. 5.4; H).

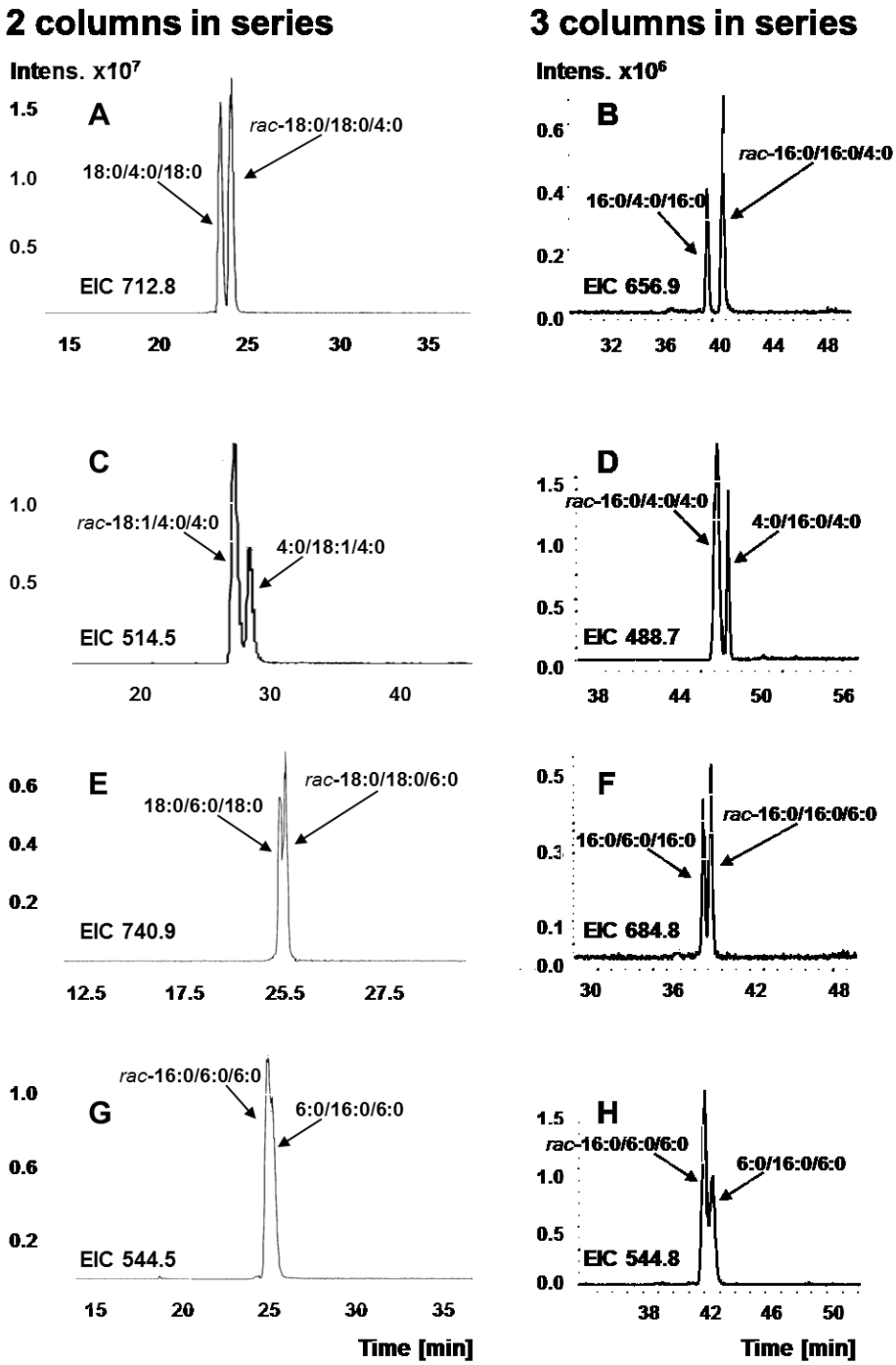

Figure 5.4. Extracted ion chromatogram (EIC) of ammonium adducts of short-chain TAG species in standard mixtures and separation of their regioisomers on two or three np-HPLC columns in series. A = Butyroyldistearoylglycerol, B = Butyroyldipalmitoylglycerol, C = Dibutyroyloleoylglycerol, D = Dibutyroylpalmitoylglycerol, E = Caproyldistearoylglycerol, F = Caproyldipalmitoylglycerol, G,H = Dicaproylpalmitoylglycerol. (Figure adapted from Figs. 2 and 4 in **IV**, and from Fig. 1 in **V**.)

Identification of MC-, LC-, and acetate TAG species by np-HPLC–ESI–MS/MS^2. Resolution and identification of medium- and short-chain molecular species of TAGs 36:0 in BF (fraction 2) using the data from ESI–MS^2 spectra recorded from the peaks ***a***–***e*** of the EIC of ammonium adduct *m/z* 656.9 are demonstrated in Figure 5.5. From the tandem mass spectra it could be deduced that in the peak ***a*** eluted medium-chain TAGs together with trilaurin. In the peak ***b*** and ***c*** eluted caproate and butyrate TAG species, respectively. Further, regioisomers of acetate TAG eluted in the last two peaks (***d***, ***e***). The MS^2 spectra showed relatively high intensity $[(M+NH_4)–NH_3–FA]^+$ ions at *m/z* 495.3, 439.4, and 411.5 which were formed *via* the loss of respective fatty acids 8:0, 12:0, and 14:0. The relatively high intensity of these ions suggested that they originated from *sn*-1(3) positions. In addition, low intensity [(M+NH4)–NH3–FA]$^+$ ions at *m/z* 467.5, 383.2, and 355.2 were most probably due to the loss of 10:0, 16:0, and 18:0, respectively, from *sn*-2 position. Hence, possible acyl chain isomers of TAG 36:0 are 12:0/12:0/12:0, 14:0/10:0/12:0, 14:0/14:0/8:0, 12:0/16:0/8:0, 10:0/18:0/8:0, and 10:0/16:0/10:0. The most intense fragment ions in the peak ***b*** are in ascending order *m/z* 411.5, 383.4, and 523.5 which refer to the loss of (ammonia and) fatty acids 14:0, 16:0, and 6:0, respectively. Low relative intensity of *m/z* 411.5 suggested cleavage of 14:0 from *sn*-2 position, hence the main acyl chain isomer in peak ***b*** is 16:0/14:0/6:0. In the peak ***c***, *m/z* 551.6 and *m/z* 383.3 are the most abundant ions in the MS^2 spectra suggesting that the main butyrate acyl chain isomer is 16:0/16:0/4:0. However, small amount of *m/z* 411.5 and *m/z* 355.4 indicates existence of another butyrate isomer, 18:0/14:0/4:0. Even though resolution on a np-HPLC column and MS^2 analysis showed no *sn*-2 isomer of butyrate and caproate TAGs, they showed existence of both acetate regioisomers of TAG 36:0 in the peaks ***d*** and ***e***. The high relative abundance of *m/z* 579.6 indicates that *sn*-1(3) isomers elute in the later peak. High intensity of *m/z* 383.4 and 355.4 suggested that structure of the acetate TAGs in the peaks ***d*** and ***e*** was 18:0/2:0/16:0 and (18:0/16:0/2:0 + 16:0/18:0/2:0), respectively.

Figure 5.6 illustrates resolution and identification of even-numbered long-chain TAGs of BF on np-HPLC–ESI–MS^2. In Figure 5.6 (A), EIC of ammonium adduct of TAG 50:1 (*m/z* 850.9) showed two peaks (***a***, ***b***). Product ion tandem mass spectra revealed that the acyl chain isomers of TAG 50:1 with one 20:0 acyl group in the molecule eluted in the first peak (***a***) and the rest long-chain isomers of TAG 50:1 eluted in the second peak (b). In MS^2 spectra from the peak ***a***, the fragment ions originated from the loss of 12:0, 14:0, 14:1, 16:0, 16:1, 18:1, and 20:0 (+ ammonia) were detected. From these, the most abundant were *m/z* 521.6 (from 20:0) and 551.6 (from 18:1) resulting in the following structures of TAG species: 20:0/12:0/18:1, 16:0/20:0/14:1 (+20:0/16:0/14:1), and 20:0/14:0/16:1. Figure 5.6 (B) shows the EIC of *m/z* 935 (TAG class 56:1) and product ion tandem mass spectra at 40.6 and 41.9 min (peaks ***c*** and ***d***). In both peaks, the same $[(M+NH_4)–NH_3–FA]^+$ ions, *m/z* 605.7, 633.8, and 635.7 were detected indicating the loss of FAs 20:0, 18:0, and 18:1, respectively, from the ammonium adduct. However, the relative intensity of *m/z* 605.7 is markedly lower in the spectra from the peak ***d*** suggesting that FA 20:0 originated from *sn*-2 position. The most probable structures of the acyl chain isomers of TAG 56:1 were (20:0/18:0/18:1 + 20:0/18:1/18:0) and 18:1/20:0/18:0 in the peaks ***c*** and ***d***, respectively. Hence, the resolving power of the np-HPLC column used was high enough to separate regioisomers of 20:0/X/X and X/20:0/X from each other.

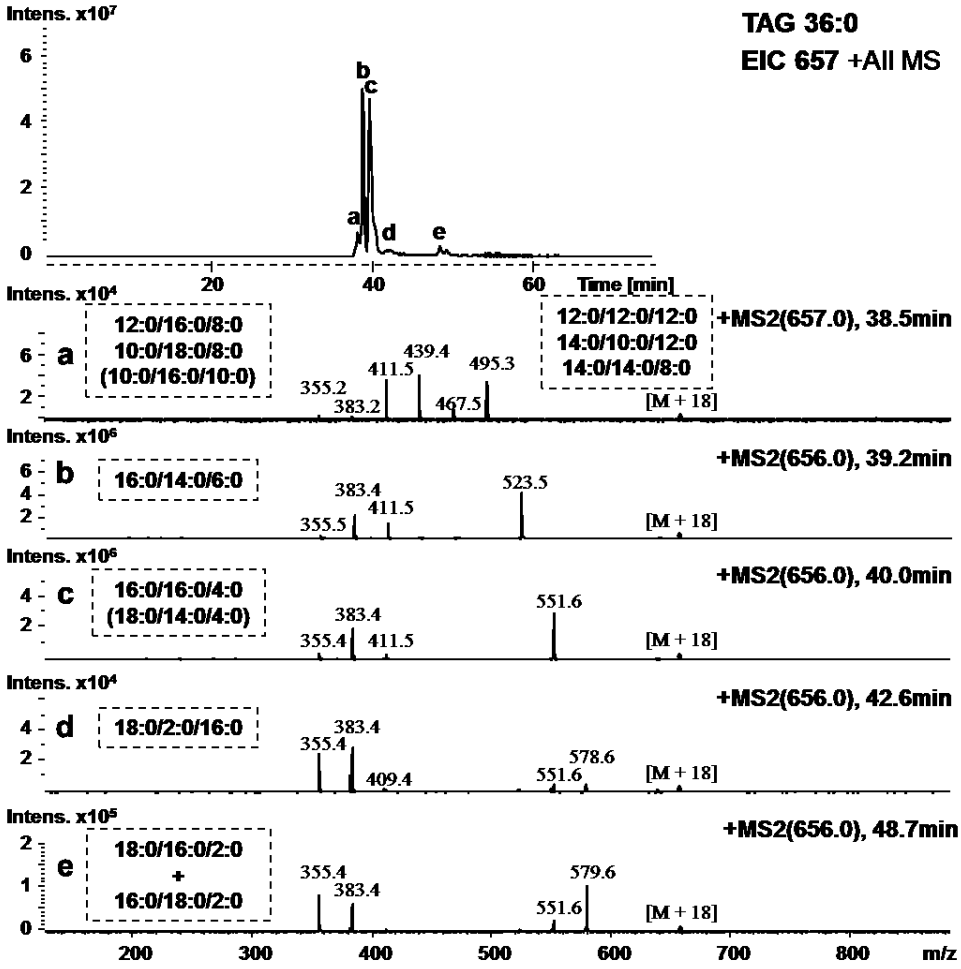

Figure 5.5. Extracted ion chromatogram (EIC) of the ammonium adduct of TAG 36:0, *m/z* = 656.9. An example of the identification of medium- and short-chain TAG species (including regioisomers of monoacetate TAG) in the fraction II of butterfat (**VII**) from the data of tandem mass spectra of the peaks ***a–e*** of EIC. (Figure adapted from Fig. 2b in **VII**.)

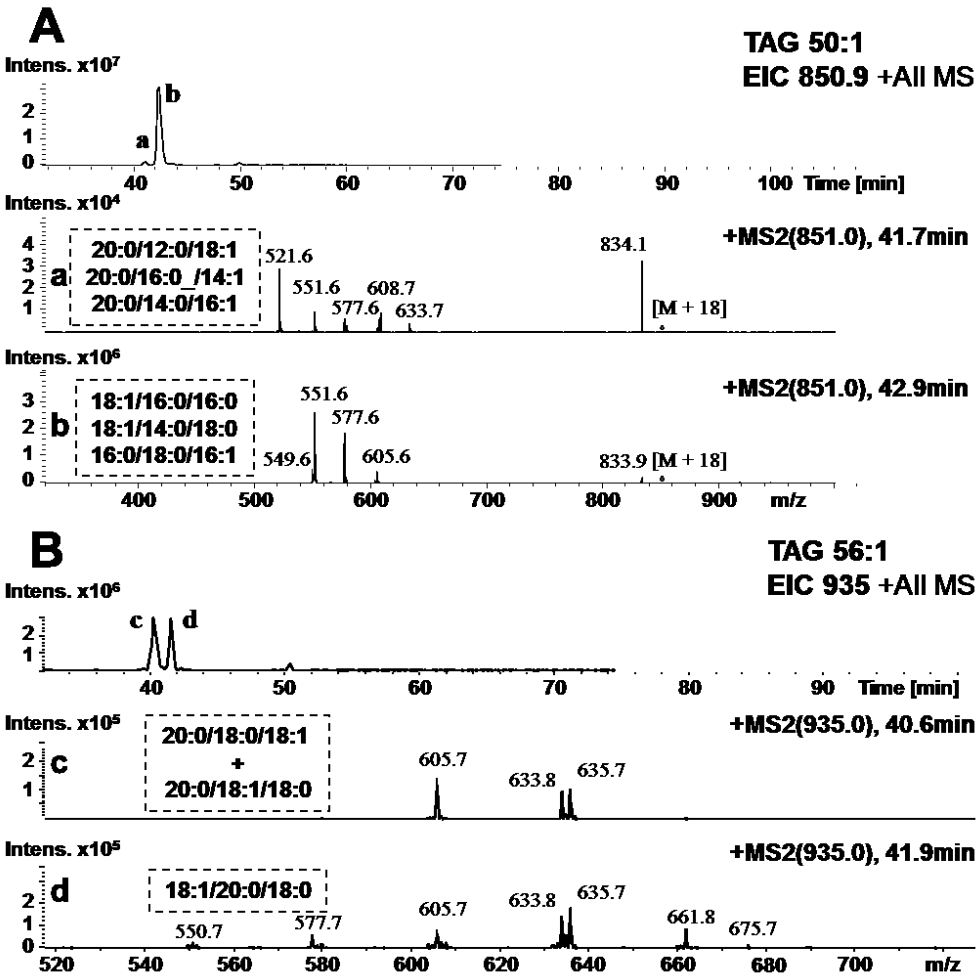

Figure 5.6. Extracted ion chromatograms (EIC) of the ammonium adducts of TAG 50:1, *m/z* = 850.9 (**A**), and TAG 56:1, *m/z* = 935.0 (**B**). Examples of resolution and identification of acyl chain and regioisomers of long-chain TAG species in butterfat from the data of tandem mass spectra of the peaks ***a–d***. (Figure adapted from Figs. 4 and 5 in **VII**.)

5.4 Quantification of the molecular species of triacylglycerols

5.4.1 Determination of molar correction factors

Empirical correction factors (ECF) for fatty acid methyl esters (FAMEs) in gas-liquid chromatography using FID. A commercial FAME standard (GLC-74; Nu-Chek-Prep, Inc.) was used for the determination of CFs. The results of six successive analysis of the standard

Table 5.9 Empirical correction factors (ECFs) for weight-% of fatty acids (FAs) determined by gas-liquid chromatography repeating the analysis of a FA methyl ester standard (GLC-74, Nu-Chek-Prep) six times (n = 6). (Table adapted from Table 2 in **I**.)

FA	ECF	S.D.
4:0	1.64	0.037
6:0	1.26	0.025
8:0	1.12	0.022
10:0	1.05	0.020
12:0	1.02	0.019
14:0	1.02	0.017
16:0	0.99	0.010
18:0	1.00	0.000
20:0	1.02	0.011
22:0	1.05	0.012
18:1	0.99	0.002
18:2	1.05	0.003
18:3	1.17	0.003

are listed in Table 5.9. ECFs were in accordance with the theoretical correction factors (Christie 1989a) used for FID-GC analysis, except that ECFs for methyl butyrate and polyunsaturated methyl esters were slightly higher. The reproducibility of determinations was adequate for whole range of FAME, however, the highest variation was observed in the analysis of very short-chain and long-chain FAMEs.

Molar correction factors (MCF) for TAG species analyzed by gas-liquid chromatography using FID. Use of experimental CFs in quantification of BF is essential due to the wide range of different molecular species of TAGs. MCFs for monoacid TAG species were determined by repeating six times the analysis of TAG mixture containing seven monoacid TAG species (24:0, 30:0, 36:0, 42:0, 48:0, 54:0 and 54:3) and giving the MCFs in relation to TAG 36:0 (Table 5.10, **I**). ECFs showed only a moderate increase with highest (TAG 54:0) and lowest (TAG 24:0) molecular weight TAGs (Table 5.10, **I**). As ECFs determined by linear calibration were converted to respective ECF values (*i.e.*, related to TAG 36:0), increase in ECFs of high molecular weight TAGs was even more moderate suggesting improved stability of newer phenyl(65%)methylsilicone columns (Table 5.10, **III**).

Effect of the concentration of calibration mixtures on MCFs was assessed by linear calibration using five concentrations of the mixture of nine monoacid TAGs (12:0, 18:0, … , 54:0, 54:3) in which the relative concentration of each analyte was 1, 2, 4, 10, and 20, and the range of $[C]_{TAG(i)}$ was 62.5–1250 or 125–2500 mg/5 mL. In addition, equal amount of internal standard (IS) (trinonanoin, [C] = 167 μg/5 mL) was added in each TAG mixture. Triplicate measurements were performed and the ratios of $n_{analyte}$ / n_{IS} and those of $A_{analyte}$ / A_{IS} were plotted according to an example shown in Figure 5.7 A. The linear regression curve (y = ax) was calculated and the slope (a) was used as MCF for TAG species. Very high average coefficient of determination (r^2_{AVG} = 0.997, n = 9) for all ACN:DB classes of TAGs (Table 5.10) suggested an excellent reproducibility of measurements and high linearity of

Table 5.10 Empirical molar correction factors (MCFs) for selected monoacid TAG species determined by gas-liquid chromatography using six separate determinations (**I**) or linear calibration (n = 15) method (**III**). (Table adapted from Table 3 in **I**, and from Table 4 in **III**.)

		I[1]		**III**			
ACN:DB[2]	TAG species	MCF	S.D.	MCF	Error	r^2 [3]	$MCF_{36:0}$[4]
12:0	4:0/4:0/4:0			2.165	0.039	0.995	2.670
18:0	6:0/6:0/6:0			1.405	0.022	0.997	1.732
24:0	8:0/8:0/8:0	1.294	0.021	1.020	0.011	0.999	1.258
30:0	10:0/10:0/10:0	1.104	0.018	0.904	0.008	0.999	0.111
36:0	12:0/12:0/12:0	1.000	0.000	0.811	0.006	0.999	1.000
42:0	14:0/14:0/14:0	0.939	0.015	0.759	0.009	0.998	0.936
48:0	16:0/16:0/16:0	1.021	0.034	0.736	0.016	0.993	0.908
54:0	18:0/18:0/18:0	1.569	0.129	0.864	0.018	0.994	1.065
54:3	18:1/18:1/18:1	1.721	0.342	0.905	0.017	0.995	1.116

[1] Original publication
[2] ACN:DB = Number of acyl carbons:number of double bonds
[3] r^2 = coefficient of determination
[4] MCF values are given in relation to tridodecanoylglycerol (TAG 36:0), *cf.*, MCF in (**I**)

the MCFs, even though two different phenyl(65%)methylsilicone columns had to be used in the analysis. In addition, Figure 5.7 B shows that MCFs for monoacid TAG species had a parabolic dependence on the ACN, which enabled to extrapolate MCFs for the other ACN classes of TAGs. Further, only a moderately higher MCF for unsaturated TAG 54:3 than for saturated TAG 54:0 (Table 5.10) resulted in the use of the same MCFs for TAG species with the same ACN but a different degree of unsaturation in quantification of TAGs.

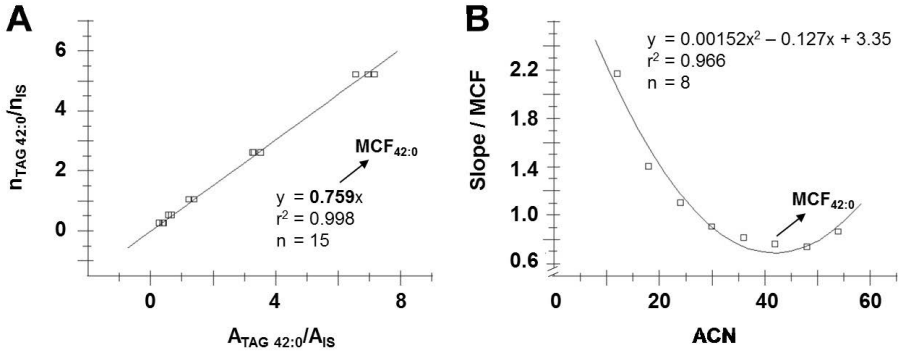

Figure 5.7. (A) Determination of empirical molar correction factor (MCF) for tritetradecanoylglycerol (TAG 42:0) as an example of using linear calibration method in quantitative gas-liquid chromatography. $A_{TAG\ 42:0}$ / A_{IS} = the ratio of the area of TAG 42:0 to the area of internal standard (IS, trinonanoylglycerol); $n_{TAG\ 42:0}$ / n_{IS} = molar ratio of TAG 42:0 to IS. **(B)** Dependence of MCF on the number of acyl carbons (ACN) of saturated TAGs determined by nonlinear curve fitting; n = number of determinations; r^2 = coefficient of determination. (Figure adapted from Figs. 1 and 2 in **III**.)

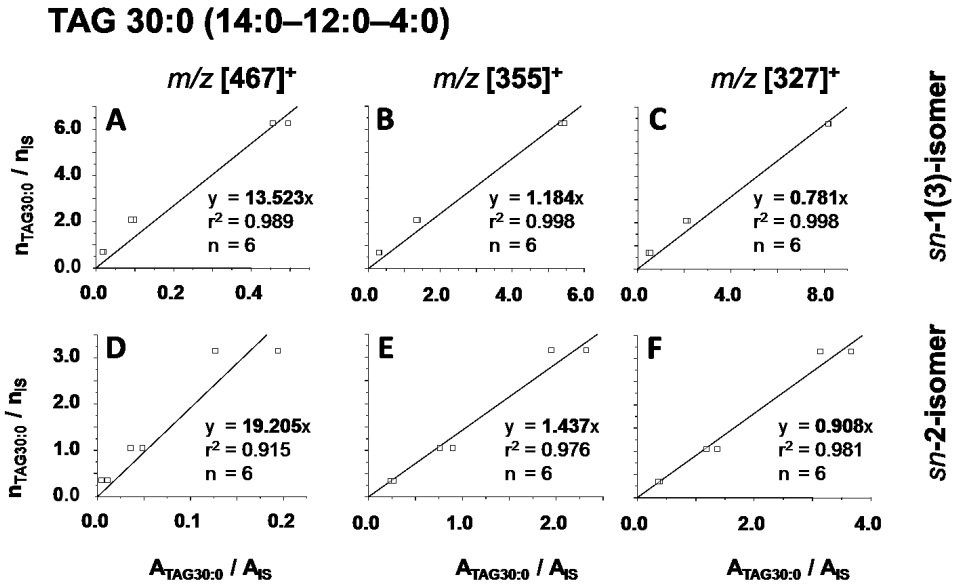

Figure 5.8. An example of the determination of empirical molar correction factor (MCF) for [M–RCOO]$^+$ ions of the regioisomers of triacid short-chain TAG species using linear calibration method in quantitative GC–EI–MS. Determination of MCFs for *sn*-1(3)-isomers (4:0/12:0/14:0 + 4:0/14:0/12:0 + 14:0/12:0/4:0 + 12:0/14:0/4:0) is shown in the plots A–C and that for *sn*-2 isomers (12:0/4:0/14:0 + 14:0/4:0/12:0) in the plots D–F. $A_{TAG\ 30:0}$ / A_{IS} = the ratio of the area of [M–RCOO]$^+$ ions (*m/z* [467]$^+$, [355]$^+$, [327]$^+$) to the area of internal standard (trinonanoylglycerol, *m/z* [355]$^+$); $n_{TAG\ 30:0}$ / n_{IS} = molar ratio of TAG 30:0 to IS; n = number of determinations; r^2 = coefficient of determination. (Figure adapted from Fig. 1 in **VI**.)

Molar correction factors (MCF) for [M–RCOO]$^+$ ions of TAG species analyzed by gas-liquid chromatography–EI–mass spectorometry. Molecular species of TAGs in 28 randomized standard mixtures of known composition were used in the determination of MCFs for [M–RCOO]$^+$ ions (see **VI**). In GC–EI–MS, MCFs were determined by a linear calibration method according to the same principles described above in the GC–FID analysis. However, only duplicate measurements and three different molar ratios of analyte/internal standard (n_i / n_{IS}) were used in linear calibration. Concentration of internal standard (trinonanoin) was 59.0 mmol/L in each mixture and the relative concentrations of the analytes were 1, 3, and 9. The molar amount of each TAG species (n_i) in the randomized TAG mixtures, including regioisomers of SC-TAGs, were determined by GC (Appendix 1, **III**). However, the theoretical molar ratio (2.0:1.0) of *sn*-1(3) and *sn*-2 isomers was used to substitute for the determined molar ratio, as the experimental ratio was < 1.9:1.0 or > 2.1:1.0. The integrated area of [M–RCOO]$^+$ ions (A_i, A_{IS}) was determined from selected ion chromatograms of EI–GC. Figure 5.8 illustrates an example of the use of linear calibration for the determination of MCFs for the regioisomers of a triacid SC-TAG (butyroyllauroyl-myristoylglycerol) in GC–EI–MS. The molar ratios of the analyte (4:0–12:0–14:0) to IS (9:0/9:0/9:0) were determined in three different concentrations of standard mixture and plotted against the area ratio of three [M–RCOO]$^+$ ions of the analyte (*m/z* [467]$^+$, [355]$^+$, and [327]$^+$ which are formed after the cleavage of 4:0, 12:0, and 14:0, respectively) to the

$[M–RCOO]^+$ ion of IS (*m/z* $[355]^+$), after which the linear regression curve (y = ax) was calculated. The slope (a) of the curve was the MCF for each $[M–RCOO]^+$ ion. In addition, Figure 5.8 demonstrates a couple of general trends: (i) The higher the *m/z* value of $[M–RCOO]^+$ ion was, the higher was the MCF derived from the same TAG species (Appendix 2 and Table 5.11); (ii) the MCFs for *sn*-2 isomers of SC-TAGs were higher than those of *sn*-1(3) isomers of the same SC-TAG (Table 5.11). However, few exceptions were observed (Appendix 2 and Table 5.11).

Altogether, experimental MCFs for $[M–RCOO]^+$ ions were determined for 147 and 79 ion species derived from EI–MS of 69 saturated and 35 unsaturated molecular species of TAGs, respectively (Appendix 2). MCFs varied from 0.366 to 15.190, from 0.275 to 12.992, and from 0.577 to 41.621 for saturated, monoene, and polyene triacylglycerols, respectively. The reproducibility of GC–EI–MS analysis was not markedly affected by the wide variation of the size of $[M–RCOO]^+$ ions, concentration, and origin of the $[M–RCOO]^+$ ions. The coefficient of determinations (r^2) was on the average (±S.D.) 0.975 (±0.043) for saturated TAGs and 0.963 (±0.115) for unsaturated TAGs. Further, r^2 was lower than 0.9 in only nine cases (Appendix 2).

In addition, experimental MCFs were determined for 136 $[M–RCOO]^+$ ions originated from 26 regioisomer pairs of mono short-chain TAG species (Table 5.11). MCFs of the *sn*-1(3) isomers of SC-TAGs were relatively similar to the MCFs of other TAGs ranging from 0.406 to 13.523, from 0.438 to 11.393, and from 0.609 to 31.555 for saturated, monene, and polyene SC-TAGs, respectively (Table 5.11). In general, the variation of MCFs was markedly higher for the *sn*-2 isomers varying between 0.578 and 19.205, 0.461 and 26.547, 0.522 and 104.898 for saturated, monoene, and polyene TAGs, respectively, as shown in Table 5.11. Coefficient of determination was 0.948 ± 0.126 and 0.890 ± 0.210 for *sn*-1(3) and *sn*-2 isomers of mono SC-TAGs, respectively, indicating slightly lower repeatability than for other TAGs which was probably due to the lack of baseline resolution, hence, more challenging integration of ion chromatograms.

Although, a significant number of MCFs for $[M–RCOO]^+$ ions were empirically determined, MCFs for several major TAG species of BF had to be extrapolated on the basis of the reasonable dependences of empirical MCFs. For example, Figure 5.9 demonstrates a general exponential decay dependence of the MCFs for different $[M–RCOO]^+$ ion on the ACN of TAGs providing a handy tool for deducing missing MCF from the known number of acyl carbons of TAG. Other trends were observed in the ratio of the MCFs of saturated TAGs (MCF_{Sat}) to that of unsaturated TAGs (MCF_{UNSat}) with the same number of acyl carbons, which assisted to extrapolate the missing MCFs for unsaturated TAG species: The average ratio of MCF_{Sat} / MCF_{UNSat} was 1.264 ± 0.209 (n = 30) for the same $[M–RCOO]^+$ ion, for example *m/z* $[383]^+$ from TAG 10:0–10:0–18:0 and 10:0–10:0–18:1 (Appendix 2). Another interesting dependences were noticed for unsaturated TAGs, in which the *m/z* of $[M–RCOO]^+$ ion was two units lower (Type A ion) than that of corresponding saturated TAG (*e.g.*, *m/z* $[465]^+$ from CyOO *vs. m/z* $[467]^+$ from CySS), and for TAGs, in which the *m/z* (Type B ion) was four units lower than that of saturated TAG (*e.g.*, *m/z* $[548]^+$ from MPoPo *vs. m/z* $[552]^+$ from MPP).The ratio of MCF_{Sat} / MCF_{UNSat} was on the average 0.752 ± 0.226 (n = 74) and 0.410 ± 0.130 (n = 17) for type A and type B ions, respectively (Appendix 2).

Table 5.11 Empirical molar correction factors (MCFs) for $[M–RCOO]^+$ ions of the regioisomers of short-chain TAG species determined by GC–EI–MS. (Table adapted from Table 4 in **VI**.)

ACN:DB[1]	TAG species	Ion[2]	*sn*-1(3) MCF	$r^{2\,3}$	*n*	*sn*-2 MCF	r^2	*n*	Ratio
28:0	4:0-12:0-12:0	439	10.428	0.673	30	12.048	0.282	18	1.16
		327	0.473	0.867	30	0.578	0.840	27	1.22
30:0	4:0-12:0-14:0	467	13.523	0.989	6	19.205	0.915	6	1.42
		355	1.184	0.998	6	1.437	0.976	6	1.21
		327	0.781	0.998	6	0.908	0.981	6	1.16
32:0	4:0-12:0-16:0	495/6	11.090	0.972	6	18.127	0.989	5	1.63
		383	1.535	0.992	6	1.798	0.996	6	1.17
		327	0.703	0.994	6	0.944	0.966	6	1.34
32:0	4:0-14:0-14:0	495/6	7.287	0.884	23	17.239	0.724	21	2.37
		355	0.406	0.971	30	0.706	0.739	30	1.74
32:1	4:0-12:0-16:1	493/4	10.043	0.939	6	14.959	0.931	4	1.49
		381	2.402	0.995	6	2.101	0.994	6	0.87
		327	0.438	0.988	6	0.461	0.968	6	1.05
34:0	4:0-14:0-16:0	523/4	4.831	0.978	6	12.936	0.982	6	2.68
		383	0.688	0.991	6	1.354	0.986	6	1.97
		355	0.458	0.991	6	0.911	0.986	6	1.99
34:0	4:0-12:0-18:0	523/4	8.107	0.975	6	16.250	0.688	6	2.00
		411	1.605	0.991	6	2.439	0.929	6	1.52
		327	0.595	0.998	6	1.029	0.905	6	1.73
34:1	4:0-14:0-16:1	521/2	11.397	1.000	4	17.728	0.999	4	1.56
		381	2.082	0.994	6	1.973	0.997	6	0.95
		355	0.540	0.995	6	0.543	0.997	6	1.01
36:0	4:0-16:0-16:0	551/2	6.371	0.784	20	9.359	0.169	15	1.47
		383	0.435	0.967	24	0.606	0.860	24	1.39
36:0	4:0-14:0-18:0	551/2	8.972	0.997	6	18.345	0.988	6	2.04
		411	1.413	0.999	6	2.281	0.984	6	1.61
		355	0.716	0.999	6	1.136	0.988	6	1.59
36:1	4:0-14:0-18:1	549/50	10.566	0.990	6	21.351	0.985	6	2.02
		409	2.706	1.000	6	3.351	0.977	6	1.24
		355	0.555	1.000	6	0.753	0.993	6	1.36
36:1	6:0-12:0-18:1	521/2	4.543	0.983	6	13.896	0.965	4	3.06
		437	2.715	0.989	6	6.680	0.958	4	2.46
		355	0.473	0.990	6	1.152	0.943	4	2.44
36:1	6:0-14:0-16:1	521/2	7.943	0.995	6	26.547		2	3.34
		409	2.392	0.995	6	4.700	0.960	6	1.96
		383	0.590	0.996	6	1.175	0.950	6	1.99
36:2	4:0-16:1-16:1	547/8	31.555	0.229	4	104.898	0.525	4	3.32
		381	0.609	0.985	12	0.522	0.976	12	0.86
38:0	6:0-16:0-16:0	551/2	5.150	0.917	11	11.394	0.055	6	2.21
		411	0.656	0.972	18	1.048	0.740	15	1.60

ACN:DB[1]	TAG species	Ion[2]	*sn*-1(3) MCF	r^2 [3]	*n*	*sn*-2 MCF	r^2	*n*	Ratio
38:0	6:0-14:0-18:0	551/2	3.597	0.999	6	11.252	0.855	4	3.13
		439	1.355	0.998	6	3.752	0.976	4	2.77
		383	0.630	0.998	6	1.713	0.973	4	2.72
38:0	4:0-16:0-18:0	579/80	6.471	0.984	6	13.813	0.996	4	2.13
		411	1.085	0.993	6	1.492	0.995	6	1.38
		383	0.719	0.993	6	1.096	0.997	6	1.53
38:1	4:0-16:0-18:1	577/8	6.654	0.984	6	14.418	0.992	5	2.17
		409	2.055	0.996	6	2.615	0.999	6	1.27
		383	0.632	0.998	6	0.811	0.989	6	1.28
38:1	6:0-14:0-18:1	549/50	6.857	0.999	6	20.871	0.990	6	3.04
		437	2.947	0.999	6	5.720	0.994	6	1.94
		383	0.620	0.998	6	1.266	0.989	6	2.04
38:2	6:0-16:1-16:1	547/8	19.566	0.982	4	52.902		2	2.70
		409	0.666	0.989	6	0.799	0.993	6	1.20
40:0	6:0-16:0-18:0	579/80	4.844	0.980	4	10.523	0.974	4	2.17
		439	1.376	0.979	6	2.022	0.989	6	1.47
		411	0.979	0.979	6	1.434	0.992	6	1.47
40:0	4:0-18:0-18:0	607/8	9.839	0.946	12	16.623	0.944	12	1.69
		411	0.588	0.991	18	0.707	0.991	18	1.20
40:0	6:0-18:0-18:0	607/8	5.970	0.991	6	16.941	0.980	6	2.84
		439	0.961	0.992	12	1.470	0.919	12	1.53
40:1	6:0-16:0-18:1	577/8	6.743	0.944	4	19.355	0.989	4	2.87
		437	3.218	0.993	6	4.057	0.996	6	1.26
		411	1.035	0.990	6	1.317	0.993	6	1.27
40:2	4:0-18:1-18:1	603/4	16.717	0.497	8	31.325	0.173	5	1.87
		409	0.793	0.924	12	0.747	0.937	12	0.94
42:2	6:0-18:1-18:1	603/4	17.663	0.645	8	31.603	0.550	7	1.79
		437	1.093	0.768	18	1.144	0.919	18	1.05

[1] ACN:DB = Number of acyl carbons:number of double bonds
[2] $[M–RCOO]^+$ ion
[3] r^2 = coefficient of determination

Interesting trends were noticed when MCFs for the regioisomers of SC-TAGs were compared. In general, the MCFs of the *sn*-2 (= $MCF_{sn\text{-}2}$) isomer of mono SC-TAGs were higher than those of the *sn*-1(3) (= $MCF_{sn\text{-}1(3)}$) isomers. The ratio $MCF_{sn\text{-}2}$ / $MCF_{sn\text{-}1(3)}$ was 1.94 ± 0.54 (n = 16) and 2.72 ± 0.50 (n = 10) for monobutyrate and monocaproate TAGs, respectively (Table 5.11). Further, the ratio of the MCF for the sum of both regioisomers (= $MCF_{(sn\text{-}1(3)+sn\text{-}2)}$) to the $MCF_{sn\text{-}1(3)}$ was 1.175 ± 0.071 (n = 36) and 1.139 ± 0.120 (n =33) for the saturated and unsaturated mono SC-TAGs, respectively (Table 5.11). The ratio for the other regioisomer, $MCF_{(sn\text{-}1(3)+sn\text{-}2)}$ / $MCF_{sn\text{-}2}$, was respectively 0.701 ± 0.155 (n = 36) and 0.710 ± 0.217 (n = 33) for saturated and unsaturated mono SC-TAGs (Table 5.11).

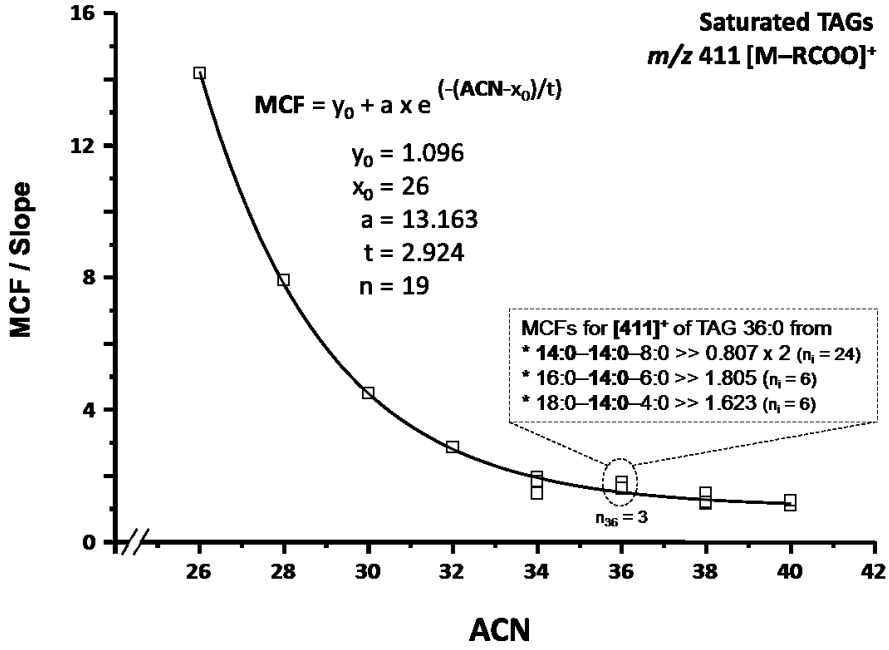

Figure 5.9. An example of using empirical molar correction factors (MCFs) for *m/z* $[411]^+$ ion and nonlinear curve fitting to extrapolate missing MCFs of saturated TAG species on the basis of acyl carbon number (ACN); n = number of all empirically determined MCFs for *m/z* $[411]^+$; n_{36} = number of data points for TAG 36:0 (*i.e.*, MCFs for different acyl chain isomers); n_i = number of empirically determined MCFs of each acyl chain isomer. (Figure adapted from Fig. 2 in **VI**.)

Molar correction factors (MCF) for $[M + NH_3]^+$ ions of ACN:DB TAG classes analyzed by np-HPLC–ESI–MS. MCFs for the correction of ion yield were determined for 20 and 42 pairs of regioisomers of SC-TAGs in randomized TAG mixtures using np-HPLC–ESI–MS with two and three columns in series, respectively (**IV, V**). MCFs were determined by linear calibration using three analyte / internal standard (IS; trinonoanoin) ratios. The areas of each TAG species (A_i) and IS (A_{IS}) were determined by integration from extracted ion chromatograms of ammonium adducts and the ratio A_i / A_{IS} of each analyte–IS pair was calculated. The molar amount of the regioisomers of SC-TAG species (n_i) were analyzed by GC (Appendix 1, III) and the ratio n_i / n_{IS} was determined. Due to the observed random composition of interesterified TAG mixtures (see above), the molar amounts of the regioisomers of SC-TAG species were calculated from the whole amount of the analyte by dividing the amount in random ratio 2:1 for *sn*-1(3) and *sn*-2 isomers, when the low resolution system of two columns in series was used. The ratios n_i/n_{IS} *vs.* A_i/A_{IS} were plotted and the linear regression curves (y = ax) were calculated and slope-values (a) were used as MCFs for the correction of ion yield (*cf.*, Fig. 5.10). As an example of the linearity of response, linear regression curves for the regioisomers of selected di- and triacid short-chain butyrate TAGs (*i.e.,* dipalmitoylbutyroylglycerol, oleoylpalmitoylbutyroylglycerol, and dibutyroylpalmitoylglycerol) are presented in Figure 5.10. In general, the regression analysis revealed excellent linearity and confirmed different MCFs for different regioisomers and acyl chain isomers of SC-TAGs. The coefficient of determination (r^2) was on the average

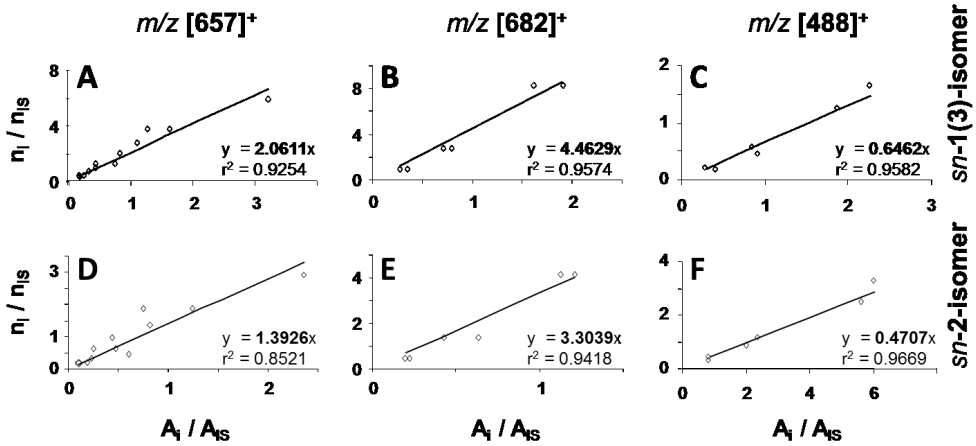

Figure 5.10. Determination of molar correction factors (MCFs) for $[M + NH_4]^+$ ions of the regioisomers of selected di- and triacid short-chain butyrate TAGs using linear calibration method in quantitative np-HPLC–ESI–MS. Determination of MCFs for correction of ion field for *sn*-1(3) isomers of dipalmitoylbutyroylglycerol (4:0/16:0/16:0 + 16:0/16:0/4:0), oleoylpalmitoylbutyroyl-glycerol (18:1/16:0/4:0 + 16:0/18:1/4:0 + 4:0/18:1/16:0 + 4:0/16:0/18:1), and dibutyroyl-palmitoylglycerol (16:0/4:0/4:0 + 4:0/4:0/16:0) is shown in the plots A,B, and C, respectively, and those for *sn*-2 isomers of 16:0/4:0/16:0 , 18:1/4:0/16:0 (+ 16:0/4:0/18:1), and 4:0/16:0/4:0 in the plots D, E, and F, respectively. A_i / A_{IS} = the ratio of the area of $[M + NH_4]^+$ ions of analyte to the internal standard (trinonanoylglycerol); n_i / n_{IS} = molar ratio of the isomers to IS; r^2 = coefficient of determination. (Figure adapted from Fig. 3 in **V**.)

0.990 ± 0.021 (n = 40) and 0.933 ± 0.058 (n = 78) when 2 columns and 3 columns in series, respectively, were used in determination (**IV, V**). The variation of the MCFs for regioisomers was higher (0.217–5.847) in the analysis using 3 columns in series than in those using 2 columns in series (0.386–2.695) which was most probably due to the increased number of different isomers and lack of the correction of integrated areas of regioisomers (**IV, V**). The difference of the MCFs of *sn*-1(3) and *sn*-2 isomers was evident in most cases. The difference varied between 1.4 and 80.4% and between 2.0 and 45.4% as 3 and 2 columns in series, respectively, were used for determination (**IV, V**).

Figure 5.11 shows polynomial dependence of MCFs for regioisomers on the ACN of the saturated diacid SC-TAGs with adequately high coefficient of determination (r^2) varying between 0.8298 and 0.9442. However, the MCFs for the regioisomers of triacid butyrate and caproate TAGs differed markedly from corresponding diacid TAG isomers (marked with open square in Figure 5.11) and MCFs of acyl chain isomers differed from each other (Fig. 5.11). For example, if triacid TAGs of *sn*-2 isomers of monobutyrate TAGs (Fig. 5.11 C) were included in the calculation of regression curve, the regression equation was $y = 0.005x^2 – 0.1995x + 2.3936$ and the coefficient of variation was lower: $r^2 = 0.7098$. MCFs for the regioisomers of monoene butyrate TAGs showed a similar polynomial dependence on ACN than saturated TAGs, but the r^2 was lower ranging from 0.6835 to 0.7395 (**V**). Further, the MCFs for the regioisomers of unsaturated monocaprate TAGs showed linear dependence on the ACN (r^2 = 0.6755–0.9291) (**V**).

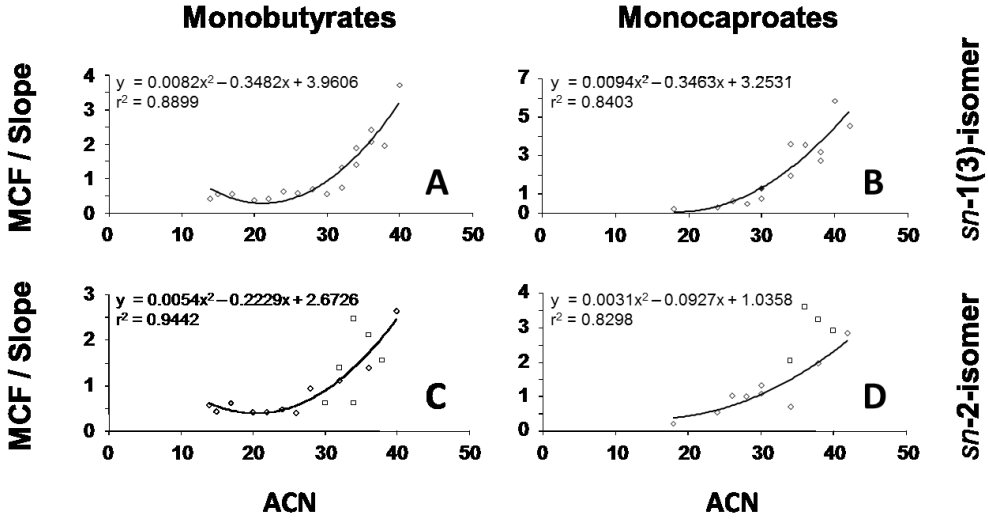

Figure 5.11. Examples of the polynomial dependence of empirical molar correction factors (MCFs) for $[M + NH_4]^+$ ions on the acyl carbon number (ACN) of saturated diacid short-chain TAG species determinded by HPLC–ESI–MS.; Open diamond = X/X/4:0-*rac*-glycerols **(A)**; Open diamond = X/X/6:0-*rac*-glycerols **(B)**; Open diamond = X/4:0/X diacid glycerols, open square = X/4:0/X' triacid glycerols **(C)**; Open diamond = X/6:0/X diacid glycerols, Open square = X/6:0/X' triacid glycerols **(D)**; X, X' = medium- or long-chain acyls; r^2 = coefficient of determination. (Figure adapted from Fig. 4 in **V**.)

In order to quantify the major ACN:DB classes in butterfat, MCFs for the correction of ion yield were determined by exploiting the calculated TAG composition of randomized butterfat (*cf.*, Kalo et al. 1986a), known amount of added internal standard (trinonanoin), and integrated areas of ammonium adduct of the main ACN:DB classes of RBF from ESI–MS ion chromatograms. The MS data was recorded from the four SPE fractions of randomized BF in duplicate by np-HPCL–ESI–MS with three columns in series and ion chromatograms of 105 most common ACN:DB classes of TAGs were extracted. The uncorrected molar amount, *i.e.*, area mol (n_{iA}) of each TAG class was calculated as follows: $n_{iA} = n_{IS} \times A_i/A_{IS}$, where n_{IS} = molar amount of IS, A_i and A_{IS} = integrated areas of TAG class and IS, respectively. The uncorrected molar amount of each ACN:DB from the four SPE fractions were added up ($n_{ACN:DB} = n_{FR1} + n_{FR2} + n_{FR3} + n_{FR4}$) after identification which based on the similar RRTs and ESI–MS^2 spectra of the $[M + NH_3]^+$ peaks in EIC of the four fractions. The uncorrected mol% (area mol%) of a TAG class was calculated in the following way: area $mol\%_{ACN:DB} = 100\% * ni_{ACN:DB} / ni_{TOT}$, where ni_{TOT} = sum of all uncorrected amounts. MCF for an ACN:DB class of TAGs was obtained by dividing the calculated (= random) mol% by area $mol\%_{ACN:DB}$ of the ACN:DB class. MCFs for regioisomers and acyl chain isomers of TAGs were calculated in a corresponding manner. The MCFs for principal ACN:DB classes and TAG species with 12–54 acyl carbons detected in BF (plus ACN:DB 25:1 and 27:1) are listed in Appendix 3. Some interesting trends were detected in the MFCs for even-numbered saturated TAGs of ACN:DB and X/X/M classes. The MCFs were relatively stable for TAGs 18:0–40:0 varying from 0.256

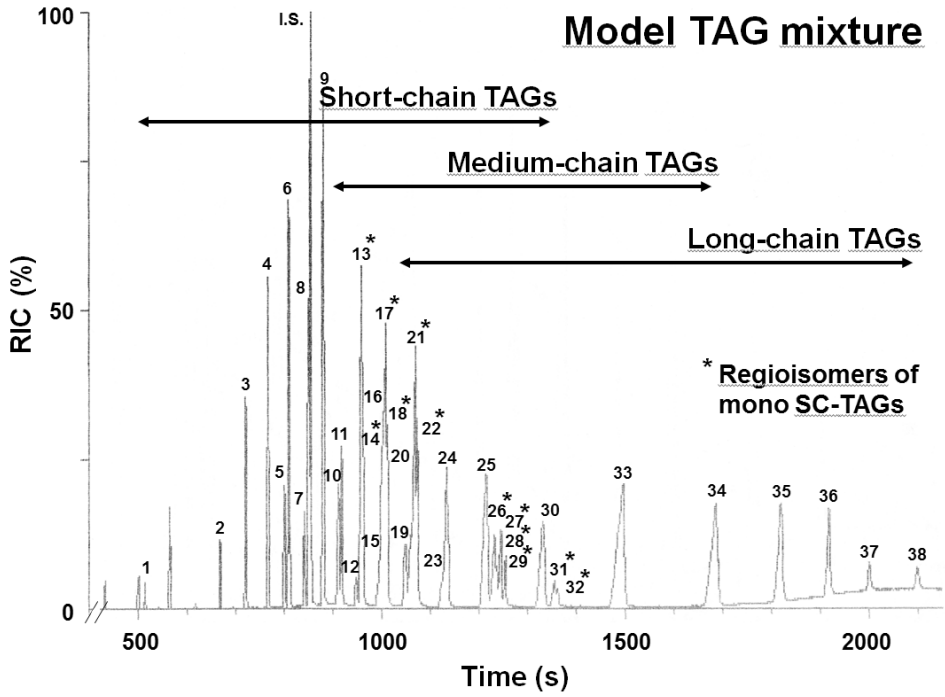

Figure 5.12. A reconstructed ion chromatogram (RIC) of the model TAG mixture. GC–EI–MS conditions are described in Section 4.2. **1:** 4:0/4:0/4:0; **2:** 6:0/6:0/6:0; **3:** 12:0-4:0-4:0; **4:** 14:0-4:0-4:0; **5:** 12:0-6:0-6:0-12:0; **6:** 16:0-4:0-4:0; **7:** 14:0-6:0-6:0; **8:** 18:0- 4:0-4:0; **9:** 12:0-12:0-4:0 + 16:0-6:0-6:0; **10:** TAG 10:0/10:0/10:0 + 12:0-12:0-6:0; **11:** 18:0-6:0-6:0; **12:** 12:0-10:0-10:0; **13:** 14:0/14:0/4:0 + 16:0/12:0/4:0; **14:** 14:0/4:0/14:0 + 12:0/4:0/16:0; **15:** 12:0-12:0-10:0; **16:** 14:0-14:0-6:0 + 16:0-12:0-6:0; **17:** 16:0/14:0/4:0 + 18:0/12:0/4:0; **18:** 14:0/4:0/16:0 + 12:0/4:0/18:0; **19:** 12:0/12:0/12:0; **20:** 16:0-14:0-6:0; **21:** 16:0/16:0/4:0 + 18:0/14:0/4:0; **22:** 16:0/4:0/16:0 + 14:0/4:0/18:0; **23:** 18:0-10:0-10:0; **24:** 16:0-16:0-6:0; **25:** 16:0-12:0-12:0 + 18:0-12:0-10:0; **26:** 18:0/16:0/6:0; **27:** 16:0/6:0/18:0; **28:** 18:0/18:0/4:0; **29:** 18:0/4:0/18:0; **30:** 14:0/14:0/14:0 + 18:0-12:0-12:0; **31:** 18:0/18:0/6:0; **32:** 18:0/6:0/18:0; **33:** 16:0-14:0-14:0 + 16:0-16:0-12:0; **34:** 16:0-16:0-14:0 + 18:0-14:0-14:0 + 18:0-18:0-10:0; **35:** 16:0/16:0/16:0 + 18:0-18:0-12:0; **36:** 18:0-16:0-16:0 + 18:0-18:0-14:0; **37:** 18:0-18:0-16:0; **38:** 18:0/18:0/18:0; **IS** = 9:0/9:0/9:0. (Figure adapted from Fig. 3 in **VI**.)

to 0.708 (mean = 0.433, and S.D. = 0.158, n = 11) as shown in Appendix 3, but increased rapidly from TAG 40:0 to 48:0 and decreased after that. Rather similar pattern for MCFs of the even-numbered monoene TAGs of the same TAG classes was observed: the MCFs for monoene TAGs were in the range of 0.227 to 0.692 (mean = 0.441, S.D. = 0.146, n = 10) for TAGs 22:1–42:1, after which the MCFs increased from ACN:DB 42:1 to 50:1, followed by decrease to TAG 54:1 (Appendix 3). The MCFs for odd-numbered saturated TAGs of ACN:DB and X/X/M classes were generally lower than those of the adjacent even-numbered TAGs, but some exceptions were detected, *i.e.*, the MCFs for TAG 25:0, 27:0, 35:0, and 37:0 (Appendix 3). In addition, the specific MCFs for regioisomers and acyl chain isomers showed definite trends. The ratio of the MCFs X/4:0/4:0 to 4:0/X/4:0 was 1.60 and 1.40 in ACN:DB classes 24:0 and 26:1, respectively (Appendix 3). Wide variation was observed in the MCFs for acyl chain isomers of even-numbered X/X/4:0 and X/X/6:0

between ACN 28 and 40. The ratio butyrate isomer / caproate isomer was on the average 2.86 ± 1.38 (n = 13) (Appendix 3).

5.4.2 Quantification of TAG species in model mixtures

Determination of TAGs in model mixtures by GC–EI–MS using MCFs for [M–RCOO]$^+$ ions. A model mixture of saturated even-numbered TAG species consisting of 20 different ACN:DB TAG classes and 45 different TAG species (excluding regioisomers) was quantified by GC–EI–MS. A reconstructed ion chromatogram (RIC) of the [M–RCOO]$^+$ ions of the TAGs in model mixture is presented in Figure 5.12. The resolution was not as high as in GC analysis (*cf.*, Fig. 5.1). However, 38 peaks were detected as shown in Figure 5.12, and several acyl chain isomers were separated to the baseline, together with the partially resolved *sn*-1(3) and *sn*-2 isomers of mono SC-TAGs marked with '*'.

Table 5.12 shows empirical molar content ([C], μmol/25 mL chloroform; as a mean of triplicate measurements) of TAG species in a model mixture determined by GC–EI–MS using MCFs for [M–RCOO]$^+$ ions, and calculated composition derived from the volumetric and gravimetric data from the preparation of the mixture. In most cases, molar content (n_i) was determined using MCFs (MCF_i) for the lowest molecular weight specific [M–RCOO]$^+$ ions due to their highest intensity in MS spectra, as follows: $n_i = (A_i * MCF_i * n_{IS}) / (A_{IS} * MCF_{IS})$, where A_i (A_{IS}) = area of the specific ion (internal standard), MCF_i (MCF_{IS}) = molar correction factor for the specific ion (IS), and n_i = molar amount of IS. The fragment ion was reckoned to be specific, if it is unique among any other adjacent isobaric TAG species or if the resolution of the GC column was high enough to enable reliable integration of the areas of the chromatographic peaks. If no specific ion could be found and resolution was poor, the molar content of TAG species was determined on the basis of a calculated area of a non-specific ion $[a_i]^+$ (= $A_{[ai]}$) according to the following hypothesis: *(i)* The area of a non-specific ion $[a]^+$ = sum of the areas of the same ion (= $A_{sum[a]}$) formed from all different isobaric and non-resolved molecular species of TAGs, hence, $A_{[ai]} = A_{sum[a]} - (A_{[aj]} + A_{[ak]}, +\ldots)$, where $A_{[aj]} + A_{[ak]}, +\ldots$ = sum of the areas of the ion $[a]^+$ cleaved from all other isobaric TAG species. *(ii)* The area of other isobaric TAGs ($TAG_{j,k,\ldots}$), *e.g.*, $TAG_j = A_{[aj]} = (n_{[bj]} * MCF_{IS} * A_{IS}) / (n_{IS} \times MCF_{[aj]})$, where $n_{[bj]}$ = molar amount of TAG_j calculated on the basis of a specific ion $[b]^+$, $MCF_{[aj]}$ = molar correction factor for a non-specific ion $[a]^+$, MCF_{IS} / n_{IS} / A_{IS} = molar correction factor / molar amount /area of internal standard, respectively.

In spite of several co-eluting acyl chain isomers of TAGs in the model mixture, all 45 different TAG species could be quantified by a combined GC and MS analysis, together with 9 *sn*-1(3)/*sn*-2 pairs of mono SC-TAGs (Table 5.12). Hence, the number of identified individual molecular species was much high higher (54 *vs*. 26) than that detected by plain high-resolution GC (**VI**), even though GC–EI–MS had much lower resolution. Variation of determinations (= RSD%) was on the average 5.3 ± 4.7% (mean ± S.D.), showing adequate repeatability of determinations (Table 5.12). However, the average RSD% was 8.6 ± 6.3 for the 9 pairs of regioisomers suggesting a more challenging integration of only partially resolved peaks of *sn*-1(3) and *sn*-2 isomers (Table 5.12).

The accuracy of the GC–EI–MS analysis was assessed by comparing the empirical composition to the calculated composition. The molar amount of the sum of all TAG species in the model mixture determined by GC–MS differed only 2% from the calculated amount (Table 5.12). The molar content of SC-, MC-, and LC-TAGs determined by GC–MS deviated 1, 3, and 7%, respectively, from the calculated molar content, indicating excellent accuracy for the determination of specific subgroups of TAG species (Table 5.12). A much higher average relative error (17%) was observed between the empirical molar amounts of individual TAG species and their calculated amounts (Table 5.12). However, the analyzed molar amount of 41% of TAG species differed from calculated amount less than 10%. Further, for 20% of TAG species, the relative error was higher than 25%.

The similar quantification method using MCFs for $[M–RCOO]^+$ ions was applied to the analysis of a more complex model mixture: TAG composition of randomized butterfat (RBF) was determined and compared to calculated random composition (**VI**). Altogether, 83, 56, and 28 individual TAG species or groups of identified TAGs were determined in the saturated, monoene, and polyene TAG fractions, respectively (data in **VI:** Tables 6–8). The average relative error between mol% of determined and calculated TAGs with ACN 22–44 (14%) was close to that observed for individual TAGs of much simpler TAG mixture above (17%). However, mol% of TAGs with 46, 52, and 54 acyl carbons differed markedly from that of calculated composition. Accuracy for the determination of subgroups of TAG species in RBF was lower than that for subgroups of model mixture probably due to the much higher number of molecular species. Total amount of saturated, monoene, and polyene TAGs differed from the calculated total amount 15%, 1%, and 41%, respectively (**VI**, Tables 6–8). Further, the average relative error for the group of abundant (> 0.5 mol%) mono short-chain TAG species was 20% (n = 24).

Determination of TAGs in a randomized model mixture by np-HPLC–ESI–MS using MCFs based on area mol% of $[M+NH_4]^+$ions and calculated random mol%. An interesterified mixture (BMO) of three equimolar monoacid TAGs (4:0/4:0/4:0 + 14:0/14:0/14:0 + 18:1/18:1/18:1) was used as model mixture to confirm the applicability of the quantification method (see Chapter 5.4.1) that was applied to the analysis of TAGs in butterfat by np-HPLC–ESI–MS (**VII**). The MS data for the ammonium adducts of the TAGs in the solution A of BMO was recorded in duplicate and the areas of the peaks of different TAG species were integrated. Uncorrected molar amount and uncorrected mol% (= area mol%) for 15 TAG species in BMO were determined and random composition (mol%) was calculated. MCF for each TAG species was calculated by dividing the mol% with area mol%. The MCFs varied between 0.320 and 3.695 (**VII**), which was expected due to the wide range of ACN:DB classes in the model mixture. The molar composition (n_i) of the solution B of BMO was determined using the MCFs, area data of EIC of ammonium adducts from a duplicate analysis (A_{iB1}, A_{iB2}), and molar amount and area data of the internal standard (trinonanoin) (Table 5.13). In addition, the accuracy of the quantification method was assessed by comparing the empirical molar composition to the calculated random composition and the molar composition determined by GC as shown in Table 5.13. The RSD% of duplicate measurements was on the average 7.7 ± 7.4% (Table 5.13). The average relative error between the molar amounts of the individual TAG species determined by

Table 5.12 Content ([C], μmol/25 mL chloroform; mol%) of the molecular species of TAGs in the standard mixture of known (calculated) composition determined by GC–EI–MS using molar correction factors for the area of $[M–RCOO]^+$ ions (n = 3). (Table adapted from Table 5 in **VI**.)

ACN:DB[1]	TAG species	RRT[2]	Peak No.[3]	EI–MS $[Ion]^+$ [4]	[C][5]	RSD(%)[6]	mol%	Calculated[7] [C]	mol%
Short-chain TAGs									
12:0	4:0/4:0/4:0[8]	0.601	1	215	0.954	3.2	0.67	0.895	0.64
18:0	6:0/6:0/6:0	0.782	2	271	1.720	2.2	1.20	1.729	1.23
20:0	12:0-4:0-4:0[9]	0.844	3	215	3.819	1.7	2.67	3.771	2.69
22:0	14:0-4:0-4:0	0.897	4	215	4.940	2.8	3.46	4.597	3.27
24:0	12:0-6:0-6:0	0.938	5	271	2.062	2.6	1.44	2.231	1.59
24:0	16:0-4:0-4:0	0.949	6	215	4.580	2.1	3.20	5.298	3.77
26:0	14:0-6:0-6:0	0.985	7	271	1.583	2.0	1.11	1.562	1.11
26:0	18:0-4:0-4:0	0.995	8	215	3.889	1.8	2.72	3.263	2.32
28:0	12:0-12:0-4:0[10]	1.029	9	327	3.456	10.1	2.42	4.233	3.01
28:0	16:0-6:0-6:0	1.032	9	271	5.929	3.4	4.15	5.797	4.13
30:0	12:0-12:0-6:0	1.037	10	355	2.179	0.8	152	2.250	1.60
30:0	18:0-6:0-6:0	1.074	11	271	1.765	2.7	1.23	1.905	1.36
32:0	14:0/14:0/4:0	1.122	13	355	3.483	11.4	2.44	3.284	2.34
32:0	16:0/12:0/4:0	1.122	13	327	4.241	3.6	2.97	3.556	2.53
32:0	14:0/4:0/14:0	1.125	14	355	1.252	7.0	0.88	1.642	1.17
32:0	16:0/4:0/12:0	1.125	14	327	1.176	21.7	0.82	1.850	1.32
34:0	14:0-14:0-6:0	1.172	16	383	2.655	4.0	1.86	1.483	1.06
34:0	16:0-12:0-6:0	1.172	16	355	4.491	3.2	3.14	4.248	3.03
34:0	16:0/14:0/4:0	1.179	17	355	1.590	3.9	1.11	3.770	2.68
34:0	18:0/12:0/4:0	1.179	17	327	2.274	2.6	1.59	1.600	1.14
34:0	16:0/4:0/14:0	1.184	18	355	1.443	10.9	1.01	1.885	1.34
34:0	18:0/4:0/12:0	1.184	18	327	1.000	19.2	0.70	0.839	0.60
36:0	16:0-14:0-6:0	1.250	20	523/24	3.431	0.4	2.40	3.088	2.20
36:0	18:0/14:0/4:0	1.250	21	355	3.504	4.4	2.45	3.062	2.18
36:0	16:0/16:0/4:0	1.250	21	551/2	2.726	8.1	1.91	3.896	2.78
36:0	18:0/4:0/14:0	1.256	22	355	2.059	6.2	1.44	1.531	1.09
36:0	16:0/4:0/16:0	1.256	22	551/2	1.339	8.0	0.94	1.948	1.39
38:0	16:0-16:0-6:0	1.325	24	411	6.045	2.0	4.23	5.130	3.65
40:0	18:0/16:0/6:0	1.440	26	411	2.083	5.8	1.46	2.207	1.57
40:0	18:0/6:0/16:0	1.446	27	411	0.717	21.4	0.50	1.104	0.79
40:0	18:0/18:0/4:0	1.456	28	411	2.231	2.2	1.56	2.195	1.56
40:0	18:0/4:0/18:0	1.467	29	411	1.286	2.0	0.90	1.091	0.78
42:0	18:0/18:0/6:0	1.584	31	439	1.307	7.6	0.91	1.245	0.89
42:0	18:0/6:0/18:0	1.591	32	439	0.711	8.1	0.50	0.608	0.43
	Σ (SC-TAG)				**87.920**		**61.51**	**88.793**	**63.24**

ACN:DB[1]	TAG species	RRT[2]	Peak No.[3]	EI–MS [Ion]+ [4]	[C][5]	RSD(%)[6]	mol%	Calculated[7] [C]	mol%
Medium-chain TAGs									
30:0	10:0/10:0/10:0	1.063	10	383	0.280	2.7	0.20	0.295	0.21
32:0	12:0-10:0-10:0	1.132	12	383[11]	0.821		0.57	0.884	0.63
34:0	12:0-12:0-10:0	1.163	15	439	1.284	8.4	0.90	0.919	0.65
38:0	18:0-10:0-10:0	1.316	23	383	0.918	4.5	0.64	0.855	0.61
40:0	18:0-12:0-10:0	1.425	25	411	1.654	5.9	1.16	1.822	1.30
46:0	18:0-18:0-10:0	1.975	34	607/8	0.903	3.1	0.63	0.927	0.66
	Σ (MC-TAG)				**5.860**		**4.10**	**5.702**	**4.06**
Long-chain TAGs									
36:0	12:0/12:0/12:0	1.228	19	439	2.332	0.2	1.63	2.787	1.98
40:0	16:0-12:0-12:0	1.420	25	439	5.018	3.0	3.51	4.828	3.44
42:0	14:0/14:0/14:0	1.553	30	495/6	2.859	0.7	2.00	2.586	1.84
42:0	18:0-12:0-12:0	1.559	30	439	2.803	3.0	1.96	2.347	1.67
44:0	16:0-16:0-12:0	1.750	33	551/2	6.925	4.9	4.85	4.685	3.34
44:0	16:0-14:0-14:0	1.750	33	523/4	5.687	5.6	3.98	4.919	3.50
46:0	18:0-14:0-14:0	1.970	34	495/6[11]	3.098		2.17	2.641	1.88
46:0	16:0-16:0-14:0	1.970	34	523/4	4.632	7.3	3.24	5.154	3.67
48:0	16:0/16:0/16:0	2.125	35	551/2	4.494	2.4	3.14	4.170	2.97
48:0	18:0/18:0/12:0	2.129	35	523/4	2.972	1.2	2.08	2.120	1.51
50:0	18:0-18:0-14:0	2.240	36	607/8	2.574	5.1	1.80	3.039	2.16
50:0	18:0-16:0-16:0	2.240	36	579/80	2.100	4.5	1.47	2.130	1.52
52:0	18:0-18:0-16:0	2.339	37	579/80	1.593	6.9	1.11	2.111	1.50
54:0	18:0/18:0/18:0	2.453	38	607/8	2.069	6.3	1.45	2.409	1.72
	Σ (LC-TAG)				**49.156**		**34.39**	**45.926**	**32.70**
	Total				**142.936**		**100.00**	**140.421**	**100.00**

[1] ACN:DB = Number of acyl carbons:number of double bonds

[2] RTT = Relative Retention Time; the retention time of internal standard (TAG 27:0, $[355]^+$) = 1.000.

[3] For peak numbers see Figure 5.1

[4] The specific $[M–RCOO]^+$ ion, the area of which was used for determinations and calculations

[5] Average of triplicate measurements; $[C]_i = ([C]_{IS} * A_i * RF_i) / (A_{IS} * RF_{IS})$, where $[C]_{IS}$ = concentration of internal standard (IS); A_i = the area of the specific ion of an analyte; A_{IS} = the area of the specific ion of IS; RF_i = molar correction factor for the specific ion of an analyte; RF_{IS} = molar correction factor of IS = 1.000

[6] The relative standard deviations (%)

[7] Calculation is based on volumetric and gravimetric measurements in preparation of the model TAG mixture and known molar composition of component TAG mixtures

[8] TAG structure with slashes (/; *e.g.,* 4:0/4:0/4:0) indicates known (regioisomeric) structure

[9] Structure/regioisomer (*e.g.*, 12:0-4:0-4:0) is not known

[10] Underlined structure indicates that more than one TAG species are eluted in the same peak

[11] n = 1

ESI–MS and those calculated according to the random distribution was 12.4 ± 9.2% (Table 5.13). However, the total molar amount of the 15 TAG species was almost the same in the calculated and determined composition. The relative difference between determined and calculated molar amounts of SC-TAGs and LC-TAGs was 1.7 and 3.4%, respectively, indicating excellent accuracy for the determination of large subgroups of TAGs. The molar amounts of TAG species determined by quantitative ESI–MS and GC methods were rather similar: the average relative error was 15.4% for individual TAG species (Table 5.13). The ratio of *sn*-1(3) to *sn*-2 isomers of the short-chain TAG species was on the average 2.0 (S.D. = 0.4; n = 10) as was expected for the randomized TAG mixture.

Table 5.13 Molar amount (n_i) of the molecular species of TAGs in a randomized standard mixture with a known (calculated) composition determined by LC–ESI–MS using molar correction factors based on the use of internal standard (9:0/9:0/9:0), random composition of respective TAG mixture and uncorrected molar amount (area mol) of ammonium adducts of TAG species. (Table adapted from Table 1 in **VII**.)

ANC:DB[1]	TAG species	ESI–MS n_i (B1)	n_i (B2)	n_i (avg)	%RSD[2]	CALC n_i[3]	GC n_i[4]	Δ (%) MS *vs.* Calc.[5]	MS *vs.* GC
12:0	4:0/4:0/4:0	0.078	0.098	0.088	15.9	0.082	0.041	7.2	53.4
22:0	14:0/4:0/4:0	0.151	0.174	0.163	10.5	0.176	0.231	8.0	2.9
	4:0/14:0/4:0	0.074	0.077	0.076	2.6	0.088		16.2	
26:1	18:1/4:0/4:0	0.133	0.190	0.162	24.8	0.186	0.230	15.0	8.5
	4:0/18:1/4:0	0.089	0.091	0.090	0.2	0.093		3.2	
32:0	14:0/4:0/14:0	0.118	0.122	0.120	2.5	0.094	0.099	21.4	17.5
	14:0/14:0/4:0	0.194	0.172	0.183	8.7	0.189	0.159	3.1	13.1
36:1	18:1/4:0/14:0	0.195	0.186	0.191	3.1	0.200	0.204	4.8	7.1
	18:1_/14:0/4:0[6]	0.404	0.396	0.400	1.3	0.399	0.356	0.2	11.0
40:2	18:1/4:0/18:1	0.085	0.093	0.089	6.7	0.106	0.096	18.7	7.9
	18:1/18:1/4:0	0.220	0.242	0.231	6.9	0.211	0.175	8.6	24.2
	Σ(SC-TAG)			1.791		1.822	1.591	1.7	11.2
42:0	14:0/14:0/14:0	0.117	0.104	0.111	8.1	0.101	0.110	8.3	0.5
46:1	18:1/14:0/14:0	0.272	0.206	0.239	19.8	0.322	0.341	35.4	43.6
50:2	18:1/18:1/14:0	0.422	0.423	0.423	0.2	0.340	0.398	19.4	5.8
54:0	18:1/18:1/18:1	0.148	0.140	0.144	3.5	0.120	0.138	16.6	4.2
	Σ(LC-TAG)			0.915		0.883	0.987	3.4	7.9
	Total	2.700	2.714	2.707		2.706	2.578	0.0	4.7

[1] ACN:DB = Number of acyl carbons:number of double bonds

[2] %RSD = Relative standard deviation (%): %RSD = (100 * S.D.)/mean

[3] Calculated composition of the randomized standard mixture based on the molar amount of component standard mixtures of monoacid TAGs (4:0/4:0/4:0, 14:0/14:0/14:0, 18:1/18:1/18:1)

[4] Gas chromatographic analysis on a polarizable phenylmethylsilicone capillary column (**III, VI**)

[5] Difference in the molar amount of TAG species determined by ESI–MS *vs*. GC or calculated amount

[6] 18:1_/14:0/4:0 indicates 18:1/14:0/4:0 + 14:0/18:1/4:0

Table 5.14 Proportion (mol%) of triacylglycerol classes in butterfat determined by three chromatographic and mass spectrometric methods. ESI–MS = normal-phase liquid chromatography–electrospray mass spectrometry; EI–MS = gas chromatography–electron impact mass spectrometry; GC = high-resolution gas chromatography. **III**, **VI**, and **VII** refer to original publications.

TAG class		ESI–MS (**VII**) mol%	EI–MS (**VI**) mol%	Norm.[1] mol%	GC (**III**) mol%	Norm. mol%
Saturated TAGs	even-numbered	34.42	44.89	38.72	39.13	37.42
	NIMS[2]	2.61			5.69	
	odd-numbered	1.98				
	NIMS	1.02				
Monoene TAGs	even-numbered	32.93	34.06	29.38	36.95	35.33
	NIMS	3.05			3.61	
	odd-numbered	1.78				
	NIMS	0.70				
Polyene TAGs	even-numbered	18.88	21.02	18.13	14.08	13.47
	NIMS	2.36			0.23	
	odd-numbered	0.17				
	NIMS	0.07				
Total		100.0	100.0		99.7	
Identified even-numbered TAGs		86.2	100.0	86.2	90.2	86.2

[1] Total amount of identified TAG species is normalized to 86.2 mol% (= total amount of TAGs determined by np-HPLC–ESI–MS)
[2] NIMS = not identified molecular species

5.4.3 Quantification of TAG species in butterfat

Molar composition of the TAG species in BF was determined by three different chromatographic and mass spectrometric methods using appropriate specific MCFs. If no specific MCF was available, MCF was extrapolated in GC (**III**) and GC–EI–MS analysis (**VI**) as described in Chapter 5.4.1. In np-HPLC–ESI–MS analysis (**VII**), the MCFs of butyrate TAGs were used for the MCFs of acetate TAGs with the same ACN and, correspondingly, MCFs for TAGs with 54 acyl carbons were exploited for MCFs of TAGs with ACN 55–57 and the same degree of unsaturation. For other TAG species, MCF of corresponding ACN:DB class substituted for specific MCF. Prior to the analysis, the TAGs of BF were fractionated according to the degree of unsaturation (**III, VI**) or molecular weight (**V**, **VII**). In GC and GC–MS methods (**III, VI**) only even-numbered TAG species were identified and quantified, but odd-numbered TAGs were included in the analysis when the HPLC–MS methods (**V, VII**) were used. Special attention was paid on the acyl chain isomers and regioisomers of short-chain TAGs.

Table 5.14 summarizes the proportion (mol%) of saturated, monoene, and polyene TAGs in butterfat. The proportion of identified even-numbered saturated TAGs varied between 34.4 and 38.7 mol% of all TAG species. Even-numbered saturated TAGs were the most abundant

TAG class in butterfat regardless of the analysing method. The variation in the proportions of even-numbered monoene TAGs (29.4–35.3 mol%) and polyene TAGs (13.5–18.9 mol%) was at the same level than that of saturated TAGs.

Proportion (mol%) of the identified even-numbered molecular species of saturated, monoene, and polyene TAGs in butterfat determined by the three methods (ESI–MS, EI–MS, and GC) is summarized in Tables 5.15–5.17, respectively. The most probable structure of the regioisomers of several TAG species determined by ESI–MS^2 is also depicted. In addition, the columns '1-2-3A' and 'S-M-L TAG classes' in Tables 5.15–5.17 show categorization of TAG species according to the number of different fatty acyls in TAG molecule and the carbon chain length of the three fatty acyls in TAGs, respectively.

As classification of TAGs according to the number of acyl carbons (ACN) and the number of double bonds (DB) was used, identified saturated even-numbered TAGs were detected between ACN 18 and 56 (+ ACN 12), monoene TAGs between ACN 24 and 56, and polyene TAGs between ACN 36 and 56 in the present study (Tables 5.15–5.17). DB of identified polyene TAGs was in the range of two to five. All quantification methods showed that the most abundant ACN classes of saturated TAGs in butterfat were in order ACN 36:0, 38:0, and 34:0 composing together 15.7–18.8 mol% of all TAGs (Table 5.15). The abundance profile of monoene TAGs was slightly different with two distinct maxima at ACN 38:1–40:1 and at ACN 50:1 (Table 5.16). The most abundant ACN class was ACN 38:1 (5.4–6.0 mol%). The proportion of ACN 40:1 and 50:1 varied between 3.6 and 4.1 mol%, and 3.6 and 4.9 mol%, respectively. The abundance profile of ACN classes of polyene TAGs was slightly leaned towards high molecular weight TAGs. All methods showed that ACN 52:2 (3.3–3.7 mol%) and 50:2 (2.1–3.5 mol%) were the most prominent ACN classes of polyene TAGs in butterfat (Table 5.17).

Classification of TAGs according to the number of different esteried fatty acids in TAG molecule (*cf*., Tables 5.15–5.17) provided a useful piece of information about the TAG structure of milk fat. The HPLC–ESI–MS analysis showed that practically all monoacid TAGs of ACN:DB classes 12:0–54:0 (except 24:0) and 54:3 existed in butterfat, but only 4:0/4:0/4:0, 6:0/6:0/6:0, and 18:0/18:0/18:0 could be quantified (0.2 mol% in total) as individual TAG species (Tables 5.15 and 5.17). However, the most abundant individual molecular species of monoacid TAGs were determined as a major TAG in groups of mono-, di-, and triacid TAG species as follows: 14:0/14:0/14:0, 2.8 mol% (a group of six major TAGs); 16:0/16:0/16:0, 2.4 mol%; 18:1/18:1/18:1, 1.6 mol% (last two groups consisted of one minor and two major TAGs) (Tables 5.15 and 5.17). Respectively, triolein (1.0 mol%), tripalmitin (0.5 mol%) and trimyristin (0.3 mol%) were shown to be the three most abundant monoacid TAG species in EI–MS analysis (Tables 5.15 and 5.17). These observations suggested that the real amount of monoacid TAGs in analyzed butterfat was 2–3 mol% in total. In GC and GC–EI–MS analyses, the proportion of diacid and triacid TAGs ranged from 25 to 29 mol% and from 43 to 47 mol%, respectively, confirming that triacid TAGs were the most abundant TAG class composing 50–55% of all individually determined even-numbered TAGs in butterfat. In HPLC–ESI–MS analysis, the proportion of di- and triacid TAGs determined as individual TAG species was definitely lower (6 and 25 mol%, respectively), however, their proportion as mixed TAG groups was considerable (48 mol%).

Table 5.15 Composition (mol%) of the identified molecular species of saturated even-numbered TAGs in butterfat determined by normal-phase liquid chromatography–electrospray mass spectrometry (ESI–MS), gas chromatography–electron impact mass spectrometry (EI–MS), and gas chromatography (GC). **III**, **VI**, and **VII** refer to original publications. (Table adapted from Table 5 in **III**, Table 6 in **VI**, and Table 3 in **VII**.)

ACN:DB[1]	TAG species or *Σ(TAG groups)*	S-M-L TAG classes[2]	1-2-3A[3]	ESI–MS (**VII**) mol%	±S.D.[4]	EI–MS Norm.[5] (**VI**) mol%	±DEV[6]	GC Norm. (**III**) mol%	±DEV
12:0	4:0/4:0/4:0[7]	S-S-S	1	0.0692	±0.0057				
	12:0 total			**0.07**					
18:0	6:0/6:0/6:0	S-S-S	1	0.0288	±0.0029			0.07	±0.03
	18:0 total			**0.03**				**0.07**	
20:0	*6:0/10:0/4:0*[8]	S-S-M	3	0.0022	±0.0004			0.14	±0.05
	20:0 total			**0.00**				**0.14**	
22:0	*4:0/16:0/2:0*	S-S-L	3	0.0019	±0.0002				
	14:0/4:0/4:0	S-S-L	2	0.0027	±0.0003			0.02	±0.02
	12:0/6:0/4:0	S-S-L	3	[9]		0.00		0.04	±0.04
	(8:0/8:0/6:0)[10]	S-M-M	2						
	Σ(Co)			0.0103	±0.0018				
	22:0 total			**0.01**		**0.00**		**0.06**	
24:0	*16:0/6:0/2:0*	S-S-L	3	0.0083	±0.0004				
	16:0/4:0/4:0	S-S-L	2	0.0217	±0.0042	0.01	±0.00	0.03	±0.00
	4:0/16:0/4:0	S-S-L	2	0.0014	±0.0004				
	6:0/14:0/4:0	S-S-L	3	0.0156	±0.0021				
	6:0-6:0-12:0[11]	S-S-L	2			0.02	±0.00		
	14:0/6:0/4:0	S-S-L	3						
	(12:0/8:0/4:0)	S-M-L	3			0.02	±0.00		
	Σ(Co+Cy)			0.0148	±0.0021				
	4:0-10:0-10:0	S-M-M	2			0.01	±0.01		
	6:0-8:0-10:0	S-M-M	3						
	8:0-8:0-8:0	M-M-M	1			0.00	±0.00	0.08	±0.00
	24:0 total			**0.06**		**0.05**		**0.11**	
26:0	*18:0/4:0/4:0*	S-S-L	2	0.0038	±0.0009			0.08	±0.01
	16:0/6:0/4:0	S-S-L	3	0.0427	±0.0079	0.12	±0.00	0.09	±0.00
	6:0/16:0/4:0	S-S-L	3	0.0163	±0.0021				
	12:0-8:0-6:0	S-M-L	3						
	10:0-10:0-6:0	S-M-M	2			0.01	±0.01		
	14:0-6:0-6:0	S-S-L	2					0.07	±0.00
	14:0/8:0/4:0	S-M-L	3						
	12:0/10:0/4:0	S-M-L	3			0.01			
	Σ(B, other)			0.0497	±0.0067				
	26:0 total			**0.11**		**0.14**		**0.23**	
28:0	*18:0/2:0/8:0*	S-M-L	3						
	16:0/2:0/10:0	S-M-L	3						
	Σ(Ac)			0.0018	±0.0003				
	18:0/6:0/4:0	S-S-L	3			0.08	±0.00	0.04	±0.00
	(16:0/6:0/6:0)	S-S-L	2			0.03		0.18	±0.01
	Σ(di-sc 28:0)			0.0260	±0.0050				

ACN:DB[1]	TAG species or *Σ(TAG groups)*	S-M-L TAG classes[2]	1-2-3A[3]	ESI–MS **(VII)** mol%	±S.D.[4]	EI–MS **(VI)** Norm.[5] mol%	±DEV[6]	GC **(III)** Norm. mol%	±DEV
	16:0/8:0/4:0	S-M-L	3			0.16	±0.00		
	(14:0/10:0/4:0)	S-M-L	3						
	Σ(B)			0.0982	±0.0139				
	14:0/10:0/4:0	S-M-L	3			0.43	±0.03		
	(12:0/12:0/4:0)	S-L-L	2			0.04		0.19	±0.00
	(12:0/10:0/6:0)	S-M-L	3						
	(16:0/8:0/4:0)	S-M-L	3						
	(12:0_/8:0/8:0)[12]	M-M-L	2						
	Σ(B+Co)			0.1849	±0.0156				
	8:0/14:0/6:0	S-M-L	3						
	8:0/12:0/8:0	M-M-L	2			0.02	±0.00	0.03	±0.00
	Σ(Co+Cy)			0.0095	±0.0018				
	10:0/12:0/6:0	S-M-L	3						
	14:0/8:0/6:0	S-M-L	3					0.03	±0.00
	10:0/8:0/10:0	M-M-M	*2*						
	Σ(Co+Cy)			0.0136	±0.0023				
	28:0 total			**0.33**		**0.76**		**0.47**	
30:0	*18:0/10:0/2:0*	S-M-L	3						
	16:0/12:0/2:0	S-L-L	3						
	14:0/14:0/2:0	S-L-L	2						
	Σ(Ac)			0.0071	±0.0008				
	18:0/8:0/4:0	S-M-L	3			0.04	±0.01		
	16:0/10:0/4:0	S-M-L	3			0.47	±0.01		
	14:0_/12:0/4:0	S-L-L	3			0.32	±0.03	0.65	±0.02
	Σ(B)			0.4314	±0.0131				
	10:0/14:0/6:0	S-M-L	3						
	8:0/16:0/6:0	S-M-L	3			0.10	±0.00		
	8:0/14:0/8:0	M-M-L	2						
	Σ(Co+Cy)			0.0076	±0.0000				
	6:0-12:0-12:0	S-L-L	2			0.44	±0.08		
	14:0/10:0/6:0	S-M-L	3						
	16:0_/8:0/6:0	S-M-L	3						
	Σ(Co)			0.0448	±0.0003				
	8:0-10:0-12:0	M-M-L	3			0.03	±0.01		
	14:0/8:0/8:0	M-M-L	2					0.14	±0.01
	10:0/10:0/10:0	M-M-M	1					0.06	±0.03
	Σ(Cy+Ci)			0.0345	±0.0021				
	6:0-6:0-18:0	S-S-L	2			0.21	±0.05	0.12	±0.01
	30:0 total			**0.53**		**1.61**		**0.98**	
32:0	*16:0_/12:0/4:0*	S-L-L	3			1.09	±0.13		
	14:0/14:0/4:0	S-L-L	2			0.52		1.50	
	18:0_/10:0/4:0	S-M-L	3			0.19	±0.02		
	Σ(B)			1.3877	±0.0493				
	16:0/10:0/6:0	S-M-L	3			0.43	±0.02		
	14:0/12:0/6:0	S-L-L	3						
	12:0/12:0/8:0	M-L-L	2			0.32	±0.02		

ACN:DB[1]	TAG species or *Σ(TAG groups)*	S-M-L TAG classes[2]	1-2-3A[3]	ESI–MS **(VII)** mol%	±S.D.[4]	EI–MS **(VI)** Norm.[5] mol%	±DEV[6]	GC **(III)** Norm. mol%	±DEV
	Σ(Co+Cy)			0.1944	±0.0066				
	16:0-8:0-8:0	M-M-L	2					0.34	
	14:0/8:0/10:0	M-M-L	3						
	10:0/14:0/8:0	M-M-L	3						
	12:0/12:0/8:0	M-L-L	2						
	12:0/10:0/10:0	M-M-L	2					0.11	
	Σ(Cy+Ci)			0.1070	±0.0037				
	32:0 total			**1.69**		**2.54**		**1.95**	
34:0	*18:0/12:0/4:0*	S-L-L	3			0.38		3.26	±0.01
	16:0/14:0/4:0	S-L-L	3			2.19			
	Σ(B)			3.3376	±0.2675				
	18:0/10:0/6:0	S-M-L	3			0.21			
	16:0/12:0/6:0	S-L-L	3			0.50	±0.00		
	14:0/14:0/6:0	S-L-L	2					0.70	±0.00
	Σ(Co)			0.3605	±0.0415				
	18:0-8:0-8:0	M-M-L	2			0.20	±0.01		
	14:0/8:0_/12:0	M-L-L	3						
	12:0/12:0/10:0	M-L-L	2					0.30	±0.02
	10:0/14:0/10:0	M-M-L	2			0.39	±0.00		
	16:0/8:0/10:0	M-M-L	3						
	Σ(Cy+Ci)			0.1747	±0.0024				
	34:0 total			**3.87**		**3.86**		**4.26**	
36:0	*18:0_/16:0/2:0*	S-L-L	3	0.0704	±0.0466				
	18:0/2:0/16:0	S-L-L	3	0.0266	±0.0000				
	16:0/16:0/4:0	S-L-L	2			4.10			
	18:0/14:0/4:0	S-L-L	3			1.36	±0.04		
	Σ(B)			5.2455	±0.2829			5.39	±0.16
	18:0_/12:0/6:0	S-L-L	3			0.03			
	16:0/14:0/6:0	S-L-L	3			2.21	±0.03	1.70	±0.06
	Σ(Co)			1.3493	±0.0362				
	10:0/18:0/8:0	M-M-L	3						
	10:0/16:0/10:0	M-M-L	2						
	12:0/12:0/12:0	L-L-L	1					0.69	±0.00
	14:0/8:0/14:0	M-L-L	2			0.09	±0.02		
	16:0/8:0/12:0	M-L-L	3			0.18			
	10:0/14:0/12:0	M-L-L	3			0.53	±0.01		
	Σ(Cy+Ci+La)			0.2445	±0.0095				
	36:0 total			**6.94**		**8.50**		**7.78**	
38:0	*18:0/16:0/4:0*	S-L-L	3	2.8515	±0.1077	2.37	±0.07	2.37	±0.06
	18:0/14:0/6:0	S-L-L	3			0.36	±0.01		
	16:0/16:0/6:0	S-L-L	2			2.08	±0.03		
	Σ(Co)			1.3270	±0.0227			2.22	±0.03
	18:0/10:0/10:0	M-M-L	2						
	18:0/12:0/8:0	M-L-L	3			0.35	±0.07	0.71	±0.01
	16:0/14:0/8:0	M-L-L	3			0.34			

ACN:DB[1]	TAG species or *Σ(TAG groups)*	S-M-L TAG classes[2]	1-2-3A[3]	ESI–MS **(VII)** mol%	±S.D.[4]	EI–MS Norm.[5] mol%	**(VI)** ±DEV[6]	GC Norm. mol%	**(III)** ±DEV
	14:0/12:0/12:0	L-L-L	2			0.12	±0.07		
	16:0_/12:0/10:0	M-L-L	3			0.07	±0.05	0.48	±0.04
	14:0/14:0/10:0	M-L-L	2			0.71	±0.07		
	Σ(Cy+Ci+La)			0.5656	±0.0125				
	38:0 total			**4.74**		**6.40**		**5.78**	
40:0	*18:0/18:0/4:0*	S-L-L	2	0.7203	±0.0192	0.36	±0.01	0.36	±0.03
	18:0/16:0/6:0	S-L-L	3	0.8382	±0.0154	0.93	±0.01	0.95	±0.03
	16:0/12:0/12:0	L-L-L	2			0.16	±0.00		
	14:0/12:0/14:0	L-L-L	2			0.28	±0.06		
	14:0/18:0_/8:0	M-L-L	3			0.18	±0.03	0.88	±0.02
	16:0/16:0_/8:0	M-L-L	2			0.49	±0.03		
	18:0/12:0/10:0	M-L-L	3			0.22		1.25	±0.09
	16:0/14:0/10:0	M-L-L	3			1.14	±0.02		
	Σ(Cy+Ci+La)			1.1329	±0.0285				
	40:0 total			**2.69**		**3.76**		**3.44**	
42:0	*20:0_/18:0/4:0*	S-L-L	3	0.0023	±0.0002				
	18:0/18:0/6:0	S-L-L	2	0.0416	±0.0047	0.00		0.17	±0.01
	18:0/12:0/12:0	L-L-L	2			0.25	±0.03		
	16:0/12:0_/14:0	L-L-L	3			0.53	±0.02	2.42	±0.19
	14:0/14:0/14:0	L-L-L	1			0.29	±0.01		
	16:0/16:0_/10:0	M-L-L	2			1.65	±0.09		
	16:0/8:0/18:0	M-L-L	3			0.30	±0.03	0.43	±0.03
	14:0_/18:0/10:0	M-L-L	3			0.22	±0.01		
	Σ(Cy–M)			2.7736	±0.0081				
	42:0 total			**2.82**		**3.23**		**3.02**	
44:0	18:0_/14:0_/12:0	L-L-L	3			0.65	±0.03		
	16:0/12:0/16:0	L-L-L	2			0.56	±0.16	1.85	±0.12
	16:0/14:0/14:0	L-L-L	2			0.79	±0.07	0.52	±0.05
	18:0/10:0/16:0	M-L-L	3			0.56	±0.06		
	Σ(Ci+La+M)			2.1933	±0.1471				
	8:0-18:0-18:0	M-L-L	2					0.15	±0.01
	44:0 total			**2.19**		**2.56**		**2.52**	
46:0	16:0/12:0/18:0	L-L-L	3			0.47	±0.08		
	18:0/14:0/14:0	L-L-L	2			0.80	±0.01		
	16:0/16:0/14:0	L-L-L	2			1.46	±0.09		
	18:0/10:0/18:0	M-L-L	2			0.03	±0.02		
	Σ(Ci+La+M)			2.5632	±0.0737			2.32	±0.16
	46:0 total			**2.56**		**2.75**		**2.32**	
48:0	20:0/12:0/16:0	L-L-L	3						
	20:0/14:0/14:0	L-L-L	2						
	20:0/10:0_/18:0	M-L-L	3						
	Σ(20:0)			0.0277	±0.0032				
	18:0/14:0/16:0	L-L-L	3			1.10	±0.21		
	16:0/16:0/16:0	L-L-L	1			0.53	±0.04		
	(18:0/12:0/18:0)	L-L-L	2			0.02	±0.02		

ACN:DB[1]	TAG species or *Σ(TAG groups)*	S-M-L TAG classes[2]	1-2-3A[3]	ESI–MS (**VII**) mol%	±S.D.[4]	EI–MS (**VI**) Norm.[5] mol%	±DEV[6]	GC (**III**) Norm. mol%	±DEV
	Σ(M+P+S)			2.3901	±0.1632			1.91	±0.10
	48:0 total			**2.42**		**1.64**		**1.91**	
50:0	20:0/12:0/18:0	L-L-L	3						
	20:0/14:0/16:0	L-L-L	3						
	Σ(20:0)			0.0996	±0.0075				
	18:0/16:0/16:0	L-L-L	2			0.65	±0.16		
	(18:0/14:0/18:0)	L-L-L	2			0.09	±0.07		
	Σ(P+S)			2.0429	±0.1615			1.48	±0.09
	50:0 total			**2.14**		**0.73**		**1.48**	
52:0	20:0/16:0/16:0	L-L-L	2						
	20:0/14:0/18:0	L-L-L	3						
	Σ(20:0)			0.1168	±0.0069				
	18:0/16:0/18:0	L-L-L	2	0.9151	±0.0718	0.16	±0.09	0.72	±0.04
	52:0 total			**1.03**		**0.16**		**0.72**	
54:0	18:0/18:0/18:0	L-L-L	1	0.1150	±0.0127			0.19	±0.04
	54:0 total			**0.12**		**0.00**		**0.19**	
56:0	*20:0/18:0/18:0*	L-L-L	2	0.0160	±0.0004				
	18:0/20:0/18:0	L-L-L	2						
	16:0/22:0/18:0	L-L-L	3						
	16:0/24:0/16:0	L-L-L	2						
	Σ(P+S)			0.0436	±0.0069				
	56:0 total			**0.06**		**0.00**		**0.00**	
	TOTAL			**34.42**		**38.72**		**37.42**	

[1] ACN:DB = Number of acyl carbons:number of double bonds

[2] Classification of TAGs according to the carbon chain length of esterified acyls. S, M, and L denote short-chain acyls (2–6 acyl carbons), medium-chain acyls (8–10 acyl carbons), and long-chain acyls (12–24 acyl carbons), respectively

[3] Classification of TAGs based on the number of different acyls in molecule. 1 = monoacid TAG, 2 = diacid TAG, and 3 = triacid TAG

[4] The sum of the molar amounts (mol%) of SPE fractions 1–4 ± standard deviation (S.D.). The S.D. was calculated using equation (Harris 1987) $S.D. = \sqrt{(S.D._1^2 + S.D._2^2 + S.D._3^2 + S.D._4^2)}$ in which $S.D._1$, $S.D._2$, $S.D._3$, and $S.D._4$ are standard deviations for molar amounts of SPE fractions 1–4, respectively; see **VII** for details

[5] The mol% of the identified even-numbered TAG species is normalized to the amount (86.2 mol%) of the identified even-numbered molecular species of TAGs determined by ESI–MS

[6] Average of duplicate measurements ± individual measurement. If no DEV was shown, only one measurement was used

[7] Structure in normal font indicates predominant regioisomer

[8] Structure in *italics* indicates regioisomer identified by MS^2

[9] Vertical line indicates which TAG species are combined to the amount (Σ/mol%) of different TAG groups

[10] ( ) = minor molecular species in a group of TAG species with the same retention time

[11] Structure (*e.g.*, 6:0-6:0-12:0) is not determined by MS^2

[12] For example, 12:0_/8:0/8:0 indicates 12:0/8:0/8:0 + 8:0/12:0/8:0

When even-numbered TAGs were categorized on the basis of the carbon chain length of esterified fatty acids (*cf*., Tables 5.15–5.17), ten different TAG classes could be differentiated: (1) S-S-S, (2) S-S-M, (3) S-S-L, (4) S-M-M, (5) S-M-L, (6) S-L-L, (7) M-M-M, (8) M-M-L, (9) M-L-L, and (10) L-L-L, in which S, M, and L denote short-chain acyls (2–6 acyl carbons), medium-chain acyls (8–10 acyl carbons), and long-chain acyls (12–24 acyl carbons), respectively. In this classification, short-chain TAG species belong to categories (1)–(6), medium-chain TAG species to categories (7)–(9), and long-chain TAG species solely to category (10). According to this categorization, the most abundant even-numbered TAG classes in butterfat were S-L-L and L-L-L (Tables 5.15–5.17) which contained 30–37 and 30–34 mol% of TAG species in butterfat, respectively. According to EI–MS and GC determinations, another abundant TAG class was M-L-L (9–13 mol%). In ESI–MS determination, the proportion of pure M-L-L TAG class was low (0.3 mol%), however, *ca*. 17 mol% of butterfat TAGs consisted of combined TAG classes (M-L-L + S-L-L) and (M-L-L + L-L-L). The same three TAG classes were the only categories, in which at least one TAG species in each unsaturation category (*i.e*., saturated, monoene and polyene TAGs) were detected by all analytical methods (GC, EI–MS, ESI–MS). TAG species in every TAG classes were detected solely by the HPLC–ESI–MS method. Contrary to ESI–MS, no TAG species were detected by GC in the TAG class S-M-M and, respectively, by GC–EI–MS in the TAG classes S-S-S, S-S-M, and M-M-M (Tables 5.15–5.17).

The molar amount of SC-, MC-, and LC-TAGs varied in the range 34.0–39.5, 1.1–13.7, and 29.1–33.6 mol% (Tables 5.15–5.17) of all TAGs, respectively, depending on the analytical method used. The significantly lower proportion of MC-TAGs (1.1 mol%) determined by HPLC–ESI–MS than other methods (11.3/13.7 mol%) resulted from the fact that relatively high proportion of MC-TAGs (17.1 mol%) was quantified as TAG groups consisted of both MC- and LC-TAGs.

The total amount of the identified SC-TAGs with two or three butyryl or caproyl acyls varied between 0.3 and 0.9 mol% in np-HPLC–ESI–MS studies (Tables 5.15–5.17, Table 3 in **V**), which was at the similar level as detected in GC–MS analysis (0.7 mol%). In GC analysis, the proportion of di- and tri-SC-TAGs was more than double (1.5 mol%) (Tables 5.15–5.17). The ESI–MS^2 analysis revealed the existence of two monoacid SC-TAG species (tributyrate and tricaproate) in the TAG category S-S-S (Table 5.15), but no dibutyrocaproates/butyrodicaproates were detected in this small TAG category. In addition, di-SC-TAGs included the most infrequently occurring TAG class in the butterfat (S-S-M), in which only one TAG species was identified and quantified, *i.e.*, *sn*-2 isomer of the butyroylcaproylcaprioylglycerol (Table 5.15, Table 3 in **V**). In the TAG category S-S-L, the np-HPLC–ESI–MS^2 analysis showed 9 butyrocaproate, 6 dibutyrate, and 3 dicaproate isomers (Tables 5.15–5.17). Among these molecular species, the number and molar proportion L/S/S regioisomers was markedly higher than that of S/L/S isomers (L denotes any long-chain acyl, S = 4:0/6:0). Altogether, five S/L/S isomers of SC-TAGs were detected (Tables 5.15–5.17). The molar ratio of four identified pairs of regioisomers [L/S/S:S/L/S] ranged from 0.9 to 15.5 (mean 6.5) (Tables 5.15–5.17) and from 1.3 to 13.5 (mean 7.8) (Table 3 in **V**) in three and two columns HPLC–ESI–MS methods, respectively.

The total proportion of monobutyryl and monocaproyl TAGs was shown to be 33.7–34.7 mol% in HPLC–ESI–MS analysis (Tables 5.15–5.17, Table 4 in **V**). In addition, 3.5 mol% of monobutyrates and monocaproates was quantified as TAG groups together with molecular species of MC- and LC-TAGs (Tables 5.15–5.17) resulting in a total amount of 37.2–38.2 mol%. Hence, molar proportion of monobutyrates and monocaprates (38.0–38.2 mol%) determined by GC/GC–EI–MS was close to the results of ESI–MS analysis (Tables 5.15–5.17). Proportion of monobutyryl TAGs was calculated to be 71 and 67% of the total amount of mono-SC-TAGs in ESI–MS and GC/EI–MS studies, respectively (Tables 5.15–5.17). Altogether, 92, 65, and 36 different monobutyryl and monocaproyl TAGs belonging to S-L-L and S-M-L classes were determined by ESI–MS, EI–MS, and GC analysis, respectively, as individual molecular species or as a member of a group of TAGs (Tables 5.15–5.17). Alternatively, the abundance and number of identified TAG species in the TAG class of S-M-M were very low. In the ESI–MS analysis, the sole identified saturated monocaproate was in *sn*-1(3) configuration (Table 5.15). Further, essentially all of the regioisomers of TAGs in S-L-L and S-M-L classes were *sn*-1(3) isomers. Only two pairs of the regioisomers of monocaproates were detected and quantified as a member of groups of other TAGs and traces of one *sn*-2 isomer of monobutyryl TAGs and that of monocaproyl TAGs were observed (Tables 5.15–5.17).

Short-chain TAG species containing at least one acetyl acyl group were analyzed solely by np-HPLC–ESI–MS system using three (**V, VII**) and two columns (**V**) in series. The total molar amount of acetate TAGs in butterfat was 0.2 mol% in the TAG classes S-S-L, S-M-L, and S-L-L (Tables 5.15–5.17). The ACN:DB classes of saturated acetate TAGs (Table 5.15, Table 5 in **V**) varied between 22:0 and 36:0 (except 32:0–34:0) and those of monoene TAGs (Table 5.16, Table 5 in **V**) between 24:1 and 38:1 (except 32:1–34:1). One diene acetate TAG (38:2) was also detected (Table 5.17). Altogether, 14 and 22 monoacetyl TAG species were identified by three and two HPLC columns methods, respectively (Tables 5.15–5.17, Table 5 in **V**). However, marked variation was observed in the molecular species of acetate TAGs. In ESI–MS^2 analysis with three HPLC columns in series only one saturated acetobutyrate (4:0/16:0/2:0) and one acetocaproate (16:0/6:0/2:0) were detected in the TAG class S-S-L (Tables 5.15–5.17). In two columns in series (Table 5 in **V**), altogether eight saturated and unsaturated TAG species (4 acetobutyrates and 4 acetocaproates) were found. However, no diacetates were discovered in butterfat. In three columns method, 64% of the 14 acetate TAG species belonged to the S-L-L class (Tables 5.15–5.17), but in two columns method, 55% of TAGs belonged to the S-M-L class (Table 5 in **V**). The molar proportion of the sum of X/X/2:0 regioisomers (X denotes any other acyl group than 2:0) to that of X/2:0/X isomers was 2 (Tables 5.15–5.17). When two columns in series were used in HPLC–MS, the respective ratio of area proportions was close to 10 (Table 5 in **V**).

HPLC–ESI–MS, GC–EI–MS, and GC methods revealed 86, 49, and 34 molecular species of MC-TAGs, respectively (Tables 5.15–5.17). However, due to the relatively high number of different TAG species and relatively low resolution of acyl chain isomers of MC- and LC-TAGs on chromatographic columns, determination of the molar amount of individual TAG species was challenging. Obviously, the most abundant MC-TAG class was M-L-L (Tables 5.15–5.17). Altogether, 62 different molecular species of TAGs were detected by

Table 5.16 Composition (mol%) of the identified molecular species of monoene even-numbered TAGs in butterfat determined by normal-phase liquid chromatography–electrospray mass spectrometry (ESI–MS), gas chromatography–electron impact mass spectrometry (EI–MS), and gas chromatography (GC). **III**, **VI**, and **VII** refer to original publications. (Table adapted from Table 6 in **III**, Table 7 in **VI**, and Table 3 in **VII**.)

ACN:DB[1]	TAG species or *Σ(TAG groups)*	S-M-L TAG classes[2]	1-2-3A[3]	ESI–MS (**VII**) mol%	±S.D.[4]	EI–MS Norm.[5] (**VI**) mol%	±DEV[6]	GC Norm. (**III**) mol%	±DEV
24:1	16:1-4:0-4:0[7]	S-S-L	2			0.00	±0.00	0.01	
	24:1 total					**0.00**		**0.01**	
26:1	*18:1/4:0/4:0*[8]	S-S-L	2	0.0232	±0.0039				
	4:0/18:1/4:0	S-S-L	2	0.0033	±0.0003				
	16:1/6:0/4:0	S-S-L	3	[9]				0.11	±0.01
	12:0/10:1/4:0	S-M-L	3						
	Σ(Other 26:1)			0.0052	±0.0008				
	26:1 total			**0.03**		**0.00**		**0.11**	
28:1	16:1-8:0-4:0	S-M-L	3			0.06	±0.00		
	(16:1/6:0/6:0)[10]	S-S-L	2					0.25	±0.01
	18:1/6:0/4:0	S-S-L	3						
	(6:0/18:1/4:0)	S-S-L	3						
	(14:0/10:1/4:0)	S-M-L	3						
	(10:1/14:0/4:0)	S-M-L	3						
	Σ(B+Co)			0.0099	±0.0008				
	28:1 total			**0.01**		**0.06**		**0.25**	
30:1	*18:1/8:0/4:0*	S-M-L	3			0.00	±0.00	0.20	
	16:0/10:1/4:0	S-M-L	3						
	12:1_/14:0/4:0[11]	S-L-L	3						
	14:1_/12:0/4:0	S-L-L	3						
	Σ(B)			0.0759	±0.0053				
	18:1/6:0/6:0	S-S-L	2	0.0061	±0.0000	0.17	±0.00	0.29	±0.01
	8:0/18:1/4:0	S-M-L	3						
	14:1/8:0/8:0	M-M-L	2						
	Σ(Cy+B)			0.0239	±0.0050				
	16:1-10:0-4:0	S-M-L	3			0.00			
	30:1 total			**0.11**		**0.17**		**0.49**	
32:1	*14:1/14:0/4:0*	S-L-L	3						
	16:0_/12:1/4:0	S-L-L	3						
	12:0_/16:1/4:0	S-L-L	3			0.01	±0.00	0.50	±0.01
	18:1/10:0/4:0	S-M-L	3			0.31	±0.06	0.30	±0.01
	Σ(B)			0.6882	±0.0147				
	16:1-8:0-8:0	M-M-L	2			0.04	±0.00		
	18:1-8:0-6:0	S-M-L	3			0.03	±0.03		
	16:1-10:0-6:0	S-M-L	3			0.09	±0.05		
	32:1 total			**0.69**		**0.49**		**0.79**	
34:1	*18:1_/12:0/4:0*	S-L-L	3			0.84		0.76	±0.01
	(16:1_/14:0/4:0)	S-L-L	3			0.13	±0.03		
	(14:1_/16:0/4:0)	S-L-L	3						
	Σ(B)			0.6661	±0.0301				
	14:1/14:0/6:0	S-L-L	3						

ACN:DB[1]	TAG species or *Σ(TAG groups)*	S-M-L TAG classes[2]	1-2-3A[3]	ESI–MS **(VII)** mol%	±S.D.[4]	EI–MS Norm.[5] **(VI)** mol%	±DEV[6]	GC Norm. **(III)** mol%	±DEV
	10:0/18:1/6:0	S-M-L	3			0.03	±0.03	0.58	±0.01
	Σ(Co)			0.1002	±0.0011				
	18:1/8:0/8:0[12]	M-M-L	2			0.03	±0.00	0.07	±0.00
	16:1/8:0/10:0	M-M-L	3						
	14:1/10:0/10:0	M-M-L	2						
	Σ(Cy+Ci)			0.0962	±0.0060				
	16:1-12:0-6:0	S-L-L	3			0.29			
	34:1 total			**0.86**		**1.32**		**1.41**	
36:1	*16:0_/18:1/2:0*	S-L-L	3	0.0408	±0.0071				
	18:1/14:0/4:0	S-L-L	3			2.32	±0.35	2.47	±0.04
	(16:0_/16:1/4:0)	S-L-L	3			0.30		0.08	±0.04
	Σ(B)			1.9628	±0.0680				
	18:1/12:0/6:0	S-L-L	3			0.18	±0.04	0.72	±0.07
	16:0/14:1/6:0	S-L-L	3						
	14:0/16:1/6:0	S-L-L	3						
	14:0/10:1_/12:0	M-L-L	3						
	Σ(Co+10:1)			0.4816	±0.0162				
	18:1/10:0/8:0	M-M-L	3	0.0743	±0.0011				
	16:1-12:0-8:0	M-L-L	3			0.02	±0.01		
	16:1-10:0-10:0	M-M-L	2			0.09	±0.01	0.15	±0.00
	36:1 total			**2.56**		**2.91**		**3.41**	
38:1	*18:1/18:0/2:0*	S-L-L	3	0.0163	±0.0000				
	18:1/2:0/18:0	S-L-L	3	0.0290	±0.0033				
	18:0-16:1-4:0	S-L-L	3			0.16	±0.01		
	18:1/16:0/4:0	S-L-L	3	4.0781	±0.1684	4.36	±0.08	4.59	±0.05
	18:1/14:0_/6:0	S-L-L	3			0.78	±0.17	1.09	±0.01
	16:1/16:0/6:0	S-L-L	3						
	18:1/10:0/10:0	M-M-L	2						
	14:0/14:1/10:0	M-L-L	3						
	12:0/14:1/12:0	L-L-L	2						
	Σ(Co+Ci+La)			0.9735	±0.0223				
	18:1/12:0/8:0	M-L-L	3						
	16:1_/14:0/8:0	M-L-L	3			0.24		0.35	±0.01
	14:1/16:0/8:0	M-L-L	3						
	16:1/12:0/10:0	M-L-L	3			0.16	±0.02		
	18:1/10:0/10:0	M-M-L	2			0.03			
	Σ(Cy+Ci)			0.2889	±0.0164				
	38:1 total			**5.39**		**5.72**		**6.03**	
40:1	*18:1_/18:0/4:0*	S-L-L	3	1.9919	±0.0177	1.18	±0.11	1.47	±0.00
	18:1/16:0/6:0	S-L-L	3			2.08	±0.00	1.87	±0.00
	18:0/16:1/6:0	S-L-L	3			0.10			
	Σ(Co)			1.2996	±0.0463				
	18:1/6:0/16:0	S-L-L	3						
	16:0/16:1/8:0	M-L-L	3					0.76	
	18:1/14:0_/8:0	M-L-L	3			0.19	±0.06		
	18:1/12:0_/10:0	M-L-L	3			0.10			

ACN:DB[1]	TAG species or *Σ(TAG groups)*	S-M-L TAG classes[2]	1-2-3A[3]	ESI–MS (**VII**) mol%	±S.D.[4]	EI–MS (**VI**) Norm.[5] mol%	±DEV[6]	GC (**III**) Norm. mol%	±DEV
	16:0/14:1/10:0	M-L-L	3						
	14:0/16:1_/10:0	M-L-L	3						
	14:0/14:1/12:0	L-L-L	3						
	Σ(Co–La)			0.6793	±0.0068				
	12:0-12:0-16:1	L-L-L	2			0.00			
	40:1 total			**3.97**		**3.66**		**4.10**	
42:1	*18:1_/20:0/4:0*	S-L-L	3						
	18:0/20:1/4:0	S-L-L	3						
	Σ(B)			0.0654	±0.0041				
	18:1/18:0/6:0	S-L-L	3	1.0864	±0.0422	0.33	±0.03	1.19	±0.11
	18:1/8:0_/16:0	M-L-L	3			0.60		0.55	±0.05
	16:0/12:0/14:1	L-L-L	3						
	16:0/12:1_/14:0	L-L-L	3						
	14:0/16:1_/12:0	L-L-L	3						
	18:1/12:0/12:0	L-L-L	2			0.48			
	14:0/14:1/14:0	L-L-L	2						
	18:1/10:0_/14:0	M-L-L	3			0.19			
	18:0/14:1/10:0	M-L-L	3						
	16:0/16:1/10:0	M-L-L	3			0.46	±0.03		
	Σ(Cy–M)			1.5021	±0.0377				
	8:0-16:1-18:0	M-L-L	3			0.02			
	42:1 total			**2.65**		**2.08**		**1.73**	
44:1	18:1/8:0/18:0	M-L-L	3			0.06	±0.04		
	18:1/16:0_/10:0	M-L-L	3			1.40	±0.26	0.99	±0.01
	18:0/16:1/10:0	M-L-L	3						
	18:1/14:0/12:0	L-L-L	3			0.39	±0.09	1.67	±0.12
	14:0/16:1/14:0	L-L-L	2						
	18:0/14:1/12:0	L-L-L	3						
	16:0/16:1_/12:0	L-L-L	3						
	16:0/14:1/14:0	L-L-L	3						
	Σ(Cy–M)			2.1709	±0.0139				
	44:1 total			**2.17**		**1.85**		**2.66**	
46:1	18:1/10:0_/18:0	M-L-L	3			0.27	±0.08	2.56	±0.05
	18:1/12:0_/16:0	L-L-L	3			1.17	±0.24		
	12:0/16:1/18:0	L-L-L	3						
	18:1/14:0/14:0	L-L-L	2			1.24	±0.09		
	14:0/18:0_/14:1	L-L-L	3						
	16:0/16:1/14:0	L-L-L	3			0.30	±0.02		
	16:0/14:1/16:0	L-L-L	2						
	Σ(Ci–M/Mo)			2.6644	±0.1353				
	46:1 total			**2.66**		**2.98**		**2.56**	
48:1	18:1/14:0_/16:0	L-L-L	3			1.77	±0.19	3.73	±0.14
	16:0/16:1/16:0	L-L-L	2			0.85	±0.20		
	Σ(M+P)			3.0842	±0.2009				
	14:0-16:1-18:0	L-L-L	3			0.60	±0.10		
	12:0-18:0-18:1	L-L-L	3			0.29	±0.09		

ACN:DB[1]	TAG species or *Σ(TAG groups)*	S-M-L TAG classes[2]	1-2-3A[3]	ESI–MS **(VII)** mol%	±S.D.[4]	EI–MS **(VI)** Norm.[5] mol%	±DEV[6]	GC **(III)** Norm. mol%	±DEV
	48:1 total			**3.08**		**3.52**		**3.73**	
50:1	20:0/12:0/18:1	L-L-L	3						
	20:0/14:0/16:1	L-L-L	3						
	20:0/16:0_/14:1	L-L-L	3						
	Σ(20:0)			0.1869	±0.0507				
	18.1/14:0_/18:0	L-L-L	3			0.72	±0.17		
	18:1/16:0/16:0	L-L-L	2			2.67	±0.23	4.56	±0.13
	16:0/18:0/16:1	L-L-L	3			0.22	±0.05		
	Σ(P+S)			4.7740	±0.3820				
	50:1 total			**4.96**		**3.61**		**4.56**	
52:1	(20:0/16:0/16:1)	L-L-L	3						
	20:0/14:0/18:1	L-L-L	3						
	Σ(20:0)			0.0400	±0.0007				
	18:1/16:0_/18:0	L-L-L	3			0.96	±0.18	2.87	±0.07
	(18:0/16:1/18:0)	L-L-L	2			0.03	±0.02		
	Σ(S)			2.7534	±0.2378				
	52:1 total			**2.79**		**0.98**		**2.87**	
54:1	18:0/18:0/18:1	L-L-L	2	0.5835	±0.0317	0.03	±0.01	0.62	±0.03
	20:0/16:0/18:1	L-L-L	3						
	(20:0/16:1/18:0)	L-L-L	3						
	Σ(20:0)			0.2096	±0.0099				
	54:1 total			**0.79**		**0.03**		**0.62**	
56:1	*20:0/18:0_/18:1*	L-L-L	3	0.0906	±0.0056				
	18:1/20:0/18:0	L-L-L	3						
	(18:1/22:0/16:0)	L-L-L	3						
	Σ(22+sn-2-20)			0.1004	±0.0027				
	56:1 total			**0.19**		**0.00**		**0.00**	
	TOTAL			**32.93**		**29.38**		**35.33**	

[1] ACN:DB = Number of acyl carbons:number of double bonds

[2] Classification of TAGs according to the carbon chain length of esterified acyls. S, M, and L denote short-chain acyls (2–6 acyl carbons), medium-chain acyls (8–10 acyl carbons), and long-chain acyls (12–24 acyl carbons), respectively

[3] Classification of TAGs based on the number of different acyls in molecule. 1 = monoacid TAG, 2 = diacid TAG, and 3 = triacid TAG

[4] The sum of the molar amounts (mol%) of SPE fractions 1–4 ± standard deviation (S.D.). The S.D. was calculated using equation (Harris 1987) $S.D. = \sqrt{(S.D._1^2 + S.D._2^2 + S.D._3^2 + S.D._4^2)}$ in which $S.D._1$, $S.D._2$, $S.D._3$, and $S.D._4$ are standard deviations for molar amounts of SPE fractions 1–4, respectively; see **VII** for details

[5] The mol% of the identified even-numbered TAG species is normalized to the amount (86.2 mol%) of the identified even-numbered molecular species of TAGs determined by ESI–MS

[6] Average of duplicate measurements ± individual measurement. If no DEV was shown, only one measurement was used

[7] Structure (*e.g.*, 16:1-4:0-4:0) is not determined by MS^2

[8] Structure in *italics* indicates regioisomer identified by MS^2

[9] Vertical line indicates which TAG species are combined to the amount (Σ/mol%) of different TAG groups

[10] ( ) = minor molecular species in a group of TAG species with the same retention time

[11] For example, 12:1_/14:0/4:0 indicates 12:1/14:0/4:0 + 14:0/12:1/4:0

[12] Structure in normal font indicates predominant regioisomer

ESI–MS and they were divided evenly (21-19-22) into saturated, monoene, and polyene TAGs. In addition, the number of caprylate (26) and capriate TAGs (31) was close to the same. Further, five MC-TAGs containing fatty acyl 10:1 were detected. The molar amount of individual TAG species in M-L-L class was 9.5–12.5 mol% in EI–MS and GC determinations, but practically all MC-TAG species were quantified as members of a group of several TAG classes in ESI–MS analysis. Only a few TAG species of the TAG class M-M-L were quantified reliably as individual molecular species by np-HPLC–ESI–MS due to the reasons mentioned above. However, it was obvious that dicaprylates, dicapriates together with caprylcapriates existed in butterfat, as well as *sn*-1(3) and *sn*-2 isomers of those TAG species (Tables 5.15–5.17). The molar amount of the individually quantified TAG species of the whole class varied between 0.2 and 0.8 mol% of all TAGs in butterfat (Tables 5.15–5.17). Further, the np-HPLC–ESI–MS analysis revealed one monoacid MC-TAG (10:0/10:0/10:0) and one diacid MC-TAG (10:0/8:0/10:0) in the TAG class of M-M-M. In addition, GC–EI–MS analysis showed traces of another monoacid MC-TAG (8:0/8:0/8:0) (Table 5.15).

The TAG class of L-L-L composed of saturated and unsaturated TAG species with 36–56 acyl carbons and 0–5 double bonds (Tables 5.15–5.17). It was a very abundant TAG class (29.1–33.6 mol%) of even-numbered TAGs with the highest number of different TAG species: 124, 56, and 31 molecular species of TAGs were quantified by ESI–MS, EI–MS and GC methods, respectively (Tables 5.15–5.17). In general, the resolving power of np-HPLC columns in series and phenyl(65%)methylsilicone GC column was not high enough to separate acyl chain isomers resulting in the quantification of TAGs mostly as groups or by calculation (GC–EI–MS). In HPLC–MS analysis, however, TAG species containing very long-chain acyl groups (ACN > 18) were separated from other acyl chain isomers in most cases and 27 TAG species could be quantified in this TAG subgroup (Table 5.15–5.17). In addition, np-HPLC columns were observed to separate the regioisomers of TAGs containing FA 20:0, *e.g.*, 20:0/18:0/18:0 *vs.* 18:0/20:0/18:0 and 20:0/18:0_/18:1 *vs.* 18:1/20:0/18:0.

Several SC-TAGs were among the most frequently existing molecular species of TAGs in butterfat. The most abundant SC-TAG species, which were quantified as individual molecular species with all methods, were 18:1/16:0/4:0, 18:0/16:0/4:0, and 18:1/18:0/4:0. The molar amount of 18:1/16:0/4:0 was 4.1, 4.4, and 4.6 mol% in ESI–MS, EI–MS, and GC analysis, respectively, suggesting that butyroylpalmitoyloleoylglycerol was the most abundant TAG species in butterfat (Table 5.16). The molar amount of 18:0/16:0/4:0 varied between 2.4 and 2.9 mol%, and that of 18:1/18:0/4:0 between 1.2 and 2.0 mol% (Table 5.15). However, the most abundant saturated SC-TAG was most probably 16:0/16:0/4:0, which was determined together with another major SC-TAG (18:0/14:0/4:0). The molar amount of this pair of TAGs was 5.3–5.5 mol% (Table 5.15), of which the share of 16:0/16:0/4:0 was *ca*. 75% (estimated by GC–EI–MS) resulting in a calculated molar amount of 3.9–4.1 mol%. Other abundant butyrate TAG groups (major component) were (16:0/14:0/4:0 + 18:0/12:0/4:0) and (18:1/14:0/4:0 + 16:0/16:1/4:0), the molar amounts of which were, respectively, 3.3 and 2.0 mol% in ESI–MS, 2.6 and 2.6 mol% in EI–MS, and 3.3 and 2.6 mol% in GC analysis (Tables 5.15 and 5.16). The most abundant caproate TAG groups (major component) in butterfat were (16:0/14:0/6:0 + 18:0/12:0/6:0), (16:0/16:0/6:0

+ 18:0/14:0/6:0), and (18:1/16:0/6:0 + 18:0/16:1/6:0). The proportion of each TAG group was 1.3 mol% in ESI–MS, 2.2–2.4 mol% in EI–MS, and 1.7–2.2 mol% in GC analysis (Tables 5.15 and 5.16).

All analyzing methods indicated that the most abundant LC-TAG species was 18:1/16:0/16:0, which eluted together with two other LC-TAGs (18:1/14:0/18:0 and 16:1/16:0/18:0). In ESI–MS analysis, the molar amount of that group of LC-TAGs was 4.77 mol% (Table 5.16). However, according to EI–MS analysis, the share of 18:1/16:0/16:0 was 74%, resulting in an estimated molar amount of 3.5 mol%. Other substantial groups of monoene LC-TAGs (major component) were (18:1/14:0/16:0 + 16:1/16:0/16:0) and (18:1/16:0/18:0 + 18:0/16:1/18:0) molar amount of which varied between 2.6 and 3.7 mol%, and between 1.0–2.9 mol%, respectively, depending on the analyzing method used (Table 5.16). The most abundant polyene TAGs in butterfat (3.1–3.7 mol%) observed to be a group of LC-TAGs consisting of 18:1/16:0/18:1 and 18:2/18:0/16:0, the former being the major component (Table 5.17). In addition, another abundant (2.8 mol%) group of six polyene LC-TAGs (18:1/14:0/18:1 + 18:2/14:0/18:0 + 18:1/14:1/18:0 + 18:1/16:1/16:0 + 18:0/16:1/16:1 + 18:2/16:0/16:0) was detected by HPLC–ESI–MS (Table 5.17). Correspondingly, the molar amounts of (18:1/14:0/18:1 + 18:2/14:0/18:0) and (18:1/16:1/16:0 + 18:0/16:1/16:1) were even more prominent (*i.e.*, 2.0 and 1.5 mol%, respectively) according to GC–EI–MS analysis (Table 5.17). Further, two groups of saturated LC-TAGs with ACN 48:0–50:0 exceeded 2 mol% when analyzed by ESI–MS: (18:0/14:0/16:0 + 16:0/16:0/16:0 + 18:0/12:0/18:0) 2.4 mol% and (18:0/16:0/16:0 + 18:0/14:0/18:0) 2.0 mol% (major component) (Table 5.15).

The molar amount of any individual MC-TAG species or any group consisting solely of MC-TAGs did not exceed 1 mol%, despite the analyzing method. However, molar amount of five TAG groups, which comprised both MC- and LC-TAGs, was detected to be between 2.1 and 2.8 mol% when HPLC–ESI–MS method was used for analysis. The following even-numbered saturated TAG groups of ACN 42:0–46:0 were quanfied by ESI–MS: (18:0/12:0/12:0 + 16:0/12:0/14:0 + 14:0/14:0/14:0 + 16:0/16:0/10:0 + 16:0/ 8:0/18:0 + 14:0/18:0/10:0) 2.8 mol%, (18:0/14:0/12:0 + 16:0/12:0/16:0 + 16:0/14:0/14:0 + 18:0/10:0/16:0) 2.2 mol%, and (16:0/12:0/18:0 + 18:0/14:0/14:0 + 16:0/16:0/14:0 + 18:0/10:0/18:0) 2.6 mol%. Further, the molar amount of two groups of several monoene TAG with ACN 44:1–46:1, *i.e.*, (18:1/14:0/12:0 + 14:0/16:1/14:0 + 18:0/14:1/12:0 + 16:0/16:1/12:0 + 16:0/14:1/14:0 + 18:1/8:0/18:0 + 18:1/16:0/10:0 + 18:0/16:1/10:0) and (18:1/12:0/16:0 + 12:0/16:1/18:0 + 18:1/14:0/14:0 + 14:0/18:0/14:1 + 16:0/16:1/14:0 + 16:0/14:1/16:0 + 18:1/10:0/18:0), was 2.2 and 2.7 mol%, respectively.

The molar amount of odd-numbered TAG species was determined solely by np-HPLC–ESI–MS and the mol% of identified molecular species is listed in Table 5.18. The total amount of identified odd ACN TAGs in butterfat was 3.9 mol% (+ 1.8 mol% of unidentified TAG species) and the total number of identified molecular species was 122 (Table 5.18). The vast majority (87%) of TAG species was triacid TAGs and no monoacid TAGs was detected (Table 5.18). The proportion of short-, medium-, and long-chain TAGs was 22, 20, and 58%, respectively, indicating a higher proportion of long-chain TAGs compared to that

Table 5.17 Composition (mol%) of the identified molecular species of polyene even-numbered TAGs in butterfat determined by normal-phase liquid chromatography–electrospray mass spectrometry (ESI–MS), gas chromatography–electron impact mass spectrometry (EI–MS), and gas chromatography (GC). **III**, **VI**, and **VII** refer to original publications. (Table adapted from Table 6 in **III**, Table 8 in **VI**, and Table 3 in **VII**.)

ACN:DB[1]	TAG species or *Σ(TAG groups)*	S-M-L TAG classes[2]	1-2-3A[3]	ESI–MS (**VII**) mol%	±S.D.[4]	EI–MS Norm.[5] (**VI**) mol%	±DEV[6]	GC Norm. (**III**) mol%	±DEV
36:2	*18:1/14:1/4:0*[7]	S-L-L	3	[8]				0.14	±0.01
	18:2/14:0/4:0	S-L-L	3			0.08	±0.07		
	Σ(B)			0.2859	±0.0100				
	16:1-16:1-4:0[9]	S-L-L	2			0.03	±0.02		
	18:1/8:0/10:1[10]	M-M-L	3	0.0587	±0.0039				
	36:2 total			**0.34**		**0.11**		**0.14**	
38:2	*18:1/18:1/2:0*	S-L-L	2	0.0044	±0.0000				
	18:1/2:0/18:1	S-L-L	2	0.0250	±0.0003				
	18:1/16:1_/4:0[11]	S-L-L	3			0.14	±0.00		
	18:2/16:0/4:0	S-L-L	3			0.22	±0.00	0.12	±0.00
	Σ(B)			0.6490	±0.0169				
	18:1/14:1/6:0	S-L-L	3						
	18:2/14:0/6:0	S-L-L	3						
	18:1/10:1/10:0	M-M-L	3						
	10:0/18:2/10:0	M-M-L	2						
	Σ(Co+Ci)			0.3470	±0.1256				
	16:1-16:1-6:0	S-L-L	2			0.12	±0.02	0.32	±0.01
	38:2 total			**1.03**		**0.47**		**0.44**	
40:4	*18:3/18:1/4:0*	S-L-L	3						
	18:2/18:2/4:0	S-L-L	2						
	Σ(B)			0.1091	±0.0169				
	40:4 total			**0.11**					
40:3	*18:3/18:0/4:0*	S-L-L	3						
	18:2/18:1/4:0	S-L-L	3						
	Σ(B)			0.2550	±0.0083				
	40:3 total			**0.26**					
40:2	*18:1/18:1/4:0*	S-L-L	2			1.36	±0.00	1.06	±0.07
	18:0/18:2/4:0	S-L-L	3			0.08			
	Σ(B)			1.4125	±0.0396				
	18:2/16:0/6:0	S-L-L	3			0.17	±0.04		
	18:1/16:1/6:0	S-L-L	3			0.18	±0.04	0.29	±0.00
	16:1/16:1_/8:0	M-L-L	2						
	18:1/12:0/10:1	M-L-L	3						
	16:1/14:0/10:1	M-L-L	3						
	Σ(Co+Cy+10:1)			0.4239	±0.0103				
	14:1/16:1/10:0	M-L-L	3						
	18:1/8:0/14:1	M-L-L	3						
	Σ(Cy+Ci)			0.1221	±0.0137				
	40:2 total			**1.96**		**1.79**		**1.35**	
42:3	*18:2_/18:1/6:0*	S-L-L	3						
	(18:3_/18:0/6:0)	S-L-L	3						
	Σ(Co)			0.1461	±0.0010				

ACN:DB[1]	TAG species or *Σ(TAG groups)*	S-M-L TAG classes[2]	1-2-3A[3]	ESI–MS **(VII)** mol%	±S.D.[4]	EI–MS **(VI)** Norm.[5] mol%	±DEV[6]	GC **(III)** Norm. mol%	±DEV
	42:3 total			**0.15**					
42:2	*18:1/20:1/4:0*	S-L-L	3	0.0223	±0.0000				
	18:2_/18:0/6:0	S-L-L	3			0.06		0.07	±0.03
	18:1/18:1/6:0	S-L-L	2			0.86	±0.02	0.65	±0.02
	Σ(Co)			0.5177	±0.0235				
	18:1_/16:1/8:0	M-L-L	3						
	18:1/6:0/18:1	S-L-L	2						
	16:1/8:0/18:1	M-L-L	3						
	16:0/8:0/18:2	M-L-L	3						
	18:2/14:0/10:0	M-L-L	3						
	18:1/14:1/10:0	M-L-L	3						
	16:1/10:0/16:1	M-L-L	2						
	Σ(Co+Cy+Ci)			0.1387	±0.0094				
	42:2 total			**0.68**		**0.92**		**0.72**	
44:2	18:1/18:1/8:0	M-L-L	2						
	18:2/8:0_/18:0	M-L-L	3						
	18:1/10:1_/16:0	M-L-L	3						
	Σ(Cy+10:1)			0.1618	±0.0039				
	18:1/18:1_/8:0	M-L-L	2			0.91	±0.04	0.54	±0.01
	18:1/10:0/16:1	M-L-L	3						
	18:2/18:0/8:0	M-L-L	3						
	18:2/10:0/16:0	M-L-L	3						
	18:1/14:1/12:0	L-L-L	3						
	16:0/14:1/14:1	L-L-L	2						
	Σ(Cy+Other)			0.3916	±0.0067				
	44:2 total			**0.55**		**0.91**		**0.54**	
46:3	18:3/14:0/14:0	L-L-L	2						
	18:1/18:2/10:0	M-L-L	3						
	18:1/18:1_/10:1	M-L-L	2					0.07	±0.02
	Σ(46:3)			0.0526	±0.0345				
	46:3 total			**0.05**				**0.07**	
46:2	18:1/10:0/18:1	M-L-L	2			0.90	±0.03	0.79	±0.10
	18:0/10:0/18:2	M-L-L	3						
	18:1/14:0/14:1	L-L-L	3						
	16:1/16:1/14:0	L-L-L	2			0.26	±0.08		
	Σ(Ci+M)			0.8833	±0.0373				
	18:2-14:0-14:0	L-L-L	2						
	18:1-16:1-12:0	L-L-L	3			0.17	±0.03		
	46:2 total			**0.88**		**1.33**		**0.79**	
48:4	20:4/16:0/12:0	L-L-L	3						
	18:3/12:0/18:1	L-L-L	3						
	18:2/12:1/18:1	L-L-L	3						
	Σ(48:4)			0.0201	±0.0030				
	48:4 total			**0.02**					
48:3	14:1/18:1/16:1	L-L-L	3					0.25	±0.02
	18:1/18:2/12:0	L-L-L	3						
	Σ(48:3)			0.1091	±0.0000				

ACN:DB[1]	TAG species or *Σ(TAG groups)*	S-M-L TAG classes[2]	1-2-3A[3]	ESI–MS **(VII)** mol%	±S.D.[4]	EI–MS **(VI)** Norm.[5] mol%	±DEV[6]	GC **(III)** Norm. mol%	±DEV
	48:3 total			**0.11**				**0.25**	
48:2	18:2/14:0/16:0	L-L-L	3			1.29	±0.22	1.06	±0.02
	18:1/16:1/14:0	L-L-L	3			0.56	±0.09		
	18:1/14:1_/16:0	L-L-L	3						
	18:1/12:0/18:1	L-L-L	2			0.86	±0.03		
	16:0/16:1/16:1	L-L-L	2						
	Σ(48:2)			1.2499	±0.0380				
	48:2 total			**1.25**		**2.72**		**1.06**	
50:4	18:3/18:1_/14:0	L-L-L	3						
	18:2/18:2/14:0	L-L-L	2						
	18:1/14:1_/18:2	L-L-L	3						
	Σ(50:4)			0.1246	±0.0004				
	50:4 total			**0.12**					
50:3	18:2_/18:1/14:0	L-L-L	3			0.72	±0.02	0.31	±0.05
	(18:1/16:1/16:1)[12]	L-L-L	2						
	(18:1/14:1/18:1)	L-L-L	2						
	(18:3/18:0/14:0)	L-L-L	3						
	(18:2/18:0_/14:1)	L-L-L	3						
	(18:3/16:0/16:0)	L-L-L	2						
	Σ(50:3)			0.4795	±0.1023				
	50:3 total			**0.48**		**0.72**		**0.31**	
50:2	18:1/14:0_/18:1	L-L-L	2			1.97	±0.16	2.16	±0.08
	18:2/14:0/18:0	L-L-L	3						
	18:1/14:1/18:0	L-L-L	3						
	18:1/16:1/16:0	L-L-L	3			1.54	±0.00		
	18:0/16:1/16:1	L-L-L	2						
	18:2/16:0/16:0	L-L-L	2						
	Σ(50:2)			2.7974	±0.2199				
	50:2 total			**2.80**		**3.50**		**2.16**	
52:4	20:4/16:0/16:0	L-L-L	2						
	18:3/18:1/16:0	L-L-L	3						
	18:2/18:2/16:0	L-L-L	2						
	Σ(52:4)			0.2044	±0.0114				
	52:4 total			**0.20**					
52:3	18:2/18:1/16:0	L-L-L	3			0.15	±0.00	0.14	±0.02
	18:1/18:1/16:1	L-L-L	2						
	18:2/18:0/16:1	L-L-L	3						
	18:3/18:0/16:0	L-L-L	3						
	Σ(52:3)			0.6485	±0.4467				
	52:3 total			**0.65**		**0.15**		**0.14**	
52:2	18:1/16:0/18:1	L-L-L	2			2.86	±0.03	3.28	±0.18
	18:2/18:0/16:0	L-L-L	3			0.28			
	Σ(P)			3.7087	±0.3616				
	18:1-18:0-16:1	L-L-L	3			0.41	±0.12		
	52:2 total			**3.71**		**3.54**		**3.28**	
54:5	18:1/18:2/18:2	L-L-L	2						
	18:3/18:2/18:0	L-L-L	3						

ACN:DB[1]	TAG species or *Σ(TAG groups)*	S-M-L TAG classes[2]	1-2-3A[3]	ESI–MS **(VII)** mol%	±S.D.[4]	EI–MS **(VI)** Norm.[5] mol%	±DEV[6]	GC **(III)** Norm. mol%	±DEV
	18:1/18:1/18:3	L-L-L	2						
	Σ(54:5)			0.0979	±0.0144				
	54:5 total			**0.10**					
54:4	18:1/18:2/18:1	L-L-L	2					0.08	±0.00
	18:0/18:2/18:2	L-L-L	2						
	18:1/18:3_/18:0	L-L-L	3						
	Σ(54:4)			0.1173	±0.0129				
	54:4 total			**0.12**				**0.08**	
54:3	18:1/18:1/18:1	L-L-L	1			0.96	±0.09	0.78	±0.02
	18:1/18:2/18:0	L-L-L	3			0.22	±0.01		
	(18:0/18:3/18:0)	L-L-L	2						
	Σ(54:3)			1.5592	±0.0732				
	54:3 total			**1.56**		**1.18**		**0.78**	
54:2	18:1/18:0/18:1	L-L-L	2			0.66	±0.07	0.96	±0.05
	(18:0/18:2/18:0)	L-L-L	2			0.12			
	Σ(S)			1.3853	±0.0634				
	20:0/16:1/18:1	L-L-L	3						
	20:0/18:2/16:0	L-L-L	3						
	18:1/20:1/16:0	L-L-L	3						
	Σ(P+Po)			0.0653	±0.0002				
	54:2 total			**1.45**		**0.78**		**0.96**	
56:2	20:0/18:1/18:1	L-L-L	2						
	(20:0/18:0_/18:2)	L-L-L	3						
	Σ(20:0)			0.1552	±0.0211				
	18:1/20:1/18:0	L-L-L	3	0.1472	±0.0032				
	56:2 total			**0.30**					
	TOTAL			**18.88**		**18.13**		**13.47**	

[1] ACN:DB = Number of acyl carbons:number of double bonds

[2] Classification of TAGs according to the carbon chain length of esterified acyls. S, M, and L denote short-chain acyls (2–6 acyl carbons), medium-chain acyls (8–10 acyl carbons), and long-chain acyls (12–24 acyl carbons), respectively

[3] Classification of TAGs based on the number of different acyls in molecule. 1 = monoacid TAG, 2 = diacid TAG, and 3 = triacid TAG

[4] The sum of the molar amounts (mol%) of SPE fractions 1–4 ± standard deviation (S.D.). The S.D. was calculated using equation (Harris 1987) $S.D. = \sqrt{(S.D._1^2 + S.D._2^2 + S.D._3^2 + S.D._4^2)}$ in which $S.D._1$, $S.D._2$, $S.D._3$, and $S.D._4$ are standard deviations for molar amounts of SPE fractions 1–4, respectively; See **VII** for details

[5] The mol% of the identified even-numbered TAG species is normalized to the amount (86.2 mol%) of the identified even-numbered molecular species of TAGs determined by ESI–MS

[6] Average of duplicate measurements ± individual measurement. If no DEV was shown, only one measurement was used

[7] Structure in *italics* indicates regioisomer identified by MS^2

[8] Vertical line indicates which TAG species are combined to the amount (Σ/mol%) of different TAG groups

[9] Structure (*e.g.*, 16:1-16:1-4:0) is not determined by MS^2

[10] Structure in normal font indicates predominant regioisomer

[11] For example, 18:1/16:1_/4:0 indicates 18:1/16:1/4:0 + 18:1/4:0/16:1

[12] ( ) = minor molecular species in a group of TAG species with the same retention time

of even-numbered TAGs (Table 5.18). Odd-numbered SC-TAGs eluted in separate peaks in the following order: caproate, valerate, butyrate, and propionate TAGs. Both *sn*-1(3) and *sn*-2 isomers of mono short-chain TAGs were shown to exist. Odd-numbered LC-TAGs eluted mostly in two or three peaks. In general, TAG species with the longest odd ACN acyl group (*i.e.*, 19:0) eluted in the first peak, and those with shortest odd ACN acyl groups in last peak. Table 5.18 shows that the most abundant odd-numbered TAG group of SC-TAGs was [15:0/14:0/10:0 + 12:0/17:0/10:0 + 12:0/13:0/14:0 + 15:0/16:0_/8:0 + 16:0/16:0/7:0 + 14:0/18:0/7:0] 0.31 mol%, and that of LC-TAGs [18:1/15:0_/16:0 + 18:1/17:0_/14:0 + 16:0/17:1/16:0 + 16:0/16:1/17:0] 0.41 mol%.

Table 5.18 Composition (mol%) of the identified molecular species of odd-numbered TAGs in butterfat determined by normal-phase liquid chromatography–electrospray mass spectrometry (ESI–MS). **VII** refers to an original publication. (Table adapted from Table 3 in **VII**.)

ACN:DB[1]	TAG species or *Σ(TAG groups)*	ESI–MS **(VII)** mol%	±S.D.[2]	ACN:DB	TAG species or *Σ(TAG groups)*	ESI–MS **(VII)** mol%	±S.D.
Saturated TAGs				**Monoene TAGs**			
33:0	*15:0_/14:0/4:0*[3]	0.0765	±0.0024	**35:1**	*18:1/3:0_/14:0*	0.0029	±0.0003
	17:0_/10:0/6:0[4]	0.0160	±0.0005		*17:1/4:0/14:0*	[5]	
	33:0 total	**0.09**			*15:0/4:0/16:1*		
35:0	*16:0/16:0/3:0*				*Σ(B)*	0.0269	±0.0009
	18:0/14:0/3:0				**35:1 total**	**0.03**	
	Σ(3:0)	0.0026	±0.0000	**37:1**	*18:1/6:0/13:0*	0.1385	±0.0083
	16:0_/15:0/4:0	0.0015	±0.0000		**37:1 total**	**0.14**	
	15:0/14:0/6:0	0.0229	±0.0019	**39:1**	*17:0/18:1/4:0*	0.0768	±0.0009
	15:0/6:0/14:0	0.0150	±0.0000		*16:0/18:1/5:0*	0.0503	±0.0053
	35:0 total	**0.04**			*18:1/15:0/6:0*	0.0959	±0.0011
37:0	*18:0/16:0/3:0*	0.0151	±0.0024		**39:1 total**	**0.22**	
	17:0/16:0/4:0			**41:1**	*17:0/18:1/6:0*	0.0443	±0.0010
	18:0/15:0/4:0				18:1/15:0/8:0[6]		
	Σ(B)	0.1069	±0.0091		15:0/18:1/8:0		
	15:0_/16:0/6:0				17:0/16:1/8:0		
	14:0_/17:0/6:0				*Σ(Cy)*	0.0376	±0.0019
	Σ(Co)	0.2792	±0.0824		**41:1 total**	**0.08**	
	14:0/8:0/15:0	0.0481	±0.0039	**43:1**	*23:1/16:0/4:0*	0.0055	±0.0006
	37:0 total	**0.45**			18:0/10:1/15:0	0.0241	±0.0003
39:0	*18:0/17:0/4:0*	0.0563	±0.0009		18:1/17:0/8:0		
	16:0/17:0/6:0				15:0/18:1/10:0		
	18:0/15:0/6:0				16:1/17:0/10:0		
	Σ(B+Co)	0.0614	±0.0047		*Σ(Cy+Ci)*	0.0434	±0.0036
	14:0/18:0/7:0				**43:1 total**	**0.07**	
	16:0/16:0/7:0			**45:1**	18:1/10:0_/17:0		
	15:0_/16:0/8:0				18:0_/17:1/10:0		
	15:0/14:0/10:0				18:1/12:0_/15:0		

ACN:DB[1]	TAG species or *Σ(TAG groups)*	ESI–MS mol%	**(VII)** ±S.D.[2]
	12:0/17:0/10:0		
	12:0/13:0/14:0		
	Σ(other 39:0)	0.3091	±0.2370
	39:0 total	**0.43**	
41:0	*18:0/19:0/4:0*	0.0144	±0.0013
	18:0/17:0/6:0	0.0336	±0.0010
	18:0/6:0/17:0		
	16:0/17:0/8:0		
	16:0/9:0/16:0		
	16:0/15:0/10:0		
	14:0/17:0/10:0		
	15:0/12:0/14:0		
	Σ(other 41:0)	0.0807	±0.0029
	41:0 total	**0.13**	
43:0	19:0/8:0/16:0		
	19:0/14:0/10:0		
	Σ(19:0)	0.0050	±0.0002
	16:0/17:0/10:0		
	18:0/13:0/12:0		
	14:0/17:0_/12:0		
	15:0/12:0/16:0		
	15:0_/14:0/14:0		
	Σ(other 43:0)	0.1028	±0.0119
	43:0 total	**0.11**	
45:0	19:0/10:0/16:0		
	19:0/12:0/14:0		
	Σ(19:0)	0.0117	±0.0001
	16:0/17:0_/12:0		
	16:0/15:0_/14:0		
	14:0/17:0/14:0		
	(18:0/10:0/17:0)		
	(15:0/18:0/12:0)		
	(13:0/16:0/16:0)		
	Σ(other 45:0)	0.1236	±0.0118
	45:0 total	**0.14**	
47:0	19:0/10:0/18:0		
	19:0/12:0/16:0		
	19:0/14:0/14:0		
	Σ(19:0)	0.0265	±0.0011
	16:0/17:0/14:0		
	15:0/18:0_/14:0		
	15:0/16:0/16:0		
	Σ(other 47:0)	0.2093	±0.0085
	47:0 total	**0.24**	

ACN:DB	TAG species or *Σ(TAG groups)*	ESI–MS mol%	**(VII)** ±S.D.
	14:0/14:0/17:1		
	Σ(45:1)	0.0678	±0.0028
	45:1 total	**0.07**	
47:1	18:1/12:0_/17:0		
	18:1/15:0_/14:0		
	16:0/17:1/14:0		
	Σ(47:1)	0.1776	±0.0009
	47:1 total	**0.18**	
49:1	19:0/12:0/18:1		
	19:0/14:1/16:0		
	Σ(19:0)	0.0261	±0.0052
	18:1/17:0_/14:0		
	18:1/15:0_/16:0		
	16:0/17:1/16:0		
	16:0/16:1/17:0		
	Σ(other 49:1)	0.4121	±0.0271
	49:1 total	**0.44**	
51:1	19:0/14:0/18:1		
	(19:0/14:1_/18:0)[7]		
	(19:0/16:0/16:1)		
	Σ(19:0)	0.0515	±0.0034
	18:1/16:0_/17:0		
	18:0/17:1/16:0		
	18:1/15:0/18:0		
	Σ(other 51:1)	0.2612	±0.0084
	51:1 total	**0.31**	
53:1	17:0_/18:0/18:1	0.0369	±0.0037
	20:0/15:0/18:1		
	(20:0/16:0_/17:1)		
	Σ(20:0)	0.0081	±0.0005
	19:0/16:0/18:1		
	(19:0/16:1_/18:0)		
	(16:0/19:1_/18:0)		
	Σ(19:0+19:1)	0.0208	±0.0020
	53:1 total	**0.07**	
55:1	*19:0/18:1_/18:0*	0.0371	±0.0261
	18:1/19:0/18:0		
	18:0/19:1/18:0		
	Σ(other 55:1)	0.0725	±0.0065
	55:1 total	**0.11**	
57:1	18:0/23:1_/16:0		
	18:1/23:0/16:0		
	Σ(57:1)	0.0590	±0.0044
	57:1 total	**0.06**	

ACN:DB[1]	TAG species or *Σ(TAG groups)*	ESI–MS **(VII)** mol%	±S.D.[2]
49:0	19:0/14:0/16:0		
	(19:0/12:0/18:0)		
	Σ(19:0)	0.0581	±0.0063
	18:0/15:0_/16:0		
	16:0/16:0/17:0		
	Σ(other 49:0)	0.1770	±0.0185
	49:0 total	**0.24**	
51:0	18:0/15:0/18:0		
	18:0/17:0/16:0		
	Σ(18:0)	0.0498	±0.0058
	20:0/17:0/14:0		
	20:0/15:0/16:0		
	Σ(20:0)	0.0115	±0.0001
	19:0/14:0/18:0		
	19:0/16:0/16:0		
	Σ(19:0)	0.0378	±0.0061
	51:0 total	**0.10**	
53:0	19:0/16:0/18:0	0.0031	±0.0006
	20:0/17:0/16:0		
	20:0/15:0/18:0		
	Σ(20:0)	0.0018	±0.0002
	18:0/19:0/16:0		
	18:0/17:0/18:0		
	Σ(other 53:0)	0.0043	±0.0003
	53:0 total	**0.01**	
55:0	18:0/21:0/16:0		
	19:0/18:0/18:0		
	(20:0/17:0/18:0)		
	Σ(55:0)	0.0188	±0.0010
	55:0 total	**0.02**	
	TOTAL	**1.98**	

ACN:DB	TAG species or *Σ(TAG groups)*	ESI–MS **(VII)** mol%	±S.D.
	TOTAL	**1.78**	
Polyene TAGs			
53:2	19:1/16:0_/18:1		
	18:2/15:0/20:0		
	Σ(19:1+20:0)	0.0091	±0.0009
	18:1/18:0/17:1		
	18:0/18:2/17:0		
	Σ(other 53:2)	0.0823	±0.0137
	53:2 total	**0.09**	
57:2	18:1/21:0/18:1		
	18:1/16:0/23:1		
	18:1/21:1_/18:0		
	Σ(57:2)	0.0794	±0.0073
	57:2 total	**0.08**	
	TOTAL	**0.17**	
Odd-numbered TAGs TOTAL		**3.93**	

[1] ACN:DB = Number of acyl carbons:number of double bonds

[2] The sum of the molar amounts (mol%) of SPE fractions 1–4 ± standard deviation (S.D.). The S.D. was calculated using equation (Harris 1987) $S.D. = \sqrt{(S.D._1^2 + S.D._2^2 + S.D._3^2 + S.D._4^2)}$ in which $S.D._1$, $S.D._2$, $S.D._3$, and $S.D._4$ are standard deviations for molar amounts of SPE fractions 1–4, respectively; see **VII** for details

[3] Structure in *italics* indicates regioisomer identified by MS^2

[4] For example, 18:1/16:1_/4:0 indicates 18:1/16:1/4:0 + 18:1/4:0/16:1

[5] Vertical line indicates which TAG species are combined to the amount (Σ/mol%) of different TAG groups

[6] Structure in normal font indicates predominant regioisomer

[7] ( ) = minor molecular species in a group of TAG species with the same retention time

6 DISCUSSION

6.1 Fractionation of TAGs prior to chromatographic separation

In the present study (**I**), a simple method of low-pressure column chromatography using p-propylbenzene sulfonic acid SPE columns in silver ion form was applied to produce saturated, monounsaturated and polyunsaturated TAG fractions for further examination. In general, determination of the TAG composition of bovine milk fat (MF) is a demanding analytical task due to the numerous molecular species with a wide range of molecular weights (MWs) and varying fatty acid content. In order to increase the number of identified and quantified TAG species in MF, fractionation of TAGs is essential prior to chromatographic determination (Myher et al. 1988). Traditionally, the TAGs of MFs have been fractionated by silica TLC into two (high/low MW) (Blank and Privett 1964, Mottram and Evershed 2001) or three fractions (high/medium/low MW) (Kuksis and Breckenridge 1968, Breckenridge and Kuksis 1968b, Parodi 1980, Myher et al. 1988, 1993, Banks et al. 1989, Steele and Banks 1994) according to the MW, and by silver ion TLC (Ag-TLC) into the fractions with different degree of unsaturation and configuration of double bonds (Blank and Privett 1964, Kuksis and Breckenridge 1968, Parodi 1980, Lund 1988, Myher et al. 1988, Marai et al. 1994, Fraga et al. 1998, Robinson and MacGibbon 1998a,b, 2000, Fontecha et al. 2000), or by both methods (Blank and Privett 1964, Kuksis and Breckenridge 1968, Parodi 1980). In addition to TLC methods, rp-HPLC (Weber et al. 1988a,b, Maniongui et al. 1991, Gresti et al. 1993, Dugo et al. 2005) and Ag-HPLC (Laakso et al. 1992, Laakso and Kallio 1993a,b, Brühl et al. 1993, 1994, Dugo et al. 2006) have been used frequently as a prefractionation method and, more or less, as the first dimension of 2D chromatography to produce a high number of TAG fractions (even more than 50). However, improved TLC methods (Fraga et al. 1998, Robinson and MacGibbon 1998a,b, 2000, Fontecha et al. 2000) have shown that even Ag-TLC could fractionate MF TAGs up to nine different fractions (Robinson and MacGibbon 1998a,b, 2000) according to the degree of unsaturation, *cis–trans* isomerism, and carbon chain length of fatty acyls. Further, Ag-TLC is generally regarded as more suitable for the fractionation of highly unsaturated TAGs than low-pressure silver ion column chromatography (Morris 1966, Christie 1989a). However, Christie (1990) showed that p-propylbenzene sulfonic acid SPE columns loaded with silver ions were suitable for the fractionation of the TAGs in palm oil and cocoa butter, which are relatively saturated food fats with less complex TAG composition than that of MF. Even so, the present study (**I**) demonstrated that Christie's (1990) elution scheme had to be improved in order to get pure fractions by silver ion column chromatography from more complex TAG mixtures such as milk fat derivatives. Even though the fractionation of TAGs according to the degree of unsaturation is based on the complex formation between silver ions attached to the solid phase of SPE columns and double bonds of TAGs in the mobile phase, other interactions that are relevant to the specific TAG composition of MFs occur as well. The nonpolar nature of the p-propylbenzene ring and the mostly masked polar nature of silica gel matrix are able to affect the rate of resolution of most nonpolar and polar TAG species of MF, respectively.

The present study (**III, VI, VII**) together with several other findings (*e.g.*, Parodi 1981, Gresti et al. 1993, Laakso and Kallio 1993a, Nagy et al. 2013) confirmed that the most

abundant TAG classes of MF are saturated (S) and monoene (M) TAGs, hence, the overlapping of these fractions in the silver ion chromatography had to be minimized. In the present study, the overlapping of S and M fractions was shown to be *ca*. 8% by the TAG analysis (**I**). Much higher overlapping of the two fractions was reported in the fractionation of the most volatile 2.5% distillate of butteroil by Ag-TLC (Kuksis and Breckenridge 1968). In their studies, the M fraction contained only 18.8 mol% of MUFAs. In the present study, the molar amount of MUFA in the M fraction (= 26.0 mol%) was much closer to the theoretical content (= 33.3 mol%). The most feasible explanation for the overlapping was the high content of the polar saturated SC-TAGs with retarded elution (Kuksis and Breckenridge 1968). The present study showed a higher relative proportion of more polar short-chain FAs (4:0 and 6:0) and long-chain FA 20:0 in the monoene TAG fraction of randomized butterfat, which agreed with the explanation of Kuksis and Breckenridge (1968). Another logical reason for overlapping of TAG fractions was a different migration rate of *cis* and *trans* isomers of unsaturated TAG species. In Ag-TLC studies, Parodi (1980) and Myher et al. (1988) observed that the *cis–trans* isomerism of unsaturated TAGs had a definite effect on the migration of isomeric TAG species on $AgNO_3$-TLC plates. Parodi (1980) estimated the overlapping of *trans* monoene TAGs and *cis* monoene TAGs with diene TAGs to range from 1.2 to 11.4% and from 1.8 to 3.5%, respectively. The present work indicated similar behavior for *cis* and *trans* isomers of C18:1. The ratio of *cis*-18:1 to *trans*-18:1 was 1:1 and 10:1 in the saturated and monoene fractions, respectively, suggesting that a higher proportion of *trans* than *cis* isomers migrated ahead with saturated TAGs.

Improved and relatively simple low-pressure column chromatographic methods using plain silica gel SPE columns (**V**, **VII**) and p-propylbenzene sulfonic acid SPE columns in silver ion mode (**I**, **II**, **III**, **VI**) were used as a sole fractionation method of the TAGs in BF before np-HPLC–MS and high-resolution GC/GC–MS, respectively. In early studies concerning fractionation of milk fat, combination of silica gel prefractionation prior to $AgNO_3$ TLC was strongly recommended due to the yield of highly impure fractions otherwise (Kuksis and Breckenridge 1968). Combining silica gel and silver ion SPE chromatography in the present study would have probably increased further the number of identified TAG species. However, increased number of analytical steps might have complicated the quantification of TAGs to some extent. In addition, the high resolving power of phenyl(65%)methyl-silicone columns and that of two or three np-HPLC columns in series together with mass spectrometric methods enabled determination of a substantial number of TAG species in butterfat even after a single prefractionation step. Silver ion chromatography prior to GC was essential for the determination of elution order of acyl chain isomers and regioisomers of TAGs on phenyl(65%)methylsilicone columns without distracting overlap with TAG species of different degree of unsaturation. Further, both fractionations increased the number of TAG species above detection limit enabling detection of more rare molecular species of TAGs, for example acetate TAGs and odd-numbered TAGs as shown in **VII**.

6.2 Identification and chromatographic resolution of TAG species

Identification of TAG classes, individual molecular species, and TAG isomers in BF and standard mixtures was based on the resolving power of a polarizable phenyl(65%)-

methylsilicone capillary column or that of two or three microbore normal-phase analytical HPLC columns in series and specific ions (*m/z*) originated from EI–MS, ESI–MS/MS^2 of TAGs (**I–VII**).

In general, resolution of TAG species on a long, polar GC column was shown to be superior to that on an apolar column. In the early MF studies (Kuksis and McCarthy 1962, Kuksis et al. 1963, Kuksis and Breckenridge 1965, 1966, Murata and Takahashi 1973, Murata 1977, Parodi 1972, 1979b, Morrison and Hawke 1977), short apolar packed columns were used to separate TAGs according to the ACN. Corresponding resolution pattern was observed on a short (< 5 m) apolar capillary column (Molkentin and Precht 1994, 2000, Lozada et al. 1995, Gutiérrez et al. 2009). Later, an improved resolution of acyl chain isomers of TAGs which contained short-chain or unsaturated acyl groups was achieved as long (> 15 m) apolar columns were applied to separation of MF TAGs (Grob et al. 1980, Geeraert and Sandra 1987). However, polar phenylmethylsilicone fused-silica capillary columns provided even better resolution of TAG species and enabled the use of high column temperatures (Geeraert and Sandra 1985, 1987). Myher et al. (1988) and Kalo et al. (1989b, 1990) showed that phenyl(50%)methylsilicone columns separated TAG species in MF according to the level of unsaturation and the fatty acid composition of TAG molecules. The present study established the high resolving power of phenyl(65%)methylsilicone for substantial number of TAG species in synthesized TAG standards (**III, VI**), in BF (**III, VI**), and in modified BFs (**I, II, III, VI**).

For the first time, the present study (**II, III, VI**) demonstrated comprehensively the elution order of the isomers of isobaric TAG species on phenyl(65%)methylsilicone capillary columns using retention indices (RI) of a high number (112) of synthesized TAG species and specific fragmentation of TAGs in GC–EI–MS. Mono short- and medium-chain isobaric TAGs were shown to elute on phenyl(65%)methylsilicone columns from first to last as follows: XXX(X = 10:0–18:0) >> XX8:0 >> XX6:0 >> XX4:0, which was the same order as was previously reported on polar polyester column (Kuksis et al. 1973) and on polarizable phenyl(50%)methylsilicone column (Myher et al. 1988). Further, the analysis of several isomers of acetate and propionate TAGs on phenyl(65%)methyl capillary column showed the elution order of XX3:0 >> XX2:0 (X = 16:0–24:0, 18:1) (Huang et al. 1994). Myher et al. (1988) observed that replacing of caproyl acyl group with butyryl group in TAGs increased the equivalent carbon numbers (ECN) 0.29 units. In the present study, the RIs of isobaric mono SC-TAG species increased on the average 29 RI units when butyryl group substituted for caproyl group in TAG molecule. Correspondingly, the differences between the mean RIs of the acyl chain isomer pairs consisting XX6:0 / XX8:0 and XX8:0 / XX10:0 (X = 12:0–18:0) were 15 and 16 RI units, respectively, indicating partial chromatographic resolution. However, when the isobaric TAG species with both mono- and di-short- or medium-chain isomers existed, butyrate and caproate TAGs and caproate and caprylate TAGs were detected to elute in reverse order in some cases, *e.g.,* 12:0-12:0-4:0 before 16:0-6:0:6:0 and 14:0-14:0-6:0 before 18:0-8:0-8:0.

In the earlier studies, phenylmethylsilicone capillary columns were shown to separate the regioisomers of acetate (Myher et al. 1988, Huang et al. 1994), propionate (Huang et al. 1994), and butyrate TAGs (Myher et al. 1988, Klemann et al. 1994, Kalo et al. 1996, Angers

and Arul 1999). In the present study, several pairs (10) of the regioisomers of monocaproate TAGs were proved to resolve on a phenyl(65%)methylsilicone analytical GC-column as well as the 12 pairs of the regioisomers of monobutyrate TAGs. No regioisomers of acetate and propionate TAGs were analyzed by HR-GC in the present study. Myher et al. (1988) reported that the changing of butyryl acyl group from *sn*-1(3) position to *sn*-2 position resulted in 0.16 units increase of ECN on a phenyl(50%)methyl column. Correspondingly, the difference between the RIs of the regioisomers of monobutyrate TAGs was on the average 16 and 18 RI units for saturated and unsaturated TAGs, respectively, in the present study. For monocaproate TAGs, the respective difference was 10 and 12 RI units for saturated and unsaturated TAGs, respectively.

Normal-phase HPLC columns have been infrequently used to resolve TAG species of natural fats and oils. However, in the present study (**IV, V, VII**) the potential of np-HPLC in the analysis of intact TAG species in BF was introduced. The relatively high ability of np-HPLC columns to separate TAGs according to the descending ACN and ascending number of double bonds was demonstrated already by Plattner and Payne-Wahl (1979) and Rhodes and Netting (1988). Later, Mangos et al. (1999) reported on the separation of the regioisomers of acetate and propionate TAGs on a small particle size np-HPLC columns. Further, the regioisomers of mono- and dibutyryl and mono- and dicaproyl TAGs in structured lipids were shown to be separated at least partially on a small (3 μm/5 μm) particle size np-HPLC columns (Lee et al. 2002, 2003, 2007, 2008). In the present investigation (**IV, V, VII**), applicability of two and three microbore np-columns in series to resolve the acyl chain isomers of SC- and MC-TAGs and the regioisomers of short- and long-chain TAGs was evaluated. In the ACN range 32–42 of even-numbered TAGs, acyl chain isomers of short- and medium-chain TAG species in BF eluted in five peaks with increasing polarity on three np-HPLC columns in series as follows: medium-chain TAGs >> caproate TAGs >> butyrate TAGs >> *sn*-2 acetate TAGs >> *sn*-1(3) acetate TAGs. In the ACN range 40–46 of even-numbered TAGs, medium-chain TAG species coeluted with long-chain TAGs, which complicated identification and especially quantification of TAG species. In addition, an unexpected resolution of some long-chain TAG species containing 20:0 acyl group was observed in the ACN range 48–56. Some saturated, monounsaturated and diunsaturated molecular species of the 20:0/X/X type TAGs were shown to elute as an individual peak separated from other isobaric TAGs. In addition, some regioisomers of 20:0/X/X type TAGs (*i.e.*, *rac*-20:0/18:0/18:1 and 18:1/20:0/18:0; *rac*-20:0/18:0/18:0 and 18:0/20:0/18:0) separated from each other enabling reliable identification and quantification. Further, the separation of the regioisomers of short-chain TAGs on two and three microbore silica gel HPLC columns in series was assessed. The regioisomer of mono- and di-short-chain TAGs in which the shortest acyl chain was in the secondary position was shown to elute in the first peak on np-HPLC columns followed by the two isomers in which the shortest acyl chain was in the primary *sn*-positions. This elution pattern of the regioisomers of SC-TAGs was in accordance with the previous studies, which used a single np-HPLC column (Mangos et al. 1999, Lee et al. 2002, 2003, 2007, 2008). The resolving power of three microbore silica gel columns in series was higher than that of two columns in series. All regioisomers of short-chain butyrate TAGs had a baseline resolution (resolution value, RS = 1.5–2.9) and the regioisomers of monocaproates resolved close to the baseline (RS = 1.0–1.5) and those of dicaproates separated partially (RS = 0.5–1.3) on three silica gel

analytical np-columns in series. However, concurrently of the improved resolution of the regioisomers of butyrate and caproate TAGs on three np-columns, an impaired determination of the regioisomers of acetate TAGs occurred and the number of identified isomers, especially *sn*-1(3) isomers, of butterfat decreased. The present work showed clearly the significant separation power of several microbore silica gel columns in series, which made possible a reliable identification of isobaric TAGs in BF. Further, it enabled mass spectrometric identification of the regioisomers of SC-TAG species on the basis of the different fragmentation in ESI–MS^2.

Early MS studies of TAGs (Ryhage and Stenhagen 1960, Barber et al. 1964, Lauer et al. 1970, Aasen et al. 1970) established empirically the characteristic fragmentation of TAG species by electron ionization resulting in the production of several specific fragment ions: $[M–18]^+$, $[M–RCOO]^+$, $[M–RCOOH]^+$, $[M–RCOOCH_2]^+$, $[RCO]^+$, $[RCO + 74]^+$, and $[RCO + 128 + 14n]^+$. In the present study (**I, II, VI**), $[M–RCOO]^+$, $[RCO]^+$, $[RCO + 74]^+$, and $[RCO + 128]^+$ ions were used for identification of TAG species in BF and randomized butterfat by exploiting the calculated *m/z* values of the fragment ions. If three of the four ions were found in the full EI mass spectra of TAGs of the same retention time-window, a positive detection of the fragmented acyl group was confirmed and the acyl was used to compile a possible structure for TAG species. Due to the fact that $[RCO]^+$, $[RCO + 74]^+$, and $[RCO + 128]^+$ ions were identical for all TAG species containing the acyl in question and $[M–RCOO]^+$ ions were similar for all isobaric TAGs from which the same acyloxy ion was cleaved, fractionation by low-pressure silver ion column chromatography prior to analysis and established resolution (RIs / RRTs of standard TAG species) on a polarizable phenyl(65%)methylsilicone column were essential for the identification of the individual TAG species or the groups of TAGs of acyl chain isomers. Although *sn*-1(3) and *sn*-2 positional isomers could be differentiated by specific $[M – RCOOCH_2]^+$ ions formed exclusively by cleavage of fatty acyl group from primary positions in EI–MS (Ryhage and Stenhagen 1960, Barber et al. 1964, Lauer et al. 1970, Kallio et al. 1989), in the present study the regioisomers of short-chain TAG species were differentiated solely on the basis of the adequate chromatographic resolution of the isomers. Further, detection of specific $[M–RCOO]^+$ ions were regarded necessary for positive identification. However, when *m/z* of the $[M–RCOO]^+$ ions was higher than 600, the abundance of the ion was not high enough for reliable identification. Utilization of selected ion monitoring increased the abundance of $[M–RCOO]^+$ ions of major molecular species of TAGs in butterfat but restricted the detection of more rare TAG species with uncommon fragmentation. Identification of TAG species in BF using specific fragmentation of TAGs in GC–EI–MS enabled confirmation of the elution order of isobaric TAG isomers on a phenyl(65%)methylsilicone column and *vice versa*, adequate resolution of TAG isomers, even though slightly poorer than on the similar column in GLC–FID, was essential for the identification of regio- and acyl chain isomers of TAGs by GC–EI–MS.

In the present work, an improved method was developed for the identification of the ACN:DB classes of TAG species by np-HPLC–ESI–MS, and that of acyl chain and regioisomers of SC-TAGs according to the specific fragmentation by np-HPLC–ESI–MS^2. Applicability of ESI–MS for the analysis of TAGs was reported first time by Duffin et al. (1991), when a significant number of positive ammonium adduct ions were produced from

neutral TAG molecules by introducing TAGs into mass detector in an ammonium acetate-chloroform-methanol solution through an ESI interface. In addition, they (Duffin et al. 1991) showed that the low energy collision induced dissociation spectra of ammonium adducts consisted of the major daughter ions that were fragmented in ESI–MS^2 by cleavage of the fatty acyl groups from *sn*-1–3 positions of TAG molecules and, hence, enabled the compositional assessment of TAG species. Later, ammonium acetate (Cheng et al. 1998, Dorschel 2002, Marzilli et al. 2003), ammonium formate (Hvattum 2001, Byrdwell and Neff 2002, Malone and Evans 2004, Herrera et al. 2010, Nagy et al. 2013), and ammonium hydroxide (Leskinen et al. 2007) were used successfully for formation of $[M+NH_4]^+$ ions for the analysis of TAGs in ESI–MS. In early ESI–MS^2 studies, no differences were observed in the cleavage of acyl groups from secondary and primary positions of long-chain TAGs (Duffin et al. 1991, Cheng et al. 1998) and medium-chain TAGs (Segall et al. 2004). However, strong evidence of several studies (Hsu and Turk 1999, Hvattum 2001, Han and Gross 2001, Dorschel 2002, Byrdwell and Neff 2002, Marzilli et al. 2003, Malone and Evans 2004, Lévênque et al. 2010, Nagy et al. 2013) confirmed later that *sn*-1(3) and *sn*-2 isomers of long-chain TAGs could be differentiated on the basis of different cleavage from secondary and primary positions. Similar differentiation based on the lower loss of fatty acyls from the secondary position of TAGs has been reported for the regioisomers of LC-TAGs in other MS analysis using direct inlet LC–CI–MS (Myher et al. 1984), HPLC–APCI–MS (Motram and Evershed 1996, Mottram et al. 1997, Mottram et al. 2001, Jakab et al. 2003, Fauconnot et al. 2004, Leskinen et al. 2007), and ESI–MS^2 based on high-energy CID fragmentation of sodium and lithium adducts (Cheng et al. 1998, Hsu and Turk 1999). In addition, Kim et al. (2000) reported differentiation of the regioisomers of SC-TAGs by high-energy CID of sodiated precursor ions in FAB–MS^2. An alternative way to differentiate the regioisomers by MS was based on the specific fragment ions cleaved from the primary positions of TAGs as was noted for positive $[M–RCOOCH_2]$ ions by Ryhage and Stenhagen (1960) in their EI–MS studies and for negative [M–H–RCOO–100] ions by Kallio and Currie (1993), Kallio and Rua (1994), Kurvinen et al. (2001a,b), and Yli-Jokipii et al. (2003) in their NICI studies.

In the present study (**IV, V, VII**), a chloroform–methanol–ammonia water mixture was introduced post column into the sample flow from analytical HPLC columns in order to produce relatively clean MS spectra of $[M+NH_4]^+$ ions without extra sodium or potassium adducts or a high number of fragment ions derived from ammonium adducts. The clean MS spectra of ammonium adducts provided a solid basis for ESI–MS^2 studies, although Nagy et al. (2013) showed as well, how impurities, *e.g.,* sodium adducts could be exploited in identification of TAG species in ESI–MS^2. The present study (**IV, V**) showed that the cleavage of short-chain acyls (4:0, 6:0) from the *sn*-1(3) positions was in all cases at least 2.3 times (on the average 3.7–12.3 times) higher than from the *sn*-2 position of mono short-chain TAGs. Correspondingly, the cleavage of long-chain acyls from the primary positions of di-short-chain TAGs was shown to be on the average 4.4–16.7 times higher than from the secondary position. These observations enabled clear-cut differentiation between the regioisomers of di- and triacid mono-SC-TAGs or di-SC-TAGs. The present study (**IV**, **V, VII**) together with some other studies (Hvattum 2001, Dorschel 2002, Marzilli et al. 2003, Nagai et al. 2013) strongly suggested that the poor cleavage of fatty acyl from the secondary position of TAGs in ESI–MS^2 was actually independent of the carbon chain length of acyl

group. However, the extent of the difference between the cleavage from the *sn*-2 and *sn*-1(3) positions was affected by the carbon chain length of fatty acyl and the chromatographic and mass spectrometric conditions. For example, Nagy et al. (2013) used calculation that based on theoretical fragmentation patterns of regioisomers consisting of fatty acyls with different carbon chain length and degree of unsaturation as one criterion in identification of regioisomers of TAGs in their narp-HPLC–ESI–MS^2. In the present study (**IV**, **V**), the relative ease of the cleavage of butyryl group from the *sn*-1(3) positions was higher than that of caproyl group in all short-chain TAG classes. In addition, the average relative ease of the cleavage of fatty acids from the primary positions was higher in all SC-TAG classes when the HPLC–ESI–MS^2 system with higher chromatographic resolution (*i.e.*, three microbore normal-phase columns in series) was used. In general, the average ratio of the abundance of the ions originated from primary and secondary positions was higher in the present study than in other ESI–MS studies (Han and Gross 2001, Marzilli et al. 2003) or in CI–MS (Myher et al. 1984) and in APCI–MS (Mottram and Evershed 1996) investigations for long-chain TAGs. Further, the generally lower relative abundance of the fatty acyl originated from the *sn*-2 position enabled a relatively reliable assessment of the principal reverse isomer of triacid TAGs even if the isomers co-eluted in the same chromatographic peak as was demonstrated (**IV**) for the reverse isomers of enanthoylcaproylbutyroyl-*rac*-glycerol TAG standard (*rac*-6:0/7:0/4:0 *vs. rac*-7:0/6:0/4:0).

6.3 Quantification of TAG classes and individual TAG species

In all of the quantification methods (GC, GC–EI–MS, and np-HPLC–ESI–MS) applied in the present study, the leading principle was the use of specific molar correction factors (MCFs) for ACN:DB TAG classes and individual TAG species combined with the use of internal TAG standard. This was assessed necessary for reliable quantification due to the highly intricate TAG composition and structure of bovine BF. Mares (1988) concluded that empirical correction factors (ECFs) were essential part of reliable quantification of TAG mixture by capillary CG. Recently, Yoshinaga et al. (2017) showed concretely that CFs for 47 synthesized molecular species of TAGs (including regioisomers) varied significantly, but they noted as well that CFs for the regioisomers of TAGs did not show any marked difference in their GLC study. In the present study, commercially available monoacid TAG standards (TAG 12:0–TAG 54:0, TAG 54:3) were applied to determine ACN-based MCFs of TAGs by high-resolution capillary GC equipped with a phenyl(65%)methylsilicone column, cold on-column injection, flame-ionization detection, and constant carrier gas flow. MCFs for saturated monoacid TAGs showed a U-shaped parabolic dependence on ACN, which enabled calculation of MCFs for other ACN classes of TAGs. Further, a slightly higher MCF (*ca*. 5%) for TAG 54:3 than that for TAG 54:0 suggested only moderate thermal degradation of unsaturated TAGs which made possible to apply the same MCF to every molecular species of TAGs with the same ACN regardless of different degree of unsaturation. In addition, high linearity ($n = 15$, $r^2 = 0.993–0.999$) in linear calibration confirmed that reliable quantification could be performed even though the concentration of analytes in standard solutions varied considerably (between 62.5 and 2500 μg/5 mL). This agreed with the earlier findings of Mares and Husek (1985) who stated that ECFs were more or less independent of the concentration of TAGs in the ACN range 30–54 when rather

similar cold on-column injection and a high quality GC capillary column were used in their study.

Use of more specific MCFs was assessed necessary when mass spectrometry was applied to quantification of the isomers of isobaric TAG species. Early quantification studies of TAGs (Murata and Takahashi 1973, Murata 1977, Schulte et al. 1981, Murata and Takahashi 1977) were carried out without any correction of relative ion yield (*i.e.*, without ECFs), or ECFs were calculated merely for ACN:DB classes of TAGs (Hites 1970, Kallio et al. 1991). Later, however, several factors of TAG structure have shown to affect the relative ion yield in MS analysis which emphasized the need for the use of specific ECFs for different isomers. Some earlier MS studies showed that the size of the ions produced by different ionizing methods, the degree of unsaturation, the carbon number of fatty acyls with the same ACN:DB, and regioisomerism (Hites 1970, Lauer et al. 1970, Schulte et al. 1981, Myher et al. 1984, Duffin et al. 1991, Kallio et al. 1991, Kallio and Currie 1993) had an impact on the relative ion yield, which was confirmed with GC–EI–MS and HPLC–ESI–MS applications in the present study.

In the MF TAG studies, the finding of an adequate number of relevant TAG standards to be used for the calculation of RFs was assessed challenging due to a huge number of different TAG species in MF (Holčapek et al. 2005, Lisa et al. 2009). In the present GC–EI–MS investigation (**VI**), a novel approach to produce TAG standard mixtures of only ten different TAG species by chemical interesterification of three different monoacid TAGs was introduced. Later, Lisa et al. (2009) used similar concept to synthesize TAG standards in their Ag-HPLC–MS study of TAG isomers. This procedure (**VI**) enabled a flexible synthesis of desired TAG classes and molecular species, straightforward identification of TAGs, and excellent chromatographic resolution and integration of chromatograms, which resulted in reliable determination of MCFs for $[M–RCOO]^+$ ions of a significant number ($n = 104$) of individual molecular species of TAGs. In earlier EI and CI–MS studies, a relatively limited number of TAG standards were used for the correction of ion yield (Hites 1970, Kallio et al. 1991, Kallio and Currie 1993, Byrdwell et al. 1996, Kurvinen et al. 2001a) or ion yield was adjusted on the basis of the known or calculated TAG composition of natural oils (Hites 1970, Myher et al. 1984, Kallio et al. 1991, Kallio and Currie 1993, Byrdwell et al. 1996, Kurvinen et al. 2001a). Use of synthesized TAG standards for the determination of ECFs has been slightly controversial. Myher et al. (1984) preferred the use of synthetic TAGs for determination of ECFs to the use of the TAGs from natural sources. On the hand, Byrdwell et al. (1996) observed that the use of ECFs calculated on the basis of synthetic randomized TAG mixture resulted in a poor agreement between calculated and analyzed TAG composition of edible oil, probably due to the concentration dependence of ECFs. In the present study, however, the concentration dependence of MCFs was not confirmed, even though the variation in concentration of individual TAG species was high, which suggested feasibility of the ECFs of synthesized origin in the present study (**VI**).

Relatively high number of the empirically determined molar CFs, together with high reproducibility (average $r^2 > 0.96$) of the determination of CFs regardless of the size, concentration, and origin of specific ions, enabled reliable extrapolation of MCFs in EI–MS on the basis of ACN and selection of esterified fatty acids. In the present study, variation of

the values of ECF was broader than in CI–MS studies of Myher et al. (1984), who observed that relative CFs were in the range from 0.5 to 1.7 for TAG with ACN 48–54 (maximum six double bonds). The TAG composition and structure in the present investigation were more intricate than in their study (Myher et al. 1984), hence the highest detected MCF was 42, 47, and 72 times higher than the lowest MCF of saturated, monoene, and polyunsaturated TAGs, respectively. However, in the majority of the MCFs with the same degree of unsaturation the difference between the highest and lowest ECF values was only fourfold, which agreed with the findings of Myher et al. (1984). In addition, the ionization method (CI *vs*. EI) and detected ions (TIC *vs*. SIM) used in Myher's et al. study (1984) differed from those of the present study (**VI**).

Present investigation showed that the values of MCFs for *sn*-1(3) isomers of butyryl and caproyl TAGs were, on the average, 2–3-fold lower than those of their *sn*-2 isomers. Due to the fact that calculated MCFs were inversely proportional to the integrated areas of the peaks in SIM chromatograms, the finding suggested a lightly higher relative ion yield of $[M–RCOO]^+$ ions of short-chain acyls from *sn*-1(3) positions than *sn*-2 position. Another hypothetical reason (*cf.*, Evershed 1996) for the observed difference could be due to genuinely different response of $[M–RCOO]^+$ fragments of regioisomers in EI–MS detector owing to a slight difference in spatial structure of regioisomers. As Evershed (1996) noted, an irreversible adsorption of TAG species (*i.e.*, only one regioisomer) on the stationary phase could result in different ECFs. However, the latter reason is highly improbable, because the GC studies (**III**, **VI**) showed an expected area ratio (2:1) of *sn*-1(3):*sn*-2 isomers of SC-TAGs in randomized TAG mixtures indicating similar adsorption behavior of both regioisomers on phenylmethylsilicone columns.

In np-HPLC–ESI–MS studies (**IV**, **V**), MCFs were determined for $[M + NH_3]^+$ ions derived from SC-TAGs using synthesized TAG standards, linear calibration method, and internal TAG standard. The variation of MCF values of regioisomers was more moderate than in EI–MS studies using corresponding quantification method. The highest MCF was 7 and 27 times higher than the lowest MCF when two and three HPLC-columns were used, respectively, compared to EI–MS method, in which the highest values of MCFs of *sn*-1(3) and *sn*-2 isomers of different degree of unsaturation were 26–52 and 33–201 times higher, respectively, than the lowest values. Broader variation in the values of MCFs with the method using three HPLC columns in series was presumably due to a higher number of the pairs of the regioisomers (42 *vs*. 20) and slightly different integration of ion chromatograms. However, both methods showed high coefficient of determination ($r^2 = 0.93–0.99$) in linear calibration enabling reliable correction of relative ion yield in SC-TAG mixtures with varied concentrations. In addition, MCFs of SC-TAGs showed polynomial (or linear, in some minor cases) dependence on ACN, which enabled extrapolation of MCFs of non-synthesized SC-TAG species. The MCFs of acyl chain isomers and regioisomers (difference 1–80%) were shown to differ from each other markedly. In addition, asymmetry of SC-TAG molecule was shown to have a distinct influence on MCFs. The *sn*-2 isomers of triacid mono SC-TAGs (*e.g.*, L/S/L') were shown to have higher MCFs than diacid mono SC-TAGs (*e.g.*, L/S/L) of isobaric TAG isomers. The more substantial the difference between the carbon chain lengths of the acyls in the primary positions was the higher was MCF. The same phenomenon was observed for *sn*-1(3) isomers (L/L/S *vs.* L/L'/S) but in somewhat

less extent. This finding suggested easier formation of ammonium adducts of symmetric SC-TAG molecules than those of asymmetric molecules in ESI–MS.

MCFs determined for several synthetic butyrate and caproate TAG species (**IV**, **V**) will provide a solid basis for quantitative evaluation of over one third of all molecular species of TAGs in butterfat (*cf.*, Gresti et al. 1993) by np-HPLC–ESI–MS. However, BF contains a significant amount of other molecular species of TAGs, *i.e.*, medium- and long-chain TAGs, acetate TAGs and odd ACN TAGs. Hence, a less laborious and straightforward method to determine the MCFs for butterfat TAG was developed by modifying a method suitable for HPLC–APCI–MS analysis (Byrdwell et al. 1996). MCFs were determined by np-HPLC–ESI–MS for major ACN:DB classes (105), several isomers of LC-, MC-, and SC-TAGs, and some SC molecular species of TAGs (**VII**) using calculated TAG composition of chemically interesterified butterfat (*cf.*, Kalo et al. 1986a), added internal standard, and integrated areas of ion chromatograms of $[M + NH_3]^+$ ions. Wide variation of MCFs, which is characteristic for CFs for TAGs of butterfat regardless of quantification method, was detected, but definite trends dictated by the number of acyl carbons were observed, too. However, extrapolation of missing CFs was harder than with MCFs derived from synthetic TAGs due to a lightly less systematic dependence on ACN. Nevertheless, some particular trends in specific MCFs of SC-TAGs were observed. The ratio of MCFs of the regioisomers of dibutyrates of TAG 24:0 and 26:1 was 1.40 and 1.60, respectively. In addition, the ratio of MCFs of monobutyrates and -caproates was on the average 2.86 ± 1.38 (n = 13) in TAG with ACN 28–40. Additional benefit of using calculated composition of randomized butterfat on the basis for MCF calculation was the semi-automatic correction of isotope effect of ^{13}C, which was known to influence on quantification in direct ESI–MS studies (Han and Gross 2001, Linderborg et al. 2014). Further, the resolving power of np-HPLC columns of isologous molecular species of TAGs reduced the influence of isotope effect compared to that in direct ESI–MS.

Repeatability and accuracy of the mass spectrometric quantification methods using EI–MS and ESI–MS detection were tested with randomized multi-TAG mixtures with known (*i.e.*, calculated) composition, and with synthesized or natural origin of TAGs. Even though the model mixtures of synthesized TAGs consisted of substantial number of coeluting TAG species and several regioisomers of SC-TAGs, all TAG species and all resolved regioisomers could be quantified. In general, repeatability (RSD%) of the measurements of both methods was at the same level, *i.e.*, on the average 5.3 ± 4.7% and 7.7 ± 7.4% in EI–MS and ESI–MS, respectively. A slightly higher average RSD% (8.6 ± 6.3%) was detected for the regioisomers of SC-TAG in EI–MS analysis probably due to a partial resolution of regioisomers resulting in a slightly inaccurate integration of the peaks. However, assessed repeatability was adequate and it resembled the findings of Myher et al. (1984) in their LC–MS measurements.

Accuracy of measurements was evaluated by comparing the calculated composition of randomized model mixtures to their empirically determined composition. The calculated content of individual TAG species in model mixtures of randomized TAG standards differed from the determined content on the average 17% and 12% in EI–MS and ESI–MS quantification, respectively, and 14% in EI–MS analysis of randomized butterfat in the

range of ACN 22–44. However, the determined content of some TAG species of randomized butterfat in high molecular weight ACN:DB classes differed markedly from the calculated content. In general, accuracy of the applied methods in the present study was in accordance with the findings of LC–MS measurements of Myher et al. (1984) who used ECFs originated from the natural oils, and with those of LC–FID determinations by Byrdwell et al. (1996), who quantified randomized vegetable oil. Definitely higher (*ca.* 47%) difference between calculated and analyzed values was reported in LC–FID measurement in which ECFs derived from synthetic TAG mixture (Byrdwell et al. 1996). On the other hand, much better accuracy (*ca.* 9%) was reported by Byrdwell et al. (1996) in LC–FID when ECFs were calculated on the basis of FA composition. In their study (Byrdwell et al. 1996), however, different ECFs for regioisomers were not taken into account. In addition, it was encouraging that empirically determined total molar amount of TAGs in model mixtures of synthesized TAGs was remarkably close to the calculated total amount: In EI–MS the difference was only 2%, and in ESI–MS there were practically no difference. In addition, the determined amount of specific subgroups of TAGs was close to the calculated values. Empirical molar content of the subgroups of SC-, MC-, and LC-TAGs differed from calculated content 1–7% and 2–3% in EI–MS and ESI–MS method, respectively. A little larger deviation was observed when subgroups of TAGs of randomized butterfat were analyzed by EI–MS method. The analyzed amount of the subgroup of mono SC-TAG species in randomized butter differed from the calculated amount 20%. The determined molar content of saturated, monoene, and polyene TAGs deviated from the known amount 15, 1, and 41%, respectively.

For the determination of molar composition of TAGs in butterfat, three different quantification methods, *i.e.*, high-resolution GC, GC–EI–MS, and np-HPLC–ESI–MS, were described in the present study (**III**, **VI**, **VII**). In all three methods, the analytical approach included the use of specific molar correction factors for ACN:DB classes, molecular species, or isomers of TAGs together with the use of added internal standard. The MCFs were calculated from the known composition of synthesized TAG mixtures or that of randomized natural fat, *i.e.*, chemically interesterified butterfat, and carefully integrated areas of TAG peaks in chromatograms. The present MS investigations showed no superiority of the MCFs based on synthesized TAG species over those originated from modified butterfat and *vice versa*. Reliable quantitative results were obtained in studies with MCFs of both origin, as long as the number of the MCFs was high enough (*i.e.*, > 100) to ensure the quantification of the major TAG classes and molecular species of butterfat due to the extensive variation in their molecular size, degree of unsaturation, acyl chain isomerism and regioisomerism. A substantial number of MCFs of varying size, structure and acyl composition of TAGs enabled extrapolation of more uncommon TAG species. In GC analysis, however, a more reduced number of MCFs were enough for accurate quantification due to a lower number of resolved TAG isomers. Repeatability and accuracy assessment of MS based quantification showed rather typical values suggesting adequately reliable quantification of ACN:DB classes and molecular species of TAGs with the present methods. Further, accuracy of the determination of selected subgroups of TAGs showed promising results. All MS methods provided novel information concerning MCFs for the regioisomers of short-chain TAGs and confirmed the need to use different and specific MCFs for *sn*-1(3) and *sn*-2 isomers, for example, in quantification of TAGs of modified milk fats or those of structured fats consisting short- and long-chain acyls.

6.4 Composition and structure of the TAG species in butterfat

In the present study, molar composition and structure of TAGs were determined from specific fractions of butterfat due to vast number of different TAG classes and molecular species of untreated butterfat. Fractionation was carried out according to the degree of unsaturation or the molecular size of TAG species, which were traditional ways to fractionate milk fat (*cf.*, Blank and Privett 1964, Kuksis and Breckenridge 1968, Parodi 1980). Specific MCFs for TAG classes and those for molecular species of TAGs (see Chapter 6.3) were applied in all quantification studies (GLC, **III**; GC–EI–MS, **VI**; np-HPLC–ESI–MS, **VII**), as appropriate MCF was determined. However, several MCFs were extrapolated in GC and EI–MS quantifications according to the principles described in Chapter 5.4.1 and 6.3. In addition, MCFs of butyrate TAGs and those of TAG 54 were used as MCFs of acetate TAGs and those of TAGs with more acyl carbons than 54, respectively, in ESI–MS quantification.

General TAG composition of butterfat. All quantification methods (**III, VI, VII**) yielded similar even-numbered TAG profile (mol%) with a high maxima at the ACN range 36–40 and a slightly lower maxima at the ACN range 48–52, which is typical for MF TAGs (*e.g.*, Padley et al. 1986, Gresti et al. 1993, Destaillats et al. 2006). A slightly divergent distribution was observed by Nagy et al. (2013), who discovered a high proportion of TAG 46 and a slightly lower proportion of TAG 36. However, one reason for this slightly uncharacteristic distribution might be that a typically very abundant TAG 16:0/16:0/4:0 (ACN 36) was not detected at all, and TAG 16:0/16:0/14:0 (ACN 46) was among the most abundant (4.73 mol%) TAGs in BF (Nagy et al. 2013, supplemental material), which were extremely uncommon findings among TAG studies of MF. Further, it is important to notice that ACN profile of even-numbered TAGs is different if it is based on either weight (wt%) or mole percent (mol%). In general, the most abundant TAG classes (wt%) are usually TAG 38 (12.6–13.2%), TAG 50 (11.4–11.8%), TAG 52 (11.0–11.3%), TAG 36 (~10.5%), TAG 40 (10.0–10.5%), and TAG 48 (8.5–8.7%) (Padley et al. 1986, Destaillats et al. 2006). If ACN distribution is calculated as mol% (data from Padley et al. 1986 and Destaillats et al. 2006), the relative proportion of TAG 36–40 is increasing and the most abundant (mol%) TAG classes are TAG 38 (13.7–14.4%), TAG 36 (~12.0%), TAG 40 (10.6–11.1%), TAG 50 (10.0–10.3%), TAG 52 (9.3–9.6%), and TAG 48 (7.6–7.9%). In the present study, some variation in the molar proportion of ACN classes was observed when different quantification methods were applied, however, the proportions were close to typical values. The most abundant ACN classes (mol% of even-numbered TAGs / mol% of all TAGs) were TAG 38 (12.9–14.6% / 11.2–12.6%), TAG 36 (11.4–13.4% / 9.8–11.5%), TAG 40 (10.4–10.7% / 8.9–9.2%), TAG 50 (9.9–12.1% / 8.5–10.5%), TAG 52 (5.6–9.7% / 4.8–8.4%), and TAG 48 (8.0–9.1% / 6.9–7.9%). Further, the abundance profiles of saturated, monoene, and polyene TAGs had distinct properties. ACN profiles of saturated and polyunsaturated TAGs had one maxima at ACN:DB 36:0 (6.9–8.5 mol% of all TAGs) and 52:2 (3.2–3.7 mol%), respectively, but monene TAGs had two maxima at ACN:DB 38:1 (5.4–6.0 mol%) and 50:1 (3.6–4.9 mol%). In the study of Nagy et al. (2013), the most abundant ACN:DB classes of unsaturated TAGs were the same, *i.e.*, ACN:DB 38:1 (7.1 mol% of all TAGs) and 50:1 (4.2 mol%) for monoene TAGs and ACN:DB 52:2 (3.5 mol%) for polyene TAGs, which were close to the findings of the present study. However, the most abundant ACN:DB class

for saturated TAGs was ACN:DB 46:0 (5.9 mol%), instead of ACN:DB 36:0 (3.3 mol%) in their study (Nagy et al. 2013), probably due to the reasons mentioned above.

Results of all quantification methods (**III, VI, VII**) showed that saturated TAGs were the largest subgroup of the identified even-numbered TAGs classified by the degree of unsaturation. Proportion of saturated TAGs of all identified even-numbered TAGs varied between 40 and 45%, which was slightly higher than that of monoene TAGs (34–41%). These findings agreed relatively well with the studies of Parodi (1981), Gresti et al. (1993), Laakso and Kallio (1993a), and Nagy et al. (2013), in which the combined proportion of saturated and monoene TAGs varied between 72 and 81% of all TAGs. In the most exhaustive investigations of Gresti et al. (1993) and Nagy et al. (2013), which covered over 400 and 300 different even-numbered TAG species, respectively, the proportion of saturated and monoene TAGs of all even-numbered TAGs was observed to be equal (~ 39 mol%). In the most extensive analysis of the present study by ESI–MS, the same proportions were close to each other (40 *vs*. 38 mol%), suggesting a relatively equal share of saturated and monounsaturated TAGs in BF when high number of TAG species were included in the analysis. However, Parodi's (1981) quantitative studies of eight Australian MF samples showed that either of the two main TAG classes could be the major TAG class due to the natural variation of milk composition. In the present study, relative amount of polyene TAGs was substantially lower (16–22%) than that of saturated and monoene TAGs, but was close to 13–22 mol% observed in other studies (Parodi 1981, Gresti et al. 1993, Laakso and Kallio 1993a, Nagy et al. 2013). In ESI–MS quantification, identified odd-numbered TAGs showed similar trend: The proportion of saturated, monoene, and polyene TAGs was *ca.* 50, 45, and 5%, respectively, of all identified odd-numbered TAGs in butterfat. Rather similar distribution of odd-numbered TAGs, *i.e.*, 49, 38, and 13 mol% of saturated, monoene, and polyene TAGs, respectively, can be calculated from the supplemental material of Nagy et al. (2013). All these findings showed that the majority (mol%) of butterfat TAG molecules was unsaturated, *i.e.*, contained at least one unsaturated FA in TAG, although merely one third of all FAs was unsaturated. All three methods of the present work provided fairly comparable results of the proportion of saturated and unsaturated TAGs. However, some differences were apparent. Use of mass spectrometric detection (EI/ESI–MS) increased the proportion of detected polyunsaturated TAGs. In addition, EI–MS method seemed to promote detection of saturated TAG species. A reason for that could be the utilization of selected ion monitoring, in which the most common $[M–RCOO]^+$ ions originated from saturated TAG species were included extensively in quantification, but less comprehensive monitoring was applied to the fragments cleaved from more rare molecular species of unsaturated TAGs.

The mass spectrometric methods of the present study enabled detection of eight saturated monoacid TAG species comprising of three even-numbered fatty acyls 4:0–18:0. In addition, one major unsaturated monoacid TAG (18:1/18:1/18:1) was detected. The total amount of monoacid TAGs in BF was assessed to be between 2 and 3 mol% depending on the method used for analysis. It was slightly higher than the amount (*ca.* 1.5 mol%) of monoacid TAGs determined by Gresti et al. (1993) and Nagy et al. (2013). No other earlier studies using chromatographic and mass spectrometric methods had showed the existence of tributyrate and tricaproate TAGs in unmodified MF. However, tricaprylate (Gresti et al. 1993, Nagy et

al. 2013), tricapriate (Gresti et al. 1993, Mottram and Evershed 2001, Beccaria et al. 2014), trilaurate (Gresti et al. 1993, Nagy et al. 2013), trimyristate (Gresti et al. 1993, Mottram and Evershed 2001), tripalmitate (Myher et al. 1988, Gresti et al. 1993, Fraga et al. 1998, Nagy et al. 2013, Beccaria et al. 2014), tristearate (Fraga et al. 1998, Robinson and MacGibbon 1998b, Mottram and Evershed 2001, Beccaria et al. 2014), and trioleate (Gresti et al. 1993, Fraga et al. 1998, Robinson and MacGibbon 1998b, Mottram and Evershed 2001, Gastaldi et al. 2011, Nagy et al. 2013, Beccaria et al. 2014) have been detected in MF previously, at least as traces, and even one monoacid odd-numbered TAG (15:0/15:0/15:0) has been identified (Nagy et al. 2013).

The dominance of triacid TAGs in butterfat was evident. Their proportion (% of total number) of all identified even-numbered TAG species was assessed by MS methods, which showed that the proportion of tri-/di-/monoacid TAG species was 67%/31%/2% and 60%/38%/2% in ESI–MS and EI–MS studies, respectively. The distribution of TAG classes in ESI–MS study was identical with the distribution calculated from the data of Gresti et al. (1993). The share of triacid TAGs was 67% also in the studies of Mottram and Evershed (2001) and Gastaldi et al. (2011). Slightly lower share (63%) of triacid TAGs can be calculated from the data of Fraga et al. (1998), Robinson and MacGibbon (1998b), and Beccaria et al. (2014). The dominance of triacid TAGs in BF was also evident as their molar proportion of all TAGs was assessed. Quantification by EI–MS showed that 47 and 29 mol% of individually detected even-numbered TAGs were tri- and diacid TAGs, respectively. In addition, 8 mol% of them were quantified as several TAG groups comprised of both TAG classes. In ESI–MS study (**VII**), the amount of individually quantified tri- and diacid TAGs was only 25 and 6 mol%, respectively. However, the molar amount of the groups containing both TAG classes was 48 mol%. In the study of Gresti et al. (1993), the molar proportion of tri- and diacid TAGs was 56 and 26 mol%, respectively. Similar trend can be calculated from the data of Nagy et al. (2013), which showed that two thirds of TAGs (mol%) were triacid TAGs.

In the current study, diversity of the general composition in BF TAGs is also assessed on the basis of SML classes of TAGs, *i.e.*, according to the size and number of the three esterified FAs in TAG molecules. HPLC–ESI–MS(/MS) method showed that TAG species existed in all ten SML classes (see Chapter 5.4.3), but this was not confirmed with GC and GC–EI–MS methods. The most common SML classes were shown to be S-L-L containing one SC- and two LC-FAs (30–37 mol%) and L-L-L containing three LC-FAs (30–34 mol%), which was confirmed with all applied methods. On the other hand, only one and two molecular species of TAGs were detected in the TAG class S-S-M and M-M-M, respectively. No comprehensive studies concerning SML classes of BF TAGs have been reported, but calculations based on the investigation of Nagy et al. (2013, supplemental material) showed that S-L-L and L-L-L classes were the most abundant TAG classes in BF containing *ca*. 38 and 36 mol% of all TAG species, respectively, which resembled the findings of the present study. No TAG species were observed in the rare S-S-M and M-M-M classes in their study (Nagy et al. 2013).

Composition of individual TAG species in butterfat. In the present study, 336, 186, and 115 different molecular species of even-numbered TAGs were identified and quantified by np-

HPLC–ESI–MS(/MS), GC–EI–MS, and GLC, respectively. In addition, 122 odd-numbered TAG species were determined in ESI–MS studies. In most chromatographic and mass spectrometric investigations of MF TAGs, the number of detected TAG species has varied between *ca*. 50 and 150 depending on the origin of MF samples and the methods used for analysis (Myher et al. 1988, 1993, Spanos et al. 1995, Fraga et al. 1998, Robinson and MacGibbon 1998b, Mottram and Evershed 2001, Gastaldi et al. 2011, Beccaria et al. 2014). Maniongui et al. (1991) and Gresti et al. (1993) reported the existence of a far higher number (404) of even-numbered TAG species when they studied MF extracted by Folch method from French milk using combined methods of reversed-phase liquid chromatography and GC. The number of individual TAG species of saturated and monounsaturated TAGs in their study was at the same level as in the present study, *i.e.*, 110 *vs.* 129, and 106 *vs.* 104, respectively, but the reported number of polyunsaturated TAG species was significantly higher (188 *vs.* 103). Odd-numbered TAGs were not determined in the studies of Maniongui et al. (1991) and Gresti et al. (1993) resulting in a slightly lower total number of identified TAG species than in the present work (**VII**). Recently, Nagy et al. (2013) studied TAGs in BF using a high-resolution hybrid ESI–MS, and quantified altogether 565 molecular species of TAGs including 339 even-numbered and 226 odd-numbered TAGs. Their findings resembled those of the current study (**VII**), especially the number of even-numbered TAG species was almost identical. Even though the number of different regio- and reversed isomers in their study was substantially higher than in the present investigation (**VII**), the number of TAGs with different FA composition was nonetheless high, *i.e.*, 185 and 128 even-and odd-numbered TAGs, respectively (Nagy et al. 2013).

In the early GC–EI/CI–MS (Myher et al. 1988) and rp-HPLC–CI–MS studies (Myher et al. 1993) of the most volatile distillate of BF, 129 and 140 individual TAG species, respectively, were detected, and a significant number (32 and 42, respectively) of them were not observed in the present study. Practically all of the different TAG species were monoacetate, -butyrate, and -caproate TAGs, which might exist in concentrated amounts in a highly volatile fraction of BF and, thus, exceeded the detection threshold. Fraga et al. (1998) used Ag-TLC–GC method to analyzed butter and Robinson and MacGibbon (1998b) Ag-TLC–rp-HPLC to determine TAGs in anhydrous MF resulting in the detection of 81 and 97 different molecular species (including *trans* isomers) of TAGs, respectively, of which almost everyone was identified and quantified in the current work (**VII**). In addition, all 54 TAG species that were identified from MF by Gastaldi et al. (2011) using narp-HPLC–APCI–MS^2, were proved to exist in MF in the present study. In other recent rp-HPLC–APCI–MS milk fat studies, Mottram and Evershed (2001) and Beccaria et al. (2014) detected 118 and 139 TAGs, respectively, including both even- and odd-numbered TAG species. In both investigations, nine molecular species of TAGs, which were mainly unsaturated TAGs and odd-numbered TAGs, were not detected in the present study.

Altogether, 64 and 61 even- and odd-numbered TAG species, respectively, which were detected in the present study by np-HPLC–ESI–MS (**VII**) were not reported to exist in MF in other extensive chromatographic and/or mass spectrometric TAG studies of bovine MF (Myher et al. 1988, 1993, Gresti et al. 1993, Spanos et al. 1995, Fraga et al. 1998, Robinson and MacGibbon 1998b, Mottram and Evershed 2001, Gastaldi et al. 2011, Nagy et al. 2013, Beccaria et al. 2014). The vast majority of novel saturated TAGs were either acetate TAGs

(7 molecular species) or TAGs (9) containing one very long-chain FA (CN ≥ 20) (one SC-TAG included). In addition, two tri-SC-TAGs, two butyro-caproates, and one S-M-L type monobutyrate were detected and further, four TAG species with at least one MC-FA attached to the glycerol backbone. Slightly fewer novel monounsaturated TAG species (19 *vs.* 25) were determined. Most of them (11) were TAGs containing one very long-chain FA in the TAG molecule (this group included two mono-SC-TAGs, too). Novel short-chain monounsaturated TAG species were detected as follows: One *sn*-2 isomer of monoacetate TAG, two di-SC-TAGs, and two butyrate TAGs containing one medium- and one long-chain fatty acyl group. In addition, two novel MC-TAGs and one novel L-L-L type TAG containing FA12:1 were observed to exist in BF. A half (10) of the novel polyunsaturated TAG species were LC-TAGs, of which *ca*. two thirds contain one very long-chain FA (20:0, 20:1, 20:4). The ESI–MS studies (**VII**) showed only three novel polyunsaturated SC-TAG species, of which two were *sn*-2 isomers of monoacetate and monocaproate TAGs and one was a monobutyrate TAG with a very long-chain FA. Most of the novel medium-chain TAGs (five out of seven) contained a rare 10:1 fatty acyl in the TAG molecule. When two silica gel columns in series were used for separation of MF TAGs in ESI–MS study (**V**), additional 20 novel acetate TAGs including several *sn*-2 isomers were quantified (area%) from MF.

On the other hand, ten common molecular species of TAGs (16:0/12:0/4:0, 14:0/14:0/4:0, 16:0/14:0/4:0, 18:0/16:0/4:0, 16:0/16:0/6:0, 18:0/16:0/6:0, 16:0/16:0/8:0, 18:1/12:0/4:0, 18:1/4:0/4:0, 18:1/18:0/4:0) in the ACN:DB range of 32:0–40:0 and 34:1–40:1 were identified both in the current study and in ten other extensive investigations of MF TAGs (Myher et al. 1988, 1993, Gresti et al. 1993, Spanos et al. 1995, Fraga et al. 1998, Robinson and MacGibbon 1998b, Mottram and Evershed 2001, Gastaldi et al. 2011, Nagy et al. 2013, Beccaria et al. 2014). All of these TAGs – except 16:0/16:0/8:0 – were molecular species comprising of one SC-acyl group (4:0, 6:0) and two LC-acyl groups (even-numbered 12:0–18:0, 18:1). Further, six other SC-TAG species (16:0/16:0/4:0, 18:0/14:0/4:0, 18:1/10:0/6:0, 18:1/16:0/4:0, 18:1/14:0/6:0, 18:1/16:0/6:0) and two MC-TAG species (16:0/14:0/8:0, 16:0/14:0/10:0) were detected both in the current study and in nine out of ten other extensive MF studies (see above). For example, a very common MF TAG 16:0/16:0/4:0 (*cf.*, Table 3.3) was discovered in the present work as well as in the studies of Myher et al. (1988, 1993), Gresti et al. (1993), Spanos et al. (1995), Fraga et al. (1998), Robinson and MacGibbon (1998b), Mottram and Evershed (2001), Gastaldi et al. (2011), and Beccaria et al. (2014), but surprisingly, Nagy et al. (2013) did not report it to exist in BF. If the studies concerning a very volatile TAG fraction of MF (Myher et al. 1988, 1993) were excluded from the comparison, eleven additional TAG species were reported to exist in MF in all other extensive studies (Gresti et al. 1993, Spanos et al. 1995, Fraga et al. 1998, Robinson and MacGibbon 1998b, Mottram and Evershed 2001, Gastaldi et al. 2011, Beccaria et al. 2014), as well as in the present study. Two of the TAG species were monobutyrate TAGs (18:1-10:0-4:0, 18:1-18:1-4:0), one is a MC-TAG (18:1-18:1-10:0), and the rest were LC-TAGs containing three LC-acyl groups, *i.e.*, saturated 12:0–18:0 FAs and unsaturated 18:1 FAs.

Assessment of the most abundant TAG species in MF on the basis of quantitative studies is not always unambiguous due to different analyzing methods, varying MFs, selected percentages (wt%, mol%, area%) and grouping of several individual TAG species into a

larger TAG group. However, in several quantitative studies (Myher et al. 1988, Gresti et al. 1993, Fraga et al. 1998, Gastaldi et al. 2011, Nagy et al. 2013) the following three molecular species of TAGs were among the three most abundant TAGs (mol%) in four out of five of the studies: *i.e.*, 16:0/16:0/4:0, 18:1/16:0/4:0, and 16:0/14:0/4:0. In Nagy's et al. study (2013), 16:0/16:0/4:0 was not detected at all, and in Fraga's et al. (1998) and Gastaldi's et al. (2011) studies, 16:0/14:0/4:0 and 18:1/16:0/4:0, respectively, were not among the six most abundant TAG species. In the present study, all of the above mentioned SC-TAG species were among the most abundant TAGs regardless of the applied method (**III**, **VI**, **VII**). The other common TAGs among the six most abundant TAG species were 18:0/16:0/4:0 (Myher et al. 1988, Gresti et al. 1993, Fraga et al. 1998, Gastaldi et al. 2011), 18:1/18:1/16:0 (Gresti et al. 1993, Fraga et al. 1998, Nagy et al. 2013), 18:1/18:1/16:0 (Gresti et al. 1993, Fraga et al. 1998, Nagy et al. 2013), 18:1/16:0/16:0 (Fraga et al. 1998, Nagy et al. 2013), 18:1/16:0/14:0 (Gresti et al. 1993, Nagy et al. 2013), 18:1/14:0/4:0 (Myher et al. 1988, Fraga et al. 1998), and 16:0/16:0/6:0 (Myher et al. 1988, Gastaldi et al. 2011), most of which were among the most abundant TAG species in the present study as well. In the present investigation, the most abundant (mol%) TAG species / a group of TAG species (mean ± S.D. of the results determined by **III**, **VI**, **VII**) were 16:0/16:0/4:0 + 18:0/14:0/4:0 (5.4 ± 0.1), 18:1/16:0/16:0 + 18:1/18:0/14:0 + 16:1/18:0/16:0 (4.3 ± 0.6), 18:1/16:0/4:0 (4.3 ± 0.3), 18:1/18:1/16:0 + 18:2/18:0/16:0 (3.4 ± 0.3), 16:0/14:0/4:0 + 18:0/12:0/4:0 (3.1 ± 0.4), 18:1/16:0/14:0 + 16:1/16:0/16:0 (3.1 ± 0.6), and 18:0/16:0/4:0 (2.5 ± 0.3) (major component of the group, estimated by GC–EI–MS, **VI**). The respective contents (mol%) of the same TAG species / TAG groups in the extensive studies of Gresti et al. (1993) [and Nagy et al. (2013)] were 4.5 [1.4], 3.9 [6.6], 4.2 [5.4], 2.7 [3.5], 3.4 [3.9], 3.0 [2.9], and 2.5 [2.1]. The very low content (1.4 mol%) of (16:0/16:0/4:0 + 18:0/14:0/4:0) in the study of Nagy et al. (2013) originated solely form 18:0/14:0/4:0 because 16:0/16:0/4:0 was not detected, however, it was close to the estimated average content (1.3 mol%) of 18:0/14:0/4:0 in the present study. The contents (mol%) of the same major TAG species / TAG groups determined by three different methods (**III**, **VI**, **VII**) differed from each other on the average (%, mean ± S.D.) 15.1 ± 7.5, 10.2 ± 7.2, and 15.2 ± 16.5 as ESI–MS/EI–MS, ESI–MS/GLC, and EI–MS/GLC methods, respectively, were compared. When the results of ESI–MS study (**VII**) were compared to those of Gresti et al. (1993) and Nagy et al. (2013), the average difference between the determinations of the same major TAGs was roughly at the same level, *i.e.*, 11.4 ± 9.2 and 21.2 ± 13.1%, respectively, as was the difference between three methods of the present study (see above). The content of the major individual TAG species was estimated on the basis of their calculated (Tables 5.15–5.17, GC–EI–MS) share in the TAG groups and their average content determined by the three methods. The most abundant (> 2.5 mol%) individual TAG species in BF were 18:1/16:0/4:0 (4.3 mol%), 16:0/16:0/4:0 (4.0 mol%), 18:1/16:0/16:0 (3.2 mol%), 18:1/18:1/16:0 (3.1 mol%), 16:0/14:0/4:0 (2.6 mol%), and 18:0/16:0/4:0 (2.5 mol%).

The above findings suggested that the chromatographic and mass spectrometric methods used in the present study were capable to detect several novel TAG species at low concentrations in MF and, at the same time, they were reliable choices to determine the most frequently found TAG species in MF.

Composition and structure of short-chain TAG species of even-numbered TAGs. SC-TAGs are an important subgroup of MF TAGs due to their influence on physical and technological properties of MF, as well as on metabolism of TAGs. For example, composition and structure of SC-TAGs affect characteristic melting and crystallization behavior of MF (Timmen and Putton 1988, Walstra et al. 1995), flavor formation in milk and milk products (Urbach and Gordon 1994, Jensen 2002), and digestion and absorption of MF TAGs (Bracco 1994, Mu and Høy 2004). In the present study, use of prefractionation of MF TAGs prior to chromatographic separation and use of polar or polarizable stationary phases in chromatography enabled effective analysis of these slightly polar TAG species and their regioisomers. In general, proportion of mono- and di-SC-TAGs has been observed to be between 36 and 42 mol%, and between 0.6 and 0.8 mol% of all TAGs in MF, respectively (Gresti et al. 1993, Nagy et al. 2013). In the present investigation, as GC was used to separate TAGs (**III**, **VI**), total amount of mono-SC-TAGs was shown to be *ca*. 38 mol%. HPLC–ESI–MS method (**V**, **VII**) gave slightly lower share (*ca*. 34 mol%) of individually separated SC-TAGs, but their actual proportion was probably close to 38 mol% due to additional 3.5 mol% of quantified TAG groups which contained both SC-TAGs and MC/LC-TAGs. Anyway, the observed proportion of mono-SC-TAG was slightly higher than 36 mol% determined by Gresti et al. (1993), but lower than 42 mol%, which was calculated from the data of Nagy et al. (2013). In the MS analyses of TAGs (**V–VII**), the total amount of di-SC-TAGs in BF was shown to be less than 1 mol%, *i.e.*, 0.3–0.9 mol% depending on the analytical method, which was in accordance with the results of other extensive MF TAG analyses (see above). Higher proportion (1.5 mol%) of di-SC-TAGs, which was observed in GC analysis (**III**), was most probably due to more unspecific identification (FID) of chromatographic peaks compared to MS detection, which could result in overestimation of the proportion of di-SC-TAGs. In the present study, dibutyrate, dicaproate, and butyrocaproate TAG species were shown to exist in MF as was respectively observed in other studies, (*e.g.*, Gresti et al. 1993, Myher et al. 1993, and Nagy et al. 2013). However, acetobutyrates and acetocaproates were detected for the first time in untreated BF, but no diacetates were observed (**V**, **VII**). Later, the data from Nagy et al. (2013) supported our findings as different isomers of 16:0-6:0-2:0 were quantified in their study. The presence of the following di-SC-TAG isomers in BF have not been reported in any other extensive chromatographic or mass spectrometric studies: *rac*-6:0-10:0-4:0, *rac*-4:0-16:0-2:0, *rac*-6:0-14:0-4:0, *rac*-4:0-18:0-4:0, and *rac*-6:0-18:1-4:0. In addition, the present investigation suggested that uncommon tri-SC-TAGs could exist in MF, because small amounts of two monoacid SC-TAGs (tributyrin and tricaproin) were quantified for the first time in untreated BF (**III**, **V**, **VII**). Previously, tri-SC-TAGs have been detected only in interesterified MF (Marai et al. 1994).

The ratio of the regioisomers of di-SC-TAGs (*rac*-L(M)-S-S/*rac*-S-L(M)-S), which originated from the same molecular species of TAGs, has not been investigated thoroughly, even though, some estimates of general regioisomer ratio of di-SC-TAGs can be performed from previous studies (Myher et al. 1993). In the present study (**V, VII**), altogether five regioisomer pairs of di-SC-TAGs were detected in BF by np-HPLC–ESI–MS and the regioisomer ratio was calculated. The proportion of *rac*-L-S-S(S') was 91 mol% (mean), 61 mol% (mean), and 77 mol% for two different dibutyrates, two different butyrocaproates, and one butyroacetate, respectively. The results were similar in both two (**V**) and three

columns in series (**VII**) analysis. These findings suggested that asymmetric regioisomers of di-SC-TAGs [L(M)-S-S/L(M)-S-S'] are more common than their symmetric regioisomer pair [S-L(M)-S/S-L(M)-S'], which further contributed to the overall asymmetry of BF TAGs originated from a very specific FA distribution among *sn*-positions of TAGs and from a high proportion of tri-acid TAGs. Recently, Nagy et al. (2013) included molar composition of three regioisomer pairs of di-SC-TAG in their research data (= supplemental material), which partly supported the dominance of L-S-S type isomer. The proportion of *rac*-L-S-S(S') isomers of one acetocaproate, one dibutyrate, and one butyrocaproate were 86, 37, and 59 mol%, respectively.

GC–EI–MS (**VI**) and np-HPLC–ESI–MS (**VII**) studies confirmed that the proportion of *sn*-2 isomers of monobutyryl and -caproyl TAGs in MF was very low, as was expected on the basis of numerous regio- and stereospecific studies (Pitas et al. 1967, Breckenridge and Kuksis 1968a, Bus et al. 1976, Pfeffer et al. 1977, Parodi 1979b, 1982, Christie and Clapperton 1982, Blasi et al. 2013). GC–EI–MS (**VI**) revealed no *sn*-2 isomers in untreated MF, but few *sn*-2 butyrates and caproates were detected in np-HPLC–ESI–MS study (**VII**). Regioisomer pairs of two unsaturated caproyl TAGs (*rac*-18:1-6:0-16:0/18:1-16:0-6:0, *rac*-18:1-6:0-18:1/18:1-18:1-6:0) were identified as members of TAG groups, hence the regioisomer ratio was not assessed. Further, traces of one *sn*-2 butyryl (*rac*-18:1-4:0-16:1) and one *sn*-2 caproyl TAG (*rac*-18:1-6:0-14:0) were detected as a member of TAG groups. Nagy et al. (2013) observed a slightly higher number of regioisomer pairs of monobutyryl and monocaproyl TAGs in their ESI–MS study. The average proportion of *sn*-1(3) isomers of three monobutyrates and six monocaproates were 99.7 and 95.8%, respectively (Nagy et al. 2013). Recently, Nagai et al. (2015) used a nondestructive analysis of intact MF TAGs (chiral-HPLC–APCI–MS) and showed that butyryl and caproyl fatty acyl groups are solely esterified to the *sn*-3 position in butyroyl- and caproyldipalmitoyl-*sn*-glycerols. Findings of the present study concerning regiospecific distribution of SC-FAs in mono-SC-TAGs supported the results of above mentioned regio- and stereospecific analyses of MF TAGs, which have shown that short-chain FAs (especially butyric acid) are almost exclusively located in the *sn*-1(3) position, or merely at the *sn*-3 position.

HPLC–ESI–MS (**VII**) analyses showed that monobutyryl and monocaproyl TAGs exist in MF in S-M-M, S-M-L, and S-L-L type TAG classes. The molar ratio of monobytyrates to monocaproates was 2.0:1 in GC (**III**) and GC–EI–MS studies (**VI**), which was close to the corresponding ratio (1.9:1) calculated from the data of Nagy et al. (2013). HPLC–ESI–MS study yielded a higher ratio (2.5:1), which could be a slightly unsure result due to a considerable proportion of mono-SC-TAGs that were quantified as a member of several other TAG species. In the present work, np-HPLC–ESI–MS method enabled identification and quantification of seven monobutyryl TAGs, which were not reported to be in MF by other researchers using comprehensive chromatographic and mass spectrometric analyses (Myher et al. 1988, 1993, Gresti et al. 1993, Spanos et al. 1995, Fraga et al. 1998, Robinson and MacGibbon 1998b, Mottram and Evershed 2001, Gastaldi et al. 2011, Nagy et al. 2013, Beccaria et al. 2014). Most of the novel monobutyrates were TAG species containing a very long-chain FA (20:0, 20:1) in TAG molecule. *i.e.*, *rac*-20:0-18:0-4:0, *rac*-18:1-20:0-4:0, *rac*-18:0-20:1-4:0, and *rac*-18:1-20:1-4:0. In addition, three monobutyrates (*rac*-16:0-8:0-4:0, *rac*-8:0-18:1-4:0, and *rac*-10:1-14:0-4:0), which had a MC-FA esterified to TAG

molecule were detected. A sole novel monocaproyl TAG was a symmetrical *sn*-2 isomer of a diunsaturated TAG (*rac*-18:1-6:0-18:1).

MF TAGs with an acetyl acyl group in molecule are infrequently analyzed, even though already Parodi (1975) detected monoacetyl TAGs in MF using np-TLC and packed column GC. Later, Limb et al. (1999) succeeded to observe acetyl groups in TAGs of milk synthesizing tissue with more sophisticated analyses by ^{1}H-NMR, ^{13}C-NMR and FAB–MS(/MS) techniques, which suggested their existence also in milk itself. Earlier, several monoacetate TAGs have been determined in molecular distillates of MF in which the fraction of SC-TAGs was concentrated (Myher et al. 1988, 1993, Itabashi et al. 1993), hence the relative amount of acetate TAGs was concentrated as well. More recently, improvement of ESI–MS and APCI–MS methods have enabled detection of acetate TAGs in relatively untreated MF (Nagy et al. 2013, Beccaria et al. 2014). In the present study, fractionation of BF TAGs according to molecular weight of TAGs prior to HPLC separation and use of efficient np-HPLC–ESI–MS(/MS) resulted in detection of 0.2 mol% of acetate TAGs in BF including several novel monoacetate TAG species with both two (**V**) and three (**VII**) columns in series methods. In two HPLC-columns system (**V**), saturated and monounsaturated monoacetate TAGs in the ACN range 22–30 were detected. In addition, the overall proportion of the *sn*-2 regioisomers (*rac*-X-2:0-X; X = any other FA than 2:0) of all acetate TAGs was *ca*. 9.5% (area-%). On the other hand, in three HPLC-columns system (**VII**), acetate TAGs in the ACN range 28–38 were quantified and three regioisomer pairs of acetate TAGs (*rac*-18:0-16:0-2:0/18:0-2:0-16:0, *rac*-18:1-18:0-2:0/18:1-2:0-18:0, *rac*-18:1-18:1-2:0/18:1-2:0-18:1) were detected. The proportion of *sn*-2 isomer in these pairs was 59 mol%, which was higher than the general share (37 mol%) of *sn*-2 isomers of all acetate TAGs. The findings of the present work enhances the selection of identified and quantified acetate TAGs in MF. In earlier investigations, the detected acetate TAG species consisted only of one acetyl and two long-chain acyl groups in the ACN range 28–38 (Myher et al. 1988, Myher et al. 1993, Itabashi et al. 1993, Limb et al. 1999, Beccaria et al. 2014). In the present investigations, several S-S-L and S-M-L type monoacetates in the ACN range 22–30 were detected, in addition to S-L-L type acetates with ACN 30–38. Recently, Nagy et al. (2013) documented S-S-L type monoacete in MF (16:0-6:0-2:0) as well. Further, the acetyl group had been located in the *sn*-3 position in all S-L-L type acetates according to earlier investigations (Itabashi et al. 1993, Limb et al. 1999), which is in contrast to the findings of the current work (**V, VII**). However, in the investigation of Nagy et al. (2013) the proportion of the *sn*-2 regioisomer of monoacetate TAG (*i.e.*, *rac*-S-2:0-L) was 36 mol%, which was comparable to the calculated proportions (mol%) in **VII**. Myher et al. (1988) and Nagy et al. (2013) have detected odd-numbered acetyl TAGs in MF, but these findings could not be confirmed in the present investigation (**VII**). The results of present study indicated that the composition and structure of acetate TAGs in MF could vary more than the results of earlier investigations suggested (Myher et al. 1988, Myher et al. 1993, Itabashi et al. 1993, Limb et al. 1999, Beccaria et al. 2014). For example, S-S-L and S-M-L type monoacetate TAGs were identified instead of sole S-L-L type TAGs, and a significant proportion of the *sn*-2 regioisomers of monoacetyl TAGs was observed instead of solely the *sn*-1(3) isomers. However, it is worth to note that analysis of acetate TAGs is challenging due to their low amount in MF and specific properties. Even small changes is analytical conditions could influence on results as slightly varying results of **V** and **VII**

indicated. Anyway, altogether nine monoacetate TAGs, which were not reported to include in MF TAGs were detected by np-HPLC–ESI–MS(/MS), *i.e.*, *rac*-4:0-16:0-2:0, *rac*-18:0-2:0-8:0, *rac*-16:0-2:0-10:0, *rac*-18:0-10:0-2:0, *rac*-16:0-12:0-2:0, *rac*-14:0-14:0-2:0, *rac*-18:0-2:0-16:0, *rac*-18:1-2:0-18:0, and *rac*-18:1-2:0-18:1.

Composition and structure of medium-chain TAG species of even-numbered TAGs. MC-TAG species are TAG molecules containing 1–3 MC and 0–2 LC fatty acyls. In MF TAG studies, MC-TAGs are relatively seldom assessed as a special TAG class, even though metabolism of MC-TAGs and FAs differ from LC-TAGs and FAs, and MC-FAs are generally regarded more neutral in elevating cholesterol levels than selected LC-FAs (Keys et al. 1965, Woollett et al. 1989), and hence render them an interesting research topic. However, significant number of individual MC-TAG species has been detected in several MF studies (Myher et al. 1988, 1993, Gresti et al. 1993, Spanos et al. 1995, Fraga et al. 1998, Robinson and MacGibbon 1998b, Mottram and Evershed 2001, Gastaldi et al. 2011, Nagy et al. 2013, Beccaria et al. 2014). In the present study, 86, 49, and 34 MC-TAG species were detected by np-HPLC–ESI–MS(MS), GC–EI–MS, and GLC, respectively, which revealed some general trends in MC-TAGs of BF. GLC/GC–EI–MS studies (**III**, **VI**) suggested that the proportion of MC-TAGs in BF was 11–14 mol%. Unfortunately, proportion of individually determined MC-TAG species by np-HPLC–ESI–MS was very low, hence exact assessment of the proportion of MC-TAGs was impossible. However, ESI–MS study (**VII**) showed that M-L-L TAG class contained highest number (62) of MC-TAG species and they were evenly distributed in saturated (21), monoene (19), and polyene (22) TAG classes. The number of capriate TAGs (31) was only slightly higher than caprylate TAGs (26). Exact ratio of *sn*-1(3) to *sn*-2 isomers in mono-MC-TAG was challenging to assess, but the number of pure *sn*-1(3) isomers seemed to be higher than pure *sn*-2 isomers. In addition, significant number (22) of MC-TAG species was detected in M-M-L class as well, and all types of di-MC-TAGs (*i.e.*, dicaprylates, dicapriates, and caprylcapriates) were shown to exist in BF. Most (64%) of M-M-L type TAGs were *rac*-L-M-M type regioisomers. Further, two tri-MC-TAGs were detected by ESI–MS/MS, *i.e.*, *sn*-10:0-10:0-10:0 and *rac*-10:0-8:0-10:0. In addition, traces of *sn*-8:0-8:0-8:0 were determined by GLC/GC–MS. The number of M-M-M type TAG species is even theoretically rather restricted, but some TAG species have been discovered in MF by other research groups (Gresti et al. 1993, Mottram and Evershed 2001, Nagy et al. 2013, Beccaria et al. 2014). Existence of 14 different mono- and di-MC-TAG species in BF was confirmed for the first time by the present np-HPLC–ESI–MS(MS) study as follows (predominant regioisomer indicated): (saturated MC-TAGs) *rac*-12:0-12:0-8:0, *rac*-20:0-10:0-18:0, *rac*-12:0-8:0-8:0, *rac*-14:0-8:0-8:0, *rac*-14:0-8:0-10:0; (monoene MC-TAGs) *rac*-12:0-10:1-14:0, *rac*-10:0-10:0-18:1; (polyene MC-TAGs) *rac*-18:1-12:0-10:1, *rac*-16:1-14:0-10:1, *rac*-16:1-8:0-18:1, *rac*-18:2-8:0-18:0, *rac*-18:1-10:1-16:0, *rac*-18:1-8:0-10:1, and *rac*-18:1-10:1-10:0.

Composition and structure of long-chain TAG species of even-numbered TAGs. Molecules of LC-TAGs contain solely LC fatty acyls (CN ≥ 12), hence all LC-TAGs belong to L-L-L TAG class, which was among two most abundant SML classes of even-numbered TAGs in MF in the present study (see above). LC-TAGs regulate melting and crystallization of BF (Parodi 1981), and have a significant role in triggering crystallization in bulk fat (Gresti et al. 1993). In the present study, 124, 56, and 31 LC-TAG species were detected by

np-HPLC–ESI–MS(MS), GC–EI–MS, and GLC, respectively. However, polar stationary phases of phenyl(65%)methylsilicone columns and two or three microbore np-HPLC columns in series could not resolve individual regio- and acyl chain isomers of LC-TAGs in general, hence most LC-TAG species were quantified as a sum of several TAG species (**III**, **VI**, **VII**), or the proportion of individual TAG species was assessed by calculation (**VI**). An important exception to this observation was that three microbore np-HPLC columns in series resolved several LC-TAGs containing one very long-chain fatty acyl (CN ≥ 20) from other acyl chain isomers. Further, some regioisomers of the same LC-TAGs separated from each other, for example *rac*-20:0-18:0-18:0 from *rac*-18:0-20:0-18:0, and *rac*-20:0-18:0-18:1 from *rac*-18:1-20:0-18:0. These findings enabled detection of the following 26 LC-TAG species (predominant regioisomer indicated) and isomers with a very long-chain acyl group in the TAG molecule, which have not been reported to exist in bovine MF by other research groups (Myher et al. 1988, 1993, Gresti et al. 1993, Spanos et al. 1995, Fraga et al. 1998, Robinson and MacGibbon 1998b, Mottram and Evershed 2001, Gastaldi et al. 2011, Nagy et al. 2013, Beccaria et al. 2014): (saturated LC-TAGs) *rac*-20:0-12:0-16:0, *rac*-20:0-12:0-18:0, *rac*-20:0-14:0-18:0, *rac*-20:0-18:0-18:0, *rac*-18:0-20:0-18:0, *rac*-16:0-22:0-18:0, *rac*-16:0-24:0-16:0; (monoene LC-TAGs) *rac*-20:0-12:0-18:1, *rac*-20:0-14:0-16:1, *rac*-20:0-16:0-14:1, *rac*-20:0-16:0-16:1, *rac*-20:0-14:0-18:1, *rac*-20:0-16:1-18:0, *rac*-20:0-18:0-18:1, *rac*-18:1-20:0-18:0, *rac*-18:1-22:0-16:0; (polyene LC-TAGs) *rac*-18:2-12:1-18:1, *rac*-18:3-12:0-18:1, *rac*-20:4-16:0-12:0, *rac*-20:4-16:0-16:0, *rac*-20:0-16:1-18:1, *rac*-20:0-18:2-16:0, *rac*-18:1-20:1-16:0, *rac*-18:0-18:3-18:0, *rac*-20:0-18:0-18:2, and *rac*-18:1-20:1-18:0. In general, LC-TAGs that contain acyl groups with 20 acyl carbons or more have been determined relatively infrequently in MF, but few molecular species have been quantified by Gresti et al. (1993), Fraga et al. (1998), and Mottram and Evershed (2001) with different chromatographic and mass spectrometric methods.

Composition of odd-chain TAG species in butterfat. In the present study, a significant number (122) of odd-numbered TAGs in BF were determined by np-HPLC–ESI–MS (**VII**). Further, 61 of them were TAG species which have not shown to exist in BF in other extensive investigations (Myher et al. 1988, 1993, Gresti et al. 1993, Spanos et al. 1995, Fraga et al. 1998, Robinson and MacGibbon 1998b, Mottram and Evershed 2001, Gastaldi et al. 2011, Nagy et al. 2013, Beccaria et al. 2014). In most of the above mentioned studies the number of odd ACN TAG species was shown to vary between 1 and 25 (Myher et al. 1988, 1993, Fraga et al. 1998, Robinson and MacGibbon 1998b, Mottram and Evershed 2001, Beccaria et al. 2014), but Gresti et al. (1993) and Gastaldi et al. (2011) did not determined odd ACN TAGs at all. However, the data from the study of Nagy et al. (2013) showed even higher number (128 different TAG species / 226 different regioisomers of TAGs) of odd-numbered TAG species in MF than did the present study. The total amount of odd-numbered TAGs in MF was 7.6 mol% in the study of Nagy et al. (2013), which was close to *ca*. 5% reported in early studies (Taylor and Hawke 1975, Kallio et al. 1989). In the present study, the amount of identified odd ACN TAGs was slightly lower (3.9 mol%), but together with unidentified odd-numbered TAGs the total amount was 5.7 mol%, which was in accordance with the studies of Taylor and Hawke (1975), Kallio et al. (1989), and Nagy et al. (2013). In the early studies of the most volatile fractions of MF (Myher et al. 1988, 1993), practically all detected odd-numbered TAGs were SC-TAGs, which was probably due to restricted range of ACN of TAGs in general in the volatile fractions of MF. Contrary

to the studies of Myher et al. (1988, 1993), only unsaturated odd-numbered LC-TAGs were determined in bovine MF by Fraga et al. (1998) and by Robinson and MacGibbon (1998b). However, Mottram and Evershed (2001) and Beccaria et al. (2014) showed that both SC and LC odd ACN TAGs existed in MF, and even some MC odd-numbered TAGs were identified (Spanos et al. 1995, Beccaria et al. 2014). In the present study, the proportion of SC-, MC-, and LC-TAGs was 22, 20, and 58%, respectively, indicating a lower share of SC-TAGs compared to even-numbered SC-TAG (~38 mol%). The present investigation (**VII**) and the study of Nagy et al. (2013) confirmed the considerable diversity of odd ACN TAGs concerning the degree of unsaturation of TAGs (0–2 DBs in odd-numbered TAGs), the carbon chain length of fatty acyls (SC, MC, LC), acyl chain isomerism of isobaric TAGs, and regioisomerism of mono-SC-TAGs. In **VII** [and in Nagy et al. 2013], the ACN:DB classes of odd-numbered TAG ranged from 33:0 [29:0] to 55:0 [49:0] and from 35:1[35:1] to 57:1 [51:1] for saturated and monoene TAGs, respectively. In addition, 53:2 and 57:2 ACN:DB classes were observed for polyene TAGs in the present study (**VII**), and 39:2, 51:2, and 53:2 ACN:DB classes in the study of Nagy et al. (2013). In every ACN:DB class of identified odd-numbered TAGs (except 37:1), several (2–9; ~5 on the average) acyl chain isomers of isobaric TAGs were observed (**VII**). Similar pattern was observed in other studies, in which a substantial number of odd-chain TAGs have been detected (Myher et al. 1988, 1993, Nagy et al. 2013).

Structure and composition of TAG species and regioisomers in BF were determined by three different chromatographic and mass spectrometric methods (GLC, GC–EI–MS, np-HPLC–ESI–MS). The results suggested that the applied methods provided a reliable alternative to determine the major TAG species and TAG classes in BF. In addition, several minor TAG species that have not been detected previously in MF by other methods were identified and quantified in the present study. In particular, all methods were shown to be valuable in quantification and structural elucidation of intact regioisomers of SC-TAG species in untreated BF. These findings seemed to validate the applied methodology, in spite of the fact that all methods had key advantages and disadvantages: GLC–FID method provided a clear-cut separation of the regioisomers of SC-TAGs and otherwise logical and easily interpretable separation of TAGs after prefractionation of TAGs according to the degree of unsaturation with Ag^+-SPE columns. However, coeluting TAG species were impossible to quantify as individual TAG species. In addition to the advantages of GLC–FID, GC–EI–MS enabled identification and quantification of significant number of coeluting TAG species on the basis of characteristic fragmentation in EI and use of a high number of specific MCFs. Though, a slight overloading of GC-columns was required in GC–MS, in order to increase detection of minor TAGs, which decreased chromatographic resolution of the acyl chain isomers of TAGs and the regioisomers of SC-TAGs. Further, np-HPLC–ESI–MS(/MS) provided a powerful analytical tool to determine composition and structure of a significant number of acyl chain isomers of isobaric TAGs, the regioisomers of SC-TAGs, and the predominant regioisomer of other TAGs in BF. However, calculation of results was laborious and, due to numerous coeluting TAG species, several TAGs in BF were not quantified as individual TAG species but together with other TAG species.

7 CONCLUSIONS

Quantitative high-resolution gas-liquid chromatography (GLC), gas chromatography–electron ionization mass spectrometry (GC–EI–MS), and normal-phase high-performance liquid chromatography–electrospray ionization mass spectrometry / tandem MS (np-HPLC–ESI–MS/MS^2) were applied to determine composition and structure of intact triacylglycerol (TAG) species in bovine butterfat (BF), and particularly those of regio- and acyl chain isomers of short-chain triacylglycerols (SC-TAGs). The methods were validated by analyzing model TAG mixtures of known composition and analytical approaches were optimized especially to determine the *sn*-2 and *sn*-1(3) isomers of SC-TAG species. The main findings concerned both analytical methodology of the regio- and acyl chain isomers of SC-TAG species in general and composition and structure of TAG species and regioisomers of SC-TAGs in BF in particular.

Determination of TAG composition and structure of bovine BF is challenging due to a huge number of different TAG species and isomers. Hence, TAG analysis of BF can be facilitated by separating TAGs into less intricate fractions prior to chromatographic and mass spectrometric analysis. In the present study, a simple low-pressure column chromatographic method that exploited a solid phase extraction (SPE) column loaded with p-propylbenzene sulfonic acid sorbent in silver ion mode (Ag-SPE) was tuned up to fractionate BF into saturated, monoene, and polyene TAGs. Combining the Ag-SPE method with GLC using a polar column facilitated particularly separation of the regioisomers of SC-TAGs due to the fact that a polarizable phenyl(65%)methylsilicone column separated monobutyrate and -caproate TAG species and their regioisomers from each other and Ag-SPE fractionation prevented overlapping of the otherwise coeluting regioisomers of SC-TAGs with the same ACN but different degree of unsaturation. Further, application of the Ag-SPE and np-SPE methods prior to GLC and np-HPLC, respectively, increased content of minor TAG species in the fractions above the detection limit and resulted in increased number of identified and quantified TAG species.

The present study confirmed the elution order of regio- and acyl chain isomers of isobaric TAG species on a polarizable phenyl(65%)methyl capillary column on the basis of the retention indices (RIs) of a significant number (112) of synthesized TAG species and isomers. Further, relatively abundant empirical data demonstrated that the *sn*-2 and *sn*-1(3) isomers of monocaproate TAGs resolved on a phenyl(65%)methyl capillary column, in addition to resolution of the regioisomers of monoacetate, -propionate, and -butyrate TAGs demonstrated in other studies. As quadrupole mass detector in EI mode was combined with high-resolution GLC, coeluting acyl chain isomers of SC-TAG species could be identified on the basis of characteristic fragment ions $[M–RCOO]^+$, $[RCO]^+$, $[RCO + 74]^+$, and $[RCO + 128]^+$. Even though polar np-HPLC columns are theoretically a good choice to separate slightly polar SC-TAG species from apolar TAG species, np-HPLC has not been exploited in the analysis of SC-TAGs in MF, merely in the analysis of structured TAGs containing both SC- and LC-acyl groups. However, the present study proved the analytical potential of two and three microbore np-HPLC columns in series to resolve the regioisomers of intact mono- and dibutyrate TAGs to the baseline, monocaproate TAGs close to the baseline, and dicaproate TAGs partially. In addition, a few *sn*-2 and *sn*-1(3) isomers of long-chain TAGs

in BF containing a fatty acyl 20:0, and acetate, butyrate, and caproate TAGs were shown to resolve from each other and from the other isobaric TAGs on three np-HPLC columns in series. Combining ESI–MS^2 with np-HPLC enabled identification of coeluting acyl chain isomers and elucidation of predominant regioisomer of most TAG species in BF on the basis of specific fragment ions and different fragmentation efficiency of FAs from the *sn*-2 and *sn*-1(3) positions. The present study revealed that the abundance of the ion formed by cleavage of a short-chain fatty acid from the primary positions was at least 2.3 times higher than that from the secondary position of mono SC-TAG molecule, and corresponding difference was observed with di-SC-TAGs, which provided a clear-cut differentiation of the regioisomers of SC-TAGs.

Due to the extensive variation in composition and structure of the TAGs in bovine BF, use of specific empirical molar correction factors (MCFs) for ACN:DB classes, individual TAG species, or regioisomers was assessed essential in all three quantification methods (GLC, GC–EI–MS, np-HPLC–ESI–MS). Quantification of intact TAG species and regioisomers of SC-TAGs in BF directly by GC–EI–MS and by np-HPLC–ESI–MS using a significant number (> 100) of specific MCFs for $[M–RCOO]^+$ and $[M + NH_4]^+$ ions, respectively, rendered the methods unique applications in BF quantification. Adequately wide variety of specific MCFs in both methods provided a solid basis to extrapolate MCFs for uncommon molecular species of TAGs and demonstrated unambiguously that molecular size, degree of unsaturation, regio- and acyl chain isomerism of TAGs, and even asymmetry of isobaric TAGs affected MCFs.

Altogether, 335, 186, and 115 different even-numbered TAG species and isomers in bovine BF were quantified as individual molecular species of TAGs or as a member of small TAG groups by np-HPLC–ESI–MS, GC–EI–MS, and GLC, respectively. Further, additional 122 odd-numbered TAG species were determined by np-HPLC–ESI–MS. In particular, all methods enabled reliable quantification of the intact regioisomers of SC-TAGs in BF. Distribution of ACN:DB TAG classes in BF and selection of the major TAG species determined by all three methods were shown to resemble the findings of other extensive quantitative milk fat studies. On the other hand, np-HPLC–ESI–MS enabled determination of 64 and 61 even- and odd-numbered TAG species, respectively, which were not detected in BF previously. A high proportion of the novel TAG species was SC-TAGs including *sn*-2 isomers of monobutyryl and -caproyl TAGs, di- and tri-SC-TAG species, and monoacetate TAG species. Unique information on the composition and structure of the regio- and acyl chain isomers of acetate TAGs and on the ratio of the regioisomers of di-SC-TAGs was gathered by np-HPLC–ESI–MS/MS^2.

The present study showed that the quantitative chromatographic and mass spectrometric methods described here provided several useful alternatives to determine the regio- and acyl chain isomers of SC-TAGs in bovine BF. In addition, reliable quantifications of both major and minor TAG species in BF at the same time in the same analysis by np-HPLC–ESI–MS were demonstrated. However, the research material was rather restricted (unhydrous BF from one batch), which should be taken into account as the absolute composition and structure of bovine MF TAGs are assessed on the basis of the present data. The possible application areas of the methods will be the detection of the adulteration of bovine MF by

other edible oils and fats or by interesterified BF, the elucidation of biological and technological function of BF TAGs, monitoring of BF modification procedures, and investigation of structured TAGs with short-chain fatty acyls. However, the described quantification methods were rather laborious, which will probably restrict their applicability in practice. In future, a more comprehensive study of odd-numbered TAG species in BF by chromatographic and mass spectrometric methods might reveal new viewpoints, for example on biosynthesis of TAG species. In addition, the rapid development of chromatographic and particularly mass spectrometric methods has to be exploited in forthcoming studies of TAGs in bovine MF.

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Appendix 1

Composition of randomized TAG standards which were synthesized by chemical interesterification of equimolar amounts of three monoacid TAGs. Molar content of the standards was determined by calculation (Calc.) and by gas chromatography (GC).

	BLaM[1]			**BLaP**			**BLaS**		
	A = 4:0	B = 12:0	C = 14:0	A = 4:0	B = 12:0	C = 16:0	A = 4:0	B = 12:0	C = 18:0
mol%, A/B/C[2]	34.58	32.68	32.73	34.65	32.65	32.70	37.67	31.38	30.95
TAG species	Calc.[3] mol%	GC mol%	Diff. [4] %	Calc. mol%	GC mol%	Diff. %	Calc. mol%	GC mol%	Diff. %
AAA							5.35	1.59	70.32
AAB	12.23	11.43	6.55	12.27	10.66	13.12	13.36	12.45	6.81
AAC	12.25	11.83	3.41	12.29	10.67	13.21	13.18	11.89	9.74
ABB, *sn*-1(3) [5]	7.71	7.80	1.18	7.71	7.96	3.21	7.42	7.92	6.75
ABB, *sn*-2 [5]	3.85	4.00	3.73	3.85	4.19	8.78	3.71	4.29	15.68
ABC, *sn*-1(3)	15.44	15.22	1.41	15.44	15.01	2.81	14.63	14.43	1.42
ABC, *sn*-2	7.72	7.65	0.84	7.72	7.81	1.10	7.32	7.57	3.44
ACC, *sn*-1(3)	7.73	7.43	3.93	7.73	7.51	2.90	7.22	6.60	8.49
ACC, *sn*-2	3.87	3.81	1.33	3.87	4.38	13.31	3.61	3.25	10.06
BBB	3.64	4.47	22.81	3.63	4.40	21.29	3.09	4.64	50.29
BBC	10.94	11.09	1.32	10.91	11.72	7.45	9.14	11.46	25.30
BCC	10.96	10.99	0.32	10.93	11.64	6.47	9.02	10.30	14.24
CCC	3.66	4.27	16.70	3.65	4.06	11.33	2.96	3.61	21.90
Average [6]			2.40			7.24			10.19

	BMP			**BMS**			**BPS**		
	A = 4:0	B = 14:0	C = 16:0	A = 4:0	B = 14:0	C = 18:0	A = 4:0	B = 16:0	C = 18:0
mol%, A/B/C[2]	32.40	33.89	33.71	32.87	33.36	33.77	34.07	32.88	33.05
TAG species	Calc. mol%	GC mol%	Diff. %	Calc. mol%	GC mol%	Diff. %	Calc. mol%	GC mol%	Diff. %
AAA	3.40	1.04	69.28	3.55	2.13	40.11	3.95	1.75	55.74
AAB	10.67	9.92	7.09	10.81	9.51	12.04	11.45	8.87	22.49
AAC	10.62	9.24	12.92	10.95	9.33	14.80	11.51	8.75	24.00
ABB, *sn*-1(3)	7.44	6.62	11.11	7.32	6.09	16.72	7.37	7.01	4.77
ABB, *sn*-2	3.72	4.17	12.04	3.66	3.90	6.65	3.68	3.36	8.89
ABC, *sn*-1(3)	14.81	13.33	9.97	14.81	13.72	7.39	14.81	10.95	26.09
ABC, *sn*-2	7.40	8.13	9.78	7.41	8.31	12.18	7.40	5.57	24.81
ACC, *sn*-1(3)	7.36	7.11	3.50	7.50	6.62	11.76	7.44	6.32	15.04
ACC, *sn*-2	3.68	4.38	18.92	3.75	3.91	4.26	3.72	2.93	21.28
BBB	3.89	4.43	13.90	3.71	4.20	13.03	3.55	10.82	204.50
BBC	11.62	13.08	12.60	11.27	12.67	12.36	10.72	12.22	14.05
BCC	11.55	13.82	19.64	11.41	14.57	27.70	10.77	11.91	10.58
CCC	3.83	4.73	23.53	3.85	5.05	31.18	3.61	9.53	163.98
Average			11.76			12.58			17.20

	BLaPo			**BLaO**			**BMPo**		
	A = 4:0	B = 12:0	C = 16:1	A = 4:0	B = 12:0	C = 18:1	A = 4:0	B = 14:0	C = 16:1
mol%, A/B/C[2]	34.05	32.72	33.23	31.26	33.60	35.14	32.26	34.75	32.99
TAG species	Calc. mol%	GC mol%	Diff. %	Calc. mol%	GC mol%	Diff. %	Calc. mol%	GC mol%	Diff. %
AAA									
AAB	11.85	11.87	0.16	10.16	9.67	4.85	11.23	11.23	0.02
AAC	12.03	10.57	12.19	10.63	6.62	37.66	10.66	8.96	15.89
ABB, *sn*-1(3)	7.59	8.14	7.22	7.28	7.90	8.54	8.06	8.21	1.85
ABB, *sn*-2	3.80	4.48	18.15	3.64	4.57	25.60	4.03	4.31	6.88
ABC, *sn*-1(3)	15.42	14.95	3.00	15.23	14.20	6.72	15.31	14.41	5.89
ABC, *sn*-2	7.71	8.33	8.03	7.61	7.92	3.96	7.65	7.48	2.20
ACC, *sn*-1(3)	7.83	7.24	7.54	7.96	6.43	39.45	7.27	7.09	2.37
ACC, *sn*-2	3.91	3.58	8.56	3.98	3.24	61.40	3.63	3.61	0.75
BBB	3.65	4.49	23.15	3.91	4.82	17.21	4.34	5.33	22.66
BBC	11.11	10.96	1.36	12.28	14.89	21.27	12.37	13.13	6.19
BCC	11.28	11.01	2.41	12.84	14.74	14.79	11.74	12.46	6.15
CCC	3.82	4.38	14.64	4.48	5.00	11.69	3.72	3.78	1.68
Average			6.86			13.13			4.82

	BMO			**BPO**			**CoMS**		
	A = 4:0	B = 14:0	C = 18:1	A = 4:0	B = 16:0	C = 18:1	A = 6:0	B = 14:0	C = 18:0
mol%, A/B/C[2]	31.14	33.46	35.41	34.73	32.65	32.62	35.31	31.82	32.87
TAG species	Calc. mol%	GC mol%	Diff. %	Calc. mol%	GC mol%	Diff. %	Calc. mol%	GC mol%	Diff. %
AAA	3.02	1.58	47.77	4.19	1.47	64.97	4.40	3.80	13.65
AAB	9.73	8.98	7.78	11.81	8.54	27.69	11.90	11.42	4.05
AAC	10.30	8.92	13.40	11.80	8.67	26.55	12.29	11.50	6.46
ABB, *sn*-1(3)	6.97	6.18	11.36	7.40	6.73	9.08	7.15	6.81	4.79
ABB, *sn*-2	3.49	3.83	9.94	3.70	3.88	4.72	3.58	3.33	6.87
ABC, *sn*-1(3)	14.76	13.83	6.28	14.80	12.52	15.36	14.77	13.73	7.04
ABC, *sn*-2	7.38	7.91	7.22	7.40	7.26	1.80	7.39	6.70	9.30
ACC, *sn*-1(3)	7.81	6.79	13.11	7.39	6.59	10.81	7.63	8.02	5.14
ACC, *sn*-2	3.90	3.74	4.32	3.70	3.65	1.21	3.82	3.96	3.83
BBB	3.75	4.26	13.70	3.48	4.90	40.82	3.22	4.63	43.85
BBC	11.89	13.23	11.24	10.43	14.89	42.70	9.98	10.77	7.83
BCC	12.59	15.44	22.69	10.42	15.36	47.42	10.31	11.20	8.56
CCC	4.44	5.32	19.75	3.47	5.53	59.25	3.55	4.13	16.21
Average			10.73			18.73			6.39

	CoPS			**CoPO**			**CoMO**		
	A = 6:0	B = 16:0	C = 18:0	A = 6:0	B = 16:0	C = 18:1	A = 6:0	B = 14:0	C = 18:1
mol%, A/B/C[2]	33.45	33.57	32.99	32.65	32.31	35.03	31.09	33.36	35.53
TAG species	Calc. mol%	GC mol%	Diff. %	Calc. mol%	GC mol%	Diff. %	Calc. mol%	GC mol%	Diff. %
AAA	3.74	3.74	0.03	3.48	3.79	9.00	3.01	3.23	7.59
AAB	11.27	11.88	5.45	10.33	11.90	15.14	9.67	9.93	2.60
AAC	11.07	11.16	0.81	11.20	11.22	0.19	10.30	9.91	3.83
ABB, *sn*-1(3)	7.54	6.83	9.46	6.82	6.71	1.58	6.92	6.27	9.46
ABB, *sn*-2	3.77	3.27	13.28	3.41	3.56	4.35	3.46	3.84	11.04
ABC, *sn*-1(3)	14.82	13.54	8.62	14.78	13.32	9.90	14.74	12.77	13.35
ABC, *sn*-2	7.41	5.86	20.97	7.39	6.46	12.54	7.37	9.02	22.41
ACC, *sn*-1(3)	7.28	7.29	0.17	8.01	7.04	12.09	7.85	5.61	28.51
ACC, *sn*-2	3.64	3.56	2.20	4.01	3.85	3.99	3.92	2.85	27.36
BBB	3.78	4.17	10.29	3.37	4.11	21.82	3.71	4.02	8.26
BBC	11.15	12.48	11.90	10.97	12.03	9.63	11.86	13.29	12.08
BCC	10.96	12.37	12.85	11.89	11.93	0.28	12.63	13.97	10.56
CCC	3.59	3.84	7.04	4.30	4.08	5.01	4.49	5.29	17.87
Average			8.57			6.97			14.12

	CoLaP			**CoLaO**			**CoMPo**		
	A = 6:0	B = 12:0	C = 16:0	A = 6:0	B = 12:0	C = 18:1	A = 6:0	B = 14:0	C = 16:1
mol%, A/B/C[2]	33.26	33.56	33.18	36.54	32.39	31.07	33.36	33.86	32.78
TAG species	Calc. mol%	GC mol%	Diff. %	Calc. mol%	GC mol%	Diff. %	Calc. mol%	GC mol%	Diff. %
AAA	3.68	3.71	0.90	4.88	4.87	0.18	3.71	3.57	3.80
AAB	11.14	11.45	2.79	12.97	13.27	2.25	11.30	12.38	9.50
AAC	11.01	11.66	5.85	12.45	12.49	0.36	10.94	11.41	4.28
ABB	11.24	11.54	2.71	11.50	11.75	2.13	11.47	11.69	1.84
ABC, *sn*-1(3)	14.81	15.49	4.59	14.71	15.63	6.28	14.81	15.05	1.63
ABC, *sn*-2	7.41	6.30	14.95	7.35	6.73	8.53	7.41	8.08	9.16
ACC, *sn*-1(3)	7.32	6.97	4.79	7.05	6.85	2.94	7.17	5.99	16.40
ACC, *sn*-2	3.66	3.43	6.44	3.53	3.55	0.72	3.58	3.31	7.54
BBB	3.78	4.38	15.80	3.40	3.57	5.10	3.88	4.46	14.89
BBC	11.21	10.73	4.33	9.78	9.78	0.01	11.27	10.77	4.49
BCC	11.08	10.35	6.65	9.38	9.25	1.39	10.92	10.08	7.68
CCC	3.65	4.00	9.46	3.00	2.27	24.37	3.52	3.20	9.06
Average			5.90			2.73			6.95

	CoMP			**CyLaM**			**CyLaS**		
	A = 6:0	B = 14:0	C = 16:0	A = 8:0	B = 12:0	C = 14:0	A = 8:0	B = 12:0	C = 18:0
mol%, A/B/C[2]	35.12	32.57	32.30	33.08	33.84	33.05	35.79	32.12	32.09
TAG species	Calc. mol%	GC mol%	Diff. %	Calc. mol%	GC mol%	Diff. %	Calc. mol%	GC mol%	Diff. %
AAA	4.33	2.73	36.89	3.62	3.51	3.12	4.58	4.62	0.73
AAB	12.05	11.67	3.15	11.11	11.15	0.36	12.34	12.68	2.74
AAC	11.95	11.19	6.41	10.85	10.63	2.00	12.33	11.82	4.13
ABB	11.18	11.08	0.89	11.36	11.40	0.29	11.08	11.32	2.16
ABC	22.17	23.08	4.10	22.20	21.65	2.48	22.13	21.67	2.09
ACC	10.99	10.31	6.16	10.84	11.46	5.71	11.06	10.63	3.85
BBB	3.46	4.05	17.34	3.88	4.24	9.44	3.31	3.94	18.77
BBC	10.28	11.00	7.00	11.35	11.14	1.90	9.93	10.82	8.97
BCC	10.19	11.29	10.71	11.09	10.82	2.45	9.92	9.61	3.11
CCC	3.37	3.60	6.86	3.61	4.01	11.11	3.30	2.89	12.61
Average			5.49			2.17			3.86

	CyMP			**CyMS**			**CyLaO**		
	A = 8:0	B = 14:0	C = 16:0	A = 8:0	B = 14:0	C = 18:0	A = 8:0	B = 12:0	C = 18:1
mol%, A/B/C[2]	34.43	33.00	32.57	32.87	34.60	32.53	33.42	33.23	33.35
TAG species	Calc. mol%	GC mol%	Diff. %	Calc. mol%	GC mol%	Diff. %	Calc. mol%	GC mol%	Diff. %
AAA	4.08	3.69	9.51	3.55	3.80	7.07	3.73	4.88	30.83
AAB	11.74	11.00	6.23	11.21	10.87	3.07	11.13	11.28	1.35
AAC	11.58	10.90	5.92	10.54	9.85	6.55	11.17	10.66	4.64
ABB	11.25	11.46	1.85	11.81	12.20	3.33	11.07	11.49	3.77
ABC	22.20	21.07	5.12	22.20	20.72	6.64	22.22	15.44	30.52
ACC	10.96	11.76	7.29	10.43	10.16	2.63	11.15	10.86	2.62
BBB	3.59	3.16	11.98	4.14	5.28	27.58	3.67	5.49	49.51
BBC	10.64	11.47	7.81	11.68	12.12	3.75	11.05	11.48	3.91
BCC	10.50	11.49	9.41	10.98	11.29	2.82	11.09	12.62	13.84
CCC	3.46	4.00	15.80	3.44	3.69	7.25	3.71	5.80	56.40
Average			6.23			4.11			8.66

	CyMO			**CiLaP**			**CiLaS**		
	A = 8:0	B = 14:0	C = 18:1	A = 10:0	B = 12:0	C = 16:0	A = 10:0	B = 12:0	C = 18:0
mol%, A/B/C[2]	38.74	30.40	30.85	32.27	34.15	33.57	32.57	34.31	33.12
TAG species	Calc. mol%	GC mol%	Diff. %	Calc. mol%	GC mol%	Diff. %	Calc. mol%	GC mol%	Diff. %
AAA	5.81	5.64	2.97	3.36	5.81	72.76	3.46	3.50	1.26
AAB	13.69	12.81	6.38	10.67	9.44	11.49	10.92	10.49	3.92
AAC	13.89	12.06	13.14	10.49	9.84	6.20	10.54	10.14	3.83
ABB	10.74	11.03	2.71	11.29	9.90	12.33	11.50	10.90	5.21
ABC	21.80	19.55	10.34	22.20	18.12	18.35	22.21	21.61	2.67
ACC	11.06	10.69	3.37	10.91	10.95	0.38	10.72	10.99	2.55
BBB	2.81	3.87	37.61	3.98	7.39	85.61	4.04	4.45	10.06
BBC	8.55	9.46	10.58	11.75	10.72	8.76	11.70	12.78	9.23
BCC	8.68	10.25	18.15	11.55	10.91	5.52	11.29	11.59	2.67
CCC	2.94	4.64	57.88	3.78	6.92	83.01	3.63	3.55	2.26
Average			9.24			9.00			4.30

	CiMP			**CiLaO**		
	A = 10:0	B = 14:0	C = 16:0	A = 10:0	B = 12:0	C = 18:1
mol%, A/B/C[2]	31.88	33.95	34.17	33.11	33.45	33.44
TAG species	Calc. mol%	GC mol%	Diff. %	Calc. mol%	GC mol%	Diff. %
AAA	3.24	3.16	2.58	3.63	3.44	5.19
AAB	10.35	10.01	3.34	11.00	8.71	20.79
AAC	10.42	10.93	4.89	11.00	9.59	12.76
ABB	11.02	10.58	4.02	11.11	9.39	15.48
ABC	22.19	21.43	3.41	22.22	20.64	7.13
ACC	11.17	12.25	9.67	11.11	12.84	15.56
BBB	3.91	3.56	8.95	3.74	4.21	12.39
BBC	11.82	11.96	1.18	11.22	11.65	3.76
BCC	11.89	11.97	0.61	11.22	13.87	23.64
CCC	3.99	4.17	4.47	3.74	5.66	51.25
Average			3.88			14.16

[1] Abbreviations for acyl groups: B = 4:0, Co = 6:0, Cy = 8:0, Ci = 10:0, La = 12:0, M = 14:0, P = 16:0, S = 18:0, Po = 16:1, O = 18:1

[2] Content (mol%) of FAs (A, B, C) in the interesterified mixture of TAG at the baseline

[3] TAG composition was calculated according to the principles expressed in the equations (a)–(c): (a) AAA = A^3/10000, (b) AAB = $3A^2B$/10000, (c) ABC = 6ABC/10000, were A, B, C = content of (different) FAs expressed in mol% and AAA, AAB, and ABC are monoacid, diacid, and triacid TAGs, respectively (Kalo et al. 1986a).

[4] Difference (%) between calculated and determined (GC) content (mol%) of each TAG species. Diff (%) = 100 * ABS($mol\%_{CALC} - mol\%_{GC}$) / $mol\%_{CALC}$

[5] Content (mol%) of the regioisomers of mono short-chain TAG species. *E.g.*, A = 4:0 and B = 12:0 <=> ABB, *sn*-1(3) = *rac*-4:0/12:0/12:0; ABB, *sn*-2 = 12:0/4:0/12:0

[6] Average deviation for di and triacid TAGs (n = 7 / std mixture) ➔ monoacid TAGs excluded

Appendix 2

Molar correction factors (MCFs) for $[M–RCOO]^+$ ions of the molecular species of triacylglycerols (TAGs) determined by GC–EI–MS.

ACN:DB[1]	Saturated TAG species	Ion[2]	MCF	r^2 [3]	n[4]	ACN:DB	Unsaturated TAG species	Ion	MCF	r^2	*n*
12:0	4:0-4:0-4:0	215	3.169	0.759	36						
18:0	6:0-6:0-6:0	271	1.503	0.960	48						
20:0	4:0-4:0-12:0	327	9.448	0.989	24						
		215	0.688	0.915	24						
22:0	4:0-4:0-14:0	355	8.794	0.981	28						
		215	0.529	0.967	28						
24:0	8:0-8:0-8:0	327	1.037	0.980	36	24:1	4:0-4:0-16:1	381	10.274	0.971	12
	6:0-6:0-12:0	355	3.164	0.996	12			215	0.319	0.990	12
		271	0.875	0.975	12						
24:0	4:0-4:0-16:0	383	6.673	0.936	22						
		215	0.372	0.971	22						
26:0	6:0-6:0-14:0	383	3.686	0.972	24	26:1	4:0-4:0-18:1	409	7.959	0.942	12
		271	0.726	0.979	24			215	0.275	0.987	12
	4:0-4:0-18:0	411	7.091	0.977	18						
		215	0.366	0.924	18						
28:0	8:0-8:0-12:0	383	2.068	0.977	18	28:1	6:0-6:0-16:1	409	5.758	0.999	6
		327	1.327	0.957	18			271	0.468	0.999	6
	6:0-6:0-16:0	411	3.960	0.958	24						
		271	0.604	0.951	24						
	4:0-12:0-12:0	439	12.571	0.645	30						
		327	0.562	0.858	30						
30:0	10:0-10:0-10:0	383	0.839	0.996	24	30:1	6:0-6:0-18:1	437	4.866	0.965	18
	8:0-8:0-14:0	411	2.252	0.988	24			271	0.434	0.996	18
		327	0.939	0.964	24						
	6:0-12:0-12:0	439	6.880	0.929	12						
		355	0.683	0.972	12						
	6:0-6:0-18:0	439	3.025	0.984	12						
		271	0.450	0.994	12						
	4:0-12:0-14:0	467	15.190	0.993	6						
		355	1.265	0.999	6						
		327	0.823	0.998	6						
32:0	10:0-10:0-12:0	411	1.431	0.995	18	32:1	4:0-12:0-16:1	493/4	11.282	0.938	6
		383	1.582	0.999	18			381	2.292	0.995	6
	8:0-12:0-12:0	439	5.158	0.979	18						
		383	0.844	0.969	18						
	8:0-8:0-16:0	439	2.290	0.998	6						
		327	0.726	1.000	6						
	4:0-12:0-16:0	495/6	12.811	0.980	6						
		383	1.620	0.997	6						
		327	0.776	0.999	6						

ACN:DB[1]	Saturated TAG species	Ion[2]	MCF	$r^{2\,3}$	n[4]	ACN:DB	Unsaturated TAG species	Ion	MCF	r^2	*n*
	4:0-14:0-14:0	495/6	9.701	0.938	26						
		355	0.487	0.969	30						
34:0	10:0-12:0-12:0	439	3.314	0.987	18	34:1	4:0-14:0-16:1	521/2	12.992	1.000	4
		411	0.973	0.995	18			381	2.043	0.995	6
	10:0-10:0-14:0	439	1.833	0.996	6			355	0.540	0.995	6
		383	1.270	0.999	6		8:0-8:0-18:1	465	3.409	0.997	12
	8:0-12:0-14:0	467	4.898	0.991	6			327	0.586	0.996	12
		411	1.775	0.990	6						
		383	1.121	0.989	6						
	6:0-14:0-14:0	495/6	6.042	0.965	24						
		383	0.647	0.979	24						
	6:0-12:0-16:0	495/6	3.838	0.970	6						
		411	1.464	0.988	6						
		355	0.650	0.994	6						
	8:0-8:0-18:0	467	2.886	0.990	12						
		327	0.675	0.984	12						
	4:0-14:0-16:0	523/4	6.114	0.979	6						
		383	0.826	0.993	6						
		355	0.551	0.993	6						
	4:0-12:0-18:0	523/4	9.953	0.951	6						
		411	1.838	0.991	6						
		327	0.702	0.994	6						
36:0	12:0-12:0-12:0	439	0.723	0.936	70	36:1	4:0-14:0-18:1	549/50	12.719	0.991	6
	10:0-10:0-16:0	467	2.025	0.991	12			409	2.902	0.998	6
		383	1.081	0.992	12			355	0.609	1.000	6
	8:0-14:0-14:0	495/6	3.698	0.937	24		6:0-12:0-18:1	521/2	5.923	0.993	6
		411	0.807	0.986	24			437	3.399	0.996	6
	6:0-14:0-16:0	523/4	6.461	1.000	6			355	0.591	0.997	6
		411	1.805	0.998	6		6:0-14:0-16:1	521/2	10.490	0.999	6
		383	1.146	0.992	6			409	2.875	0.999	6
	4:0-16:0-16:0	551/2	7.866	0.793	20			383	0.711	0.996	6
		383	0.486	0.955	24	36:2	4:0-16:1-16:1	547/8	41.620	0.165	4
	4:0-14:0-18:0	551/2	10.834	0.999	6			381	0.577	0.983	12
		411	1.623	1.000	6						
		355	0.819	1.000	6						
38:0	10:0-14:0-14:0	495/6	2.557	0.999	6	38:1	4:0-16:0-18:1	577/8	8.111	0.986	6
		439	1.029	0.998	6			409	2.216	0.998	6
	12:0-12:0-14:0	467	1.490	0.984	10			383	0.683	0.998	6
		439	2.052	0.986	10		6:0-14:0-18:1	549/50	8.838	0.999	6
	10:0-12:0-16:0	495/6	2.371	0.998	6			437	3.515	0.998	6
		467	2.663	0.999	6			383	0.747	0.997	6
		411	1.255	1.000	6		8:0-12:0-18:1	521/2	3.879	0.999	6
	10:0-10:0-18:0	495/6	1.401	0.998	6			465	3.184	0.996	6

ACN:DB[1]	Saturated TAG species	Ion[2]	MCF	$r^{2\,3}$	n[4]	ACN:DB	Unsaturated TAG species	Ion	MCF	r^2	*n*
		383	0.886	0.997	6			383	0.583	0.997	6
	8:0-14:0-16:0	523/4	3.594	0.995	6		10:0-10:0-18:1	493/4	1.877	0.997	6
		439	1.628	0.998	6			383	0.678	0.993	6
		411	1.143	0.998	6	38:2	6:0-16:1-16:1	547/8	24.851	0.990	4
	8:0-12:0-18:0	523/4	4.526	0.987	6			409	0.705	0.992	6
		467	2.593	0.988	6						
		383	0.869	0.986	6						
	6:0-16:0-16:0	551/2	6.395	0.971	17						
		411	0.735	0.985	24						
	6:0-14:0-18:0	551/2	4.658	0.998	6						
		439	1.716	0.999	6						
		383	0.796	0.999	6						
	4:0-16:0-18:0	579/80	7.888	0.986	6						
		411	1.198	0.996	6						
		383	0.815	0.996	6						
40:0	12:0-12:0-16:0	495/6	1.011	0.934	18	40:1	6:0-16:0-18:1	577/8	8.576	0.953	4
		439	1.756	0.966	18			437	3.457	0.997	6
	10:0-14:0-16:0	523/4	2.685	0.998	6			411	1.116	0.997	6
		467	2.308	0.999	6		8:0-14:0-18:1	549/50	6.203	0.996	6
		439	1.679	0.998	6			465	4.212	0.998	6
	12:0-14:0-14:0	495/6	2.379	0.968	10			411	0.986	0.999	6
		467	1.311	0.982	10		10:0-12:0-18:1	521/2	3.903	0.994	6
	10:0-12:0-18:0	523/4	2.797	0.998	6			493/4	2.959	0.997	6
		495/6	1.894	0.998	6			411	0.800	0.990	6
		411	1.192	0.998	6		12:0-12:0-16:1	493/4	1.621	0.983	6
	8:0-16:0-16:0	551/2	4.121	0.991	6			439	1.200	0.950	6
		439	0.755	0.996	6	40:2	4:0-18:1-18:1	603/4	20.110	0.488	8
	8:0-14:0-18:0	551/2	5.228	0.997	6			409	0.724	0.973	12
		467	2.436	0.996	6						
		411	1.171	0.998	6						
	6:0-16:0-18:0	579/80	5.917	0.982	4						
		439	1.541	0.982	6						
		411	1.096	0.983	6						
	4:0-18:0-18:0	607/8	11.416	0.949	12						
		411	0.624	0.993	18						
42:0	14:0-14:0-14:0	495/6	0.654	0.939	84	42:1	12:0-12:0-18:1	521/2	2.331	0.985	18
	12:0-12:0-18:0	523/4	1.457	0.974	18			439	1.433	0.981	18
		439	1.882	0.965	18	42:2	6:0-18:1-18:1	603/4	18.902	0.656	8
	10:0-16:0-16:0	551/2	3.670	0.991	12			437	1.038	0.870	18
		467	1.222	0.996	12						
	6:0-18:0-18:0	607/8	7.626	0.996	6						
		439	1.096	0.991	12						
44:0	12:0-16:0-16:0	551/2	3.597	0.901	16	44:1	14:0-14:0-16:1	521/2	2.458	0.970	12
		495/6	1.036	0.911	18			495/6	1.367	0.992	12

ACN:DB[1]	Saturated TAG species	Ion[2]	MCF	r^2 [3]	n[4]	ACN:DB	Unsaturated TAG species	Ion	MCF	r^2	*n*
	14:0-14:0-16:0	523/4	1.366	0.947	24	44:2	8:0-18:1-18:1	603/4	12.214	0.974	8
		495/6	2.054	0.934	24			465	1.508	0.990	12
	8:0-18:0-18:0	607/8	8.244	0.970	10		12:0-16:1-16:1	547/8	11.517	0.999	4
		467	1.206	0.964	12			493/4	0.995	0.992	6
46:0	14:0-14:0-18:0	551/2	1.527	0.993	18	46:1	14:0-14:0-18:1	549/50	2.711	0.957	18
		495/6	1.759	0.997	18			495/6	1.340	0.960	18
	14:0-16:0-16:0	551/2	2.806	0.969	24	46:2	10:0-18:1-18:1	603/4	15.362	0.997	4
		523/4	1.090	0.980	24			493/4	1.037	0.997	6
	10:0-18:0-18:0	607/8	5.293	0.987	6		14:0-16:1-16:1	547/8	8.037	0.969	8
		495/6	0.929	0.996	6			521/2	1.073	0.985	12
48:0	16:0-16:0-16:0	551/2	0.880	0.963	63	48:2	12:0-18:1-18:1	603/4	9.062	0.979	12
	12:0-18:0-18:0	607/8	5.179	0.974	16			521/2	1.097	0.986	18
		523/4	1.014	0.925	18	48:3	16:1-16:1-16:1	547/8	2.212	0.944	14
50:0	16:0-16:0-18:0	579/80	1.533	0.980	12	50:1	16:0-16:0-18:1	577/8	2.210	0.977	12
		551/2	2.351	0.983	12			551/2	1.614	0.983	12
	14:0-18:0-18:0	607/8	3.479	0.996	18	50:2	14:0-18:1-18:1	603/4	10.956	0.963	18
		551/2	1.148	0.996	18			549/50	1.610	0.979	18
52:0	16:0-18:0-18:0	607/8	3.876	0.978	10	52:2	16:0-18:1-18:1	603/4	8.387	0.971	9
		579/80	1.692	0.982	12			577/8	2.023	0.988	12
54:0	18:0-18:0-18:0	607/8	1.675	0.965	41	54:3	18:1-18:1-18:1	603/4	3.924	0.883	38

[1] ACN:DB = Number of acyl carbons:number of double bonds
[2] $[M–RCOO]^+$ ion
[3] Coefficient of determination
[4] Number of determinations

Appendix 3

Molar correction factors (MCF) determined by np-HPLC–ESI–MS for ACN:DB classes and some TAG species. MCF = ratio of calculated composition (mol%) of randomized butterfat (RBF) to uncorrected mol% of TAG species in RBF.

ACN:DB[1]	TAG species	Area mol%[2]	Calc. mol%[3]	MCF[4]
12:0	ACN:DB	0.034	0.210	6.112
18:0	ACN:DB	0.606	0.286	0.472
20:0	ACN:DB	0.728	0.357	0.490
	6:0/10:0/4:0	*0.078*	*0.050*	*0.638*
22:1	ACN:DB	0.180	0.044	0.245
22:0	ACN:DB	1.367	0.779	0.570
	12:0/6:0/4:0	*0.067*	*0.046*	*0.684*
	14:0/4:0/4:0	*0.558*	*0.360*	*0.646*
23:0	ACN:DB	0.213	0.044	0.207
24:1	ACN:DB	0.295	0.099	0.336
24:0	ACN:DB	2.523	1.788	0.708
	X/X/4:0[5]	*0.284*	*0.144*	*0.506*
	6:0/14:0/4:0[6]	*0.379*	*0.144*	*0.379*
	16:0/4:0/4:0	*0.823*	*0.793*	*0.963*
	4:0/16:0/4:0	*0.657*	*0.396*	*0.603*
25:1	ACN:DB	0.064	0.015	0.229
25:0	ACN:DB	0.233	0.218	0.933
26:2	ACN:DB	0.310	0.084	0.269
26:1	ACN:DB	1.779	0.997	0.561
	X/X/4:0[5]	*0.129*	*0.039*	*0.299*
	18:1/4:0/4:0	*0.909*	*0.616*	*0.678*
	4:0/18:1/4:0	*0.625*	*0.308*	*0.493*
26:0	ACN:DB	4.775	1.864	0.390
	X/M[8]*/4:0*[5]	*0.380*	*0.156*	*0.410*
	16:0/6:0/4:0	*0.535*	*0.316*	*0.590*
	6:0/16:0/4:0	*0.535*	*0.316*	*0.590*
	18:0/4:0/4:0	*0.566*	*0.328*	*0.579*
27:1	ACN:DB	0.076	0.012	0.154
27:0	ACN:DB	0.013	0.038	2.979
28:1	ACN:DB	1.668	0.801	0.480
	18:1/6:0/4:0	*0.426*	*0.245*	*0.576*
28:0	*X/X/6:0*[5]	*0.838*	*0.151*	*0.180*
	X/X/4:0[5]	*1.559*	*0.748*	*0.647*
29:1	ACN:DB	0.083	0.005	0.061
29:0	ACN:DB	0.272	0.039	0.144
30:1	ACN:DB	1.394	0.546	0.391
30:0	X/X/M[5]	0.834	0.297	0.357
	X/X/6:0[6]	*0.726*	*0.177*	*0.244*

ACN:DB[1]	TAG species	Area mol%[2]	Calc. mol%[3]	MCF[4]
	X/X/4:0[5]	*1.535*	*0.583*	*0.380*
31:1	ACN:DB	0.101	0.009	0.087
31:0	ACN:DB	0.272	0.046	0.171
32:1	*X/X/4:0*[5]	*1.055*	*0.440*	*0.968*
	X/X/6:0[5]	*0.024*	*0.084*	*0.186*
32:0	*X/X/M*[5]	*0.331*	*0.090*	*0.273*
	X/X/6:0[5]	*0.849*	*0.311*	*0.366*
	X/X/4:0[5]	*1.506*	*0.938*	*0.623*
33:1	ACN:DB	0.114	0.014	0.125
33:0	ACN:DB	0.391	0.096	0.247
	X/X/6:0[5]	*0.140*	*0.025*	*0.181*
	X/X/4:0[5]	*0.194*	*0.051*	*0.261*
34:1	ACN:DB	1.944	1.079	0.555
	X/X/6:0[5]	*0.675*	*0.166*	*0.246*
	X/X/4:0[5]	*1.044*	*0.522*	*0.500*
34:0	*X/X/M*[5]	*0.622*	*0.183*	*0.294*
	X/X/6:0[5]	*1.626*	*0.296*	*0.182*
	X/X/4:0[5]	*1.812*	*1.542*	*0.851*
35:1	ACN:DB	0.187	0.034	0.183
35:0	ACN:DB	0.554	0.231	0.418
	X/X/6:0	*0.133*	*0.020*	*0.151*
	X/X/4:0	*0.198*	*0.112*	*0.563*
36:2	ACN:DB	0.653	0.307	0.470
36:1	*X/X/M*	*0.440*	*0.100*	*0.227*
	X/X/6:0[6]	*1.557*	*0.735*	*0.472*
	X/X/4:0[6]	*1.288*	*1.088*	*0.845*
36:0	*X/X/M*[5]	*1.090*	*0.279*	*0.256*
	16:0/14:0/6:0[7]	*1.417*	*0.543*	*0.383*
	X/X/4:0[5]	*1.271*	*2.062*	*1.622*
37:1	ACN:DB	0.468	0.196	0.419
37:0	ACN:DB	0.609	0.244	0.400
	X/X/6:0	*0.129*	*0.067*	*0.515*
	X/X/4:0	*0.165*	*0.050*	*0.300*
38:2	*X/X/6:0*[5]	*0.173*	*0.073*	*0.420*
	X/X/4:0[5]	*0.465*	*0.336*	*0.722*
38:1	ACN:DB	10.729	4.599	0.429
	X/X/6:0[5]	*1.998*	*0.504*	*0.252*
	18:1/16:0/4:0	*2.051*	*2.329*	*1.136*
38:0	*X/X/M*[5]	*1.332*	*0.398*	*0.299*
	X/X/6:0[5]	*2.425*	*0.822*	*0.339*
	18:0/16:0/4:0	*1.347*	*1.239*	*0.920*
39:1	ACN:DB	0.618	0.155	0.250
	X/X/6:0	0.129	0.049	0.381
	18:1/17:0/4:0	0.157	0.039	0.245
39:0	X/X/M[5]	0.132	0.057	0.433

ACN:DB[1]	TAG species	Area mol%[2]	Calc. mol%[3]	MCF[4]
	X/X/6:0[5]	*0.240*	*0.038*	*0.159*
	18:0/17:0/4:0	*0.094*	*0.020*	*0.217*
40:4	ACN:DB	0.140	0.083	0.596
40:3	ACN:DB	0.553	0.335	0.605
40:2	ACN:DB	2.580	1.749	0.678
	X/X/6:0[6]	*1.366*	*0.586*	*0.429*
	18:1/18:1/4:0	*1.082*	*0.905*	*0.836*
40:1	X/X/M[6]	1.593	0.787	0.494
	X/X/6:0[6]	*2.651*	*0.955*	*0.360*
	18:1/18:0/4:0	*1.300*	*0.963*	*0.741*
40:0	X/X/M[5]	1.860	1.218	0.655
	18:0/16:0/6:0	*1.205*	*0.494*	*0.410*
	18:0/18:0/4:0	*0.446*	*0.256*	*0.574*
41:1	ACN:DB	0.416	0.072	0.172
41:0	ACN:DB	0.443	0.117	0.265
42:3	*18:2/18.1/6:0*	*0.121*	*0.065*	*0.540*
42:2	ACN:DB	1.636	0.767	0.469
	X/X/M[6]	0.401	0.113	0.283
	X/X/6:0[5]	*1.193*	*0.395*	*0.331*
42:1	ACN:DB	2.839	1.963	0.692
	X/X/M+L/L/L[8]	1.777	1.276	0.718
42:0	X/X/M+L/L/L	1.503	1.967	1.308
	18:0/18:0/6:0	*0.114*	*0.010*	0.087
	20:0/4:0/18:0	*0.132*	*0.010*	0.078
43:1	ACN:DB	0.326	0.077	0.237
43:0	ACN:DB	0.247	0.136	0.548
44:2	ACN:DB	0.986	0.494	0.500
44:1	ACN:DB	1.690	2.037	1.206
44:0	ACN:DB	1.202	2.360	1.964
45:1	ACN:DB	0.227	0.105	0.463
45:0	ACN:DB	0.222	0.207	0.929
46:3	ACN:DB	0.307	0.138	0.449
46:2	ACN:DB	0.711	0.775	1.091
46:1	ACN:DB	1.090	2.519	2.312
46:0	ACN:DB	0.867	2.935	3.386
47:1	ACN:DB	0.167	0.204	1.218
47:0	ACN:DB	0.173	0.290	1.675
48:4	ACN:DB	0.077	0.026	0.341
48:3	ACN:DB	0.225	0.207	0.876
48:2	ACN:DB	0.507	1.105	2.178
48:1	ACN:DB	0.946	3.851	4.070
	18:1/14:0/16:0	*0.926*	*3.003*	*3.243*
48:0	ACN:DB	0.753	3.142	4.173
	20:0/X/X	0.033	0.017	0.533
	16:0/14:0/18:0[5]	*0.703*	*3.014*	*4.288*

ACN:DB[1]	TAG species	Area mol%[2]	Calc. mol%[3]	MCF[4]
49:1	ACN:DB	0.175	0.382	2.182
49:0	ACN:DB	0.155	0.227	1.469
50:4	ACN:DB	0.089	0.098	1.107
50:3	ACN:DB	0.244	0.478	1.963
50:2	ACN:DB	0.527	2.039	3.865
50:1	ACN:DB	0.867	4.767	5.498
50:0	ACN:DB	0.528	2.119	4.016
	20:0/X/X	0.028	0.032	1.151
	18:0/16:0/16:0[5]	*0.454*	*2.087*	*4.594*
51:1	ACN:DB	0.128	0.254	1.982
51:0	ACN:DB	0.111	0.085	0.766
52:4	ACN:DB	0.098	0.182	1.857
52:3	ACN:DB	0.203	0.688	3.392
52:2	ACN:DB	0.548	2.959	5.404
52:1	ACN:DB	0.665	2.794	4.198
	20:0/X/X	0.049	0.025	0.509
	18:1/18:0/16:0	0.604	2.730	4.521
52:0	ACN:DB	0.351	0.774	2.206
	20:0/X/X	0.050	0.050	1.000
	18:0/18:0/16:0	0.269	0.726	2.700
53:2	ACN:DB	0.051	0.076	1.504
53:1	ACN:DB	0.101	0.054	0.537
53:0	ACN:DB	0.116	0.012	0.104
54:6	ACN:DB	0.023	0.010	0.415
54:5	ACN:DB	0.041	0.074	1.836
54:4	ACN:DB	0.045	0.045	1.013
54:3	ACN:DB	0.172	0.871	5.054
54:2	ACN:DB	0.321	1.111	3.459
54:1	ACN:DB	0.339	0.619	1.825
54:0	ACN:DB	0.181	0.129	0.713

In this table, use of italics indicates regioisomers of (short-chain) TAG species and that of normal font a predominant regioisomer.

[1] ACN:DB = Number of acyl carbons:number of double bonds

[2] Uncorrected mol% (area mol%) of TAG species in randomized butterfat

[3] Mol% of TAG species in randomized butterfat calculated according to random distribution

[4] MCF = calc.mol%/area mol%

[5] More than one TAG species are identified

[6] Included minor impurities of other identity

[7] Included other regioisomer

[8] M denotes medium-chain acyl (C8–C10); L denotes long-chain acyl (C12–C24)