Norges miljø- og biovitenskapelige universitet

Master's Thesis 2021 60 ECTS
Faculty of Chemistry, Biotechnology and Food Science

Determining Monoacylglycerol Positional Isomers as Trimethylsilyl (TMS) Derivatives by GC-MS

Anders Moen Flytkjær
Master of Chemistry

Abstract

Monoacylglycerols (MAG) are constituents of milk lipids that are always present at low levels but also one end products of triglyceride (TAG) digestion. The most abundant type of lipids found in milk. The position at which fatty acids are bound to glycerol affects their absorption and possibly health properties (Lindmark Månsson, 2008)(Christie, 2010). Information on whether FA is bound to the outer 1(3) positions or the middle position 2- on the glycerol molecule is therefore useful. 2-MAG is believed to be main product of enzymatic digestion in the stomach and small intestine (Embleton & Pouton, 1997).

The most common method of qualitative- and quantitative fatty acid analysis is based on hydrolysis of these from their position on the parent molecule and subsequent methylation into FAME derivatives. The FAME method currently used in-house does not produce information on original position of FA but another derivatization method does. Trimethylsilyl (TMS) ester derivatives are easy and quick to produce, have characteristic MS fragments that can be used for identification, are selective on some GC columns and therefore suitable GC-MS analysis. Semi quantitative determination of MAG isomers as TMS esters could be coupled to the quantitative FAME analysis to add another layer of information

Here BSTFA:TMCS was used as derivatization agent to produce TMS derivatives of MAG standards to determine descriptive fragment ions for the 1- and 2-MAG positional isomers. Both lacked the molecular ion but had a m/z $[M-15]^+$ as largest fragment. 1-MAG had $[M-103]^+$ as a unique base peak that together with $[M-15]^+$ could be used to identify these. 2-MAG had a fragment of m/z $[218]^+$ of moderate intensity which was lacking for 1-MAG and a $[M-161]^+$ fragment that together with $[M-15]^+$ could be used to deduce identity. Fragmentation patterns and retention times were used to identify MAG isomers from two digested milk product samples. Identification was possible though no success was had in resolving all C18 species of MAG on the rtx-2330 GC column when both isomers were present. Most MAG was found in sn-1 position against the hypothesis that digestion produces predominantly 2-MAG but experiments on standards found that FA migration from sn-2 to sn-1 had happened during SPE separation of lipids. Attempts to reduce this by performing SPE at lower temperatures reduced the effect but did not remove it. It was concluded that TMS derivatization is promising tool for isomer determination but was not suitable on the rtx-2330 due to its low maximum temperature which makes SPE necessary to avoid TAG contamination. Changing SPE column to a type that not catalyze isomerization or changing GC column to a high temperature resistant type so that TAG can be injected could be possible solutions.

Acknowledgements

The semesters of 2020 and 2021 concluded while the world was filled by uncertainty and fear but also creativity and hope. Overall many people will look back on this period, if nothing else, as a weird one.

University life did not go unchanged. Campus grounds opened and closed to much confusion of its users, meetings became digital and we all had to learn the intricacies of this platform. Not all projects that were planned got to proceed and communication halted as no one could predict what was to come.

In the midst of this a big 'thank you' to the university employees who fought hard enabling students to still progress is in order. I'd also like to thank my supervisors Dag Ekeberg and Hanne Devle for making the project possible and for allowing me to make, and learn, from my own mistakes. A teaching that is often overlooked in this busy world and one I took ample opportunity to engage in.

Lastly I have to word my appreciation for the people that I've shared house and life with during this time. Who needs the outside world when the best people are found at home.

Contents

Chapter 1

Introduction

Cow milk consumed in Norway through a wide range of products and of which several could be considered traditional staples. Its lipids encompass several short and medium length fatty acids not common to other products (Lindmark Månsson, 2008). While general health effects of bovine milk consumption in adult populations might be modest - as beneficial compounds like calcium, vitamin D and *etc.* found in milk could be covered by other products or supplements (Willett & Ludwig, 2020) - there is special interest from researchers into some of the constituents that make up this liquid.

Triglycerides constitutes the largest amount of important animal and plant based dietary fats (Christie, 2010). In milk about 98% of lipids are found as triglycerides, 2% as diglycerides, 1% as phospolipids and 0.1 % as free fatty acids (*FFA*) (Lindmark Månsson, 2008). In the body triglycerides are enzymatically degraded into di- and monoglycerides as well as free fatty acids. The process takes place in both the stomach and small intestine. It involves an enzyme class named 'lipases' from which the two most important are gastric- and pancreatic lipase(s). These facilitates a hydrolysis reaction at (mostly) position 1 and 3 of the TAG molecule. Which frees a fatty acid for uptake and leaves the glycerol backbone one substituent poorer (Embleton & Pouton, 1997) (Ramirez et al., 2001).

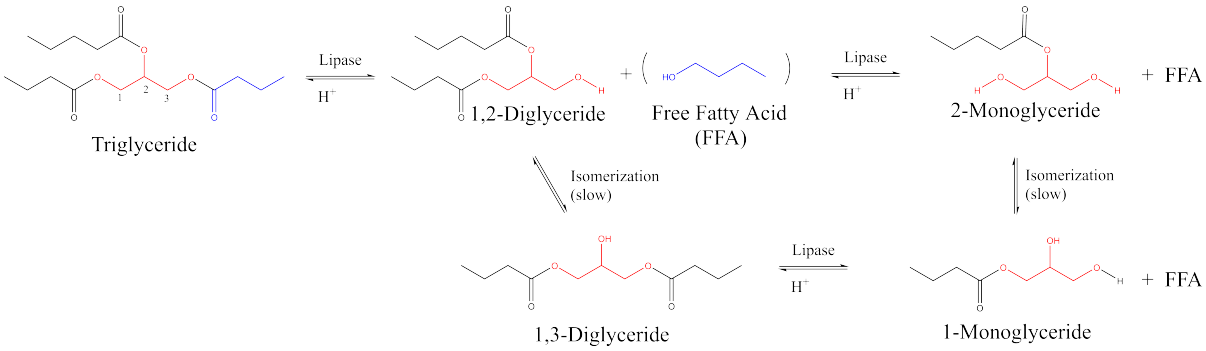


Figure 1.1: Generalized scheme for digestion of a triglyceride (here tributyrin as acyl groups). Lipase facilitates a hydrolysis reaction which frees a fatty acid (FFA) from the position 1 or 3 of the glycerol (Left-to-right). By this reaction the TAG is transformed into firstly DAG, and subsequently MAG - in addition to two FFA's. The FFA's and MAG molecule is taken up by the body (Embleton & Pouton, 1997)

The end result by complete degradation of a TAG molecule would thus be a 2-monoglyceride and two FFAs of variable types. Depending on original positons of the FAs on the precursor TAG molecule. Some amount is also believed to be completely transformed into glycerol and FFAs. As the acyl group will slowly migrate onto the positions targeted by the lipases (Michalski et al., 2013) (Zhou et al., 2021). A by product of migration is also loss of enantiomeric purity. As the FA will ultimately settle as a mix between positions 1- and 3. This could prove important if the two enantiomeric forms have different biological importance (Garcia et al., 2011a).

The acyl group in position "2", or sn-2, is telling of its further use as 2-MAG is a template from

h

Table 1.1: Typical ranges (percent of total mass) of fatty acids composition from bovine milk. Described by length of acyl carbon chain with common names. Taken from (Jensen, 2002).

Fatty Acid Type	Trivial name	Weight Percent
4:0	Butyric	2–5
6:0	Caproic	1–5
8:0	Caprylic	1–3
10:0	Capric	2–4
12:0	Lauric	2–5
14:0	Myristic	8–14
15:0	Pentadecanoic	1–2
16:0	Palmitic	22–35
16:1	Palmitoleic	1–3
17:0	Margaric	0.5–1.5
18:0	Stearic	9–14
18:1	Oleic	20–30
18:2	Linoleic	1–3
18:3	Linolenic	0.5–2

which to re-synthesize DAG and TAG molecules inside the organism. And if 2-MAG is present in suitable amounts this pathway is preferred over other alternatives. Indicating that 2-MAG is a important precursor in triglyceride synthesis within the body (Fred & Borgström, 1965) (Mitchell et al., 2008). MAG, and especially monolaurin (acyl C12), also has antimicrobial and anti viral properties that might provide a health benefit to food items containing this (Dufour et al., 2007). MAG has an additional use as an emulsifier and is the most commonly used additive for this function in food products (Chu & Nagy, 2013).
Triglycerides are considered 'simple' when they contain the same fatty acid in all positions. If two or more of the fatty acids differ it is considered mixed. Which is the case for most natural occurring TAG. In these cases the center carbon also becomes chiral and both optical as-well-as positional isomers are possible (Dewick, 2009).

A vast range of lipids are present in bovine milk. A recent study of milk triglycerides found 65 different fatty acids attached to the glycerol. And these formed thousands of unique TAG combinations or as they defined it 'species' (Liu et al., 2020). The implication for number of feasible MAG, of 65 evenly distributed and abundant FA's, would be an imminence number of possible combinations (3^{65}). In reality a small amount of species is much more frequent than others.

This is because synthesis of triglycerides in mammals is a non-random process that preferentially esterifies FA's at specific positions on the glycerol backbone. C4:0 and C6:0 strongly adhere to the sn3 position, longer still carbon numbers (from C8:0 to C16:0) has a tendency to be esterified at positions sn1 and sn2. Saturation also affects positioning and the common C18:0 is mostly added to position sn1. Whereas for C18:1 it's both at sn1 and sn3 (Lindmark Månsson, 2008). There many factors that impact the lipid composition of milk as individual genetics, feed type and seasonal variations all have an effect. Still most mass in milk lipids is from a few fatty acids that are much more prevalent than others, see table 1.1. Other than these the higher numbers found in other analysis as mentioned previously, are only present in trace amount. In addition to varying acyl length unsaturated forms are also found in both *cis* and *trans* configuration (Amores & Virto, 2019) and some branched FA types produced from bacterial activity in the rumen of the cow are also present (Yan et al., 2017).

The Norwegian Centre for Research-based Innovation (*CRI*) has funded multiple research projects under the umbrella term 'Foods of Norway'. The intent was to develop Norwegian aquaculture and agriculture as a whole through invention of new technologies. From this a project named "LipidInflammaGenes" was sprung. Aiming at uncovering the *"Effects of lipids composition and structure on digestibility and inflammation*" (Haugh, 2020). One point of interest was FA positioned at sn-2 on TAG. The identity of which could be important for potential health effects, but determination of also was an analytical problem that had to be solved.

1.1 Monoacylglycerol Analysis Overview

Development of methods for lipid determination is in no way a novel endeavour. Mass spectroscopic techniques into this subject alone have been developed and improved upon since its invention some 50 years ago (Murphy, 2020). And while it has had an big impact in lipid research, it is just one of several instruments used today (Christie, 2010).

Here the intent was *not* to produce a complete review of published research, as the volume of information even limited to milk fats simply is too large. Rather, its an attempt to draw forth some current techniques and their uses. Highlighting ones which at least partly focus on determining the monoacylglycerols.

1.1.1 Extraction and purification of lipid samples

The first step in any analysis will often be extraction the compound of interest from the environment wherein it's embedded. For extraction of total lipid content two techniques developed in the late fifties are still widely used today. These, now gold standards, are the work of 'Folch et al., 1957' and 'Bligh and Dyer, 1959' which in both cases relies on step-wise partitioning of lipids in chloroform/methanol/water mixed phases. Making both protocols a type of liquid-liquid extraction (LLE).
Lipids in a homogenized sample incubated in these solutions will ultimately be concentrated in the bottom layer (predominantly chloroform), whereas more polar compounds like metabolites, amino acids, salts etc. can be discarded with the upper phase (Eggers & Schwudke, 2016). Although both methods were developed as a way to extract lipids from fish tissue, they have been modified and extensively applied to other matrices such as milk (Amores & Virto, 2019). Most of the lipid mass is expected to be recovered for either method, although the method of 'Bligh and Dyer' is less applicable when fat percentage is above 2 (Iverson et al., 2001). Staying within the realm of *LLE* non-polar lipid types from food items have also been extracted in petroleum ether (Petrović et al., 2010) and less polar polyunsaturated MAG in ethyl acetate

Due to the range of polarities within species of MAG and FFA recoveries of these lipids with the above mentioned LLE methods are lower and the same is true for the range of species that can be extracted (Chu & Nagy, 2013).

1.1.2 Methods in Monoacylglyceride Analysis

The most common methods for qualitative and quantitative investigation of fatty acid composition is converting these into fatty acid methyl esters (FAME) that are then analyzed by GC-FID (Gas Chromatography coupled with Flame Ionization Detector) or sometimes MS (Amores & Virto, 2019). Previous to this lipid subclasses has to be separated and some popular ways of doing this is by High Performance Liquid Chromatography (HPLC), Thin Layer Chromatography (TLC) or Solid Phase Extraction (SPE) (Christie, 2010)(Amores & Virto, 2019). Fatty acids from fractions like TAG, DAG, MAG, FFA, sphingolipids, sterols etc. can then be hydrolyzed and methylated in a single step (transesterification) into FAME derivatives by several reagents like HLC/ methanol for an acid-catalyzed reaction or sodium methoxide for a base-catalyzed one, choice will depend on analytical target (Eder, 1995).
As the polar alcohol groups are replaced with non polar ethers the overall polarity of the lipid is decreased. And as a result the volatility increases. This enables the transfer of molecules into the gas phase so that GC can be utilized at reasonable temperatures. In gas phase the compounds can be separated on a reverse-phase column according to polarity more easily so than their non-derivative counter-parts (Drozd, 1986). Many GC columns have great selectivity for FAME and therefore a great number can be analzyed (Amores & Virto, 2019). One draw back is that individual FA's is removed from their original position and other techniques must be applied if this information is wanted.

Some researchers has been interested in differentiating the positions (1 and 3) at opposite ends of the glycerol in their analysis. When the FA is positioned in either of these modes, the middle carbon of the glycerol is considered a chiral center and the two enantiomers can then, in theory, be independently investigated. The earliest success into this, was the work of Brockerhoff, 1965 whom used pancreatic lipase to produce mixed 1,2- and 2,3- DAG from TAG which was then resynthesized into phospholipids. Using the stereo-specific enzyme '*Phospholipase A*' the sn-2 FA moiety from only the L-phospholipid (FA 1,2 Pho 3) was then released. By clever book keeping the initial positions could then be calculated after

quantifying the sn-1 and -2 fractions. Which was done as their FAME representatives by GC-FID.

More recently, researchers have developed non-enzymatic methods serving the same purpose by using chiral high performance liquid chromatography (HPLC) columns (Takagi & Ando, 1991)(Garcia et al., 2011b)(Deng et al., 2007). One group achieved this by partially chemically degrading TAG into DAG with ethyl magnesium bromide. Separating sn-1,3 and sn-2- fractions by thin layer chromatography (**TLC**) and subsequently resolving sn-1 and sn-3 as their 3,5-dinitrophenylurethane (3,5-DNPU) derivates by HPLC, using a chiral column and a ultraviolet detector (Takagi & Ando, 1991). Quantification was performed as before on individual fractions by FAME analysis with GC-FID.

1.1.3 TMS Derivatization in Monoacylglyceride Analysis

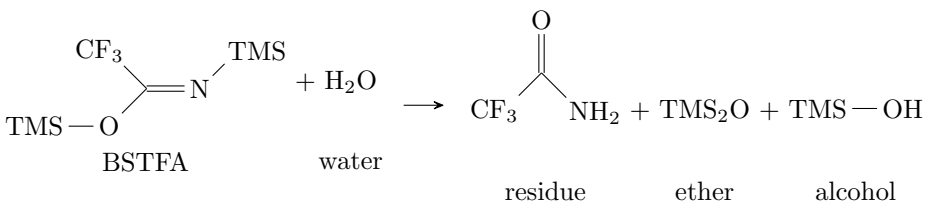
Unhindered hydroxyl groups (HO-R) like those found in MAG and DAG, readily react with common commercially available chemicals like N,O-Bis(trimethylsilyl)acetamide (BSA), hexamethyldisilazane (HDMS), trimethylsilyl chloride (TMCS) or N,O-Bistrifluoroacetamide (BSTFA) (Blau, 1993)(Pierce, 1968) (Griffiths & Wang, 2020). In this process the FA moiety is kept intact on the glycerol and its position can be determined (Destaillats et al., 2010).

Silylation agents function by substituting acidic protons with a trimethylsilyl- group ($Si-(CH_3)_3$). And can serve this purpose for a range of functional groups with the following order of ease: Alcohols (R-OH) > carboxylic acids (R-COOH) > thiols (R-SH) > amines (R-NH2) (Drozd, 1986)(Little, 1999).

The TMS derivatives formed are volatile (due to reduced dipole-dipole interaction), often have narrow and symmetric chromatographic peaks, show increased thermal stability (is suitable for high temperature GC) and in many cases (acylglycerides included) have descriptive fragments of high molecular mass and intensity which can be used to deduce structural information. An advantage of the latter is improved sensitivity and selectivity, as presence of ions at equal (higher)mass-to-charge is less likely (Halket & Zaikin, 2003). The fragmentation reactions are described in a later chapter. The TMS group will also block inter-molecular acyl group migration which is common in the acylglycerides (Boswinkel et al., 1996). Thus having the benefit of preventing isomerization during analysis following the derivation step.

A strength of this method is the simplicity and speed at which it can be performed. One method of wide use is *Cd 11b-91* recommended by the American Oil Chemist Society (AOCS) which uses BSTFA, TMCS and pyridine as solvent/catalyser to convert MAG and DAG into TMS ethers by heating at 80C for 45 min in a single step reaction before injecting directly into a GC system (Satou et al., 2017) (Destaillats et al., 2010). Variations in regards to reaction time and temperature are found throughout the literature: 70C + 20 min (Fagan et al., 2004), 60C + 60 min (Monteiro et al., 2003), 70C + 180 min (Berger & Schnelder, 1992), room temp + 30 min (Satou et al., 2017) and 40C + 16 hours (Schaaf & Dettner, 2000).

TMS derivatives and agents will react with water (see equation 1.1.3) and presence of this sample must therefore be avoided (Pierce, 1968).



(1.1)

1.2 The Problem of Acyl Migration

Blau, 1993 notes that mild silyation conditions should be considered for MAG to prevent isomerization between the 2- and 1-MAG isomers. This tendency of the acyl group to migrate from the outer position(s) on the glycerol backbone is found in both MAG (Fureby et al., 1996) and for DAG (Kodali et al., 1990). The rate of this transfer is generally higher in MAG than for DAG (Takagi & Ando, 1991). In the latter case, configuration show a tendency towards positions 1,3- over 1,2 for the acyl groups (Kodali et al., 1990). The effect was described as-early-as 1924 by Fischer, 1924. The famous Nobel price winner whose name prides the Fischer projection used to describe carbohydrates.

Temperature, solvent, pH and FA length all impact the amount of migration. This provides a challenge for storage, handling and other treatment of samples if precise information of isomer distribution is needed.

There has been observed significant isomerization of both MAG and DAG during SPE. Some studies report issues when using a silica type column versus none for a silica-diol type. With a reported change in isomer distribution just shy of 30% (Fagan et al., 2004) (Koprivnjak et al., 1997). Similarly, aminopropyl based SPE columns seem to cause about equal high rates of acyl migration. (Koprivnjak et al., 1997) (Ruiz-Gutierrez & Perez-Camino, 2000).

The reasoning for this can be found in the mechanism of this transfer reaction. Initially the free electron pair of the hydroxy- group starts an nucleophilic attack on the carbonyl carbon in the ester. Forming a five-membered ring intermediate. Which in turn is broken up by cleaving of the old carbon-oxygen ether bond resulting in the new configuration, see **??**. As primary alcohols are stronger nucleophiles than secondary ones the positional preference of the acyl group will be shifted towards these (outer positions) (Kodali et al., 1990).

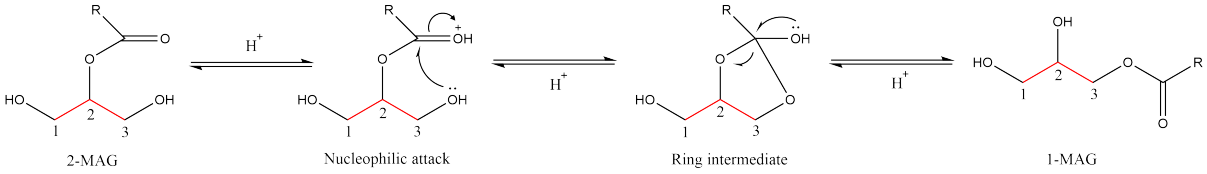


Figure 1.2: A possible mechanism for acid catalyzed acyl group migration in a monoacylglycerol (from sn2 to sn1). Figure and description based on Fureby et al., 1996.
Step 1. The carbonyl is protonted by an acid reaction.
Step 2. The adjacent hydroxyl oxygen does a nucleophilic attack the protonated carbonyl carbon and a five membered ring intermediate is formed.
Step 3. Another nucleophillic attack opens the ring and a 1-monoglyceride is now formed.

Compton et al., 2007 investigated acyl migration kinetics in solvent-free MAG. The process was found to follow a first order kinetic model on which temperature had a large effect on the reaction rate. As an average increase by factor 5,6 times was found for each 20 C increment. This effect diminished with higher temperature; with the largest increase observed being the initial one between room temperature and 40C.

Overall the solvent-free reaction was found to be quite slow. With reaction half-time (at room temperature) of 3500 hours. And much less 446 hours at 40C (Compton et al., 2007). By comparison Boswinkel et al., 1996, who investigated the migration rates of several MAG in water-saturated hexane at 30C, found reactions to proceed at about 50-100 times the rates of this. Migration rates also increase with higher carbon number of the FA moiety (at least within ranges 4-12C). Equilibrium was found to be somewhere in the ranges of 9:1 to 8:2 with the sn-1 form being favored.

1.3 EI Fragmentation of bis-TMS monoacylglycerols

EI fragmentation of bis-TMS acylglycerols share some general patterns across varying carbon length, in addition to having several ions in common with spectra of other TMS derivatives. For 1(3)- and 2-MAG a paper by Destaillats et al., 2010 has published possible structures for the diagnostic ions based on MS spectra of the C16 isomers.

With this information they succeeded in identifying additional MAG species (C16-22, unsaturated and mono- to poly saturated) from a sample using GC-MS. The molecular ion was not present, but a m/z $[M-15]^+$ likely originating from the loss of a methyl (CH_3) group was found. Elucidating the identity of unknowns firstly was initiated by using this ion to determine the fatty acid chain length. Relative abundance of this fragment ion is low (5-10%) when compared to other TMS derivates where this sometimes forms the base peak (Harvey & Vouros, 2020).

Following this, the researchers used m/z $[M-103]^+$ which forms the base peak for, and is descriptive of; the 1(3)-MAG species to determine if spectra belonged to this group. An additional ion, m/z $[205]^+$, is also found to be descriptive of 1(3)-MAG. In both cases the mechanism of formation is thought to be through alpha cleavage type reactions (see reaction **a** and **b** on figure 1.3, left) which is common among ethers (de Hoffmann & Stroobant, 2007).

As for the 2-MAG types a relative abundant ion of m/z $[218]^+$ is descriptive. Its proposed to form by a hydrogen rearrangement reaction which releases the fatty acid moiety (reaction **c** on figure 1.3, right). From this the loss of a methyl group gives rise to another less intense but also descriptive ion at m/z $[203]^+$ (reaction **c1**). At m/z $[191]^+$ is an additional descriptive ion for 2-MAG are described by Destaillats et al., 2010. Its structure has later been corrected by Harvey and Vouros, 2020 based on proton NMR data (see **e** on 1.3). No mechanism is proposed other than it involves migration of a TMS group.
Expanding the list for 2-MAG is a unique fragment with a m/z of $[M-161]^+$ which structure was proposed by Harvey and Vouros, 2020 but who did not report a mechanism (**d** on 1.3). This ion has a higher relative intensity than the $[M-15]^+$ and like this (since the FA moiety is reserved) contains information regarding FA carbon chain length, which is otherwise lacking for the other predictive ions for 2-MAG.

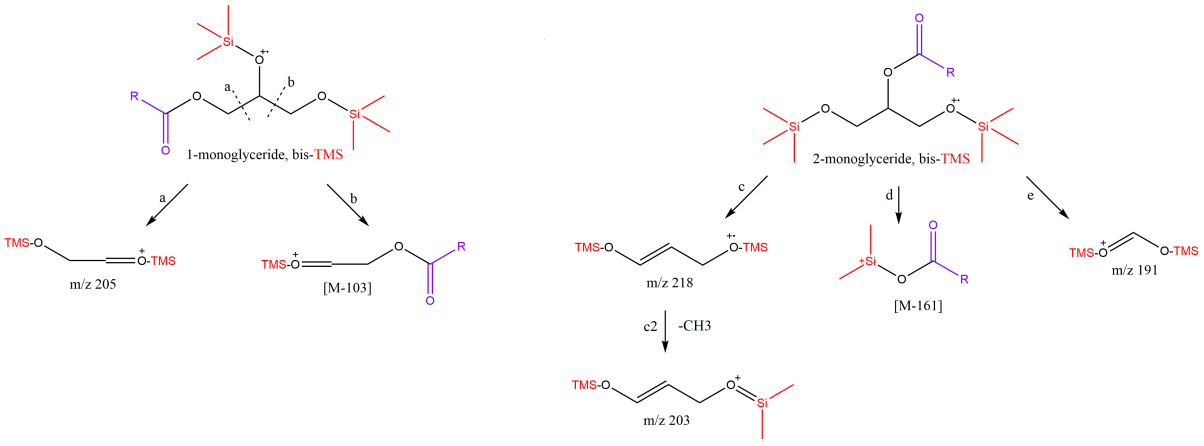


Figure 1.3: Major descriptive fragmentation patterns for 1- and 2-monoacylglycerols (left and right respectively) as bis-TMS derivatives. Structures and mechanisms from (Destaillats et al., 2010) and (Harvey & Vouros, 2020).

Some fragments shared among many TMS derivatives and based on the group itself. For primary alcohol based ethers, after an initial loss of a methyl group (reaction **f** on 1.4) hydrogen rearrangement reactions can happen to either the oxygen (**f3**) or to the silica (**f2**). Resulting in ions of m/z $[75]^+$ or $[89]^+$.

Alpha cleavage from the TMS ether (**g2**) can results in an fragment ion of m/z $[103]^+$ ion which is found in primary alcohol derivatives (Harvey & Vouros, 2020) including both 1- and 2-MAG (Destaillats et al., 2010). The abundance is higher for 2-MAG (which contains two primary alcohols). This further decomposes (**g2**) to a common fragment of m/z $[75]^+$ (Harvey & Vouros, 2020).

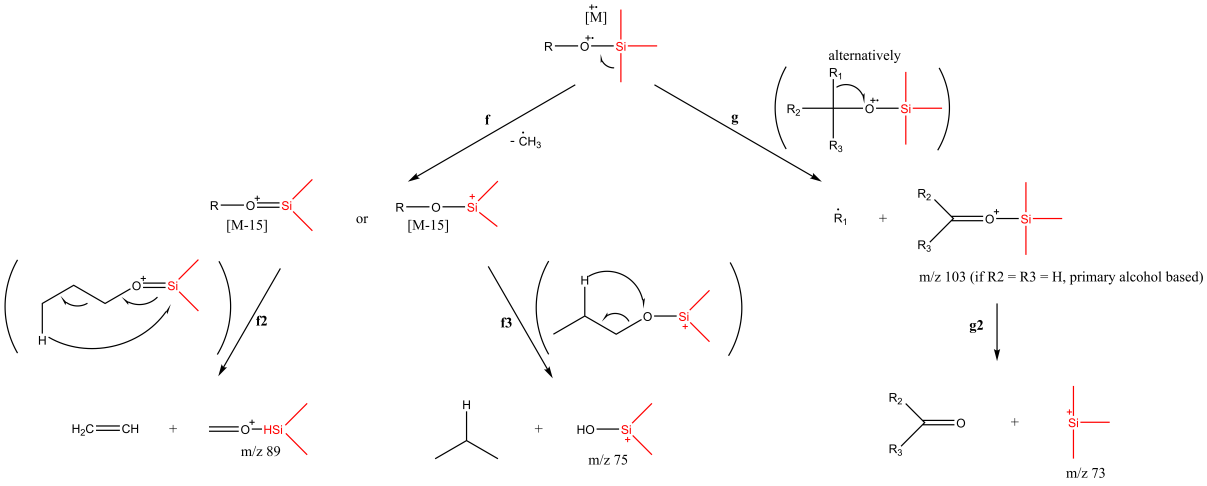


Figure 1.4: General fragments found for TMS derivates of alcohols and similar ethers (Harvey & Vouros, 2020).

1.4 Problem Statement

Knowledge on not only FA type and quantity, but also position of these on the glycerol, are important for possible health effects of MAG. This paper aims to build upon the already validated quantitative method used in routine analysis at the Department of Chemistry and Biotechnology, NMBU by determining relative abundances of the mono-acylglycerides following digestion of milk based triglycerides.

Currently the in house method used for determining fatty acids relies on extracting the total lipid mass from the sample by a modified Folchs method (As described by Devle et al., 2014). Separating, firstly, the polar lipids (eg. phospholipids and free-fatty acids) with an initial solid phase extraction (*SPE*) and secondly, utilizing another SPE step, separating the remaining neutral tri-, di, and monoglycerides (*TAG/DAG/MAG*). Finally these 'acylglycerides' are derivatized into their corresponding FA methyl esters, which in turn would be analysed by GC-MS (Devle et al., 2012).

A drawback arises from the final step. As the individual FAs are cleaved from the glycerol backbone information on their positional origin is lost.

bis-TMS derivates of MAG have previously been shown to have distinct sn-1 and -2 EI fragmentation that have been used to identify these from mixed samples by GC-MS (Destaillats et al., 2010). Here the intent was to determine intensities of *relevant fragment ions* by mass spectroscopy as-well-as gas chromatographic *rentention times*. So that MAG could be detected at the level of chain length, amount of saturation (but not cis/trans isomerism) and position on either 1(3) or 2 of the glycerol backbone. This information was determined for a selection of prepared standard solutions.

Retention times and fragmentation patterns was then applied to fractionated MAG samples from digested milk products to test feasibility of the method on a more complex matrix.

As isomerization of 2-MAG to 1-MAG was suspected to happen during current lipid fractionation process by SPE; experiments to determine whether this was an issue were also performed.

Chapter 2

Materials and methods

2.1 Chemicals and Instrumentation

Chemicals used as solvent or reagents can be found in table 2.1 and 2.2. All acylglycerol standards were purchased from Larodan, Sweden. A MAG sample derived from cream cheese (KremGo naturall, TINE), enzymatically digested by researchers from the Faculty of Chemistry, Biotechnology and Food Science (NMBU, Norway) and fractionated by master student Gard Oestboe, 2020 (after method of Haraldsen and Hausberg, 2019) was used. This had been spiked with 1-C11 and 1-C19 MAG as internal standard (I.S).

Table 2.1: Derivatizarion agent, solvents and other non lipid chemicals used

Chemical	**Retailer**	**Purity**	**CAS Number**
Chloroform	VWR Chemicals	100%	67-66-3
Methanol, 0.6% ethanol	VWR Chemicals	$\geq$ 99.8%	110-86-1
NaCl	AnalaR NORMAPUR	100%	7647-14-5
N,O-Bis(trimethylsilyl)trifluoroacetamide with trimethylchlorosilane (99/1 %)	Merck	NA	25561-30-2
n-Heptane	VWR Chemicals	$\geq$ 99%	142-82-5
n-Hexane	VWR Chemicals	$\geq$ 97%	110-54-3
Pyridine, anhydrous	Sigma-Aldrich	$\geq$ 99.8%	110-86-1

Table 2.2: Monoacylglcerol standards used in experimental work.

Compound	**Short notation**	**Retailer**	**Purity**	**CAS Number**
1-monooctanoin	1-MAG C8	Larodan	($\geq$ 99%)	26402-26-6
1-monolaurin	1-MAG C12	Larodan	($\geq$ 99%)	142-18-7
2-monolaurin	2-MAG C12	Larodan	($\geq$ 95%)	1678-45-1
1-monomyristin	1-MAG C14	Larodan	($\geq$ 99%)	589-68-4
2-monomyristin	2-MAG C14	Larodan	($\geq$ 95%)	3443-83-2
1-monopalmitin	1-MAG C16	Larodan	($\geq$ 99%)	542-44-9
2-monopalmitin	2-MAG C16	Larodan	($\geq$ 95%)	23470-00-0
2-monostearin	2-MAG C18	Larodan	($\geq$ 99%)	621-61-4
2-monoolein	2-MAG C18:1	Larodan	($\geq$ 99%)	25496-72-4
1-monolinolein	1-MAG C18:2	Larodan	($\geq$ 99%)	2277-28-3
1-monoheneicosanoin	1-MAG C21	Larodan	($\geq$ 99%)	370078-53-8

2.1.1 Gas Chromatography

Gas chromatography analysis was performed on an *Agilent* 6890N GC system, installed with a *Restek* rtx-2330 capillary column (length 60 meter, diameter 25 mm). Carrier gas was nitrogen at a flow rate of 1 mL/minute. Injection volume was 1 uL and performed by an autosampler with a mounted syringe (10 uL). Instrument was run in split mode (1:10). Injector temperature was 275C.

Oven temperature program started at 50C with an ramping increase to 275C at 10C/min. Which was used for single species tests as response curves and isomerization experiments. For determination of MAG content in digested samples the programming started at 150C and increased to 275 at 2.5C/min and 50 to 275C at 2.5C/min

2.1.2 Mass Spectroscopy

A sector instrument (EBE configuration) mass spectrometer from *Micromass*, model 'AutoSpec Ultima' was used. It run in electron impact (EI+) mode at 70 eV, source temperature was 250 C and ions was collected in full scan mode within ranges 40-600 m/z from 3-117 minutes. Spectra were collected as Total Ion Chromatograms (TIC).

2.1.3 Solid Phase Extraction

SPE was performed partly on an automated SPE-Robot (Gilson, GX-274 ASPEC, Middleton, WI, USA) and manually on a column rack connected to a pump with an valve for controlling flow. The column used was a aminopropyl type (Chromabond NH2 polypropylene, Machereye-Nagel, 500 mg, 3 mL, Dûren, Germany). Flow-rate of the mobile phases was 1.0 mL/min.

Purification of MAG from the total lipid content was achieved in two steps.

Firstly neutral, polar and phospho- lipids were separated as per Devle et al., 2014. The samples were dissolved in 1.0 mL chloroform, the column conditioned with 7,5 mL hexane, 0.5 mL sample was applied to the column. Neutral lipids was eluated with 5 mL chloroform, Polar lipids with 5 mL diethyl ether (98:2, vv) and Phospho lipids with 5 mL methanol. From the solutions containing each of these three fractions the solvent was evaporated under a nitrogen stream at 40 C. Leaving dry lipid.

Secondly the neutral lipids were separated into MAG, DAG and TAG fractions as per Haraldsen and Hausberg, 2019. The samples were dissolved in 1.0 mL hexane/chloroform/methanol (90:6::4, vvv), the column conditioned with 7,5 mL heptane, 0.150 mL sample were applied to the column, TAG was extracted with 1.0 mL heptane/diethyl ether (93:7, vv), DAG with 3.0 mL heptane/diethyl ether (93:7, vv) and lastly MAG with 3.0 mL chlorogorm/methanol (2:1, vv.). From the solutions containg each of these three fractions the solvent was then evaporated under a nitrogen stream at 40 C.

2.1.4 Preparation of trimethylsilyl (TMS) derivatives

bis-TMS derivatized MAG was produced according to AOCS Cd 11b-91 official method for MAG/DAG as it's described by Destaillats et al., 2010. Lipid samples were dissolved in 200-400 uL pyridine depending on amount. Liquid samples had the solvent evaporated under a nitrogen gas stream before being dissolved in pyridine. 50-200 uL of BSTFA:TMCS (99/1) was added to the mixture which was sealed, shaken and heated at 80C for 45 minutes. Samples were then diluted with an amount heptane depending on purpose. Stock solutions was prepared in 25 or 50 mL volumetric flasks which were then filled, other samples were prepared directly in capped GC vials and was diluted by 1 mL of heptane.

2.1.5 Software and statistics

Chromatograms and MS spectra presented were produced in MassLynx version 4.1 by *Waters* which was bundled with the GC-MS system. Peak areas were determined by the automatic integrator however baseline was sometimes manually corrected. Signal to noise ratios were determined in this software through its peak-to-peak function. Noise areas was manually selected from nearby baseline sections with no eluting analytes at lengths of least 0.5 minutes.

Standard deviations, averages, linear functions and Students t-test for two-tailed unpaired means were calculated in LibreOffice Calc (version 6.4.1.2, x64 bit) spreadsheets. Chemical structures were visualized in ChemDraw (version 20.0) by PerkinElmer. Response factors were determined as slope of molar concentration calibration curves and relative response factors calculated as RF_i divided by RF_{C16} as described by Grob and Barry, 2004.

2.2 Various determinations of MAG isomerization

2.2.1 In solvent, hexane/chloroform/methanol

The SPE protocol utilizes various volumes of a hexane/chloroform/methanol solvent to separate TAG, DAG and MAG. The lipids spend some time in this solution both during and after. E.g. waiting on the SPE-robot to finish other fractions, sample handling and subsequent solvent evaporation.

Standard solution of stereo isomeric pure 2-monomyristin (0.1 mg/mL, $\geq$ 95% purity) dissolved in hexane/chloroform/methanol (90v/6v/4v) was left at room temperature for 0, 5, 10, 15, 25, 45, 90 and 150 minutes respectably.

At each time lot an 1 mL aliquot was removed and stored in a GC vial. Solvent was evaporated under a nitrogen stream at room temperature and lipid was bis-TMS derivatized by adding 50 uL BSTFA:TMCS, 50 uL pyridine and heating to 80C for 20 minutes. The samples were subsequently diluted by 1 mL heptane before being investigated by GC-MS.

2.2.2 During lipid purification by Folch's method

2-monopalmitin standard (0,5 mL / 0,2 mg/mL) dissolved in hexane/chloroform/methanol (90v/6v/4v) was subjected to the modified Folchs extraction described by Devle et al., 2014.

The solvent was evaporated under a nitrogen stream at room temperature and the remaining lipid "purified" as per the following steps;
1: 20 mL of chloroform:methanol (2:1) was added to the sample contained in a screw cap vial, which was then sealed.
2: The vial was placed horizontally on a shaking table and shook at 350 rpm for 20 minutes.
3: 4 mL of 0.9% NaCl in denatured water was added to the solution which was then roughly shaken by hand.
4: The vial was then centrifuged at 2000 rpm for 5 minutes.
5: From the now two phases, most of the top layer was removed with a Pasteur pipette and discarded. As much as possible of the lower layer (containing lipid) was then extracted with a Pasteur pipette through the remaining top layer, and stored in a new vial.
6: The solvent was evaporated under a nitrogen stream at 40 C and lipid precipitate were bis-TMS derivatized with BSTFA:TMCS in pyridine before being investigated by GC-MS.

2.2.3 During SPE Separation of Lipid Subclasses

Determination of isomerization during SPE separation was investigated from the automated SPE protocol (robot), manually in cooler room (10C, reduced run) and manually in a walk-in freezer (-20 C). In all cases standard solutions containing 2-C16 ($\geq$ 95% purity) were used. A comparative 'before' sample was taken from the stock solutions in head of extraction work. The 'after' sample was collected from the MAG fraction following the SPE protocols described in 2.1.3. Fractions thought to contain DAG and TAG were also sampled. Both the 'before' and 'after' samples was collected in a 2 mL GC vial. Firstly solvent was removed under a nitrogen gas stream and afterwards the lipids were derivatized by adding 50 uL BSTFA:TMCS (99/1), 50 uL pyridine and heating at 80C for 40 min. Lastly the samples were diluted with 1 mL of heptane before being analyzed by GC-MS.

Chapter 3

Results and Discussion

3.1 Identification of MAG isomers as TMS derivatives by GC-MS

Determining the identity of MAG isomers from milk product samples involved several problems that had to be solved. Firstly a possible way of determining FA chain length, isomer identity and degree of saturation through chromatographic retention time and/or MS spectra analysis had to be established. High certainty of identity can be achieved by matching retention time with MS spectrum as a type of two point verification. If either information is missing or worse, contradictory, any attempt at identification becomes less persuasive.
Ideally a pure standard of each target lipid would have been run on the system to yield RT and MS spectra specific to that species for the chosen method and equipment. However selection of MAG standards, and especially 2-MAG, from chemical retailers were limited in number of types and relatively expensive for the ones available. As it was known that TMS derivatives of MAG form a homologous series in regards to RT (Isidorov et al., 2007) and can be identified from MS spectra to the level of isomer type, carbon length and amount of saturation (Destaillats et al., 2010), information from a smaller number (Table 2.2) was extrapolated to broaden the range when investigating digested samples.

A search of the NIST library (mainlib, replib 306,869 compounds) by chemical formula revealed a lack of TMS derivatives for MAG that was expected to be present in milk products. Results by former Master Student, Gard Oestboe predicted 27 different fatty acids in the MAG fraction of the same milk products (see appendix 4) from which 14 was in the library. And of these only 10 for both 1- and 2-MAG. As more than 80% was made up by C16, C18:1cis9, C14 and C18:0 alone. And here both isomers were present in the NIST library. So the most prevalent types could identified with the benefit of match factors. Still, many of the remaining minor constituents had to be identified manually.

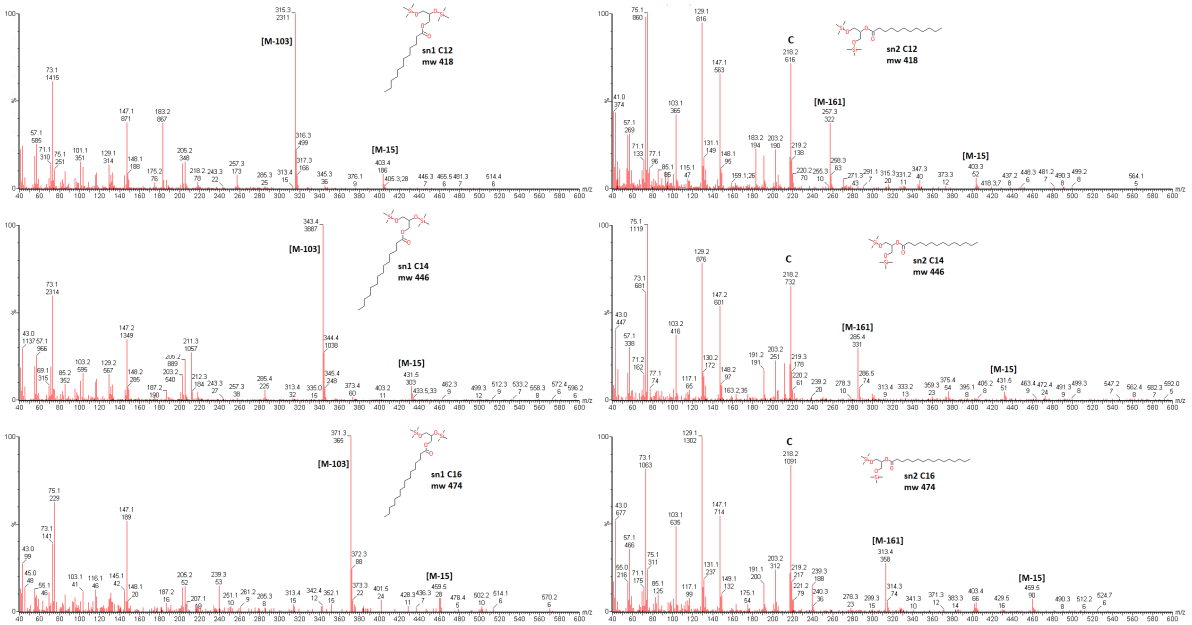


Figure 3.1: EI mass spectra for 1- and 2-MAG isomers (left and right spectra) of C12, C14 and C16. Showcasing characteristic ions for both isomers. BP was in both cases [M-15], [M-103] descriptive of sn-1 types whereas [218] and [M-161] for sn-2.

EI spectra from pairs of isomers were visually investigated (Figure 3.1) for patterns, searching for ions of reoccurring mass to charge or series related to diminishing carbon number. 11 fragments (Figure 3.2) were selected, focusing on mass to charge ions larger than 100 as they are less common in spectra of other compounds, and fragments of higher relative abundance that can be more easily discerned from background noise. A total of 35 spectra (18, 1-MAG and 17, 2-MAG), at different concentrations, for both isomers of C12, C14 and C16 as well as 1-C18:2 were selected to include variation in relative intensities.

Mass spectra for the various standards tested in this paper were in accordance with earlier published literature. For 1-MAG variants, where the FA is positioned terminal on the glycerol, base fragment was always a m/z $[M-103]^+$ type. In a few cases lower mass ions as m/z $[73]^+$, $[103]^+$, $[127]^+$ and $[147]^+$ which is present in both isomers were of comparable intensities. High concentrations seemed to overpower the detector and output would show multiple peaks at max intensity, thus data from these spectra were not considered further.
The molecular ion was not detected for any species, but a small m/z $[M-15]^+$ was present for both 1- and 2-MAG isomers at around $8\% \pm 2$ on average. For the unsaturated 1-C18:2 this peak was not discernible from background noise.

The 2-MAG isomers had higher intensities of the m/z $[218]^+$, $[M-161]^+$, 191, 129 and 103 fragments. The former was sometimes the base ion. And if not, it was one of the higher intensity peaks ($60\% \pm 20$). All except $[M-15]^+$ from the 11 tested peaks had significantly (Students t-test $p < 0.05$) different means between sn1 and sn2. Some fragment ions ($[M-235]^+$, 203 and 147) had overlapping relative intensities when standard deviations was taken into account, making isomer determination solely on these impossible or spectra with borderline values. The $[M-235]^+$ was not described in the literature. It could fit either an alpha cleave reaction from the carbonyl part of the FA ester or similar an hetero-lytic cleave from the ether type oxygen in the ester. Here, both cases would leave a charged fragment containing the FA carbon chain bound to a single oxygen atom, +O≡R. At the removal of both TMS groups and the glycerol part, as a neutral loss fragment. Since it contains the FA moiety it could still be used as evidence for identity when other fragments revealed isomerism.

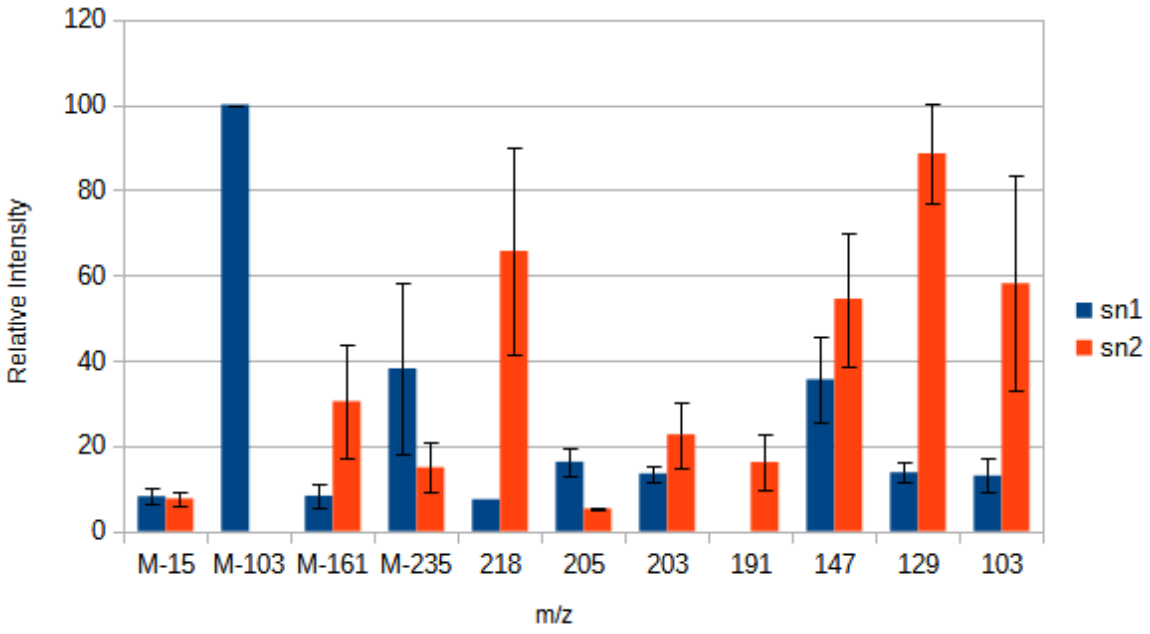


Figure 3.2: Relative intensities compared to BP of selected fragment ions from 35 spectra (18, 1-MAG and 17, 2-MAG) from different concentration of both isomers of C12, C14 and C16 as well as 1-C18:2

Using the knowledge of fragmentation sizes mentioned above (and presented in table 3.1) determining the isomer identity and carbon number in a run containing pure standards was a routine matter. Unlike Destaillats et al., 2010 whom firstly looked for the small m/z $[M-15]^+$ peak it was found to be easier initially to look for the presence of large m/z base peak that would be a suspect for $[M-103]^+$ to indicate sn1 or a somewhat intense $[218]^+$ fragment for sn2.

3.1.1 GC Retention Times and Resolution

A second puzzle piece into isomer identification was finding retention times that could be matched with MS spectra as explained above. Constructing a temperature program that had good resolution between all MAG species expected to be found in the tested dairy products was not successful. In solutions with

only saturated Problems came about when working with the digested samples that had both saturated and

Movement in a gas chromatographic system relies mostly on temperature and solid phase selectivity (Miller, 2009). Here the system was already installed with an Rtx-2330 fused-silica column from Restek, and the most conveniently method would make use of this as it was used in other routine laboratory functions. Leaving GC oven temperature programming as the means to improve system performance.

Initially a program starting at 50C increasing to 275C (maximum for column) at 10C/min was tried out on a mix containing 1- and 2-MAG of C12, C14 and C16. This ramping program is similar to what other authors have used though maximum of the column was lower than the final temperature in other published material: 340C (Fagan et al., 2004), 350C (Destaillats et al., 2010) and 360C (Satou et al., 2017).

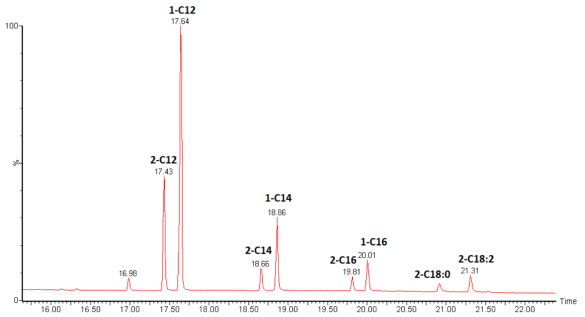


Figure 3.3: Resolved peaks of isomer pairs for C12, C14 and C16 on a temperature program starting at 50C increasing to 275C at 10C/min

All six peaks under these conditions were baseline separated and had a symmetric shape. Resolution (R) between each pair of isomers was calculated by $R = \frac{2d}{w_a + w_b}$ where d was the distance between each peak and w denoted peak width (both in minutes). Peak shape was assumed Gaussian for this calculation (an pretty close fit here) and width was estimated by drawing tangent lines at 50% peak height as described by Miller, 2009. This was done on paper with pen and ruler as the bundled software did not support easy resolution calculations.

For these three pairs at 10C/min resolution averaged 1.41 ± 0.06. Several validation criteria for resolution exist and no universal rule applies (Miller, 2009). The American Center for Drug Evaluation and Research (CDER) proposes values higher than 2 for resolution and RSD less than 1% for RT (for Drug Evaluation & Research, 1994). Which was not met for these peaks. Decreasing the ramping speed to 5C/min and 2.5C/min respectably improved resolution to 2.4 ± 0.1 and 3.74 ± 0.06 for the same peaks. But at the cost of longer run times.

To avoid broadening of peak shapes at later elution times because of eddy diffusion, and to quicken overall analysis, starting temperature was increased to 150 keeping the ramping speed of 2.5 C/min. Resolution under these conditions was intact but the total run time was much lower. Additional standard MAG had their RT determined (see table 3.1) and this temperature program seemed a good choice for carbon numbers C8-C21 based on the standards tested.

Retention time was affected by carbon number, saturation and sn-1/2 isomerism. Lower carbon numbers eluated before higher ones. And saturated before unsaturated. The sn-2 isomer always eluated first, followed by sn-1. These results were in agreement with previous studies that have shown similar behaviour for this series with a strong linear correlation to carbon number on non-polar columns (Isidorov et al., 2007) (Destaillats et al., 2010). RTs for this system were stable when injecting the same sample 10 times with relative standard deviations in ranges 0.02 - 0.03 %, see Appendix B. Which is below the minimum value of 1% recommended by CDER.

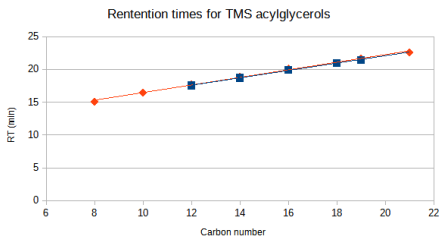


Figure 3.4: Linear relationship for carbon number for monoacylglycerol bis-TMS esters of 1-MAG (0.58x + 10.69, R^2=0.997) and 2-MAG (0.58x + 10.84, R^2=0.999)

Table 3.1: Table of MAG standards data for retention times (at 50 to 275, 10C/min), descriptive fragment ions, linear range, limits of detection/quantification and relative response factor (RRF) as molar response normalized against 1-C16.

Name	Short	Descriptive fragments (m/z)	RT (min)	Linear Range (mg/mL)	R^2	LOD/LOQ (mg/mL)	RRF
1-monocaprylin	1-C8	347 [M-15], 231 [M-103], 205	15.09	0.04 - 0.004	0.994	0.004 / 0.002	0.98
1-monodecanoin	1-C10	375 [M-15], 287 [M-103], 205	16.46	0.067 - 0.01	0.997	nd	0.69
2-monolaurin	2-C12	403 [M-15], 257 [M-161], 218	17.57	0.04 - 0.004	0.999	0.0075 / 0.005	1.37
1-monolaurin	1-C12	403 [M-15], 315 [M-103], 205	17.76	0.03 - 0.003	0.995	0.0075 / 0.005	1.18
2-monomyristin	2-C14	431 [M-15], 285 [M-161], 129	18.69	nd	nd	nd	nd
1-monomyristin	1-C14	431 [M-15], 343 [M-103], 205	18.89	nd	nd	nd	nd
2-monopalmitin	2-C16	459 [M-15], 313 [M-161], 218	19.90	0.06 - 0.001	0.999	0.0075 / 0.005	1.06
1-monopalmitin	1-C16	459 [M-15], 371 [M-103], 205	20.12	0.005 - 0.05	0.995	0.01 / 0.007	1
2-monostearin	2-C18	487 [M-15], 341 [M-161], 218	20.99	0.05 - 0.005	0.998	0.0075 - 0.005	1.04
2-monolinolein	2-C18:2	483 [M-15], 337 [M-161], 218	21.31	0.05 - 0.005	0.999	0.025 - 0.01	0.71
1-monoheneicosanoin	1-C21	529 [M-15], 441 [M-103], 205	22.63	0.1 - 0.03	0.999	0.02 / na	0.93

3.2 MAG Isomer Distribution in Digested Milk Products

Samples from two different digested Norwegian milk products were investigated to test the applicability of method on a complex sample. One from milk (bovine) cream and another from a cream cheese (TINE KremGo, naturall). 1-C11 and 1-C19 had been added as I.S for quantification in another project and was fairly intense compared to other lipids. Absolute concentration of these was not known. Quantification with a reference relates I.S response to analyte by a predetermined dose-response relationship. Which enables correction from losses during procedure (Skoog et al., 2013). Here the intent was not nearing a true concentration estimate, but finding 1- to 2-MAG isomer ratio for pairs of equal type (carbon number, saturation) in a semi quantitative manner. For this reason the I.S peaks were mostly ignored in this analysis.

3.2.1 Milk Cream

On figure 3.5 below the GC spectra for the cream milk can be seen. Twelve types of MAG was detected from C8 to C18. C11 (**b**) and C19 (**j**) was fairly intense compared to natural present MAG.

All identified peaks was 1-MAG types which did not fit the premise that enzymatic digestion by lipase leaves predominantly 2-MAG. The total number of MAG was also lower than expected. The ones that was identified were the types which are usually most prevalent indicating that the sample for some reason had low lipid content. This suspicion was reinforced by the quantification project which also found small amounts of MAG from the same sample (see appendix A). Maybe digestion was incomplete or something had happen during sample treatment. Trace amounts of 2-MAG was found by closer inspection of chromatography at expected RTs but at S/N levels less than three and peak areas outside expected linear ranges. Isomer ratios could therefore not be established.

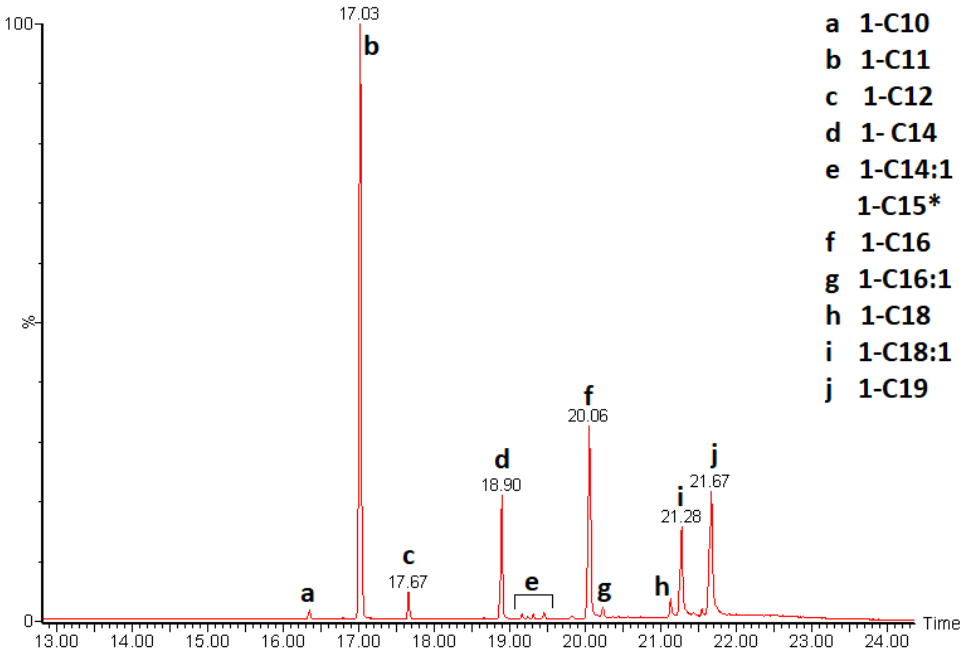


Figure 3.5: Chromatographic peaks for monoacylglycerols as TMS esters in a digested cream sample. Identified by retention times and mass spectra: **a** 1-C10, **b** 1-C11, **c** 1-C12, **d** 1-C14, **e** 1-C14:1, **f** 1-C16, **h** 1-C18, **i** 1-C18:1, **j** 1-C19. **Multiple peaks see text*

Retention times and mass spectra matched for almost all peaks which made identification fast and easy. One exception this was two smaller peaks at 19.32 and 19.46 minutes which both matched 1-C15 in the library with similar match factor scores (791 versus 821). Expected RT was calculated to be 19.54 which matched best with the second peak which also had a little higher match score in the library search. Closer inspection of the spectra (Figure 3.6) revealed that they were very similar in regards to [M-103] and [M-15], following the pattern found for 1-MAG. Notable was a less intense fragment with m/z 225, [M-235] for the earlier eluting peak compared to the following. Based on the fragment intensities of other

simple 1-MAG (Figure 3.2) this further supports the peak at 19.46 being 1-C15.
As spectra from the other peak otherwise fits an 1-MAG type it would have been strange if it was 2-MAG (which does elude a little before 1-MAG) or an pollutant. Since the calculated molecular weight does not fit an unsaturated fatty acid cis/trans isomerism was not probable either. Bacteria in the rumen of dairy cows produce a number of odd- and branched fatty acids (OBCFA) which are present in milk. This group of mostly saturated FA, like the non branched types, can be found as glycerol esters but are different from the straight chain FA in that they contain a methyl group close to the terminal FA carbon (Yan et al., 2017). The main types are C15 and C17 including iso and anteiso branched forms of these. Others are iso- C13 and C14 (Fievez et al., 2012). It is plausible that branched C15 MAG would have slightly different RT but similar EI spectra and could have been present in this sample. No peaks where found around the expected RT for C17 (20.79min). But as discussed above something was thought to have caused a lack of lipids in this sample and would maybe not have been detectable had they been present.

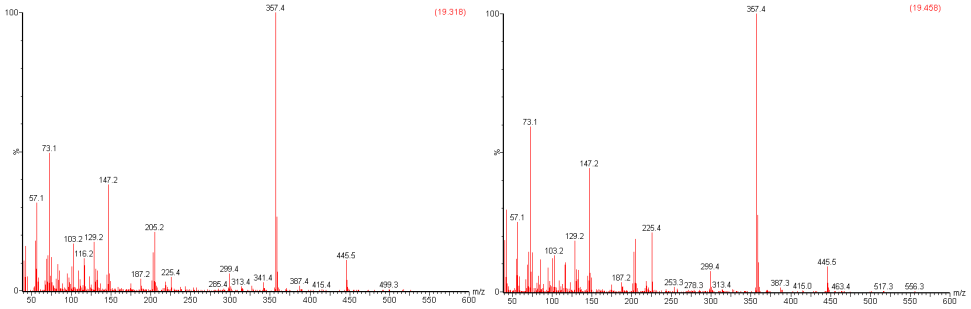


Figure 3.6: MS spectra of two C15 suspect peaks from the milk cream chromatogram at RT 19.32 and 19.46 min following the EI fragmentation pattern for sn-1 type MAG.

3.2.2 Cream Cheese, KremGo

Unlike the milk cream sample from the previous section a digested cream cheese product contained more types of FA and at a higher concentration. Less abundant FA were identified first and the sample was diluted by n-heptane by a factor of 10. So that FA of higher concentration as 1-C16 or 1-C18 were within the linear ranges that had been determined for some standards (see table 3.1). MAG content from the quantification project (Appendix A) found 22 FAME types which for TMS MAG would have meant a total of 44 if both sn-1 and -2 was present. Though many of these were only found at trace levels and only the more predominant isomer would be found. Here a total of 19 were identified. 13 sn-1 and 6 sn-2 between C8 and C18 (I.S not included in count). Identification was based on MS spectra and relative RT patterns more than absolute RT values as there was experimented with multiple temperature programs to increase resolution and isomer distribution had been shifted towards sn-1 during SPE pretreatment. For stronger evidence of identity a mix of known standards would have been run previous to the digested sample at the optimized temperature program. This is best performed not to long before as some day-to-day variation can occur.

Semi quantitative distribution of 1- and 2-MAG could only be done for isomers of equal carbon numbers if both was present. This were possible for C10 (91/9 %), C12 (92/8 %), C14 (92/8 %), C16 (92/8 %), C18 (95/5 %) and C18:1 (94/6 %). Similar ratios were found for the internal standard, C11 (91/9 %). Like the previous product lipids from cream cheese had much less sn-2 type MAG than expected. As the ratios were similar both between carbon numbers and to what was found for the I.S standard (known to be sn-1) it seemed reasonable to conclude that isomerization had taken place, and reached an equilibrium at some point during sample treatment. And thus results were not representative for the real distribution that would occur after digestion. Also for this reason variation between replicates were not considered further. As this would not reveal more than a equilibrium estimate under these conditions. Plausible explanations for this isomerization was investigated and discussed in the following section.

Multiple peaks were identified as 1-C15 and 1-C17 from the MS spectra. Both had three resolved peaks with trace level peak areas which all eluated between neighboring carbon numbers. As previously discussed these could be branched forms of equal carbon number - the presence of exactly multiple C15 and C17 peaks, which are the most common branched forms in milk, support this. But without proper

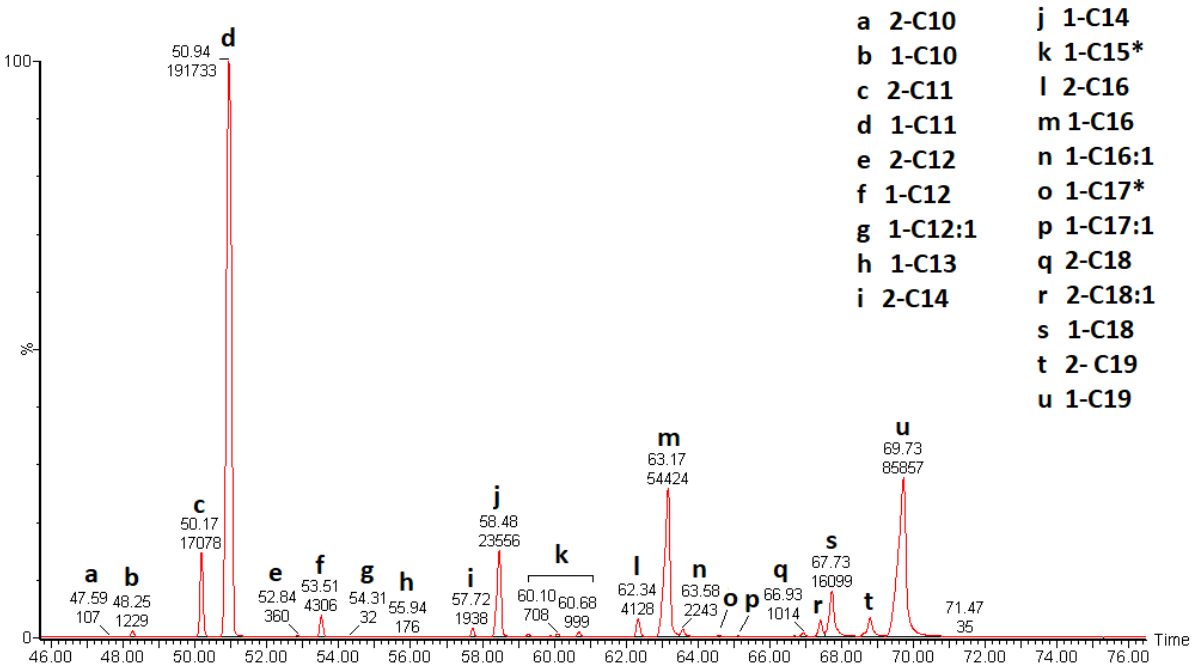


Figure 3.7: Chromatogram from a digested cream cheese sample (KremGo) with peaks from different MAG as bis-TMS esters identified from RT and MS spectra. Temperature program 50-275C at 2.5C/min. **Multiple peaks see text*

standards to determine RT and perhaps unique fragmentation ions this is within the realm of speculation.

Comparing the total FA all but one major (over 1 %) constituents that had been as FAMEs where also found in at least one form as TMS derivates. Only C18:2 (expected about 2%) was not detected. The time range at which it would be expected was already filled as both isomers of the I.S, C19 had very broad peaks in the undiluted sample. A chromatographic column has a limited number of surface space where analyte can interact with the stationary phase (SP). If sample concentration is too high retardation from SP interactions which is the basis of separation will not affect all molecules equally. Which leads to peak broadening (Miller, 2009). 2-C19 could be expected to hide 1-C18:2 (RT 68.80 compared to expected RT 68.71-68.83 for 1-C18:2). And in the diluted sample where the C19 peak eluted over a smaller time frame C18:2 might not have been detectable at all. Searching for [M-103] ion at m/z of 395, that would have been the most intense ion for 1-C18:2, did not reveal any additional information. In some cases it is possible to quantify overlapping analytes from their unique fragment ions (de Hoffmann & Stroobant, 2007), but here it was not detected. Running a sample without C19 or reducing the isomerisation issue so only sn-1 C19 is present could be solutions.

1-C18 and 1-C18:1 were also not baseline separated. This made determination of peak areas (and thus concentration) less reliable as actually determining where one peak ends and another starts becomes dubious. As a result quantification was harder. Inserting various isotermal mid temperature ramping holds of 2-5 minutes length at elution temperatures of 30- to 45C earlier than found values was attempted to solve this. Introducing wait times on temperature ramping after injection was also attempted. But none of the adjustments improved resolution between the pairs. After many attempts it seemed improbable to achieve proper resolution when MAG of equal carbon length with multiple regio- and saturation isomers are present. Litterature on TMS-MAG known by the author have mostly been concerned with a limited number and maybe because of this rarely report problems with separation. Wan et al., 2007 whom tested three different GC columns for resolution of TMS MAG esters for the analysis of vegetable oils had similar issues. Two of the columns (DB-17HT and CP-TAB-CB) were unable (like this study) to separate C18 and C18:1, while the other (DB-5HT) could, but had low resolution between C18:1 and C18:2. Interestingly Destaillats et al., 2010 whom also used the DB-5HT column had overlapping peaks for C18, C18:1 and C18:2 regio isomers, but also did run a much simpler GC oven ramp program (180-350 at 30C/min versus more complex 75C for 3 min, then 75-150 at 10C/min, then 150-250C at 5C/min and lastly 250-370C at 10C/min).

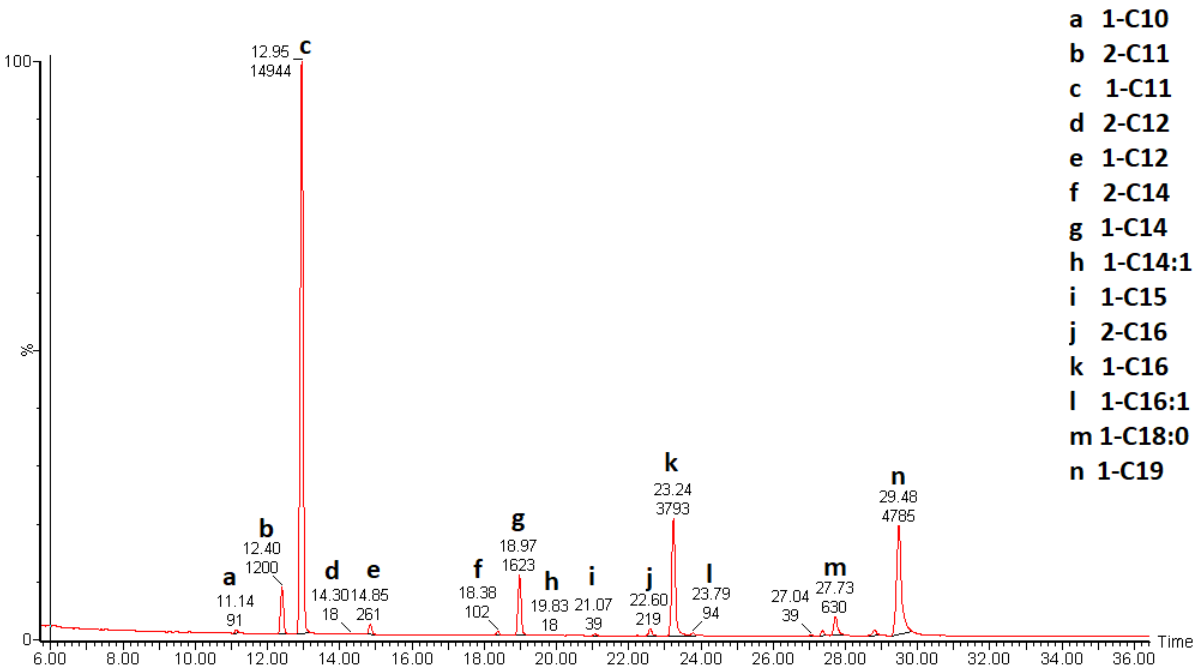


Figure 3.8: Chromatogram from a digested cream cheese sample (KremGo) with peaks from different MAG as bis-TMS esters identified by RT and MS spectra. Diluted by factor 10. Temperature program 150-275C at 2.5C/min.

Results here with the rtx-230 column indicated that this had similar resolving power as the above mentioned options described by others. Depending on the target species. Though no column seemed a catch all choice. Maximum column temperature for the rtx-2330 is 275C which are considerably lower than final temperatures for the above mentioned studies. The strength of a higher final temperature is the ability to run TAG also present in the sample through in a timely manner (Wan et al., 2007). Separating the lipids beforehand would therefore not be needed. This was not possible with the rtx-2330. Also, both SPE and GC on rtx-230 was a part of the quantification method currently employed, and determining isomer distribution would be easiest on the same equipment/samples. However doing so was found to come with issues regarding isomerization as expanded upon in the next section.

3.3 Various determinations of MAG isomerization

Isomerization from 1- to 2-MAG was suspected to happen during sample treatment. Analysis of MAG standards showed that they eluded as a lone peak corresponding to a single isomer at concentrations suitable for injection on the system. It was known that some types of SPE columns (especially amino-propyl types) are known to catalyse migration of the acyl group (Fagan et al., 2004). This same column type was used here to isolate MAG from DAG/TAG and neutral lipids from more polar.

3.3.1 During SPE separation of lipid subclasses

Treating a 2-C16 standard solution with the automated SPE robot confirmed the results from other authors that this process will lead to heavy isomerization, see 3.9. Analysis of the 2-C16 standard without SPE (green/middle) had mostly a large peak corrosponding sn-2 isomer determined from spectrum matching with the NIST library (match factor)and a smaller (RT = 24.89, 4.7% of total area) peak following this. This was in line with the quality promised by the manufacturer where 2-MAG is at worst 95% pure. In comparison the post SPE analysis (red/front) chromatogram showed two larger peaks with a isomer distribution corresponded to 66% for sn-1 and 33% for sn-2. Lipids from digested samples had likely changed from sn-2 to sn-1 in the same manner.

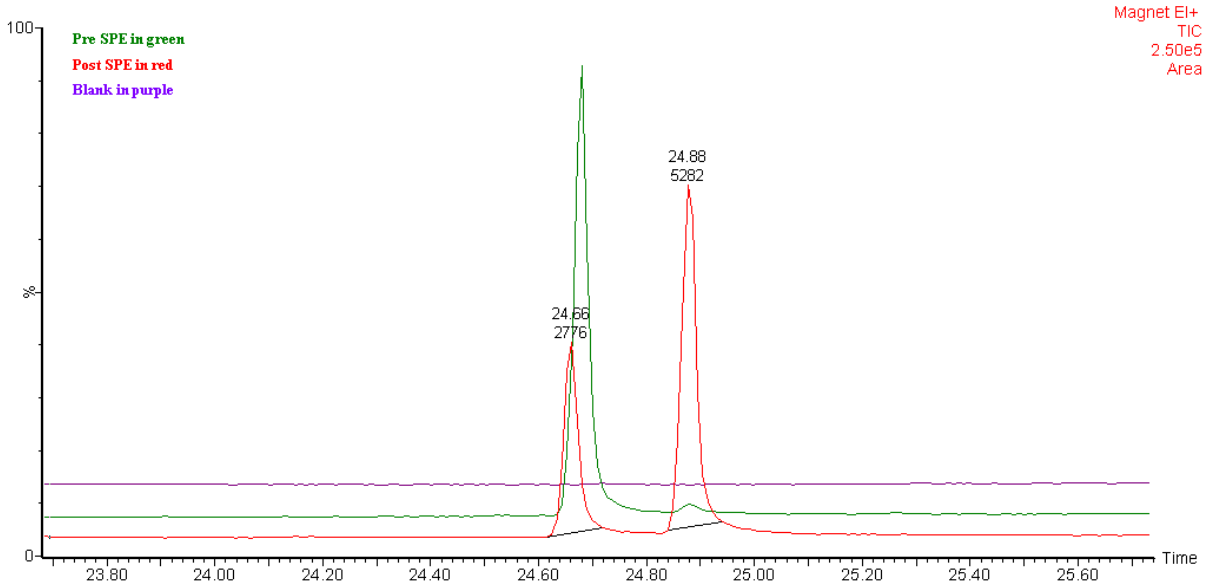


Figure 3.9: Chromatograms for a standard solution of 2-MAG C16 subjected to automated SPE program for fractionating neutral lipids. Lipid solution sampled and TMS derivatized analysed both pre- and post SPE.

The method was attempted again in a cooler room with a ambient temperature of 10C to test if this could reduce the problem, see figure 3.10. Only a single SPE run was performed and it was done manually on a mounted rack with a valve controlling the mobile phase speed. The altered conditions meant time spent by analyte in contact with the solid phase was so slightly different from the automated protocol. Never the less it was determined suitable to determine if further experiments would be needed. The pre analysis did not reveal any sn-1 in the standard, which was the case earlier. Likely due to a lower total concentration. Post SPE showed peaks for both isomers with a distribution of 89% for sn-1 and 11% for sn-1. Lowering the temperature seemed to decrease isomerization, but not enough to avoid it at 10C. It was attempted to SPE fractionate MAG already derivatized into bis-TMS ethers and thereby circumvent isomerization problems. But in all cases bis-TMS MAG was re-found in the TAG fraction (results not shown). Which was not surprising as one of the points of derivatization is lowering the polarity for better GC performance.

Another attempt was made in a walk-in freezer with ambient temperatures of -20C, see figure 3.11. The full double SPE fractionation was simulated manually. Results at this temperature showed that isomerization still would happen here. No sn-1 was detected in the control not subjected to SPE but two peaks corresponding to 91% sn-2 and 9% sn-2 was found in the post sample. This were much

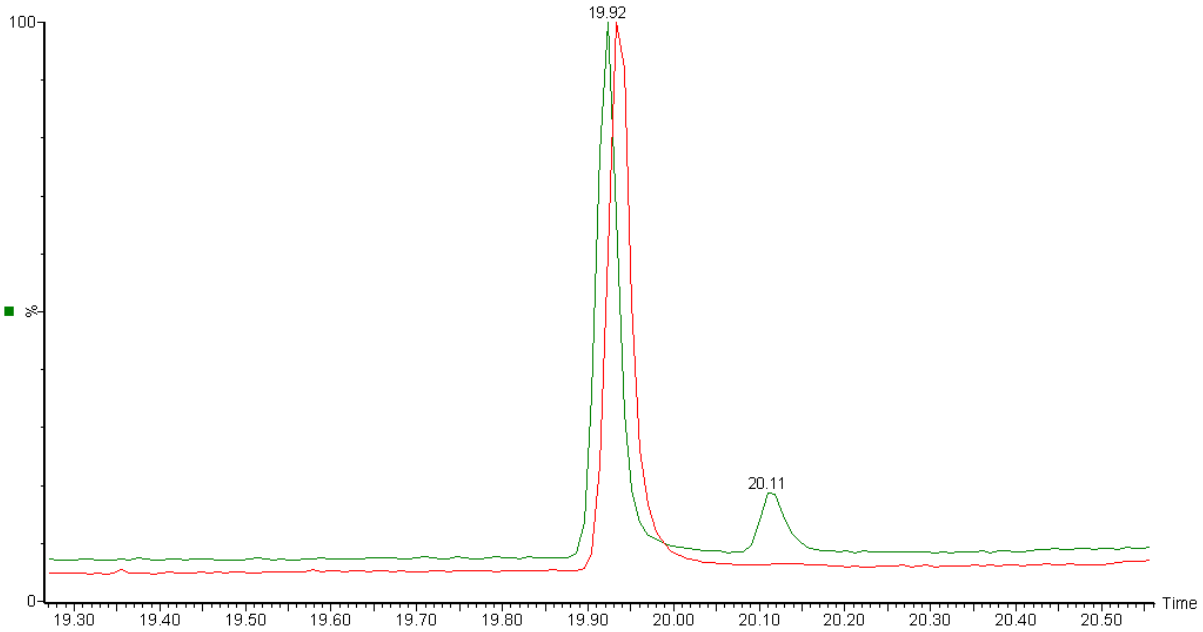


Figure 3.10: Chromatogram for a standard solution of 2-MAG C16 subjected to a manual SPE program for separating charged from neutral lipids. Performed in cooler room at 10C. Lipid solution sampled and TMS derivatized analysed both pre- and post SPE.

less so than what was found at laboratory room temperature and as such an improvement. Practical aspects of fractionating in a freezer and then still not completely being rid of the issue made this solution unattractive.

3.3.2 In solvent and during Folchs Extration

Though most of the isomerization was believed to happen during SPE fractionation some could also occur at other points. Isomerization has been decribed in litterature for MAG in both its dry form and when in solvent (Compton et al., 2007). A standard 2-C14 was left in a hexane/chloroform/methanol solution for varying amounts of time before being analysed. This would mimic conditions between the two SPE steps where the lipids was kept in this solvent. For the extraction step which precedes SPE, lipid is treated in a chloroform/methanol mix which was also tested.

For bis-TMS MAG in hexane/chloroform/methanol solvent left in a fume hood at lab conditions small peaks was found for have formed after 45 minutes with earlier times having some visual peaks at S/N less than 3 or no distinguishable peaks at all. In both cases these were not considered. The sum of integrals-to-integral percentage for the sn-1 isomer peaks was 1.2%, 1.5% and 1.7% for 45-, 90- and 150 minute reaction times. No detectable sn-1 peak was found after 24h storage at 5C for this solution. Experiment was halted after 150 minutes as this was already longer than real analysis time. During extration by Folchs method a polar chloroform/methanol mix was used, and included mechanical treatments with rough shaking and centrifugation. Here no sn-1 was detected after treatment. Total run time for this extraction is, at about 30 minutes, less than the time it took to detect isomerization in the solvent experiment and compared to the migration rates known from litterature little isomerization was expected.

From this experiment it seems that migration from 2- to 1-MAG is much less of an issue in solvent than it was for the SPE. However amounts determined was within the range of what the manufacturer estimated could be present (less than 5%) in the standards. Analytically this was a problem as results could be interpreted in two ways. Either low amounts of isomerization had happened, or some amount of 1-MAG was always present but only detected in some samples due to variations in noise. A better solution, if quantitative representation of solvent isomarization was wanted, might have been to test on a more equal concentration of 2- and 1-MAG. So that all peaks are discernable from background noise. And to include replicates for determining variation between and within sets.

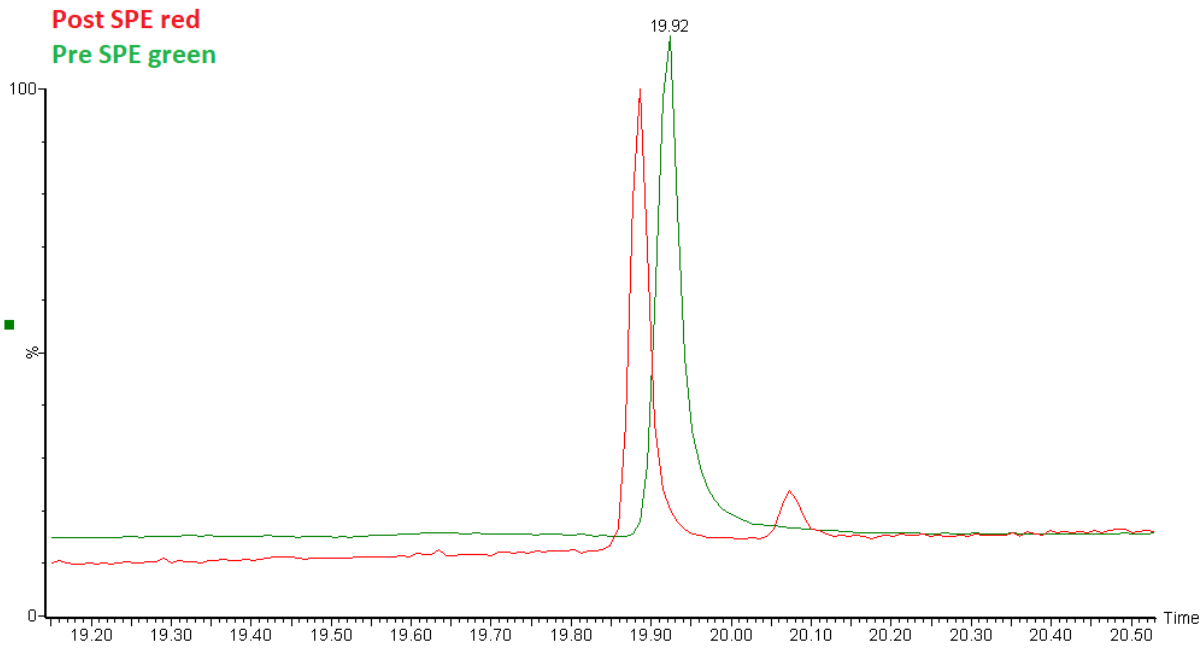


Figure 3.11: Chromatogram for a standard solution of 2-MAG C16 subjected to entire SPE fractionation protocol as described in Materials and Methods. Performed in walk-in freezer at -20C. Lipid TMS derivatized analysed pre- and post SPE to test for isomerization.

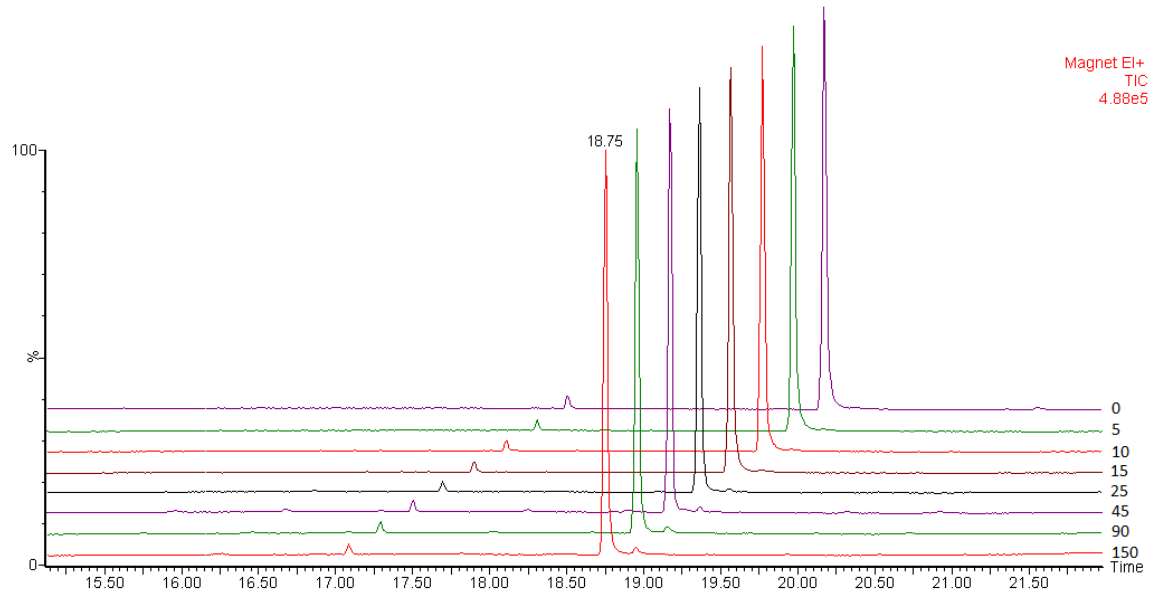


Figure 3.12: Chromatograms for 2-C14 MAG stored in hexane/chloroform/methanol (90/6/4, v/v/v) at room temperature for increasing amount of times, before being 2TMS derivatized. 0-, 5-, 10-, 15-, 25-, 45-. 90- and 150 minutes respectably. Baseline shifted (x and y axis) for better comparison.

3.4 Method Validation and Optimization

Method validation is crucial in analytical science and comprehensive validations focus on well defined parameters to evaluate methods. A list of common validation parameters include *"selectivity, specificity, linearity, accuracy, precision, recovery, LOD/LOQ, detection capability, robustness, stability, comparisons of alternative methods, safety- and economical concerns"* and combining these parameters gives an estimate on how reliable and suitable a method is for its intended use (Peris-Vicente et al., 2015).

Here time constrains and to a higher degree earlier results limited the proceeding validation work. Estimation of isomerization from 2- to 1-MAG could be seen af part of validation process and had showed that the current method was not suitable for intended use on digested samples.

3.4.1 On semi-Quantitative Isomer Distribution Estimation

When determining isomer ratios an assumption had to be made towards the general system response. It was expected that sn-1 and 2- isomers of equal carbon number would have similar responses and also that this would not change drastically with acyl group length. This assumption enables peak areas for individual analyte to be compared with sum of total peak area to calculate isomer ratios (Ping et al., 2018). For large diverse chemical groups this is generally not so accurate with some dependence of the detector used (Jordi et al., 2020). Better accuracy can be achieved when a individual response factor is applied to each analyte (Grob & Barry, 2004). But would also have required high quality standards for each target compound.

Calibration curves was constructed for 9 MAG standards (see table 3.1) and molar concentration response factors relative to 1-C16 was calculated. 1-C16 was chosen as it is common to be found at high concentrations and is somewhat in the middle of carbon number range expected to be found in milk MAG, C8-C20 (Lindmark Månsson, 2008). If the above mentioned assumption would have been true, RRF for these MAG should have been similar. Here they varied from 0.69 to 1.37. Though for a smaller selection: 1-C8, 1,2-C16, 2-C18 and 1-C21 it was closer with a range between 0.93 and 1.06.

Usually errors introduced in sample handling is larger than what comes about from MS analysis (de Hoffmann & Stroobant, 2007). This was probably also the case here and the major cause of variation in RRF. Seemingly there was no correlation with carbon length or isomerism as both 1-C8, 1-C21 and 2-C16 had closer RRF to 1-C16 than either 1- or 2-C12. One stock was made for each MAG and replicates for the calibration curves came from the same stock. So mismatched concentration estimates would have affected comparison between groups but not replicates. Main source of error was believed to have come by weighing in lipid standards on a micro weight and transferring this to other containers, an procedural error which was further increased by inexperience of the researcher. Saturation and acyl chain length both factor into melting point and cis monounsaturated FA of C18 or lower would melt at/or below room temperature whereas a saturated C16 will be solid (Christie, 2010). This meant that the various reference MAG behaved differently. Stock solutions were made by weighing between 1 and 10 mg depending on how much was available at the time. And relative error of the weighing step would have been expected to be larger when smaller amounts were measured (Skoog et al., 2013). Triple replicates for calibration curves was done for 1-C10, 1-C12 and 1-C21 but the remaining were done as single point curves. Which does not describe variation and give much worse accuracy. Expanding with more replicates as-well-as MAG types was planned, but were halted due to time constraints.

Another issue was found for the 1-C12 calibration curve where instrument errors meant that measurement of replicate series got delayed a day. The later analyzed series had higher peak area for the same concentration which seemed to indicate growing response over time, see section *Stability and Accuracy*. Which was a possible contributor to its poor RRF. In many cases standards were ordered in small quantities so experiments could not be repeated. Increasing number of replicates, amount of lipid weighed in for all calibrations and producing a stock solution for each replicate would benefit future analysis and better showcase the error from sample preparation. And so would reducing the time between sample preparation and injection.

FID is the most common detector in quantitative analysis with GC (Grob & Barry, 2004) and GC-FID methods for TMS derivatives of FFA (Wan et al., 2007) and MAG (Fagan et al., 2004) have been

found to have very similar response across varying acyl chain length and saturation/unsaturation. The theoretical background for this detector response differs from EI-MS as the FID is related to effective carbon number (ECN) of the molecule whereas EI-MS specific molecular response is described by its electron ionization cross section. An experimentally determined value (Göröcs et al., 2013). Response factors can therefore not be transferred between the two. A idea for future analysis could be to use both FID and GC detectors in a compound analysis. Multi detector analysis often give better results (Jordi et al., 2020) and would combine the great sensitivity and close relative reponses of bis-TMS MAG by FID with the identification potential of GC from fragmentation patterns.

3.4.2 Stability and Accuracy

In a study by Wan et al., 2007 peak areas for TMS derivatized FFA increased over time at a small rate of less 1% in a few hours and 3-4% over a few days when BSA was used as silylation agent. For this reason they switched to the weaker HDMS/TFA which caused them less problems in regards to this.

Similar issues were found to happen here between replicates for one series where pressure drop in the GC delayed measurement of later runs by about a day. The capped GC vials containing the samples had been left on the auto-sampler at room temperature. Peak area at each level had increased from the value of previous series and concentration calculated for each would therefore be different. Relative standard deviation was also high and averaged 17% across all three calibrations. When each point was transformed into a relative of the total peak area for the series (figure 3.13, left) all curves was found to be more similar. So it seemed that relative relationships were intact which was positive for determining isomer ratios. But something to be aware of for calibration measurements. Another solution containing 1- and 2-MAG that had previously been used in resolution testing and stored a in a fridge (about 5C) was injected 10 consecutive times from the same GC vial that was left on the autosampler rack, see Appendix B. Here the individual peaks kept stable with the larger peaks having a RSD of about 2-3% with the largest RSD (8%) found 2-C16. Though peak areas were lower than the LOQ determined from the 2-C16 calibration. The overall sum of peak areas had a RSD of 2% and therefore it seemed that bis-TMS MAG could stay stable at room temperatures for enough time to run several samples. Leaving the solution to react overnight should maybe be considered.

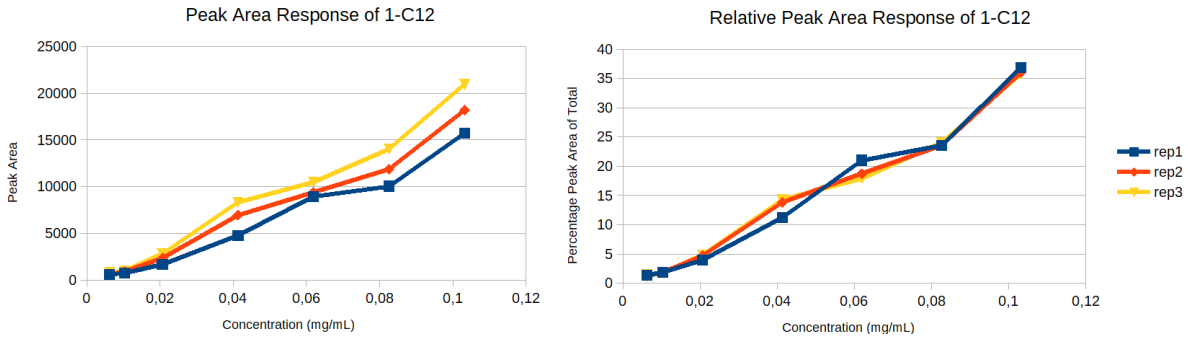


Figure 3.13: Response as peak area (right) or as peak area relative to total (left) for three series of a 1-C12, bis-TMS MAG stock solution. Run over 2 days with rep1 being the earliest and rep3 latest.

3.4.3 Derivatization Step Conditions

Derivation method utilized in this paper was one recommended by the American Oil Chemists Society (AOCS, Official Method Cd 11b-91) for MAG and DAG analysis, as described by Destaillats et al., 2010. Since both temperature and reaction times found in other research of similar compounds varied a 3x3 experiment was set to test both parameters at 3 levels each with 3 replicates for variation. Temperature tested was: 20, 40 and 80C and reaction time: 10, 30, 60 minutes. Average response was the highest when derivatization conditions were 80C for 60 minutes. Statistically this mean was significantly different from the second highest from 80C/30min ($p=0.01$), as calculated by unpaired two-tailed Students T-test, and about 7% larger. Injection needle bent from an auto-sampler malfunction for replicates of 80C/10min conditions and had to be replaced. Vial septum had been breached so that the sample were exposed to air and was therefore discarded. At lower temperatures variation within replicates was larger and mean

response much lower. It was decided to stay with the high temperature as described in the method. As time of reaction only had a modest effect between 30 and 60 minutes and both had similar low RSD it was decided to stick with the 45 minutes reaction time also described in the method.

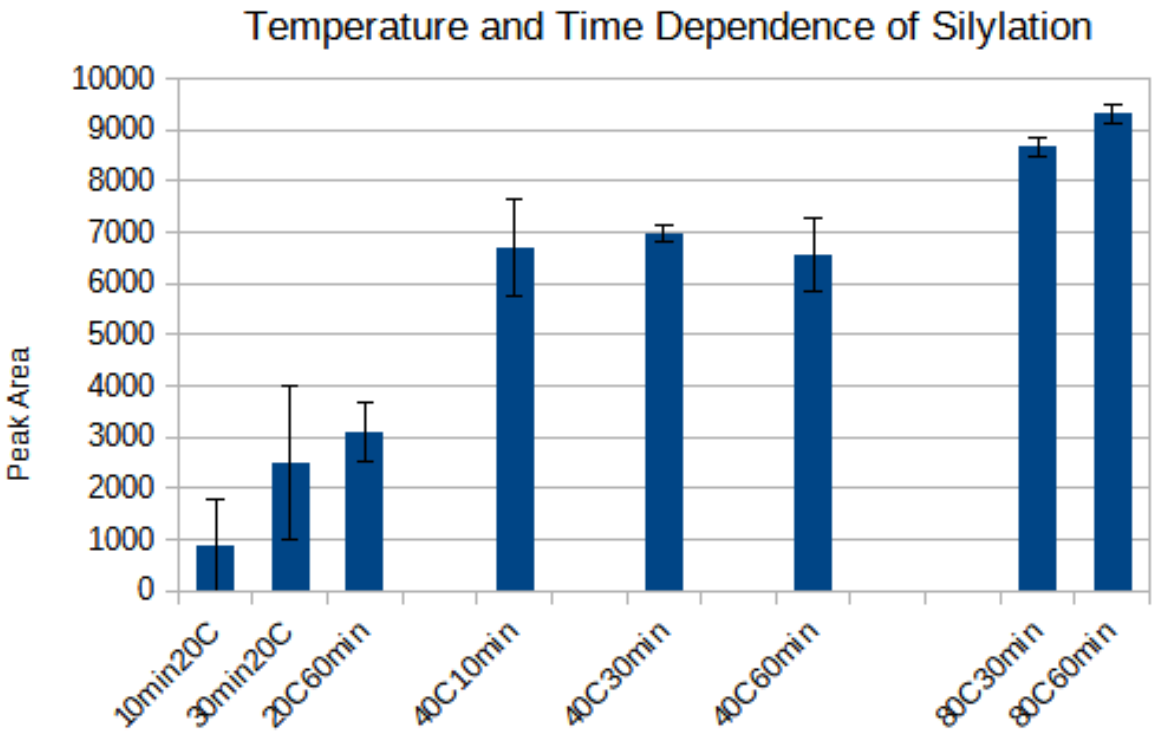


Figure 3.14: Response as peak area with standard deviation for 1-C14 MAG stock solution, bis-TMS derivatized under different reaction time and temperature conditions before GC-MS injection.

3.4.4 Procedural Considerations

The addition of a TMS group to a molecule is a one step reaction. The most common process described in literature is adding derivatization agent (BSTFA) and solvent/catalyst (pyridine) to dry MAG. Heating the mixture for an amount of time before diluting it with a solvent. However it has been shown that pyridine can sometimes hinder the reacion and/or give rise to unwanted chromatographic peaks (Pierce, 1968). It was attempted to follow the same protocol excluding pyridine and instead dissolving directly in n-heptane. Peak areas for peaks derivatized without pyridine were around 10 times lower and while they might have reached an equilibrium in time, it was decided to stick with the protocol as written as it was quicker and had no negatives.

Chapter 4

Conclusion and Future Work

Determining MAG isomers as bis-TMS derivatives by GC-MS had potential to be a quick and easy method, with total analysis time of less than a few hours, that could be coupled with the routine quantitative analysis already in use.

Isomerization from the sn-1 to 2- isomer was found to happen during SPE separation of lipid subclasses. A step that was unavoidable since maximum GC column temperature of the rtx-2330 at 275C, was lower than what was required to eluate TAG. Which would then contaminate the system. While performing SPE at lower temperatures did reduce the effect it did not remove the problem to an acceptable degree. And was otherwise unpractical. Determining true isomer distribution was not possible because of this fact, as exemplified by analysis of two digested samples which both were found to contain mostly sn-1 type MAG. Much against the expected hypothesis that lipase activity during digestion cleaves two FA's from outer positions of TAG and leave these as-well-as 2-MAG as end products.

A few solutions to the isomerization issue could be imagined. Using high temperature GC so the sample could be injected without having to isolate the lipid classes would circumvent the SPE step entirely. As an additional benefit of this is the ability to simultaneously detect DAG and TAG in the same run as other researchers have described (Satou et al., 2017) (Nang Lau et al., 2005). The downside would be a more complex matrix that could be expected to come with its own issues of increased interference. Another solution could have been changing the stationary phase for SPE separation to a di-ol type. Which has been shown to cause less isomerization than aminopropyl based columns (Ruiz-Gutierrez & Perez-Camino, 2000). And otherwise proceed with GC-EI/MS as described in this paper. Determining MAG as bis-TMS esters was for the most part achieved but with some issues regarding GC resolution of C18 species for the rtx-2330 column. Which was also known to be true for other columns (Satou et al., 2017). Another possible solution to the isomerization issue could be switching to a high temperature GC column so that it is not necessary to avoid TAG by using SPE. Simultaneous detection of MAG, DAG and TAG is also possible on such columns (Nang Lau et al., 2005).

If one of the solutions discussed is found acceptable and implemented TMS derivatization could be a useful tool in isomer determination. Fragmentation patterns allow for easy identification of both isomer as-well-as FA length/saturation and many of the descriptive ions have high m/z and therefore less likely to be misinterpreted with other chemicals. When a working method is found extensive validation should be performed on its entirety.

Bibliography

Amores, G., & Virto, M. (2019). Total and free fatty acids analysis in milk and dairy fat. *Separations*, *6*(1), 14. https://doi.org/10.3390/separations6010014

Berger, M., & Schnelder, M. P. (1992). Enzymatic esterification of glycerol ii. lipase-catalyzed synthesis of regioisomerically pure 1 (3)-rac-monoacylglycerols. *Journal of the American Oil Chemists' Society*, *69*(10), 961–965. https://doi.org/10.1016/S0141-0229(01)00344-1

Blau, K. (1993). *Handbook of derivatives for chromatography*. Wiley.

Bligh, E. G., & Dyer, W. J. (1959). A RAPID METHOD OF TOTAL LIPID EXTRACTION AND PURIFICATION. *Canadian Journal of Biochemistry and Physiology*, *37*(8), 911–917. https://doi.org/10.1139/o59-099

Boswinkel, G., Derksen, J. T. P., vant Riet, K., & Cuperus, F. P. (1996). Kinetics of acyl migration in monoglycerides and dependence on acyl chainlength. *Journal of the American Oil Chemists Society*, *73*(6), 707–711. https://doi.org/10.1007/bf02517944

Brockerhoff, H. (1965). A stereospecific analysis of triglycerides. *Journal of Lipid Research*, *6*(1), 10–15. https://doi.org/https://doi.org/10.1016/S0022-2275(20)39632-2

Christie, W. (2010). *Lipid analysis : Isolation, separation, identification and lipidomic analysis*. Oily Press, an imprint of PJ Barnes; Associates.

Chu, B.-S., & Nagy, K. (2013). Enrichment and quantification of monoacylglycerols and free fatty acids by solid phase extraction and liquid chromatography–mass spectrometry. *Journal of Chromatography B*, *932*, 50–58. https://doi.org/10.1016/j.jchromb.2013.05.026

Compton, D. L., Vermillion, K. E., & Laszlo, J. A. (2007). Acyl migration kinetics of 2-monoacylglycerols from soybean oil via 1h NMR. *Journal of the American Oil Chemists Society*, *84*(4), 343–348. https://doi.org/10.1007/s11746-007-1049-1

de Hoffmann, E., & Stroobant, V. (2007). *Mass spectrometry: Principles and applications, 3rd edition*. Wiley.

Deng, L., Nakano, H., & Iwasaki, Y. (2007). Direct separation of regioisomers and enantiomers of monoacylglycerols by tandem column high-performance liquid chromatography. *Journal of Chromatography A*, *1165*(1-2), 93–99. https://doi.org/10.1016/j.chroma.2007.07.073

Destaillats, F., Cruz-Hernandez, C., Nagy, K., & Dionisi, F. (2010). Identification of monoacylglycerol regio-isomers by gas chromatography–mass spectrometry. *Journal of Chromatography A*, *1217*(9), 1543–1548. https://doi.org/10.1016/j.chroma.2010.01.016

Devle, H., Ulleberg, E. K., Naess-Andresen, C. F., Rukke, E.-O., Vegarud, G., & Ekeberg, D. (2014). Reciprocal interacting effects of proteins and lipids during ex vivo digestion of bovine milk. *International Dairy Journal*, *36*(1), 6–13. https://doi.org/10.1016/j.idairyj.2013.11.008

Devle, H., Vetti, I., Naess-Andresen, C. F., Rukke, E.-O., Vegarud, G., & Ekeberg, D. (2012). A comparative study of fatty acid profiles in ruminant and non-ruminant milk. *European Journal of Lipid Science and Technology*, *114*(9), 1036–1043. https://doi.org/10.1002/ejlt.201100333

Dewick, P. M. (2009). *Medicinal natural products*. https://doi.org/10.1002/9780470742761

Drozd, J. (1986). *Chemical derivatization in gas chromatography*. https://doi.org/10.1016/S0021-9673(00)95303-2

Dufour, M., Manson, J. M., Bremer, P. J., Dufour, J.-P., Cook, G. M., & Simmonds, R. S. (2007). Characterization of monolaurin resistance in enterococcus faecalis. *Applied and Environmental Microbiology*, *73*(17), 5507–5515. https://doi.org/10.1128/AEM.01013-07

Eder, K. (1995). Gas chromatographic analysis of fatty acid methyl esters. *Journal of Chromatography B: Biomedical Sciences and Applications*, *671*(1-2), 113–131. https://doi.org/10.1016/0378-4347(95)00142-6

Eggers, L. F., & Schwudke, D. (2016). Liquid extraction: Folch. In M. R. Wenk (Ed.), *Encyclopedia of lipidomics* (pp. 1–6). Springer Netherlands. https://doi.org/10.1007/978-94-007-7864-1_89-1

Embleton, J. K., & Pouton, C. W. (1997). Structure and function of gastro-intestinal lipases. *Advanced Drug Delivery Reviews*, *25*(1), 15–32. https://doi.org/10.1016/S0169-409X(96)00488-7

Fagan, P., Wijesundera, C., & Watkins, P. (2004). Determination of mono- and di-acylglycerols in milk lipids. *Journal of Chromatography A*, *1054*(1), 251–259. https://doi.org/10.1016/j.chroma.2004.04.052

Fievez, V., Colman, E., Castro-Montoya, J., Stefanov, I., & Vlaeminck, B. (2012). Milk odd-and branched-chain fatty acids as biomarkers of rumen function—an update. *Animal Feed Science and Technology*, *172*(1-2), 51–65. https://doi.org/10.1016/j.anifeedsci.2011.12.008

Fischer, E. (1924). Wanderung von acyl bei den glyceriden, 643–655. https://doi.org/10.1007/978-3-642-51364-0_93

Folch, J., Lees, M., & Sloane Stanley, G. H. (1957). A simple method for the isolation and purification of total lipides from animal tissues. *J Biol Chem*, *226*(1), 497–509. https://doi.org/10.1016/S0021-9258(18)64849-5

for Drug Evaluation, C., & Research. (1994). *Reviewer guidance - validation of chromatographic methods*. Center for Drug Evaluation and Research.

Fred, K., & Borgström, B. (1965). Quantitative study of the pathways of triglyceride synthesis by hamster intestinal mucosa. *Biochimica et Biophysica Acta (BBA) - Lipids and Lipid Metabolism*, *98*(3), 520–531. https://doi.org/10.1016/0005-2760(65)90148-7

Fureby, A. M., Virto, C., Adlercreutz, P., & Mattiasson, B. (1996). Acyl group migrations in 2-monoolein. *14*(2), 89–111. https://doi.org/10.3109/10242429609106879

Garcia, P., Franco, P., Alvarez, R., & de Lera, A. R. (2011a). Separation of regioisomers and enantiomers of underivatized saturated and unsaturated fatty acid monoacylglycerols using enantioselective hplc. *Journal of Separation Science*, *34*(9), 999–1003. https://doi.org/10.1002/jssc.201000833

Garcia, P., Franco, P., Alvarez, R., & de Lera, A. R. (2011b). Separation of regioisomers and enantiomers of underivatized saturated and unsaturated fatty acid monoacylglycerols using enantioselective hplc. *Journal of separation science*, *34*(9), 999–1003.

Göröcs, N., Mudri, D., Matyasi, J., & Balla, J. (2013). The determination of gc–ms relative molar responses of some n-alkanes and their halogenated analogs. *Journal of chromatographic science*, *51*(2), 138–145. https://doi.org/10.1093/chromsci/bms118

Griffiths, W. J., & Wang, Y. (2020). *Lipidomics - current and emerging techniques*. Royal Society of Chemistry.

Grob, R. L., & Barry, E. F. (Eds.). (2004). *Modern practice of gas chromatography 4th*. John Wiley & Sons, Inc. https://doi.org/10.1002/0471651141

Halket, J. M., & Zaikin, V. G. (2003). Derivatization in mass spectrometry—1. silylation. *European Journal of Mass Spectrometry*, *9*(1), 1–21. https://doi.org/10.1255/ejms.527

Haraldsen, S., & Hausberg, I. (2019). Methode refinement for solid phase extraction(spe) of mono-, di- and triacylglyserols using automated spe.

Harvey, D. J., & Vouros, P. (2020). Mass spectrometric fragmentation of trimethylsilyl and related alkylsilyl derivatives. *Mass spectrometry reviews*, *39*(1-2), 105–211. https://doi.org/10.1002/mas.21590

Haugh, A. (2020). *Effects of lipids' composition and structure in meat and dairy foods on digestibility and low-grade inflammation in cells, animals and humans (lipidinflammagenes)*. https://www.nmbu.no/en/projects/node/34902

Isidorov, V., Rusak, M., Szczepaniak, L., & Witkowski, S. (2007). Gas chromatographic retention indices of trimethylsilyl derivatives of mono-and diglycerides on capillary columns with non-polar stationary phases. *Journal of Chromatography A*, *1166*(1-2), 207–211. https://doi.org/10.1016/j.chroma.2007.07.047

Iverson, S. J., Lang, S. L., & Cooper, M. H. (2001). Comparison of the bligh and dyer and folch methods for total lipid determination in a broad range of marine tissue. *Lipids*, *36*(11), 1283–1287. https://doi.org/10.1007/s11745-001-0843-0

J. J. Myher, L. M., & Kuksis, A. (1974). Identification of monoacyl- and monoalkylglycerols by gas–liquid chromatography–mass spectrometry using polar siloxane liquid phases. *Journal of lipid research*, *15*, 586–592. https://doi.org/10.1016/S0022-2275(20)36760-2

Jensen, R. G. (2002). The composition of bovine milk lipids: January 1995 to december 2000. *Journal of dairy science*, *85*(2), 295–350. https://doi.org/10.3168/jds.S0022-0302(02)74079-4

Jordi, M. A., Rowland, K., Liu, W., Cao, X., Zong, J., Ren, Y., Liang, Z., Zhou, X., Louis, M., & Lerner, K. (2020). Reducing relative response factor variation using a multidetector approach for extractables and leachables (e&l) analysis to mitigate the need for uncertainty factors. *Journal*

of pharmaceutical and biomedical analysis, *186*, 113334. https://doi.org/10.1016/j.jpba.2020.113334

Kodali, D. R., Tercyak, A., Fahey, D. A., & Small, D. M. (1990). Acyl migration in 1, 2-dipalmitoyl-sn-glycerol. *Chemistry and Physics of Lipids*, *52*(3-4), 163–170. https://doi.org/10.1016/0009-3084(90)90111-4

Koprivnjak, O., Conte, L., Boschelle, O., & Morassi, S. (1997). Validation of diglyceride contents in cheeses using co-ordinated chromatographic techniques. *Zeitschrift fur Lebensmitteluntersuchung und -Forschung A*, *204*(6), 429–432. https://doi.org/10.1007/s002170050107

Lindmark Månsson, H. (2008). Fatty acids in bovine milk fat. *Food and nutrition research*, *52*(1), 1821. https://doi.org/10.3402/fnr.v52i0.1821

Little, J. L. (1999). Artifacts in trimethylsilyl derivatization reactions and ways to avoid them. *Journal of Chromatography A*, *844*(1-2), 1–22. https://doi.org/10.1016/s0021-9673(99)00267-8

Liu, Z., Li, C., Pryce, J., & Rochfort, S. (2020). Comprehensive characterization of bovine milk lipids: Triglycerides. *ACS omega*, *5*(21), 12573–12582. https://doi.org/10.1021/acsomega.0c01841

Michalski, M.-C., Genot, C., Gayet, C., Lopez, C., Fine, F., Joffre, F., Vendeuvre, J.-L., Bouvier, J., Chardigny, J.-M., Raynal-Ljutovac, K., et al. (2013). Multiscale structures of lipids in foods as parameters affecting fatty acid bioavailability and lipid metabolism. *Progress in lipid research*, *52*(4), 354–373. https://doi.org/10.1016/j.plipres.2013.04.004

Miller, J. (2009). *Chromatography : Concepts and contrasts*. Wiley-Blackwell. https://doi.org/10.1365/s10337-010-1544-7

Millqvist, A., Adlercreutz, P., & Mattiasson, B. (1994). Lipase-catalyzed alcoholysis of triglycerides for the preparation of 2-monoglycerides. *Enzyme and Microbial Technology*, *16*(12), 1042–1047. https://doi.org/10.1016/0141-0229(94)90140-6

Mitchell, D., Rodriguez, J., Carriere, F., & Krieger, N. (2008). Determination of the quantitative stereoselectivity fingerprint of lipases during hydrolysis of a prochiral triacylglycerol. *Journal of Biotechnology*, *135*(2), 168–173. https://doi.org/10.1016/j.jbiotec.2008.03.013

Monteiro, J. B., Nascimento, M. G., & Ninow, J. L. (2003). Lipase-catalyzed synthesis of monoacylglycerol in a homogeneous system. *Biotechnology letters*, *25*(8), 641–644. https://doi.org/10.1023/a:1023016215537

Murphy, R. C. (2020). Lipid mass spectrometry: A path traveled for 50 years [e4492 JMS-19-0184.R1]. *Journal of Mass Spectrometry*, *55*(5), e4492. https://doi.org/10.1002/jms.4492

Nang Lau, H. L., Puah, C. W., Choo, Y. M., Ma, A. N., & Chuah, C. H. (2005). Simultaneous quantification of free fatty acids, free sterols, squalene, and acylglycerol molecular species in palm oil by high-temperature gas chromatography-flame ionization detection. *Lipids*, *40*(5), 523–528. https://doi.org/10.1007/s11745-005-1413-1

Peris-Vicente, J., Esteve-Romero, J., & Carda-Broch, S. (2015). Validation of analytical methods based on chromatographic techniques: An overview. *Analytical separation science*, 1757–1808. https://doi.org/doi.org/10.1002/9783527678129.assep064

Petrović, M., Kezić, N., & Bolanča, V. (2010). Optimization of the gc method for routine analysis of the fatty acid profile in several food samples. *Food Chemistry*, *122*(1), 285–291. https://doi.org/10.1016/j.foodchem.2010.02.018

Pierce, A. E. (1968). *Silylation of organic compounds - a technique for gas-phase analysis*. Pierce Chemical Co.

Ping, B. T. Y., Aziz, H. A., & Idris, Z. (2018). Comparison of peak-area ratios and percentage peak area derived from hplc-evaporative light scattering and refractive index detectors for palm oil and its fractions. *Journal of oleo science*, *67*(3), 265–272. https://doi.org/10.5650/jos.ess17164

Ramirez, M., Amate, L., & Gil, A. (2001). Absorption and distribution of dietary fatty acids from different sources [Nutrition and Infant Pathology]. *Early Human Development*, *65*, S95–S101. https://doi.org/10.1016/S0378-3782(01)00211-0

Ruiz-Gutierrez, V., & Perez-Camino, M. (2000). Update on solid-phase extraction for the analysis of lipid classes and related compounds. *Journal of Chromatography A*, *885*(1), 321–341. https://doi.org/10.1016/S0021-9673(00)00181-3

Satou, C., Goto, H., Yamazaki, Y., Saitou, K., Matsumoto, S., Takahashi, O., Miyazaki, Y., Ikuta, K., & Yajima, Y. (2017). Modified gas chromatographic method to determine monoacylglycerol and diacylglycerol contents in edible fats and oils. *Journal of Oleo Science*, *66*(6), 601–606. https://doi.org/10.5650/jos.ess16143

Schaaf, O., & Dettner, K. (2000). Polyunsaturated monoglycerides and a pregnadiene in defensive glands of the water beetle agabus affinis. *Lipids*, *35*(5), 543–550. https://doi.org/10.1007/s11745-000-554-6

Schummer, C., Delhomme, O., Appenzeller, B., Wennig, R., & Millet, M. (2009). Comparison of MTBSTFA and BSTFA in derivatization reactions of polar compounds prior to GC/MS analysis. *Talanta*, *77*(4), 1473–1482. https://doi.org/10.1016/j.talanta.2008.09.043

Skoog, A. D., West, M. D., Holler, J., & Crouch, R. S. (2013). *Fundamentals of analytical chemistry, 9th edition*. Mar Finch.

Takagi, T., & Ando, Y. (1991). Stereospecific analysis of triacyl-sn-glycerols by chiral high-performance liquid chromatography. *Lipids*, *26*(7), 542–547. https://doi.org/10.1007/BF02536601]

Wan, P. J., Dowd, M. K., Thomas, A. E., & Butler, B. H. (2007). Trimethylsilyl derivatization/gas chromatography as a method to determine the free fatty acid content of vegetable oils. *Journal of the American Oil Chemists' Society*, *84*(8), 701–708. https://doi.org/10.1007/s11746-007-1101-1

Willett, W. C., & Ludwig, D. S. (2020). Milk and health. *New England Journal of Medicine*, *382*(7), 644–654. https://doi.org/10.1056/NEJMra1903547

Yan, Y., Wang, Z., Wang, X., Wang, Y., Xiang, J., Kothapalli, K. S., & Brenna, J. T. (2017). Branched chain fatty acids positional distribution in human milk fat and common human food fats and uptake in human intestinal cells. *Journal of Functional Foods*, *29*, 172–177. https://doi.org/10.1016/j.jff.2016.12.024

Zhou, H., Zhang, Z., Lee, W. J., Xie, X., Li, A., & Wang, Y. (2021). Acyl migration occurrence of palm olein during interesterification catalyzed by sn-1, 3 specific lipase. *LWT*, *142*, 111023. https://doi.org/10.1016/j.lwt.2021.111023

Appendix A. MAG from Digested Samples by FAME

Shown below is the monoacylglycerols content of three Norwegian milk based products. This was the work (not yet published) of Gard Østboe together with the Faculty of Chemistry, Biotechnology and Food Science, NMBU. Permission was given to include the results here. The products had been in vitro digested by researchers from the department of Food Science and subsequently given to Gard. Total lipids were extracted by the Folch methods described earlier in this paper. These were isolated into TAG/DAG/MAG fractions by SPE. Each fraction was derivatized into corresponding methyl esters and the content of these was analysed by GC-MS (GC column rtx-2030, Quadrupole MS). Only the results from MAG fraction is included here.

MAG	Kremgo	SD	Milk Cream	SD
C4:0	0,16 %	0,05 %	0,27 %	0,25 %
C6:0	0,38 %	0,10 %	0,27 %	0,25 %
C8:0	0,59 %	0,30 %	0,37 %	0,14 %
C10:0	2,20 %	1,04 %	1,87 %	0,40 %
C12:0	3,21 %	1,18 %	2,53 %	1,11 %
C13:0	0,09 %	0,02 %	0,10 %	0,04 %
C14:0	13,34 %	1,64 %	13,19 %	1,78 %
C14:1cis9	1,82 %	0,24 %	1,34 %	0,14 %
C15:0	1,00 %	0,09 %	1,08 %	0,06 %
C15:1cis9	0,00 %	0,00 %	0,00 %	0,00 %
C16:0	34,40 %	1,62 %	41,50 %	0,41 %
C16:1cis9	2,07 %	0,85 %	1,23 %	0,74 %
C17:0	0,85 %	0,52 %	0,32 %	0,12 %
C17:1cis10	0,24 %	0,02 %	0,13 %	0,04 %
C18:0	6,96 %	1,15 %	12,07 %	2,38 %
C18:1trans9	0,33 %	0,11 %	0,17 %	0,06 %
C18:1cis9	29,42 %	4,03 %	21,51 %	2,32 %
C18:2trans9,12	0,08 %	0,03 %	0,23 %	0,07 %
C18:2cis9,12	2,02 %	0,09 %	0,55 %	0,44 %
C18:3cis6,9,12	0,00 %	0,00 %	0,00 %	0,00 %
C20:0	0,00 %	0,03 %	0,60 %	0,54 %
C18:3cis9,12,15	0,01 %	0,02 %	0,04 %	0,05 %
C20:1cis11	0,66 %	0,04 %	0,32 %	0,06 %
C21:0	0,00 %	0,00 %	0,29 %	0,21 %
C20:2cis11,14	0,00 %	0,00 %	0,00 %	0,00 %
C20:3cis8,11,14	0,07 %	0,01 %	0,00 %	0,00 %
C22:0	0,10 %	0,03 %	0,04 %	0,04 %

Figure 1: Fatty acids of the MAG fraction for three different products following chemical digestion. Measured as methyl ester derivates by Gard Oestboe

Appendix B - Robustness Test

The same stock solution was run 10 times in a row and each MAG peak was integrated to test for instrument stability in regards to peak area response. Run at temperature program 50 to 275C with an increase of 10C/min.

Table B1. Stability for the same stock solution containing several MAG standards run 10 consecutive times.

	Peak Areas						
Repetition	2-C12	1-C12	2-C14	1-C14	2-C16	1-C16	Sum
1	2032	5209	302	1228	189	430	9390
2	2056	5254	364	1230	186	466	9556
3	1996	5017	371	1299	194	458	9335
4	1989	5015	331	1221	178	395	9129
5	1999	5073	354	1231	188	449	9294
6	2111	5200	324	1221	223	489	9568
7	2062	5109	307	1203	201	457	9339
8	2076	5194	353	1223	218	483	9547
9	2093	5295	334	1224	220	503	9669
10	2129	5199	358	1332	219	497	9734
Average	2054,3	5156,5	339,8	1241,2	201,6	462,7	9456,1
STDEV	49	97	24	41	17	33	188
R.STDEV %	2	2	7	3	8	7	2

Table B2. Stability of retention times for a stock solution containing several MAG standards run 10 consecutive times.

	Rentention Times					
Repetition	2-C12	1-C12	2-C14	1-C14	2-C16	1-C16
1	17,47	17,68	18,72	18,93	19,89	20,08
2	17,47	17,69	18,73	18,93	19,9	20,09
3	17,47	17,68	18,72	18,93	19,89	20,08
4	17,47	17,69	18,72	18,93	19,89	20,08
5	17,47	17,68	18,72	18,93	19,89	20,08
6	17,47	17,68	18,72	18,93	19,89	20,08
7	17,47	17,69	18,72	18,93	19,89	20,08
8	17,47	17,68	18,71	18,92	19,88	20,07
9	17,48	17,69	18,73	18,94	19,9	20,09
10	17,48	17,69	18,72	18,93	19,89	20,09
Average	17,472	17,685	18,721	18,93	19,891	20,082
STDEV	0,004	0,005	0,006	0,005	0,006	0,006
R.STDEV %	0,024	0,030	0,030	0,025	0,029	0,031

Norges miljø- og biovitenskapelige universitet
Noregs miljø- og biovitskapelege universitet
Norwegian University of Life Sciences

Postboks 5003
NO-1432 Ås
Norway