Analytical Characterization of Camel Meat and Milk Fat

A thesis submitted for the degree of Doctor of Philosophy

by

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The aim of this study was to characterize the fat composition of camel milk fat and camel hump fat and thereby assist in the technological development of camel milk and meat based products. The latter is of major interest in parts of Africa where the camel resides in large numbers. A combination of silver-ion and reversed phase HPLC enables the isolation of triglycerides according to their saturation class and carbon number. Subjection of the isolated triglycerides to enzymatic splitting with lipase and subsequent analysis of the free fatty acids generated enables a positional analysis of the main triglyceride components and thus a characterization of the fat. The analytical techniques employed may equally well be applied to other fats or oils to allow their characterization.

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APCI	Atmospheric pressure chemical ionisation
CBE	Cocoa butter extender
CBR	Cocoa butter replacer
CHD	Coronary heart disease
CI	Chemical ionisation
CN	Carbon number
DCL	Double chain length
DSC	Differential scanning calorimetry
EI	Electron ionisation
ESI	Electron spray ionisation
FA	Fatty acid
FAME	Fatty acid methyl ester
FFA	Free fatty acid
FID	Flame ionisation detector
GC	Gas chromatography
HDL	High density lipids
HPLC	High performance liquid chromatography
HT-GC	High temperature gas chromatography
IR	Infra red
LDL	Low density lipids
MS	Mass spectrometry
O	Oleic acid
P	Palmitic acid
R	Functional group
RI	Refractive index
S	Stearic acid
SIM	Single ion monitoring
TCL	Triple chain length
TIC	Total ion count
TG	Triglyceride
TLC	Thin layer chromatography
TMSH	Trimethylsulphonium hydroxide
UV	Ultraviolet

Abbreviations with reference to triglycerides:

S	Saturated
M	Monounsaturated
D	Diunsaturated
U	Unsaturated

1.0 The Camel

In spite of its economic and ecological advantages, the virtues of the camel are almost unknown outside the communities where it is used and until now it has received little attention in comparison to other domestic animals. Until the early seventies research on camel milk was limited to studies on composition and yields. In the early eighties, studies on the physical / chemical properties of camel milk were intensified and some of the technological aspects of its utilisation were also addressed. However, such studies are still fragmentary and by no means systematic.

During the past few years there has been a significant onset of desertification, resulting from a shortage of rainfall in certain regions in Africa. Under such conditions traditional livestock suffers considerably in comparison to camels. Today in Eastern Africa the camel is replacing cattle in many areas. Aiding this process by appropriate research and development on camels could help to improve results in terms of human nutrition and in generating a cash income in the rural economy.

In all animal-rearing societies, the majority of milk was traditionally consumed in the form of fermented milk products. Fermentation is the only means of preserving milk under warm conditions. There is much scope for the technological development of acceptable camel milk products. To assist in the development of such products it is essential that the milk be thoroughly characterised in terms of its chemical composition. As cow milk has been investigated in depth for several decades, it is logical to regard it as a reference point and thus as a comparison.

1.1 Camels, their use and distribution

The extant Camelidae are classed in two genera. The Old World genus of Camelus is generally accepted as comprising of two species: C. dromedarius, the dromedary (one-humped or Arabian camel) and C. bactrianus, the Bactrian (or two-humped) camel. The habitat of the dromedary is Northern Africa, the Near East and west-central Asia. The Bactrian camel lives in the colder areas of southern Russia, Mongolia, east-central Asia and

China. In the New world there also exists a single genus of the Cameliae. comprising four species. Two, *Lama guanacoe*, the guanacoe, and *L.vicugña*, the vicuña, are wild and two, *L. glama*, the llama, and *L. pacos*, the alpaca, are domesticated. Both genera belong to the sub-family Camelinae of the family Camelidae.

The dromedary was first domesticated some 4000 years ago {1}, although exact information is not available. The general theory is that domestication first occurred in southern Arabia . The dromedary appears to have been domesticated in connection with the trade in spices, incense and possibly salt, and this was certainly its principal use around 3000 years ago.

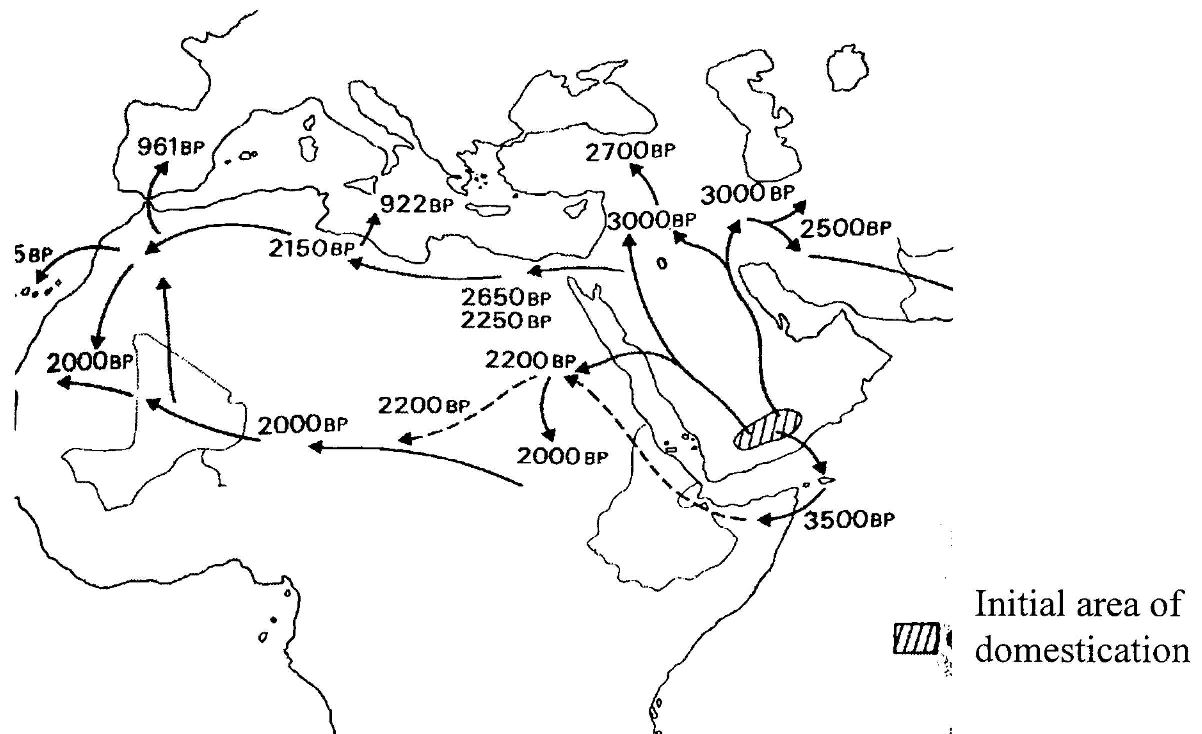


Figure 1: The centre of domestication and subsequent spread of the dromedary {1}
(BP = before present)

In all the areas where the dromedary is found today, with one exception, it fulfils an economic role as a riding or transport animal. The exception is Somalia where the principal use of the camel is as a provider of milk; here it serves in a subsidiary capacity as a pack animal for moving camp but seldom as a riding animal. The present study concentrates exclusively on this species of camel.

1.2 Physiology of the camel

The camel, with few exceptions, is found in areas where low annual rainfall occurs in a relatively short period. This is followed by a long dry season which is hot for most of the time and lasts for over 8 months of the year. These are the prevailing conditions in the palaearctic deserts of northern Africa and western and central Asia. The few areas outside this region where the camel has been introduced or has penetrated are climatically similar.

The success of the camel in climates hotter and drier than other domestic animals can tolerate is due to its peculiar physiology. The requirements for survival in hot arid areas are in general similar to those in other terrestrial environments. The body temperature must be controlled and water must be conserved and, in large mammals particularly, there is often interaction and a trade-off between the two. Small mammals escape the worst effects of temperature and dehydration by burrowing into the soil, but obviously an animal the size of a camel cannot do this. In most mammals fat is spread over the body surface just under the skin. This reduces the rate of evaporation of sweat. In the camel the fat is concentrated in the hump, which enables sweat to be evaporated easily over the rest of the body surface. The camel's coat is fairly sparse, which allows sweat to evaporate from the surface of the skin. In mammals with very thick coats evaporation occurs at the ends of the hairs, a less efficient process.

The combined attributes of the camel give it an advantage over other mammals. For example, the camel can lose 25 per cent of its body weight over a period of time, without losing its appetite for food and can then make up this amount in just 10 minutes by drinking. In contrast, the majority of animals die when they lose 12-15 per cent of their body weight {1}.

In other animals water lost is drawn from the body tissues, the interstitial tissues and the blood plasma. As a result the blood becomes viscous and the heart can no longer pump it fast enough to transfer the deep body heat to the surface for evaporation. In such a situation explosive heat death may occur. In camels very little water is drawn from the blood, which

remains fluid and can thus continue its function of heat transfer. One of the most important water conservation measures in the camel is the reduction in urine flow. Other adaptations to the desert environment in camels are listed below:

1. The body temperature can vary over a wide range under conditions of dehydration; the large mass of the camel acts as a heat buffer.

2. The camel is able to concentrate its urine to a considerable extent; urea is reabsorbed from the intestines and transferred back to the stomach for reconversion to protein.

1.3 Milk production of the camel

Estimates of lactation length vary from 9 to 18 months (although there are reports of camels in Kenya still being milked after 22 months) and total lactation yields range from about 800 litres to 3600 litres. These figures translate into daily yields of between 2.8 and 11.0 litres {1}. Undoubtedly, milk yields fall between the extremes quoted, but most authorities are extremely vague as to the method of estimation.

Yields depend on many factors, including the number of times an animal is milked (some camel owners milk six times a day, some only once every 2-3 days), the food it obtains, the climate and the frequency of watering.

1.4 Composition of camel milk fat

Camel milk fat consists of up to 98% of lipids in the form of triglycerides. Lipids serve as an energy source, act as a solvent for fat-soluble vitamins and supply essential fatty acids. The triglycerides contain a variety of fatty acids and are accompanied by small amounts of di- and mono-acylglycerols, cholesterol, free fatty acids and phospholipids {2}.

The fat content of camel milk varies between 1.7 and 4.2%, as determined by the author. Most of the data available on camel milk, with respect to fat, refers only to the total fatty

acid composition. It is known that up to 98% of the fatty acids are bound in the form of triglycerides, on which to date little has been reported.

The fatty acid composition of milk fat has a significant influence on the technological and sensory properties of milk products, e.g. cheese and butter. In order to gain a deeper understanding of the fat composition and its technological properties, characterization of the fat is necessary. Recent advances in liquid chromatography {3-6} have made it possible to separate individual triglycerides from an initial mixture of several hundred.

The fatty acid composition of camel milk is characterized by a lower proportion of saturated short chain fatty acids, e.g. butyric acid, and higher concentrations of some long chain fatty acids, e.g. stearic acid and palmitoleic acid, in comparison to cow milk. This has been reported several times in the literature {7, 8}, from which the data below have been taken.

Table 1: A comparison of camel milk fat and cow milk fat in terms of fatty acid composition {8}.

Fatty acid	Camel milk fat (%)	Cow milk fat (%)
C4:0	-	3.5
C6:0	-	2.1
C8:0	0.1	1.4
C10:0	0.12	2.1
C12:0	0.77	3.1
C14:0	10.1	10.4
C14:1	1.86	1.70
C15:0	1.62	2.44
C16:0	26.6	26.60
C16:1	10.40	1.70
C17:0	1.21	1.62
C18:0	12.2	7.86
C18:1	26.3	29.0
C18:2	2.94	3.20
C18:3	1.37	1.10
C20:0	0.57	0.11
C22:0	0.08	0.23
C22:1	0.57	-

2.0 Triglycerides and fatty acids

2.1 Nutritional and physiological aspects of fatty acids

The relationship between the fatty acid composition of a fat and its melting is apparent from the melting points of individual fatty acids shown in Table 2 below.

Table 2: The melting points (M.p.) of some common saturated and unsaturated fatty acids.

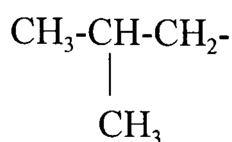
Saturated fatty acid	M.p. (°C)	Unsaturated fatty acid	M.p. (°C)
Butyric (C4:0)	-7.9	Elaidic (C18:1 *trans*)	43.7
Caproic (C6:0)	-3.4		
Caprylic (C8:0)	16.7	Oleic (C18:1 *cis*)	10.5
Capric (C10:0)	31.6	Linoleic (C18:2 *cis,cis*)	-5.0
Lauric (C12:0)	44.2	Linolenic (C18:3 *cis,cis,cis*)	-11.0
Myristic (C14:0)	54.1		
Palmitic (C16:0)	62.7		
Stearic (C18:0)	69.6		
Arachidic (C20:0)	75.4		

The low melting temperature that characterizes oils is associated with either a high proportion of unsaturated fatty acids, e.g. corn oil and olive oil, or a high proportion of short chain fatty acids, e.g. cow milk fat and coconut oil.

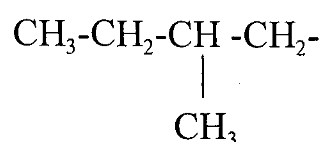
The insertion of a *cis* double bond has a dramatic effect on the shape of the molecule, introducing a kink of about 42° into the otherwise straight hydrocarbon chain. The insertion of a *trans* double bond has very little effect on the conformation of the chain and therefore very little effect on the melting temperature. For example, elaidic acid, the trans isomer of oleic acid, has similar physical properties to stearic acid.

The milk fats of ruminants are characterized by their high proportion of short chain fatty acids. These are formed from the anaerobic fermentation of carbohydrates such as cellulose by the microorganisms of the rumen. These microorganisms are also the source of the very small proportions of branched-chain fatty acids that occur in cow milk fat. Branched chain

fatty acids usually belong to the *iso* series, which have their hydrocarbon chains terminated:

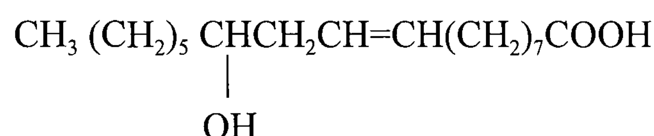


or to the *anteiso* series:

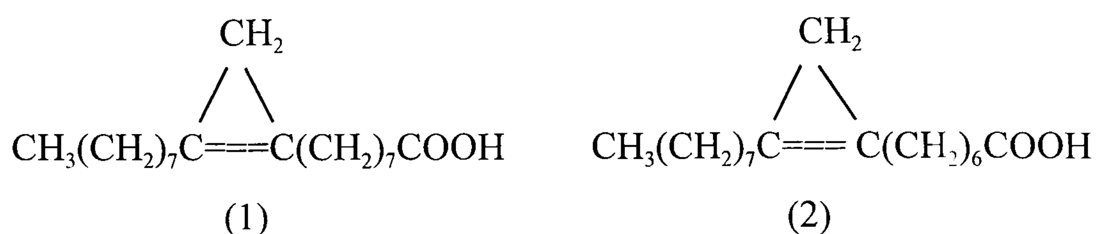


Human milk fat, like that of other non-ruminant species such as the pig, is rich in linoleic acid. Cod liver oil is most familiar as a source of vitamin D, but fish oils are particularly interesting for the diversity of long chain, highly unsaturated fatty acids they contain. The extremely cold environments of fish such as cod and herring may be a reason for the nature of fish lipids.

There are a number of fatty acids with unusual structures which are characteristic of particular groups of species or plants. For example, petroselenic acid (18:1 n-12) is found in the seed oils of celery, parsley, and carrots. Ricinoleic acid makes up about 90% of the fatty acids of castor oil.



Fatty acids containing cyclopropene rings are mostly associated with bacteria, but traces of sterculic acid (1) and malvalic acid (2) are found in cotton seed oil. They are toxic to non-ruminants, and the residual oil in cottonseed meal is sufficient to have an adverse effect on poultry fed on the meal. It must be assumed that the low levels man has consumed for many years in salad dressing and margarine manufactured from cotton seed oil have had no deleterious effects.



Fatty acids in the form of the triglycerides of the dietary fats and oils provide a major proportion of energy requirements as well as, when in excess, contributing to the unwelcome burden of superfluous adipose tissue. Cow milk fat, for example, provides a rapid source of energy due to its high proportion of short chain fatty acids. Such fatty acids are preferred by people suffering from steatorrhoea as they do not require bile for reabsorption. The same applies to medium chain fatty acids which occur in high concentrations in goat milk fat {9}. In contrast camel milk fat contains high levels of monounsaturated fatty acids, which according to Feldmann et al {10} are more easily absorbed than their saturated counterparts. It is believed that the protein to which the fatty acid is attached following resorption forms a stronger bond with unsaturated than with saturated fatty acids and that this applies to all chain lengths {11}. D. Kritchevsky {12} has quantified this theory by calculating a "coefficient of digestibility" for various triglycerides and fatty acids. For example, tristearin (18:0/18:0/18:0) has a value of 14%, while triolein (18:1/18:1/18:1) possesses a value of 99%.

Rats fed on a totally fat-free diet show a wide range of acute symptoms affecting the skin, vascular system, reproductive organs, and lipid metabolism. Similar skin disorders have occurred in children subjected to a fat-free diet. The symptoms in rats can be eliminated by feeding linoleic or arachidonic acids (which in consequence became known for a time as vitamin F) and it is generally accepted that 2-10 g of linoleic acid per day will meet an adult human's requirements.

The identification of these two 'essential fatty acids' in the 1930s preceded by some 25 years their identification as precursors of a group of animal hormones, the *prostaglandins*. Although normally regarded as the province of biochemists interested in metabolic pathways, an insight into the various interconversions of the unsaturated fatty acids is nevertheless relevant to food chemistry, especially the grouping of unsaturated fatty acids into three series, known as ω-3, ω-6 and ω-9. The numbering of the carbon atoms in the fatty acid chain starts with the carboxyl carbon as '1'. However, when the structural relationships of unsaturated fatty acids are being considered, it is usual to classify them by the distance of the last double bond from the terminal methyl group of the chain, the so-called ω-carbon.

Animals, including humans, readily synthesise saturated fatty acids up to C18. However desaturation, i.e. the insertion of double bonds, can usually only take place between carbons 9 and 10, or much more rarely at other positions nearer to the carboxyl group. This means that animals are unable to synthesise linoleic acid but humans are able to convert linoleic acid into arachidonic acid. Arachidonic acid is not found in plants. This is of little consequence to humans but of great significance to cats, which in the course of evolution have lost, but not missed, the ability to convert linoleic acid into arachidonic acid. Being carnivores, cats have always been able to obtain sufficient arachidonic acid from their diet.

The usual isomer of linolenic acid, sometimes referred to as α-linolenic acid (18:3 n-3), is formed in plants and algae by insertion of a third double bond into linoleic acid. α-Linolenic acid is the starting point for the formation of other ω-3 fatty acids. Fish consume algae which contain α-linolenic acid. Thus fish oil, e.g. herring, mackerel and salmon provide a source of this acid in the human diet.

The prostaglandins are now recognised as being just one class of a group of hormones known as the eicosanoids. Other eicosanoids include the thromboxanes and leucotrienes. There are a great many different prostaglandins, but their formation always follows the same pattern. The positions of the double bonds in the fatty acids is critical with respect to their synthesis. Eicosanoids, which are derived from ω-3 fatty acids, such as prostaglandin E_3, have an additional double bond. Although the list of physiological activities in which prostaglandins are involved continues to grow, they are best known for their role in inflammation and the contraction of smooth muscle. Thromboxanes are involved in the aggregation of platelets, part of the process of blood clot formation.

Since the early work of Dyerberg et al {13} on Greenland Eskimos, evidence has accumulated to show that ingestion of a diet rich in ω-3 fatty acids has beneficial effects on the lipid profile {13-15}, blood pressure {16}, blood viscosity {17}, bleeding time {16} and inflammatory and immunological mechanisms. The ω-3 fatty acids exert their action through three different mechanisms. Firstly, eicosapentaenoic acid (20:5 n-3) competes with arachidonic acid (20:4 n-6) for the synthesis of prostaglandins (PG) and thromboxanes

(TX), as the same cyclooxygenase pathway is used to convert eicosapentaenoic acid into PG_3 and TX_3 and arachidonic acid into PG_2 and TX_2. Inhibition of PG_2 and TX_2 synthesis by eicosapentaenoic acid results in a shift toward a non-aggregatory status, because TXA_3 is a weaker aggregating agent than TXA_2, whereas PGI_2 and PGI_3 have a similar antiaggregatory effect. Secondly, eicosapentaenoic acid replaces arachidonic acid in the membranes of platelets, which reduces arachidonic acid availability and thus diminishes PG_2 and TX_2 synthesis. Finally, the release of arachidonic acid from phospholipids, in particular from platelet membranes, is inhibited by PG_3 and TX_3. These complex interactions between the ω-6 and ω-3 fatty acid families have led investigators to attempt to determine the optimal ω-6 : ω-3 molar ratio in the diet.

Lipid emulsions are regularly used post-operatively to supply energy and essential fatty acids. To benefit from the ω-3 long chain fatty acids and thus decrease the risk of postoperative thrombosis, these acids must be added directly into lipid emulsions. When fish oils are administered by the oral route, changes in platelet composition and function are detected only after several weeks {18}. With intravenous infusion, a faster effect may be expected. Recently, a marine fish oil emulsion that contains relatively large amounts of eicosapentaenoic acid and docosahexaenoic acid has been developed by a manufacturer and is now available for clinical research purposes. A short term intravenous infusion of such an emulsion has brought about a modification of the platelet composition {19}.

In comparison to goat and cow milk fat, camel milk fat contains high concentrations of oleic acid (28 mol %), similar to that for human milk (34 mol %) {3}. Oleic acid is believed to be of biological importance. During the symposium for the American Society for Clinical Nutrition in Dallas in 1993, S. M. Grundy {20} stressed the role of this fatty acid in metabolism. Oleic acid itself has no influence on the serum cholesterol level, but assists in maintaining a normal LDL (low-density lipoprotein) level and is thus tends to prevent against arteriosclerosis. Most medium and long chain saturated fatty acids however increase the cholesterol level, an exception being stearic acid (C18:0), due to its slow resorption {20}. However, the involvement of dietary fatty acids in the occurrence of atherosclerosis and particularly heart disease is a complex issue. Nutritional guidelines focus on reducing the contribution that fats make to the total energy content of the diet and

on lowering the proportion of saturated fatty acids in dietary fat. However, it is becoming increasingly clear that the apparently simple relationships between dietary lipids, blood cholesterol levels, and the risk of coronary heart disease are not simple at all. A diverse range of other `risk factors´ and refinements is being identified which help to explain the apparent inconsistencies of the so-called `Lipid Hypothesis´ of coronary heart disease (CHD). While some of these risk factors still have a clear nutritional element others, such as geography, smoking, and inherited characteristics, do not.

Thus when the fatty acids in the diet are being considered it is now thought important to differentiate between saturated fatty acids of different chain length, as well as between the mono- and poly-unsaturated. Long chained FA are formed in the intestines (especially C16:0) by the digestion of TGs and combine with calcium or magnesium ions, forming insoluble salts which cannot be reabsorbed. In infants, especially the newly born, it is possible that such insoluble salts lead to the formation of a bolus which may obstruct the peristaltic movement of the intestine. The latter can be life threatening. Human milk contains high concentrations of calcium, but approximately 70% of the C16:0 FA is present in the 2 position in the triglycerides. Because pancreatic lipase cleaves the TG specifically in the 1 and 3 positions, hardly any C16:0 is released in the form of a FA, but remains bound within a 2-monoglyceride which does not build salts with calcium {3, 21}. In cow and camel milk fat, however, only 40% of C16:0 is present in the 2-position, (see Table 19, page 63). Thus it follows that these milk types are not ideally suited for infant feeding.

In contrast, the formation of insoluble salts in adults has hardly any negative influence on the peristaltic movement of the intestines, but binds C16:0 released from the 1 or 3 positions. This in turn tends to lower the level of blood cholesterol, of which C16:0 is a precursor {20}.

The pattern of unsaturation is also important, as indicated by the remarkably low incidence of arterial disease amongst Eskimos, in spite of a diet that appears to break all the usual nutritional rules. However, their traditional diet, though extremely fatty, is very rich in polyunsaturated fatty acids of the ω-3 series. Eicosanoids from ω-3 fatty acids are

generally less potent than those from ω-6 fatty acids in promoting the formation of the blood clots that are involved in CHD. This observation is the basis of recommendations that one should eat more fatty fish, such as herring and mackerel.

The observation that the occurrence of CHD is linked to the peroxidation of blood, has drawn attention to the role of the antioxidant vitamins, A, E; and C, and hence consumption of fruit and green vegetables. There are now indications that many of the differences in the incidence of CHD between population groups that defy explanation in terms of dietary fats (e.g between the north and south of the British Isles) can be correlated with the intake of fruit and green vegetables.

Table 3: The fatty acid composition of commonly encountered fats and oils (mol %) {22}.

Fatty acid	Beef fat	Lard	Cow milk fat	Human milk fat	Herring oil	Maize oil	Cocoa butter	Coconut fat	Palm oil	Olive oil
C4:0			9							
C6:0			5							
C8:0			2					12		
C10:0			4	1				8		
C12:0			3	3				49		
C14:0	4	2	10	5	7			16		
C14:1	1		2	1					1	
C15*	2		1	1						
C16:0	28	26	23	26	13	14	29	7	48	11
C16:1	5	4	2	5	9					1
C17*	1		1	1						
C18:0	20	15	12	7	1	2	35	2	6	3
C18:1	34	44	23	37	12	34	32	5	34	79
C18:2	3	9	2	11	2	48	3	1	11	5
C18:3	2		1	1		1				1
C18:4										
C20:0						1	1			
C20:1					19					
C20:4										
C20:5					8					
C22:1					25					
C22:5					2					
C22:6					2					

The presence of *trans* acids shows that margarine may not be all that it is claimed to be. Table 3 shows that contrary to common belief, animal fats can be highly unsaturated (e.g. fish oils) and plant fats can be largely saturated (e.g. coconut fat).

The observation that the level of cholesterol in the diet has little or no influence on the levels of cholesterol in the blood is widely ignored by the advertisers of products derived from plant oils. Hence the evidence that changing one's diet by replacing butter with margarine actually lowers the risk of CHD by a significant amount is also less clear cut than the advertisements for polyunsaturated-rich margarine imply.

2.2 Oxidation of unsaturated fatty acids

Rancidity is a familiar indication of the deterioration of fats and oils. In dairy fats rancidity is usually the result of hydrolysis of the triglycerides (lipolysis) by microorganisms so that odorous short chain fatty acids are liberated. In contrast, in other fats and oils and the fatty parts of meat and fish rancidity is the result of autoxidation of the unsaturated fatty acids. The sequence of reactions is traditionally presented in three stages, initiation, propagation, and termination. The initiation reactions give rise to small numbers of highly reactive fatty acid molecules that have unpaired electrons, *free radicals.* These are shown as R· in Figure 2 (page 17). The dot (·) shows the presence of an unpaired electron and the R denotes the remainder of the species. Free radicals are very short-lived, and highly reactive, as they seek a partner for their unpaired electron. In the propagation reactions atmospheric oxygen reacts with these radicals to generate peroxy radicals, ROO·. These are also highly reactive and go on to react with other unsaturated fatty acids, generating *hydroperoxides,* ROOH, and another free radical, R·. This free radical can then repeat the process, giving a chain reaction. The hydroperoxide also breaks down to give other free radicals. These too behave much like ROO·. The result is that an ever increasing numbers of free radicals accumulate in the fat, which absorbs considerable quantities of oxygen from the air. Eventually the concentration of free radicals reaches a point at which they start to react with each other to produce stable end-products, a process known as termination reactions. The build up of the stable end products is responsible for the familiar rancid taste.

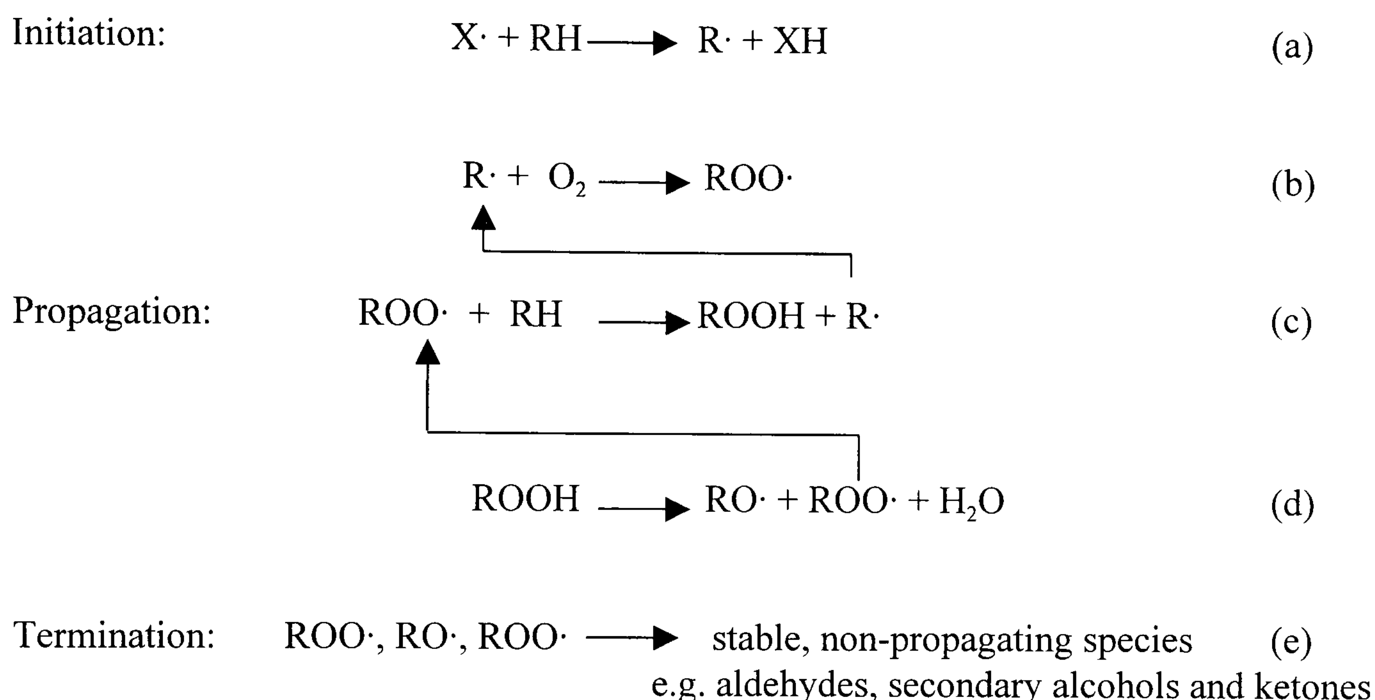


Figure 2: A summary of the reactions involved in the autoxidation of unsaturated fatty acids.

2.3 Physical aspects of triglycerides with respect to food

Early attempts to separate the component glycerides of fats, by the laborious process of fractional crystallisation from acetone solutions at low temperatures, made it clear that fats consist of a high number of species of triglycerides. Fats and oils became recognised as clearly defined mixtures of mixed and simple triglycerides.

If one considers a simple fat with only three fatty acids, there should be 27 possible triglycerides. However, as the 1- and 3 positions of the glycerol molecule are usually treated as indistinguishable, the total comes down to 18. It is important to note that the isomeric pairs of triglycerides, such as POP and PPO, are clearly differentiated. Such an abundance of data for a single fat makes it necessary to simplify triglyceride composition data when comparing different fats. The most striking feature is that fats of fairly similar fatty acid composition, such as lard and cocoa butter, may have very different triglyceride compositions. In lard there is a definite tendency for unsaturated fatty acids to occupy the outer positions of the glycerol molecule, whereas in cocoa butter the reverse is true. This difference is in fact a general one between animal and plant fats.

If the fatty acid residues at the two outer positions are different, then the triglyceride molecule is asymmetric and optical activity would be expected. It has been shown that a particular species of triglyceride in a natural fat may occur as a single enantiomer, as opposed to a racemic mixture. This indicates that the 1 and 3 positions are differentiated during triglyceride biosynthesis. In many fats there is in fact very little difference between the fatty acid compositions of these two positions but in some there is a marked difference, e.g. short chain fatty acids in cow milk fat are almost exclusively in the *sn*-3 position {21, 23, 24}. Milk fats are exceptional in that their triglycerides fall into three broad classes. In the first, all three positions are occupied by long chain fatty acids. The second class has long-chain acids at the 1 and 2-positions but short chain at 3. The third class has medium chain fatty acids at the 1- and 2-positions and medium or short chain fatty acids at the 3-position.

The determination of the triglyceride composition of a fat is particularly difficult. The need to distinguish between isomeric pairs of glycerides such as `SUS' and `SSU', crucial to the difference between cocoa butter and lard, for example, makes special demands on chromatographic procedures. Thin layer chromatography using silica gel impregnated with silver nitrate has been very successful as it will resolve these isomers {30}. The silver-ions are complexed by the double bonds of unsaturated triglycerides, whose mobility is thus reduced.

For the food chemist the melting and crystallisation characterisation of a fat are physical properties of prime importance. Although the melting points of pure triglycerides are a function of the chain lengths and degree of saturation of the component fatty acids, much as one might expect, the melting behaviour of fats is rather complex. Since natural fats are mixtures, each component having its own melting point, a fat does not have a discrete melting point but rather a `melting range′. At temperatures below this range all the component triglycerides will be below their individual melting points and the fat will be completely solid. At the bottom of the range the lowest melting types, those of lowest molecular weight or most unsaturated, will liquify. Some of the remaining solid triglycerides will probably dissolve in this liquid fraction. As the temperature is raised, the proportion of liquid to solid rises and the fat becomes increasingly plastic until, at the

temperature arbitrarily defined as the melting point, there is little or no solid fat left. This melting behaviour is illustrated in Figure 3 (page 23) for cow and camel milk fat.

A second complication is that triglycerides are polymorphic, i.e. they can exist in several different crystalline arrangements, each with its characteristic melting point, X-ray crystallographic pattern, and infrared spectrum. The three principal forms are known as α, ß, and ß′. The melting points of these forms of a number of triglyceride species are given in Table 4 below.

Table 4: The melting points (°C) of the polymorphic forms of some triglycerides {22}.

	Polymorphic form		
Fatty acid	α	β	β′
Tricaprin	-	-	32
Trilaurin	14	34	44
Trimyristin	32	44	56
Tripalmitin	44	56	66

When a melted triglyceride is cooled rapidly, it solidifies in the lowest melting, unstable α-form. If this is slowly heated, it will melt and then resolidify in the β′-form. Repetition of this procedure will bring about a transition to the final, stable β form. The β-form is also obtained by recrystallisation from solvent. Mixed triglycerides with an asymmetrical distribution of saturated and unsaturated fatty acids, i.e. SSU or UUS, have a stable ß'-form. Although each form, and many of their variations, have been characterised in terms of X-ray diffraction patterns and infrared spectra, the actual arrangements of the triglyceride molecules in the crystals remain to be found, with the exception of a handful of purified triglycerides.

The triglyceride molecules are arranged in layers with the fatty-acid chains packed closely together and running perpendicularly (in the α-forms) or obliquely (in the β′and β-forms)

to the parallel planes of the glycerol groups and the planes of terminal methyl groups. In crystallographic terms the α-forms are hexagonal, the β′-forms orthorhombic, and the β-forms triclinic. One layer may have a depth corresponding to the length of two or three fatty acid chains, known, respectively, as DCL (double chain length) or TCL (triple chain length) forms with the triglyceride molecules overlapping. In general the TGL forms are found where one of the three fatty acids attached to the glycerol structure differs markedly from the other two in terms of unsaturation or chain length, and the TCL form is seen to make for easier packing.

In a natural fat the occurrence of large numbers of different types of triglyceride will make the situation rather more complicated, but as a rule one polymorphic type predominates. Important examples of β-types are cocoa butter, coconut oil, corn oil, groundnut oil, olive oil, palm kernel oil, sunflower oil, and lard; β′-types include cotton seed oil, palm oil, rape seed oil, beef fat (tallow), herring oil, whale oil, and cow milk fat.

The usefulness in a particular application is crucially dependent on its melting and crystallisation characteristics. Fats to be spread on bread or blended with flour, etc., in cake or pastry require the plasticity that is associated with a wide melting range. The term `shortening′ is nowadays applied to all manufactured fats and oils except those such as the margarines and `low-fat′ spreads that have a significant content of non-fatty material. The literal use of the term refers to the tendency of shortenings to reduce the cohesion of the wheat gluten strands in baked goods and thereby `shorten′ or soften them. The β′-tending fats crystallise in small needle-like crystals. At the correct mixing temperatures these fats consist of these crystals embedded in liquid fat matrix, giving a soft plastic consistency ideal for incorporating air bubbles and suspending flour and sugar particles. The β-tending fats form large crystals, which give grainy textures (compare the textures of beef and pork dripping). Though difficult to aerate, they are valuable in pastry making. If shortenings are blended from mixtures of β and β′, the latter type dictates the crystallisation pattern.

Cocoa butter is another good example of the importance of melting properties to a foodstuff - in this case chocolate. The reason for the most notable feature of chocolate, its

sharp melting point (it melts in your mouth but not in your hand !), has already been considered, but we also expect chocolate to have a very smooth texture and a glossy surface. Cocoa butter can occur in six different polymorphic states with melting points ranging from 17.3 °C to 36.4 °C. Only one of these (a β-3 type, melting point 33.8 °C) has the desired properties, and the special skill of the chocolate maker lies in ensuring that the fat is in this particular state in the finished product. This is achieved by tempering. The liquid chocolate is cooled to initiate crystallisation and reheated to just below the melting point of the desired polymorphic type so as to melt out any of the undesirable types. The chocolate is then stirred at this temperature for some time in order to obtain a high proportion of the fat as very small crystals of the desired type when it is finally solidified in the mould or when it is used for coating biscuits or in confectionary.

Chocolate that has been incorrectly tempered or subjected to repeated fluctuations in temperature, as in, for example, a shop window, develops a bloom. This is a grey film which resembles a mould growth but is actually caused by the transition of some of the fat to a more stable polymorphic form which crystallises out on the surface. The migration of triglycerides from the nut centres of chocolates or the crumb of chocolate-coated biscuits can cause similar problems. Milk fat is an effective bloom inhibitor and is often included in small amounts in plain chocolates, besides accounting for about one quarter of the total fat in a typical milk chocolate. Cocoa butter is obviously a very difficult fat to handle successfully in a domestic kitchen, and chocolate substitutes are marketed for home cake decorating etc. These contain fats such as hardened (i.e. partially hydrogenated) palm kernel oil. Although they have an inferior, somewhat greasy texture, these artificially modified fats only have a single polymorphic state and therefore do not present the cook with the problems of tempering.

A fat such as hardened palm kernel oil is referred to as a cocoa butter replacer (CBR). Obviously such a fat cannot be used in combination with cocoa butter, as its melting properties are different. A novel enzymic proccess for producing cocoa butter extenders (CBE), which can be blended with cocoa butter but are cheaper, is now in commercial use.

In this enzyme-catalysed transesterification reaction a fraction of palm oil rich in POP triglycerides is mixed with stearic acid. The mixture is diluted with hexane and then pumped slowly through a bed of polymer beads in a reactor vessel at approximately 60 °C. The beads are coated with a fungal lipase. Like pancreatic lipase this enzyme only attacks the ester linkages at the 1 and 3 positions of glycerides. However, in the nearly total absence of water, the equilibrium position of the reaction is such that the enzyme catalyses not only the removal of fatty acids from the 1 and 3 positions but also the reverse reaction. The end result is the formation of a fat with almost identical proportions of POP, POS and SOS compared to natural cocoa butter.

2.4 A comparison of the melting points of camel and cow milk fat

Milk fat has no sharp, well-defined melting point, and melts over a wide temperature range. Generally, milk fat is liquid at and above body temperature and completely solidified below -40° C. At intermediate temperatures it is a mixture of solid and liquid triglycerides. The content of solid fat in the mixture is an important parameter for the technological properties of milk fat. Rüegg and Farah {25} carried out a study to determine the melting thermograms and ratios of solid to liquid in camel milk fat using differential scanning calorimetry (DSC).

The melting thermograms were recorded in the temperature range –50° to 50° C. Melting started at approximately -26° C and was complete below 43° C. Figure 3 (page 23) shows typical melting thermograms obtained with dehydrated butter fat prepared from camel and cow milk.

The thermogram for camel fat differed in shape and did not show the peak around 15° C which is typical of the middle-melting fraction of the cow milk. The profiles are very similar to the distribution of triglycerides in the respective milk fats (see Figures 18 and 20, pages 93-94).

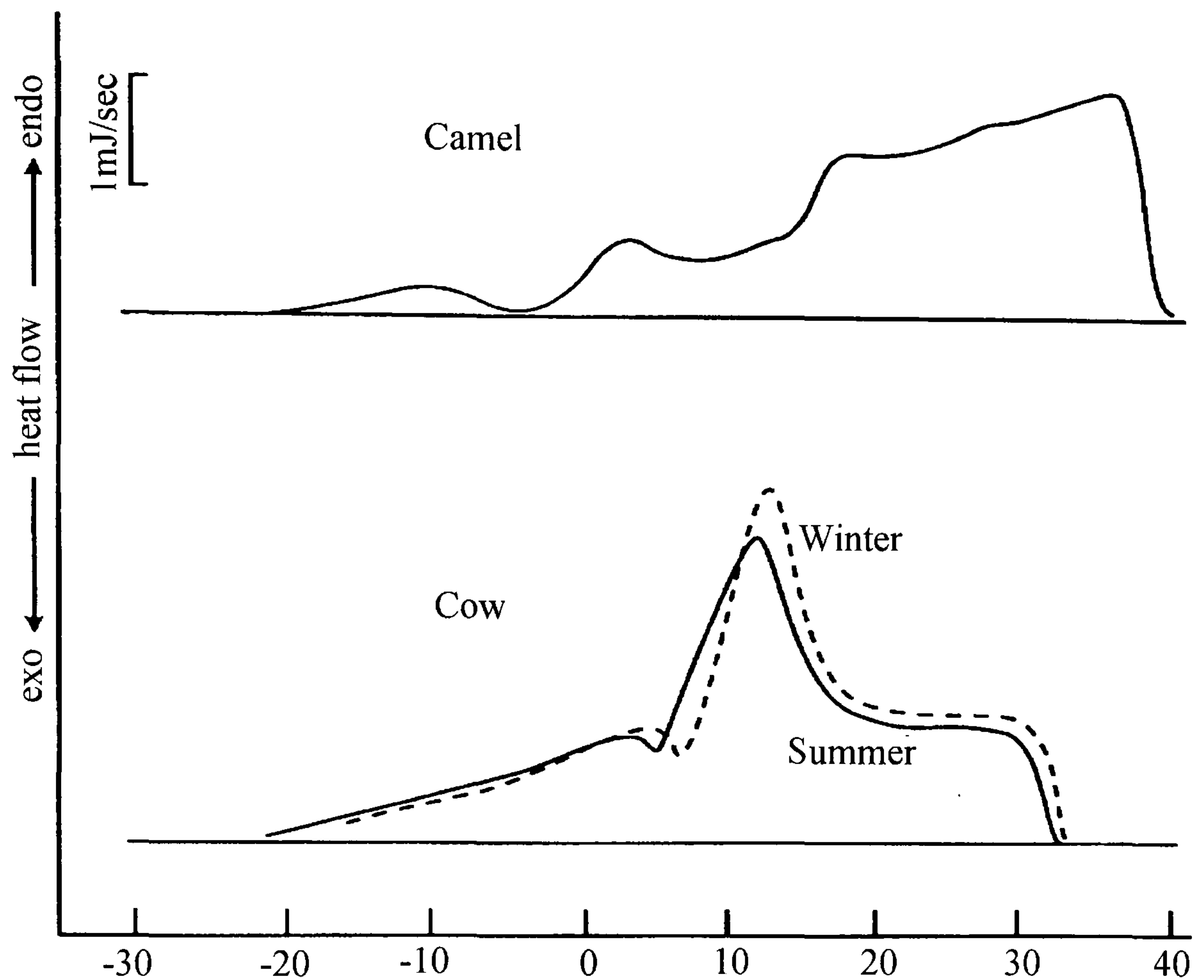


Figure 3: Differential scanning calorimetry thermogram of butter fat of camel and cow milk{23}.

The different amounts of low, medium and high melting fractions of triglycerides are consistent with the differences in the fatty acid composition between cow and camel milk (see Table 6, page 48). In camel milk fat the proportion of high melting fractions is high and those of low and medium melting fractions are low compared with cow milk fat, Figure 3.

Figure 3 also displays the difference between winter and summer butter with reference to cow milk. The seasonal variations in milk composition are well documented {26, 27}. Concentrate-rich dairy rations and feeding with silages (esp. maize) are the reasons for increasing proportions of medium –long chain fatty acids (myristic and palmitic acid) and decreasing proportions of stearic, oleic and linoleic acid in milk fat. Human nutritional studies have shown that such fat increases the LDL-cholesterol and decreases the HDL-

cholesterol concentrations {28}. Furthermore, an increasing number of dairy cows are fed with silages throughout the year which are poor in unsaturated fatty acids due to fermentation processes. In conventional farms the energy supply was improved proportionally to milk yield enhancement. Conversely, energy deficiency, especially in the first part of lactation, leads to an increase in unsaturated fatty acids in milk fat. This is due to decomposition of depot fat in a situation of negative energy balance.

From the typical curve for camel and cow milk fat, as shown in Figure 4 below, the difference in churnability between camel and cow milk fat can be explained. Butter from camel milk contains a significantly higher portion of solid fat over the entire melting range relative to butter from cow milk.

The optimal churning temperature for camel milk, about 25 °C, and for cow milk, about 10-14 °C, are in both cases the temperature at which about equal proportions of the fat are liquid and crystalline.

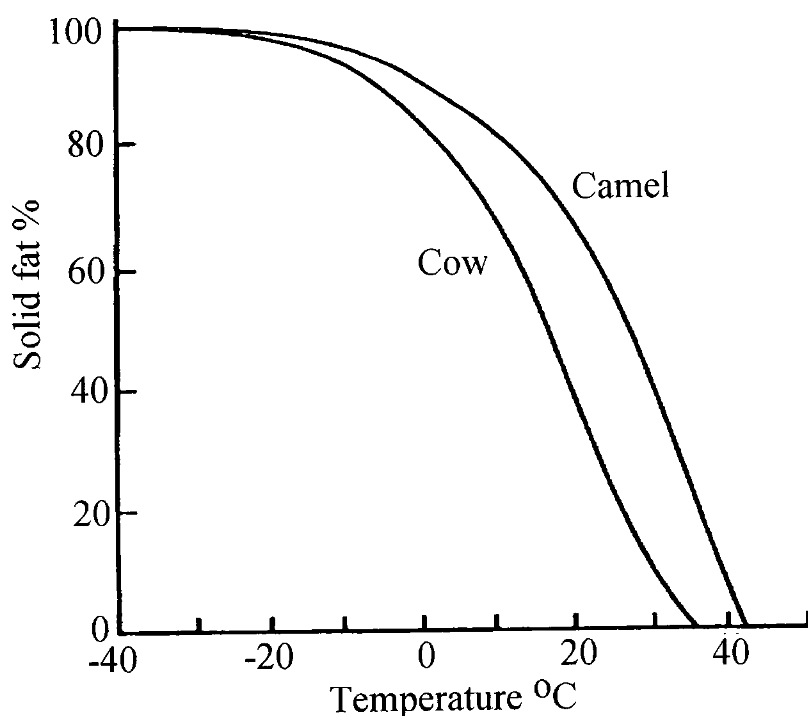


Figure 4: Melting curves of camel butter fat compared with typical curve for bovine butter fat. Percentage of solid fat as a function of temperature {25}.

3.0 Analysis of triglycerides and fatty acids

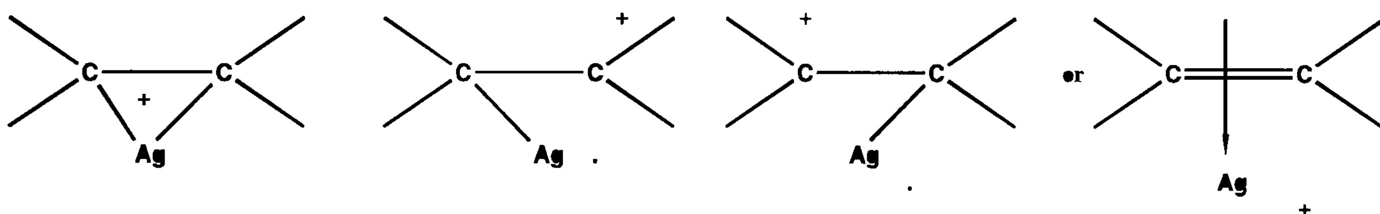
3.1 Separation of triglycerides using reversed phase HPLC

For the analysis of TGs with HPLC it is common to use reversed phase material (RP-18), in which silica gel is coated with a hydrophobic layer consisting of an aliphatic hydrocarbon with a chain length of 18.

The use of a solvent gradient, temperature gradient or a combination of both {3} enables separation of TGs according to their CN. It should be noted however that each double bond has the effect of causing a reduction in retention time approximately equivalent to two carbon atoms. Thus to separate TGs according to their CN it is important to ensure that the class type is homogenous, e.g. SSS, SSM and SMM. The latter can be achieved by separation on a silver-ion column.

3.2 Separation of triglycerides according to the degree of saturation using silver-ion chromatography

The complex formed between an olefin and silver-ion (argentation) may be represented as follows:



The stability of such complexes has been determined by partitioning the olefin between carbon tetrachloride and aqueous silver nitrate {29}. The overall energetics of hydrogenation and argentation are similar and what has been deduced concerning hydrogenation applies for argentation {29}. It has been found that *cis* olefins complex with silver-ions more tightly than do the *trans* isomers, propably because complex formation brings more relief of strain with the *cis* isomers {29}.

The impregnation of silica gel with silver nitrate makes it possible to separate compounds according to the number of double bonds and their geometry {30}. This has been used for the separation of triglycerides with 0,1,2 and 3 double bonds. It has also enabled the separation of triglycerides which differ merely in their *cis* and *trans* configuration with respect to a double bond, see Figure 5 below.

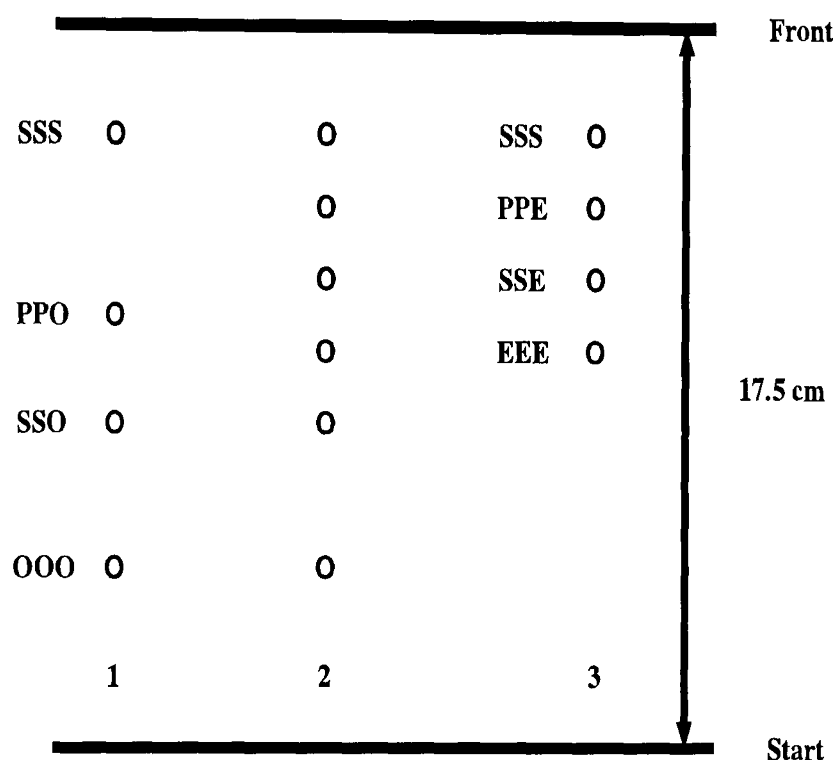


Figure 5: Separation of triglycerides on a silver nitrate impregnated silica gel plate {30}. Eluent: benzene. Adsorbent: silica gel 30:70 impregnated with silver nitrate, Run time: approx. 40 min. (S = stearic acid (C18:0), P = palmitic acid (C16:0), O = oleic acid (C18:1 c-9), E = elaidic acid (C18:1 t-9).

B. de Vries extended the above method to column chromatography {31}. Using heptane / 2M silver nitrate in glycol monoethyl ether and triethylene glycol as an eluent, it was possible to separate methyl oleate and methyl elaidate, with partition coefficients of 3.2 and 4.8, respectively.

A new chromatographic adsorbent, silica impregnated with a solution of silver nitrate and dried at 120°C, displays highly selective adsorption properties with respect to the geometry and the number of carbon-carbon double bonds of related unsaturated lipids. Elutions have been carried out with solutions of benzene or diethyl ether in light petroleum. The fractions

obtained have been identified by GC and IR. Most of them were more than 95% pure. Even in the case of elaidodipalmitin and oleodipalmitin, the degree of separation obtained was better than 90% {32}.

The detection and quantification of fatty acids, especially by UV absorption, is difficult. One method of improving the latter has been the derivatisation of fatty acids into UV absorbing esters, e.g. *p*-bromophenacyl esters {33}.

Reversed phase HPLC separates esters according to their chain length (or CN) and partially resolves *cis*- and *trans*-isomers; this technique has accordingly received much attention. This mode of chromatography is based largely on differences in partition coefficients. It is reasonable to assume that a combination of both partition behaviour and complexing ability would be a more efficient method of separating *cis*- and *trans*-isomers. The solubility of silver salts in the eluting solvents that are commonly used for reversed-phase chromatography (water : methanol mixtures) is small but sufficient for complex formation. The latter combined with partition can be used for the chromatography of monoenoic fatty acid *p*-bromophenacyl esters, using a nonpolar hydrocarbon as the stationary phase.

C. Scholfield separated fatty acid methyl esters using a silica column impregnated with silver nitrate {34}. This method has the advantage that silver ions do not come in contact with components of the HPLC pump. When silver ions come into contact with metals other than precious metals they form amalgams which can cause serious damage to HPLC pumps.

Silicic acid was coated with silver nitrate as follows. In a 100 ml round bottomed flask, a solution of 2 g silver nitrate in 50 ml dry acetonitrile was added to 10 g of 24-44 μm Biosil A. Solvent was removed under vacuum at 60°C in a rotary evaporator. A 2 mm i.d. x 61 cm stainless steel column with 10μm fritting at the detector end was filled with 1.85 g of the packing. The column was only able to resolve approximately 50 chromatograms before having to be discarded.

Long chain fatty acid methyl esters were separated on the basis of number, position and geometric configuration of double bonds with a silver nitrate silicic acid column and benzene as the mobile phase. Saturated esters are eluted first, followed by methyl elaidate and then methyl oleate. Geometric isomers of methyl 9,12-octadecadienoate and of methyl 9,15-octadecadienoate were also separated {34}.

R. Battaglia and D. Fröhlich were able to separate *cis*- and *trans*-monounsaturated fatty acids using silica which was impregnated with silver nitrate {35}. The column was prepared as follows: 10 g Spherisorb S5W were suspended in 100 ml of water, in which varying amounts of silver nitrate (e.g. for a 20% column 2.0 g) were dissolved. The suspension was dried in a rotary evaporator under vacuum at 60°C to a thick paste, which was further dried in an oven at 130°C for 2hrs at atmospheric pressure. A slurry was prepared of the impregnated silica in dioxan and aggregates were dispersed by immersing the flask in a ultrasonic bath. The slurry was then packed into a column (250 x 5mm) with hexane as the pressure liquid. Finally the column was flushed with 300 ml UV-grade hexane. The mobile phase consisted of a mixture of hexane and tetrahydrofuran (approximately 99:1).

Whereas the separation of *cis*- and *trans*-isomers was easily achieved on impregnated Partisil 20, it was not possible to separate the positional double bond isomers on this material, even by increasing the silver-load to 4% and decreasing the particle size to 5 µm. However, treatment of the spherical, porous silica (Spherisorb S5W) with silver nitrate produced an improvement in terms of the separation ability. With this material it was possible to separate the methyl esters of margarine fats into the positional isomers of the *trans* C18:1 and *cis* C18:1 groups.

Unfortunately silver nitrate bled slowly from the columns prepared by C. Scholfield and D. Fröhlich {6}and this was responsible for contamination of the fractions collected and of the spectrophotometer cells. In an attempt to circumvent this problem, silver-loaded macroreticular ion-exchange resins were packed into HPLC columns, and were found to give good resolution of methyl ester derivatives of unsaturated fatty acids {36}. Unfortunately, rather polar mobile phases such as methanol were required to elute

polyunsaturated components and this caused some hydrolysis or transesterification of intact lipids such as triacylglycerols. A rather similar approach, but with custom-packed columns containing a silica gel based ion-exchange resin loaded with silver ions, was used to effect separation of a limited range of unsaturated fatty acids {37}.

Many analysts do not have access to specialised column packing facilities. Hence it would be advantagous to be able to load pre-packed ion-exchange columns in *situ* with silver ions. Powell {38} was able to accomplish this merely by pumping silver nitrate through a column. A good resolution of prostanoids and a limited range of polyunsaturated fatty acids was obtained.

In 1987 Christie made a breakthrough by developing a silver-ion loaded column which did not suffer from bleeding and could be subjected to a high number of analysis without loss in performance {6}. A column containing Nucleosil™ 5SA was loaded with silver-ions by simply injecting silver nitrate solution *via* a Rheodyne valve, thus minimizing contact between silver nitrate and the HPLC equipment. The column was used for the separation of methyl esters of unsaturated fatty acids and, with aprotic mobile phases, the separation of molecular species of triacylglycerols.The column was first tested with fatty acid methyl esters, using mobile phases similar to those employed by others in related work, i.e. with gradients of methanol to acetonitrile. The light scattering detector was used as its response is not influenced by gradient mobile phases and is non-specific, i.e. it detects any substance being eluted from the column. Some degree of separation according to the chain length of the fatty acids was observed, longer chain components being eluted first.

Results obtained with a gradient using 1,2-dichloromethane and acetone as the mobile phase and with a sample of palm oil triacylglycerols showed good resolution of the principal components with zero to three double bonds in total in the fatty acyl chains, see Figure 6 below.

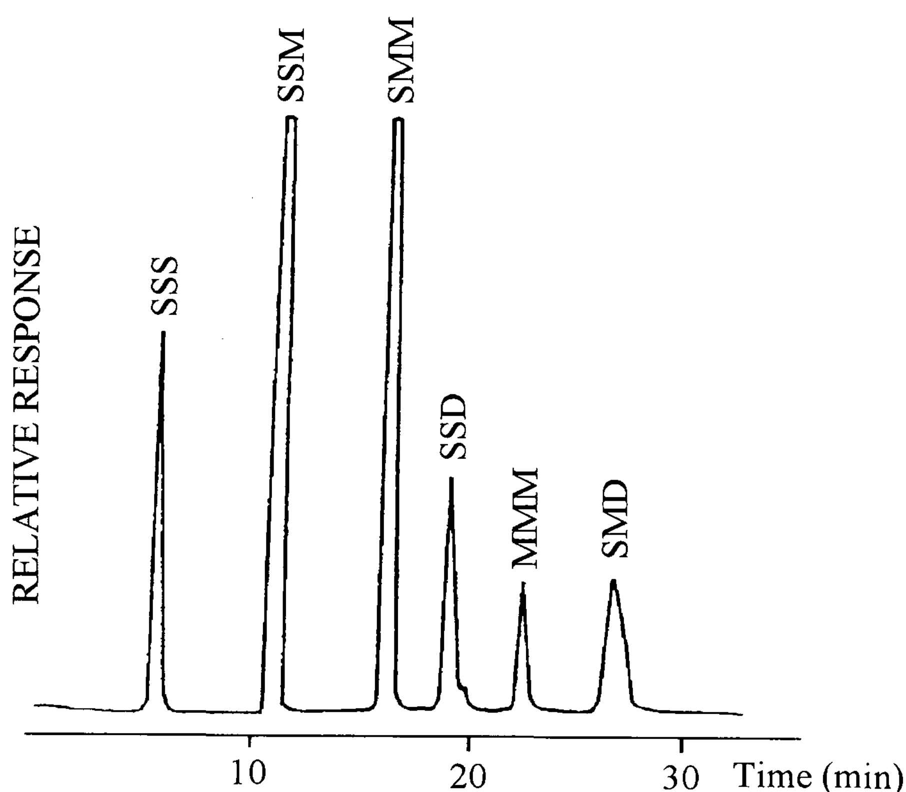


Figure 6: The separation of molecular species of triacylglycerols from palm oil (0.6 mg) by silver-ion HPLC, with a linear gradient of 1,2-dichloromethane to acetone over 30 min at a flow rate of 0.75 ml/min as the mobile phase, and using a light scattering detector. The abbreviations S, M and D refer to species containing saturated, monoenoic and dienoic fatty acid residues, respectively {6}.

In 1988 Christie published further results, demonstrating further progress in the separation of triglycerides using silver-ion HPLC {39}.

3.3 The use of high temperature gas chromatography for the analysis of triglycerides

The analysis of triglycerides using high temperature gas chromatography is a quick and convenient method for the determination of the distribution of triglycerides according to the combined chain lengths of the fatty acid moieties or carbon number (CN). A significant advantage of employing high temperature gas chromatography with a non-polar column, e.g. DB1-HT (dimethylpolysiloxane) over HPLC methods employing RP-18 material is that the retention times are hardly influenced by double bonds. Hence by using a suitable

standard it is easy to classify triglycerides according to their CN, regardless of whether they are saturated or highly unsaturated.

3.4 Analysis of free fatty acids (FFAs)

Although free fatty acids (FFAs) are usually present only at low levels in dairy products, they play an important role in terms of organoleptic quality. FFAs are formed mainly as a result of lipolytic enzyme action on glycerides. Many methods for the determination of the individual FFAs in dairy products have been published. Most of these involve lipid extraction, isolation of FFAs and gas chromatographic quantification of the individual FFA.

Several methods have been used to extract FFAs from dairy products. Salih et al. {40} and Needs et al. {41} have obtained satisfactory results by extracting FFAs from milk with acidified diethyl ether. It should be noted that these procedures are (nearly) quantitative for the higher FFAs. However, for the lower FFAs (< C8:0) partitioning of these acids between the organic and the aqueous phases may influence the quantitative results in the analysis of milk and other products containing significant quantities of water. Some of these procedures give satisfactory results, but most of them are time consuming.

Recent developments in capillary gas chromatography have made it possible to separate underivatized FFAs with chain lengths from C2:0 to C20:0 in one run. Tedious procedures, such as the quantitative conversion of lower FFAs to fatty acid methyl or ethyl esters, are therefore unnecessary. A method developed by C. Jong and H. Badings {42} describes such a procedure.

3.5 The determination of the fatty acid compostion of triglyceride samples

Before the fatty acid composition of a lipid can be determined by gas chromatography, it is necessary to prepare the comparatively volatile methyl ester derivatives of the fatty acid components. Propyl and butyl derivatives may also be prepared if work is being focussed on short chain (< C6:0) fatty acids. Numerous methods are available {43, 44}; no single

method will suffice for all circumstances and thus the method best suited for the situation must be selected.

Several methods, including those employing trimethylsulphonium hydroxide (TMSH) and sodium methoxide, have been used for the preparation of FA methyl esters for gas chromatography {45}.

3.6 Detectors for the detection of triglycerides

There are currently five types of detector which have been used for the detection of triglycerides, all of which have some form of limitation or drawback{46}: IR, transport flame ionisation, refractive index, UV and light scattering detection. Apart from refractive index detectors all are compatible with gradient elution, although IR detection suffers from base-line drift problems and UV detection is incompatible with acetone, one of the most suitable solvents. Transport flame ionisation detectors have not been available for a long time, so there is little experience with these. A reported drawback of transport flame ionisation is poor sensitivity {46}. The fifth type of detection, light scattering, can be used with gradient elution using any volatile solvent and, as in the case with transport flame ionisation, the solvent is evaporated prior to detection of the analyte. However, unlike transport flame ionisation, all the eluate may be analysed and hence the sensitivity is better.

3.6.1 The light scattering detector for the detection of triglycerides after separation using HPLC

The eluent stream enters the detector at the top of the evaporation chamber. The column outlet is connected *via* a small bore stainless steel capillary tube. The solvent/solute is fed into the nebuliser assembly where a Venturi jet operated by pressurised air or inert gas atomises the solution into a uniform dispersion of droplets which then passes as a plume into the evaporator.

Figure 7 below is a schematic representation of a light scattering detector.

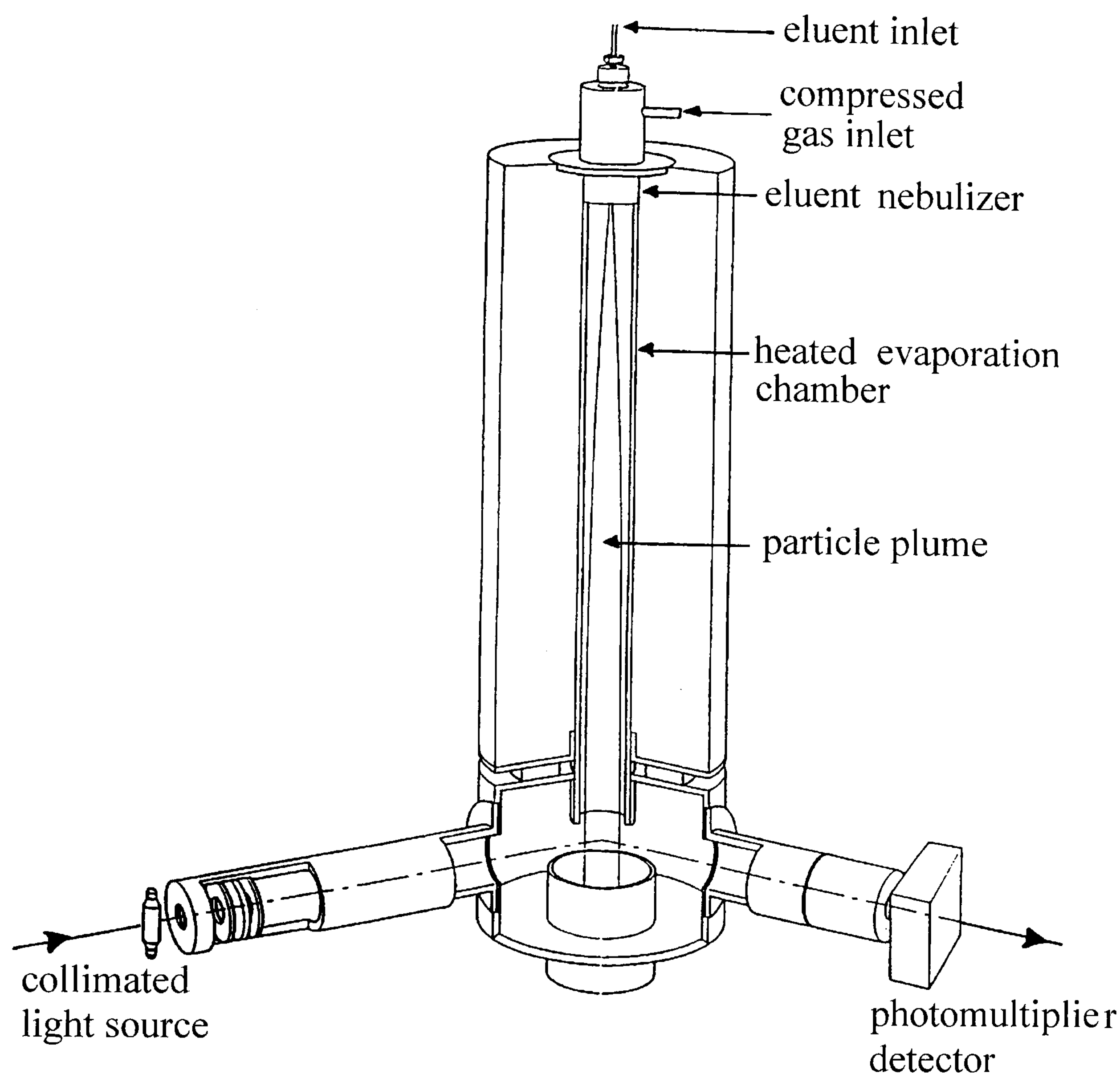


Figure 7: The principle of the evaporative light scattering detector {47}.

After nebulisation the atomised spray passes down the evaporation chamber, mainly due to the influence of the nebuliser gas flow. Its passage is assisted by the design of the exhaust section of the instrument. A small fan at the base of the exhaust chamber produces a slight negative pressure in the evaporation chamber; this ensures that the atomised spray is drawn down the evaporation chamber; the exhaust is then vented at the rear of the instrument.

Light from a lamp is collimated and passed through the instrument at right angles to the direction of the gas flow. A light trap is located opposite the source of light to eliminate internal reflections within the instrument body. When pure solvent is being evaporated, only its vapour passes through the light path and the amount of light scattered to the

photodetector is small and gives a constant response. When a non-volatile solute is present, a particle cloud passes through the light path, causing light to be scattered. This scattered light enters the optical aperture of the detection system and generates a signal response from the photomultiplier in real time. The quantity of light detected is dependent on the solute concentration and solute particle size distribution. As the detection process is affected by the size of the atomised droplets, the rate of evaporation and the nebuliser gas flow, it is imperative to maintain steady conditions, both internally and externally to the instrument.

There are four main processes by which the path of electromagnetic radiation or light can change direction when passing through a medium containing a suspended particulate phase. These are:

Rayleigh Scattering
Mie Scattering
Reflection
Refraction

The importance of each of these processes depends on the ratio of the radius of the particle (r) to the wavelength (λ) of the incident light. Rayleigh scattering is predominant when r/λ is about $5x10^{-2}$. When r/λ is greater than $5x10^{-2}$ but less than 0.5 the particles no longer behave as point sources, and Mie scattering becomes predominant. Once r/λ approaches 1.0 reflection and refraction begin to prevail.

In order to decide which mechanism is predominantly responsible for the "scattering" observed in the instrument, an estimate of r/λ must be made {47}, with aid of the equation given below:

$$D_o = (585\sqrt{\sigma} \;/\; u\sqrt{\rho}) + 597(\mu \;/\; \sqrt{\sigma})^{0.45} \times (1000Q \;/\; Q_a)^{1.5} = n_a D^3 / n_a D^2$$

Where:

D_o = mean drop diameter (= 2r)

n_a = number of drops in the size range, with diameter D

σ = liquid surface tension

ρ = liquid density

μ = liquid viscosity

u = relative viscosity between the gas stream and the liquid stream

Q = volumetric flow rate of liquid

Q_a = volumetric flow rate of gas

The particulate size may be varied by altering the gas velocity and also the initial solute concentration. Experimental work and calculations have shown that in most cases r/λ is ≥ 1.0. This suggests that the "scattering" is most predominantly due to reflection and refraction for all but the smallest particles.

Hence the mean particle diameter will determine which are the main scattering phenomena observed. Particle diameter is a function of the sample composition, concentration, solvent and the gas and liquid flow rates. This relationship gives the instrument maximum sensitivity around $r/\lambda = 4$. Detection declines rapidly when values for r/λ are above 5 or below 2.5. When $r/\lambda < 2.5$ the interference effects typical of Mie scattering cause the deflected light to be low in intensity at the angles of measurement. As the particle increases in size, reflection and refraction become dominant and sensitivity increases. A further increase in the particle size causes the ratio of surface area to volume to decrease and sensitivity decreases.

3.6.2 Mass spectrometry for the detection of triglycerides after separation using HPLC and GC

Atmospheric pressure chemical ionization (APCI) mass spectrometry has been investigated as a method for the detection of triglycerides after separation by high performance liquid chromatography {48-50}.

Initially, individual homogeneous (monoacid) triglyceride standards containing fatty acids with zero to two double bonds were analysed, to demonstrate the quality of mass spectra obtained using an APCI interface. The mass spectra showed that minimal fragmentation occurs, resulting primarily in diglyceride $[M - RCOO]^+$ ions. The degree of unsaturation within the acyl chains had a marked effect on the proportion of diglyceride ions to $[M + 1]^+$ ions formed in the APCI source {48}. The triglycerides containing singly unsaturated fatty acids gave diglyceride ions as the base peak, and $[M + 1]^+$ ions with an intensity of 20 to 28% with reference to the base peak. Only diglyceride ions were observable in the spectra of triglycerides containing saturated fatty acids.

Electrospray ionisation (ESI) has also been attempted for the analysis of triglycerides infused *via* a syringe pump {51}. Spectra obtained using ESI contained only quasimolecular ions, with no fragmentation. This lack of fragmentation can result in ambiguity in structural assignments for triglycerides of identical molecular weight. Tandem mass spectrometry/mass spectrometry (MS/MS) could provide the additional structural information necessary to identify triglyceride species uniquely. Collision-induced dissociation can also be used to produce lower molecular weight ions. The flow rates used for syringe pump infusion are very low compared to those in a conventional HPLC system. The necessity of an ionic buffer in the effluent for ESI will affect the types of columns and solvents which may be used.

APCI, like ESI, is an atmospheric pressure ionisation method. However, unlike ESI, APCI uses a corona needle discharge to impart charge onto vapourised molecules which are sprayed from a capillary inlet. These are swept into the high vacuum region of the mass spectrometer through a capillary bleed. Also unlike ESI, APCI requires no buffers in solution in order to produce efficient fragmentation. This lack of need of a buffer allows neutral, nonpolar molecules to be analysed as easily as more polar molecules which are ionisable in solution. An APCI interface allows direct introduction of HPLC column effluent at a rate of up to 2 ml/min. Due to the low level of fragmentation, APCI is an effective method for identification of TG species separated using HPLC.

APCI-MS has already been used as a detection method following the separation of triglycerides on a silver-ion column {50}. The m/z values of $[M + H]^+$ ions unambiguously define the number of acyl carbons and double bonds in the molecule. The identification of relatively saturated triacylglycerols may be problematic, because they do not yield any or only a weak $[M + H]^+$ ion. Thus the identification of milk fat triacylglycerols may be difficult and additional information such as retention time data would be required. The mass spectra of triacylglycerols achieved by APCI-MS are affected by the structure of the molecule, e.g. the degree of unsaturation and the regiospecific distribution of fatty acids between the *sn*-2 and *sn*-1/3 positions, as well as by the instrumental parameters of the mass spectrometer, such as capillary and vapourizer temperatures. At the present stage of the method, no attempt has been made to distinguish regioisomeric triacylglycerols in complex mixtures, although differences have been measured in the relative abundances of $[M - RCOO]^+$ ions formed by a loss of a fatty acyl residue from the *sn*-2 position and the *sn*-1/3 positions of triacylglycerol reference components. The regiospecific analysis of triacylglycerols would require extensive optimization and calibration of the system. The silver-loaded cation exchange column has shown potential to separate, at least partially, unsaturated regioisomeric triacylglycerols by using a ternary solvent gradient {50}. Similar results have been reported earlier using an isocratic eluent system consisting of acetonitrile and hexane {52}. The molecular weights of components eluting in the same peak in silver-ion HPLC may be differentiated according to their $[M + H]^+$, however, a single $[M - RCOO]^+$ ion could be a fragment formed by a loss of one fatty acyl residue from any one of several molecules. It may be possible to modify the elution system and ionization conditions so that triacylglycerols yield only abundant ions consisting of the whole molecule without fragmentation. Unfortunately, in such a situation the information on molecular association of fatty acids is lost. Although quantitation may cause difficulties, the on-line detection of triacylglycerols by APCI-MS after separation by silver-ion HPLC provides valuable information for structure elucidation. Mass spectra yield information on both the molecular weight and molecular association of fatty acids of triacylglycerols.

Some work has already been carried out on the analysis of TGs with a low CN extracted from cow milk fat using GC-MS (CI) {53}. One of the major problems facing the analysis of TGs using GC is their low volatility. In recent years the temperature range of high

temperature capillary columns has improved significantly enabling the separation of TGs with a CN exceeding 57. Hence it is now possible to carry out high temperature GC coupled to an MS (CI) detector. Similar fragmentation of TGs has been reported {53} as described for HPLC- APCI above. Methane may be used as a reagent gas for chemical ionisation {53, 54}, this can result in a high proportion of the ion species $[RCO]^+$ and $[RCO + 74]^+$. These are useful in determing the fatty acid residues. Isobutane chemical ionisation yields less fragmentation, with a two- to five-fold increase in molecular ion $[MH]^+$ {54, 55}.

3.7 Stereospecific analysis of triglycerides

The convention of Hirschmann {56} has been universally adopted for numbering the three hydroxyl groups of glycerol. If the central carbon atom of the glycerol molecule is viewed with the C -- H bond pointing away from the viewer, then each of the three remaining bonds leads to an hydroxyl group, see below. Hirschmann proposed that the three hydroxyl groups viewed in this manner be numbered in clockwise order, with the 2 position already defined as the hydroxyl attached directly to the central carbon atom (see Figure 8 below). This is equivalent to a standard Fischer projection in which the middle hydroxyl group is located on the left side of the glycerol carbon chain (see Figure 8 below).

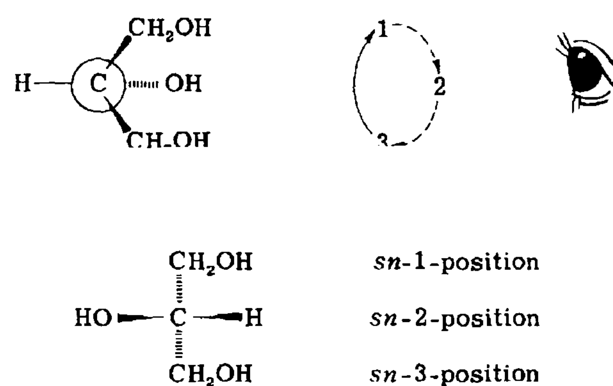


Figure 8: Schematic diagram illustrating the Hirschmann stereospecific numbering convention and the Fischer planer projection of glycerol.

Chemical and enzymatic deacylation techniques can be used to determine the fatty acids esterified at the *sn*-2- and the combined *sn*-1,3-positions of a triglyceride sample. For a

complete analysis of triglyceride positional isomers, however, it is necessary to distinguish between the *sn*-1- and *sn*-3-positions, which are stereochemically distinct. Such a procedure, called "stereospecific analysis," was first introduced in 1965 by Brockerhoff {57}. He demonstrated that the fatty acid compositions of the *sn*-1- and *sn*-3-positions could be separately analyzed based on the hydrolytic stereospecificity of phospholipase A for synthetic phospholipids derived from the original glycerides. The following year, Lands *et al* {58} introduced an alternative method for distinguishing the *sn*-1- and *sn*-3-fatty acids, utilizing the stereospecific phosphorylation of derived diglycerides by diglyceride kinase. Stereospecific analysis techniques have subsequently been improved and applied to a wide variety of samples, contributing greatly to the understanding of the triglyceride composition of natural fats.

Stereospecific analysis of triglycerides involves three basic reactions :

(a) Degradation of the triglycerides to diglycerides
(b) Phosphorylation of the diglycerides to produce phospholipids
(c) Hydrolysis of the phospholipids by phospholipase A

After each reaction, the products are separated by TLC on silicic acid and analyzed where necessary for their fatty acid composition. Many variations and combinations of reactions (a), (b), and (c) are possible; but the sequences most frequently employed are the *sn*-1,2(2,3)-diglyceride method of Brockerhoff {57}, the *sn*-1,3-diglyceride method of Brockerhoff {59}, and the procedure of Lands et al {58}.

Although originally developed using 1 g triglyceride samples, the procedure may be applied to 20-50 mg samples in the same manner as the *sn*-1,2(2,3)-digyceride method, as modified by Christie and Moore {60}.

It is evident from the above discussion that numerous alternatives are available in selecting a suitable procedure for stereospecific analysis of triglycerides. The specific choice usually depends on whether accuracy, speed, or the determination of minor fatty acids is the principal goal.

3.8 Deacylation of triglycerides using lipase (positional analysis)

Pancreatic lipase possesses near-absolute specificity for the hydrolysis of primary ester linkages in triglycerides. Studies {44} indicate >97% specificity for the *sn*-1,3-positions, and any release of *sn*-2-position fatty acids is generally attributed to acyl migration or to contamination with a non-specific lipase.

However, this approach is not generally recommended for the following reasons {44}:

1. The fatty acid specificities of pancreatic lipase often cause the free fatty acid composition to vary according to the extent of hydrolysis.
2. If any monoglyceride is completely hydrolyzed to glycerol (1-2% is usual), the FAs in the *sn*-2 position will lead to erroneous results.

Pancreatic lipase does not differentiate between the *sn*-1 and *sn*-3 positions. However, FAs with double bonds near to the carboxyl group are split off more slowly, which is a problem when analysing fish oils {3}. Short chained FAs on the other hand are split off more rapidly.

More widely accepted is a method {3, 44} in which 50 mg fat (triglyceride) samples are subjected to deacylation using pancreatic lipase. After a short reaction time (35-45 seconds) the reaction is stopped and the reaction products are separated using TLC. Triglycerides, 1,2- and 2,3-diglycerides and 2-monoglycerides are isolated and their fatty acid composition is analysed using gas chromatography. For each FA the proportion in the *sn*-2 position is calculated from the mol % in the 2-monoglycerides and the triglyceride, as follows:

$$\text{Proportion in position 2} = \frac{\text{2-Monoglyceride (mol \%)} \times 100}{\text{Triglycerides (mol \%)} \times 3}$$

If a FA is distributed evenly in the *sn*-1,2 and 3 positions, this will give a result of 33% using the above equation.

Because only small amounts of triglyceride fractions (ca. 2 mg) could be isolated using the preparative HPLC techniques available it was necessary to apply a method which could be scaled down to function with small amounts of sample (1-2 mg). Additionally, because the aim of the work was to investigate a large number of samples, a rapid and simple method was required. A method which is able to differentiate between FAs in the *sn*-2 and *sn*-1(3) positions is sufficient.

The FA composition of camel milk and meat fat consists of FAs ranging from C14:0 to C18:1. This narrow range of FAs is ideal for deacylation using lipase, as the specificity with reference to a particular FA is very limited {44}.

4.0 Aims of the work

The present thesis is part of an EEC project (EC-contract No. TS3-CT 94-0339, Use of camels in arid regions as potential sources for milk and meat products) covering the following themes:

A. Analysis and technology of camel meat {61-63}.

B. Analysis and technology of camel milk {8, 25, 64-78}.

C. Camel milk and meat productivity and camel nutrition {7, 65, 79}.

This work concentrates on themes A and B. The aim of the present study was to develop and apply analytical techniques for the characterization and determination of the free fatty acids and triglycerides in camel milk and camel hump fat, with the aim of improving camel milk and meat products.

The approach used for the triglyceride analysis was the sequential application of the following techniques:

- Silver-ion chromatography (separation according to level of saturation)
- Separation of above fractions according to carbon number using reversed phase chromatography
- Positional analysis

Further studies were carried out on the use of mass spectrometry as a means of detection following separation using HPLC or GC.

5.0 Determination of free fatty acids

Recent developments in capillary gas chromatography make it possible to analyze underivatized fatty acids with chain lengths from C:2 to C:20 in one run. A direct procedure has the advantage that the risk of contamination *via* other lipid compounds which contain FA residues, e.g. triglycerides, diglycerides, monoglycerides and phospholipids, is reduced. A procedure developed by Jong and Badings {42} was modified and adapted for this work. However, instead of using a conventional FID detector an MS detector with an EI source was used.

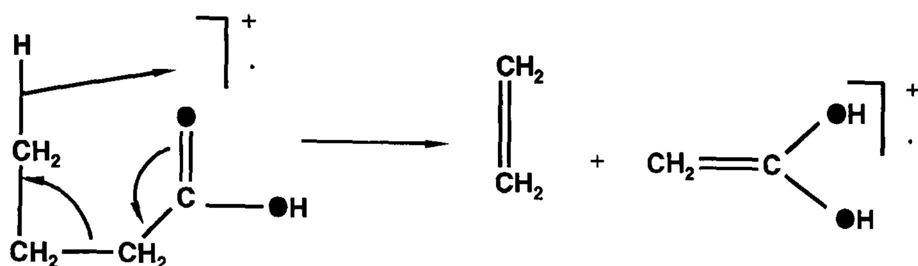
5.1 Fragmentation of free fatty acids using an MS detector with an EI source

Samples were measured using a GC-MS, in which the MS detector was fitted with an EI source. The standard was initially measured using SCAN mode, and the following fragments, confirmed by literature {54, 80, 81} were detected in all of the fatty acids measured:

mass (m/z)	fragment
60	$C_2H_4O_2^+$
73	$[(CH_2)_2COOH]^+$

Fragment m/z = 60 - McLafferty rearrangement

The McLafferty rearrangement is shown by a large number of classes of compounds, including fatty acids. This involves the transfer of a hydrogen radical in a six-membered transition state, to an atom which must be joined to the adjacent atom of the six by a double or triple bond. The case with reference to carboxylic acids is shown below. This produces the characteristic peak for acids unbranched at the α-carbon (m/z 60).



A peak at m/z 45 (CO_2H^+) also occurs for many acids. For longer chain fatty acids the fragment m/z 43 ($C_3H_7^+$) was also detected. The above fragment masses were selected for the SIM mode of detection. The latter in conjunction with known elution times, obtained *via* a suitable standard, was used for the determination and quantification of the fatty acids. Figure 13, page 90 represents an equimolar standard, used for the determination of free fatty acids in camel milk.

5.2 Free fatty acids in camel milk

Although FFAs are usually present only at low levels in dairy products, they play an important role in terms of organoleptic quality. FFAs are formed mainly as a result of lipolytic enzyme action on glycerides. The extent of lipolysis has been used as an indicator of the degree of ripening of some types of cheese, such as Italian and blue cheeses {82, 83}.

In milk, there is a partition equilibrium between FFAs in the fat and in the plasma phase. The shorter the chain length, the higher the proportion of the FFA in the plasma. The latter is less relevant for camel milk, in which the proportion of FAs with chain lengths less than C14 is low. With decreasing pH, more of the FFAs go into the fat phase. Hence for good quantitative results complete extraction of all lipid material must be achieved. Upon addition of ethanol the lipoprotein complex breaks up and thus the lipids (FFA) become available in a free form.

An FFA which does not occur naturally in milk can be used as internal standard to correct for losses during extraction. Jong and Badings {42} claimed that short chain FFAs experienced losses in the aqueous phase and hence utilised additional short chain FAs as internal standards.

Table 5: The concentration of FFAs determined in nine camel milk samples, three camels milked on three occasions, from the Ol Maisor Ranch in Kenya.

Camel 98								
								Average
Milking date	24/04/96		29/04/96		02/05/96			
fatty acid	μmol/l	%	μmol/l	%	μmol/l	%		g/l
C14:0	52.8	8.9	44.1	12.1	44.2	10.0		0.011
C16:0	158.2	26.8	110.3	30.4	131.2	29.8		0.034
C16:1	75.1	12.7	38.1	10.5	44.4	10.0		0.013
C18:0	127.4	21.5	60.7	16.7	82.0	18.6		0.026
C18:1	176.5	29.9	109.5	30.1	138.5	31.4		0.040
C18:2								
C18:3								
Total	590.3	100.00	362.8	100.00	440.4	100.00		0.124
Camel 179								
Milking date	22/04/96		24/04/96		29/04/96			
fatty acid	μmol/l	%	μmol/l	%	μmol/l	%		g/l
C14:0	76.3	13.83	61.4	8.44	36.7	9.56		0.013
C16:0	162.9	29.52	143.3	19.67	101.5	26.44		0.035
C16:1	71.6	12.99	216.3	29.70	25.3	6.61		0.027
C18:0	111.2	20.15	121.3	16.65	80.1	20.87		0.030
C18:1	129.8	23.52	164.4	22.57	125.4	32.68		0.040
C18:2			10.9	1.51	6.5	1.70		0.002
C18:3			10.7	1.47	8.2	2.16		0.003
Total	552.0	100.00	728.5	100.00	384.0	100.00		0.149
Camel 180								
Milking date	24/04/96		29/04/96		02/05/96			
fatty acid	μmol/l	%	μmol/l	%	μmol/l	%		g/l
C14:0	50.2	11.85	85.4	11.95	41.9	7.69		0.014
C16:0	107.6	25.36	190.4	26.63	121.5	22.28		0.036
C16:1	106.8	25.18	62.3	8.72	156.5	28.70		0.028
C18:0	62.0	14.63	126.0	17.63	82.3	15.10		0.026
C18:1	87.3	20.59	230.6	32.25	138.3	25.36		0.043
C18:2	10.1	2.38	20.2	2.83	4.6	0.86		0.003
C18:3								
Total	424.3	100.00	715.0	100.00	545.4	100.00		0.149

The internal standards allow the determination of the partition coefficient between the organic and aqueous phases. The use of short chain FFAs as internal standards is not necessary with respect to camel milk, for the reasons mentioned above.

The results from the analysis of nine camel milk samples, three camels milked on three occasions, gave an average value of 141mg/l FFA for camel milk. The individual results may be seen in Table 5 above.This can be compared with the value of 120 mg/l FFA reported by Jong and Badings {42} for cow milk.

The milk samples were collected from camels on the Ol Maisor Ranch in Kenya. A significant difference between the results obtained by Jong C and Badings H {42} for cow milk and the results shown in Table 5 for camel milk is the absence of short chain FFAs in camel milk. As expected the proportions of the individual FFAs reflects the proportion of triglyceride bound FAs in the respective milk types.

Free fatty acids in cheese form during ripening and are precursors of methyl ketones, alkanes, lactones and aliphatic and aromatic esters, all of which are characteristic aroma components in cheeses. Moreover, short chain fatty acids themselves contribute directly to aroma in many aged cheeses. The concentration of free fatty acids increases to a greater or lesser extent during ripening depending upon the intensity of lipolytic activity {84}.

Hard cheeses produced from camel milk in the experimental dairy at Hohenheim University, Stuttgart compared poorly in terms of aroma to the same type of cheese produced from cow milk. This is most probably due to the lack of short chained FFAs present, as discussed above.

6.0 The determination of the fatty acid composition of triglyceride samples

The fatty acid composition of all triglyceride samples was carried out according to the method employing TMSH outlined in Section 17.2.

6.1 Preparation of fatty acid methyl esters using trimethylsulphonium hydroxide (TMSH)

Most procedures for the preparation of fatty acid methyl esters suffer from the fact that they require substantial amounts of sample (over 10 mg) and have many steps, including an extraction.

The major advantages of the method employing TMSH, developed by Schulte and Weber {45}, are that no extraction procedure is necessary and after a short reaction time the solution can be analysed directly without having to remove excess reagent. These factors make it possible to scale down the procedure so that the final volume of the solution is 20 µl and this makes it possible to measure less than 5 µg of a typical FA. Using such a method, a triglyceride fraction separated on an analytical HPLC column (25 cm x 4 mm) can be collected and analysed for its FA composition.

After selection of the TMSH method, a validation was carried out, see Appendix, page 120. The coefficient of linear regression R was better than 0.999 for all the FAs investigated, ranging from C6:0 to C22:0.

To enable the quantification of the FAs in the triglyceride samples, the necessary response factors were determined using a 37 component FAME mix (No. 4-7885 Supelco, USA), see Figure 14 in the Appendix, page 90. The values are displayed in Table 29 in the Appendix, page 111.

6.2 Comparison of the fatty acid compositon of milk fat from cow, goat and camel

In agreement with other authors {2, 85}, the fatty acid composition of cow, camel and goat milk fat have a characteristic pattern for each type of animal (see Chromatograms 15 to 17 in the Appendix, pages 91-92). Table 6 below displays the relative concentrations of the individual fatty acids contained in the three milk types.

Table 6: The fatty acid composition of cow, goat and camel milk fat (mole %).

Fatty acid	Cow milk fat	Goat milk fat	Camel milk fat
C4:0	6.0	3.2	-
C6:0	2.5	2.3	0.2
C8:0	2.0	3.5	-
C10:0	4.0	12.0	0.3
C11:0	0.1	0.2	-
C12:0	4.4	5.8	1.2
C13:0	0.1	0.5	0.6
C14:0	12.7	14.1	11.3
C14:1	1.2	0.3	0.8
C15:0	1.4	1.2	1.7
C15:0	0.3	0.3	0.5
C16:0	28.5	25.1	29.3
C16:1	1.8	0.9	8.5
C17:0	0.6	1.3	1.2
C17:1	0.4	-	1.1
C18:0	11.7	7.9	11.7
C18:1	19.3	17.8	28.0
C18:2	2.9	3.4	3.6
C18:3	0.1	0.2	-

The relative quantities of saturated, monounsaturated and polyunsaturated fatty acids in cow, goat and camel milk fat, displayed in Table 7 below, shows that camel milk fat contains a higher proportion of monounsaturated fatty acids. This is confirmed by the iodine number, which has a value of 49 for camel milk fat and 25-28 for cow milk fat {2}. The iodine number is a value which reflects the number of double bonds contained in the respective fat type.

Table 7: Relative quantities of saturated, monounsaturated and polyunsaturated fatty acids in cow, goat and camel milk fat.

Fatty acid type	cow milk fat	goat milk fat	camel milk fat
saturated	74.0	77.1	57.5
monounsaturated	23.0	19.3	38.9
polyunsaturated	3.0	3.6	3.6

The viscosity of the fat is influenced strongly by the proportions of short chain and unsaturated fatty acids. From Table 6 it can be deduced that goat, cow and camel milk fat contain 27.5, 19.1 and 2.3%, respectively, of fatty acids with a chain length less than C14. The latter is largely responsible for camel milk fat having a significantly higher melting point (41.4°C) compared to cow milk fat (28-38°C) {2, 25}. Although the high proportion of unsaturated fatty acids in camel milk has the effect of reducing the melting point, the low level of short chain fatty acids appears to be the dominating factor.

Experimental work at Hohenheim University in the production of hard cheeses from camel milk has revealed that the cheese produces an unpleasant fatty taste in the mouth. Because the melting point of the fat is above that of the body temperature (37°C), it cannot melt in the mouth and thus produces a fatty film in the mouth. Butter produced from camel milk has proved to possess similar properties.

A disadvantage of unsaturated fatty acids is their susceptibility to oxidation by air (oxygen), catalyzed by light. Technological work carried out at Hohenheim University has shown that hard cheeses produced from camel milk rapidly develop a taste of oxidation on the exposed surface after having been sliced. After a week of exposure the cheese is rendered inedible.

6.3 The fatty acid composition of camel hump fat

Below in Table 8 are the fatty acid compositions of camel hump, beef and porcine fat. In all fat types the three main FA components are palmitic, stearic and oleic acid.

In Table 9 below it is clearly demonstrated that camel hump fat contains a higher proportion of saturated fatty acids than fats from other sources, as previously reported {61}. This partially accounts for the fact that camel hump fat has a higher melting point than, for example, porcine fat. Porcine fat differs from the other fat types in that it contains high amounts of linoleic acid, which would contribute to a lower melting point of the fat. It is common knowledge that pig fat is the most widely used fat in meat products, mainly due to its organoleptic qualities.

Table 8: The fatty acid composition of camel hump fat, beef fat and porcine fat (mole %).

Fatty acid	Camel hump fat		Beef fat	Porcine back fat
	Experimental	Lit. {62}	Lit. {22}	Lit {86}
C6:0	0.3			
C8:0	0.2			
C10:0	3.6			
C11:0	0.6			
C12:0	0.5	0.4		
C13:0	0.2			
C14:0	6.3	6.3	4	1.6
C14:1	1.1	1.9	1	
C15:0	1.7	1.4	2	
C15:1	0.4			
C16:0	33.8	30.0	28	29.1
C16:1	1.5	4.0	5	2.5
C17:1	0.7	0.5		
C18:0	25.9	24.9	20	18.0
C18:1	18.1	26.5	34	40.7
C18:2	0.5	1.8	3	8.1
C18:3	0.8		2	
C20:0	0.5			
C20:1	0.5			
C22:0	0.6			
C20:5	0.3			
C22:6	0.3			
Other FAs	1.6	2.3	1	0

Table 9: The proportion of saturated to unsaturated fatty acids in camel hump fat, beef fat and porcine fat (mole %).

Fatty acid	Camel hump experimental	Camel hump Lit. {62}	Beef fat Lit. {22}	Porcine fat Lit. {86}
Saturated	74.2	63.0	54	48.7
Unsaturated	24.2	34.7	45	51.3

7.0 The use of high temperature GC for the analysis of triglycerides

Figure 31 (page 100) represents a high temperature GC chromatogram of an equimolar triglyceride standard (SSS), whose composition is given in Table 23 (page 67). Using such a standard it was possible to determine response factors for the individual triglycerides, enabling the quantitative analysis of lipid samples.

7.1 The use of high temperature GC for the determination of the distribution of triglycerides according to their CN in cow, goat and camel milk fat, pig back fat and camel hump fat

Following extraction (Section 16.0) and isolation (Section 16.3) of the triglycerides, samples were subjected to high temperature GC according to the parameters given in Section 18.0.

The distribution of triglycerides in terms of CN for cow, goat and camel milk fat, pig back fat and camel hump fat is shown in Table 10 below. The respective chromatograms (excluding pig back fat), Figures 18-21, may be seen in the Appendix, pages 93-94.

The results in Table 10 below show that in camel milk fat the main triglyceride fractions range from CN = 49 to 55. In cow and goat milk fat the triglyceride fractions are distributed over a much broader range i.e. CN = 37 to 55 and the main fractions are CN = 39 to 41 and CN = 41 to 45 respectively. The variations in CN with reference to the triglyceride fractions in the five fat types is due to the variations in fatty acid composition (see Sections 6.2 and 6.3).

The main triglyceride fractions for pig back fat and camel hump fat range from CN = 53–57 and 51-57 respectively. Although the average CN of the triglycerides in pig back fat is slightly higher than that of camel hump fat, its melting point is nevertheless significantly lower due to a higher proportion of unsaturated fatty acids, see Table 9 above.

Table 10: The relative distribution (mole %) of triglycerides in cow, goat and camel milk fat, camel hump fat and pig back fat according to their CN. (1 = Jersey, 2 = Holstein, ** = other triglycerides)

CN	Pig back fat	Camel hump fat	Cow milk fat			Goat milk fat		Camel milk fat
	Expt.	Expt.	Expt.	Literature {85}		Expt.	Literature {85}	Expt.
				(1)	(2)			
29						0.6	0.3	
31			0.9	0.5	0.5	1.4	0.8	
33			1.8	1.0	0.7	2.7	1.7	
35			3.3	2.2	1.3	4.3	2.6	
37			5.1	7.3	3.5	7.7	2.9	1.4
39			10.9	14.4	9.4	9.5	5.3	2.1
41			12.1	13.7	15.9	11.8	10.7	1.4
43			9.6	9.3	11.6	13.8	12.8	0.6
45			7.7	7.0	5.7	12.3	9.3	1.1
47		0.6	6.4	6.7	3.6	9.5	7.8	3.4
49	0.5	4.4	7.3	7.2	3.8	6.3	5.8	8.6
51	2.3	14.4	8.1	8.2	5.6	5.0	2.7	16.8
53	15.1	27.8	9.2	8.2	10.9	5.0	6.4	22.9
55	66.2	28.1	7.6	5.1	14.7	3.9	12.7	20.7
57	15.9	11.8	4.5	1.0	0.5	1.2	10.5	5.7
**	0	12.9	5.5	8.2	12.3	5.0	7.7	15.3

8.0 Separation of triglycerides using silver-ion chromatography

As described in detail in Section 3.2, silver-ion chromatography enables the separation of triglycerides according to their degree of saturation or isomeric status.

8.1 Separation of cow, camel milk and camel hump fat on a silver-ion column

Following extraction (Section 16.0) and isolation (Section 16.3) of the triglycerides, samples were subjected to silver-ion chromatography (see Section 19.2 for chromatographic conditions).

Using an analytical silver-ion column it was possible to separate samples of cow milk, camel milk and camel hump fat into different classes of triglycerides, see Figures 22-24 in

the Appendix, pages 95-96. Integration of these chromatograms made it possible to deduce the proportion of the main fractions in the total fat, see Table 11 below.

Isolation of the peaks followed by gas chromatographic analysis enabled determination of their FA composition, hence their identification.

Using the preparative silver-ion column, 300-400 mg (cow milk fat, camel hump fat) or 800 mg (camel milk fat) of purified fat extract was dissolved in sufficient dichloromethane to give a concentration of 16 mg/150 µl, which was injected onto the column. After 20-25 runs for cow and camel hump fat or 50 runs for camel milk fat, the pooled fractions were isolated from the solvent using a rotary evaporator and weighed; the results are presented in Table 11 below.

Table 11: Yields and % of the major TG classes in cow, camel milk fat and camel hump fat which were isolated using a preparative silver-ion column.

	Cow milk fat		Camel milk fat		Camel hump fat	
Triglyceride Fraction	Yield (mg)	% total fat	Yield (mg)	% total fat	Yield (mg)	% total fat
SSS	100.0	47.6	204.5	44.0	82.3	39.4
SSM(cis)	63.5	30.2	87.9	18.9	63.0	30.1
SMM(cis,trans)	3.7	1.8	11.3	2.4	21.8	10.4
SMM(cis,cis)	18.9	9.0	71.8	15.4	13.7	3.4
Total	189.0	88.6	375.5	80.9	180.8	83.3

The results show clearly that the main TG classes in the above fats are SSS and SSM, which account for 62.9 %, 69.5 % and 77.8 % of total TG in camel milk, camel hump and cow milk fat, respectively. The main single fraction is SSS, which makes up 39.4 - 47.6% of the fat. This is characteristic for animal based fats and differentiates them from most plant fats. The third most significant fraction in the above fats is SMM, 10.8%, 13.8% and 17.8% in cow milk, camel hump and camel milk fat respectively. The higher proportion of SMM in camel milk fat can be traced to the higher proportion of unsaturated fatty acids compared to cow milk fat (see Table 7, page 48).

Analysis of the isolated fractions using gas chromatography enabled computation of their FA composition. The results are shown in Table 12 below.

Table 12: The composition of the isolated triglyceride peaks in Table 11 in terms of saturated, monounsaturated, diunsaturated fatty acids. * = polyunsaturated FAs

	Cow milk fat Fatty acid classes %				Camel milk fat Fatty acid classes %				Camel hump fat Fatty acid classes %			
Triglyceride	S	M	D	*	S	M	D	*	S	M	D	*
SSS	97.3	2.4	0.3	-	96.9	3.1	-	-	95.0	4.5	0.4	0.1
SSM(cis)	60.3	38.8	0.4	0.5	70.8	29.2	-	-	65.3	34.0	0.6	0.1
SMM(cis, trans)	35.3	64.3	0.4	-	41.8	58.2	-	-	33.4	65.3	0.6	0.1
SMM(cis,cis)	30.8	68.2	0.9	0.1	37.2	62.8	-	-	37.5	61.5	0.7	0.3

8.2 Separation of triglycerides in plant based oils using a silver-ion column

To demonstrate the ability of silver-ion chromatography to separate a broad range of oils or fat types, a selection of plant based oils/fats were selected and analysed.

Olive oil, thistle seed oil, sunflower oil and coconut fat were purified as described in Section 16.3, dissolved in dichloromethane and analysed on an analytical silver-ion column. Figures 25-28 in the Appendix, pages 97-98, display the triglyceride classes in each of the samples. Table 13 below displays the fatty acid composition of the above fats, their composition can readily be associated with the triglyceride classes they constitute, Figures 25-28.

Figure 28 displays the triglycerides in coconut fat and Table 13 below shows their composition. This is an example showing that in some cases, plant based fats may contain a higher proportion of saturated fatty acids than animal based fats.

Table 13: The fatty acid composition of coconut fat, olive oil, sunflower seed oil and thistle seed oil (mole %).

Fatty acid	Coconut fat	Olive oil	Sunflower seed oil	Thistle seed oil
C6:0	1.1			
C8:0	12.4			
C10:0	8.0			
C12:0	48.6	0.3		
C14:0	15.7	0.1		0.1
C16:0	6.6	11.8	5.9	7.4
C16:1		0.9		0.1
C18:0	2.7	2.9	3.9	2.5
C18:1	4.0	73.7	22.4	12.3
C18:2	0.9	8.0	66.1	76.6
C18:3		0.7	0.4	0.2
C20:0		0.5	0.3	0.4
C20:1		0.3	0.2	
C20:3				
C20:5		0.8		
C22:0			0.5	0.2
C24:0			0.2	0.1
C24:1				0.1

9.0 Separation of triglycerides using reversed phase chromatography

Below, Figure 9 is an example of a chromatogram of a TG standard of the class SSS (the CN includes the three carbon atoms in the glycerol structure). For reasons explained in Section 3.1, it is important to ensure that the TG class under investigation is homogenous in terms of saturation, otherwise an overlapping of peaks having identical CNs may occur.

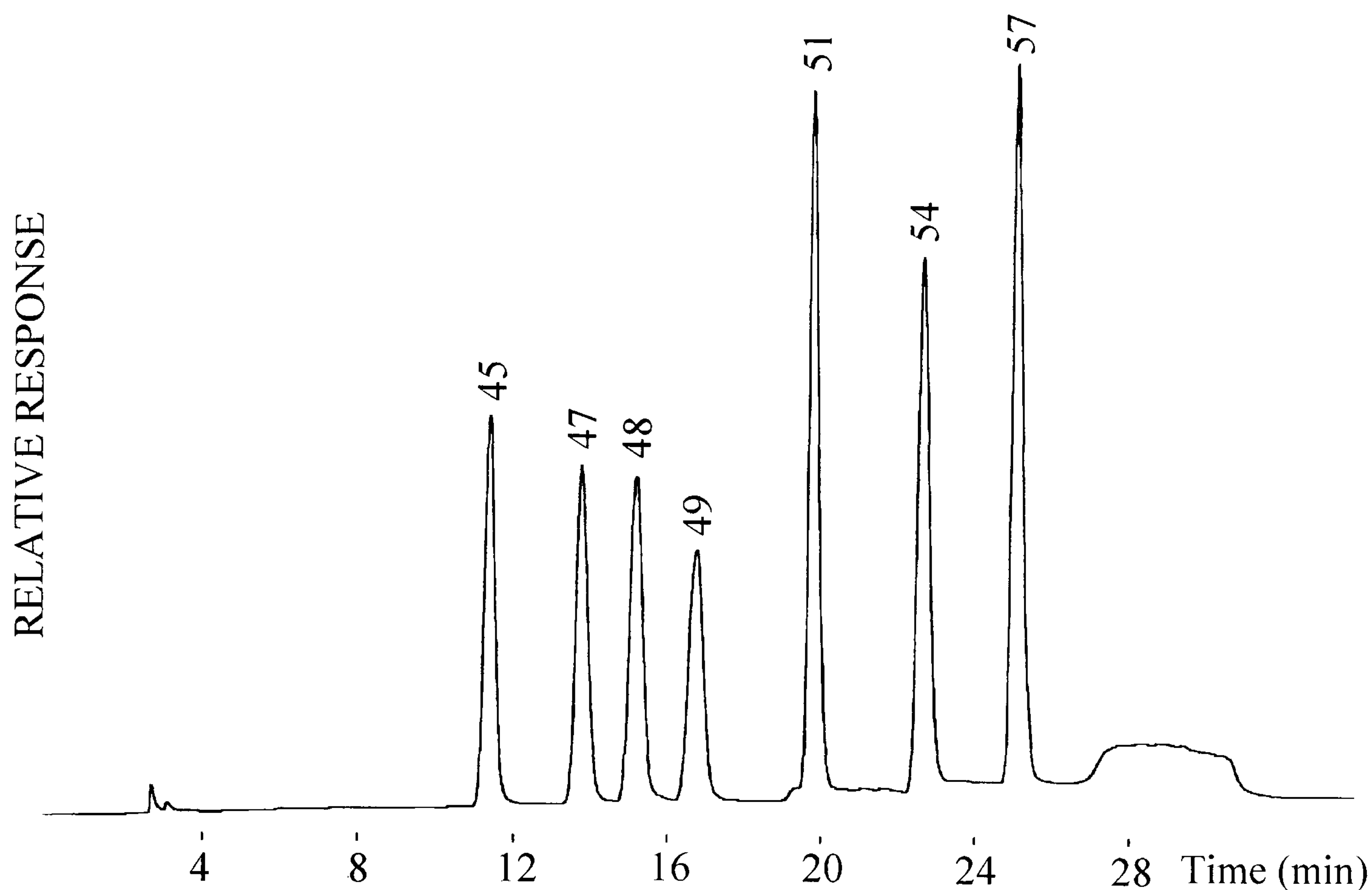


Figure 9: HPLC chromatogram (20 µl) of a 20 mmol/l equimolar TG standard (SSS) on a Nucleosil 100 C18 5 µm preparative column (250 x 8 mm), see Section 19.3 for chromatographic conditions.

9.1 Separation of silver-ion fractions of cow, camel milk fat and camel hump fat using preparative reversed phase chromatography

Using a Nucleosil 100 C18 5 µm (250 x 8 mm) preparative column it was possible to separate TG classes, e.g. SSS, SSM and SMM, into TG sub fractions according to their CN, see Tables 30-32 in the Appendix, pages 112-119. The maximum loading per run was approximately 6 mg; thus a 80 mg fraction could be separated over approximately 15 runs. Below, Figure 10 is a chromatogram of the triglyceride class SSS from camel hump fat which was isolated on a preparative silver-ion column. The fractions CN = 49-55 were isolated.

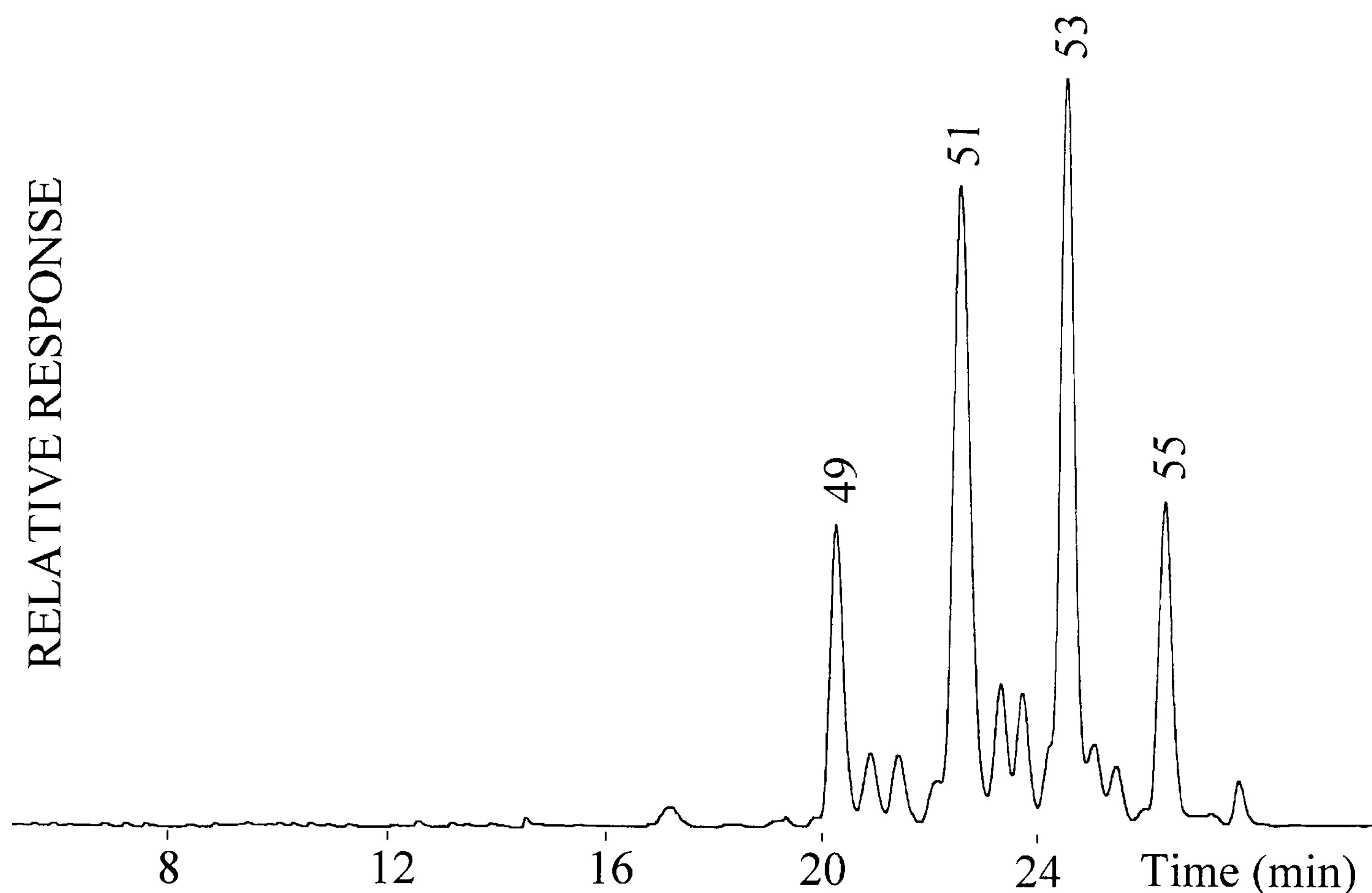


Figure 10: A chromatogram of the TG fraction SSS of camel hump fat (6mg) on a Nucleosil 100 C18, 5 µm preparative column (250 x 8 mm); see Section 19.3 for chromatographic conditions.

The triglyceride classes isolated on the silver-ion column were subjected to high temperature GC-analysis to determine the single triglyceride sub-fractions in terms of CN and their relative proportion with respect to the total fraction. Figures 29 and 30 in the Appendix, page 99, show the analysis of the fraction SSM(cis) in camel milk fat using high temperature GC analyis and RP HPLC. From the chromatograms it is evident that the method employing high temperature GC provides a better resolution of the sub-fractions. Thus the latter provides a convenient and accurate method to enable the identification and quantification of the sub-fractions within the triglyceride class, e.g. SSM(cis) camel hump fat. The results in Table 14 below show that the quantification of triglyceride sub-fractions determined using high temperature GC analysis or deduced from weighing the fractions collected using preparative RP HPLC lead to similar results.

Table 14: A comparison of the quantification of the isolated fractions in camel milk fat using high temperature GC analysis and by weighing the fractions collected on the RP preparative HPLC column. The yields are the quantities isolated.

Triglyceride fraction (CN)	% from GC chromatogram	% from weighing collected fraction	Yield (mg) (collected fraction)
SSS			
47	10.3	14.6	3.05
49	27.9	27.8	5.78
51	24.7	21.5	4.48
53	16.9	15.8	3.30
Total	79.8		
SSM (cis)			
49	19.8	20.6	4.96
51	31.8	31.6	7.59
53	22.9	22.6	5.43
55	8.3	8.0	1.91
Total	82.8		
SMM (cis,cis)			
51	18.9	14.5	2.14
53	36.6	34.7	5.11
55	27.4	34.2	5.04
57	4.6	4.1	0.6
Total	87.5		

The proporton of the main sub-fractions with respect to the total fraction was determined using high temperature GC analysis and not HPLC, as a light scattering detector displays poorer linearity in terms of response than an FID. Hence it was deduced that the total weight of the isolated fractions in TG class SSS (16.61 mg) represented 79.8% of the total SSS.

The above method was adopted for all further calculations with respect to the quantification of the sub-fractions. The proportion of each of the main triglyceride sub-fractions in camel milk fat in terms of g/100 g fat are shown in Table 15 below.

Table 15: The proportion of each isolated triglyceride sub-fraction in camel milk fat in terms of g/100 g fat.

Triglyceride fraction (CN)	Sub-fraction according to CN (g/100 g fat)	Whole fraction (g/100 g fat)
SSS		44.0
47	6.4	
49	12.2	
51	9.5	
53	7.0	
SSM(cis)		18.9
49	3.9	
51	6.0	
53	4.3	
55	1.5	
SMM(cis,cis)		15.4
51	2.2	
53	5.3	
55	5.3	
57	0.6	
Total	64.2	78.3

All the isolated fractions were tested with high temperature GC analysis for purity in terms of contamination with other triglyceride fractions possessing a different CN. Figure 32 in the Appendix, page 100, is a chromatogram of the triglyceride fraction CN = 55, SSS from camel hump fat. All the fractions collected had a purity exceeding 90 %. The main risk of contamination was from fractions with a lower CN; this could largely be avoided by not overloading the RP-18 preparative column.

10.0 Positional analysis of triglycerides using lipase

Experimental work was conducted to determine the free fatty acids generated, using commercially available triglyceride standards and lipase *pseudomonas sp.* Analysis of the free fatty acids showed that the fatty acid composition remained constant and was representative of the fatty acids in the *sn*-1(3) position.

Experimental work was conducted to optimise the lipase concentration in the buffer solution and the incubation time in order to obtain a sufficient concentration of FFAs, representative of the *sn*-1(3) position.

1.5 mg of the standards C 14/16/16 and C14/14/16 were dissolved in 1 ml hexane and added to 750 µl buffer containing 1 and 10 µg/ml lipase, respectively. After reaction times of 0.5, 1.0, 1.5, 2.0 and 2.5 hrs. the reaction was stopped by the addition of HCl; the FFAs generated were isolated by means of TLC and analysed using gas chromatography.

Gas chromatographic determinaton of the FA composition of the sample prior to enzymatic splitting and the FFAs isolated on the TLC plate enabled the deduction of the proportion of the respective FAs in the *sn*-1(3) position.

The triglyceride C17:0/C17:0/C17:0 was used as an internal standard and applied at a concentration of 0.39 µmol/ml; this is equivalent to 1.17 µmol/ml C17:0 as the FA. Because the triglyceride is monoacid, 67 % of C17:0 (as FA) is present in the *sn*-1(3) position.

By measuring the relative concentrations (with respect to C17:0) of the FFAs isolated on the TLC plate, the proportion of the individual FAs in the *sn*-1(3) position may be deduced as follows:

$$X = \frac{Z \times 0.67 \times 100}{Y}$$

X = Proportion of FA in the *sn*-1(3) position (%)

Y = Concentration with respect to internal standard **prior** to enzymatic splitting

Z = Concentration with respect to internal standard **following** enzymatic splitting

Below are the relative proportions of the FFAs C14:0 and C16:0 determined in the *sn*-1(3) positions using buffer containing 1 and 10 µg/ml lipase with respect to differing incubation times (triglyceride standard C 14/16/16).

Table 16: Relative proportions (%) of the FFAs C14:0 and C16:0 determined in the *sn*-1(3) positions using buffer containing 1 and 10 μg/ml lipase with respect to different incubation times (triglyceride standard C 14/16/16)(See Section 20.0 for conditions).

Lipase conc.	FA	0.5 hr	1.0 hr	1.5 hr	2.0 hr	2.5 hr
10 μg/ml	C14:0	33%	30%	33%	34%	34%
	C16:0	67%	70%	67%	66%	66%
1 μg/ml	C14:0	31%	33%	45%	26%	23%
	C16:0	69%	67%	55%	74%	77%

The results in Table 17 below express the results in table 16 in terms of μmol/ml.

Table 17: Relative concentrations (μmol/ml) of the FFAs C14:0 and C16:0, split from the *sn*-1 (3) positions using buffer containing 1 and 10 μg/ml lipase, with respect to differing incubation times (triglyceride standard C 14/16/16)(See Section 20.0 for conditions).

Lipase conc.	FA	0.5 hr	1.0 hr	1.5 hr	2.0 hr	2.5 hr
1 μg/ml	C14:0	0.25	0.13	0.14	0.10	0.15
10 μg/ml	C14:0	0.38	0.35	0.44	0.65	0.62
1 μg/ml	C16:0	0.55	0.27	0.17	0.29	0.49
10 μg/ml	C16:0	0.78	0.81	0.89	1.25	1.23

The results in Table 17 above show that an incubation time of 2.0 hrs and a lipase concentration of 10 μg/ml are the optimum conditions under which to split the FAs in the *sn*-1(3) position. The proportion of C14:0 to C16:0 (and *vice versa*) should theoretically be 50:50. However, based on the following information triglyceride C 14/16/16 also contains C 16/14/16:

1. If the lipase had a preference for either C14 or C16, then the proportion C14 to C16 would change with time. This was not the case with respect to the results for 10 μg/ml lipase in Table 16.

2. If the lipase was splitting FAs in the *sn* 2 position this would generate either monoglycerides or 1,3-diglycerides. Repeated TLC analysis revealed that neither 1,3-diglycerides or monoglycerides were present.

Further investigations of the activity of the lipase with respect to triglyceride standard C 14/14/16 produced the results shown in Table 18 below.

Table 18: Relative proportion of the FFAs C14:0 and C16:0 determined in the *sn*-1(3) positions using buffer containing 10 μg/ml lipase with respect to different incubation times (triglyceride standard C 14/14/16)(See Section 20.0 for conditions).

Lipase conc.	FA	0.5 hr	1.0 hr	1.5 hr	2.0 hr	2.5 hr
10 μg/ml	C14:0	46%	49%	49%	53%	53%
	C16:0	54%	51%	51%	47%	47%

The results in Table 18 show a stable relationship between C14:0 an C16:0, approaching a theoretical ratio of 50:50 for each of the FAs. As for the triglyceride C 14/16/16, no monoglycerides or 1,3-diglycerides were detected after separating the reaction products using TLC.

10.1 Overall positional analysis of the distribution of fatty acids in cow milk fat, camel milk fat, and camel hump fat

The fat samples were extracted and purified according to the methods in Sections 16.0 and 17.3 and then subjected to enzymatic splitting as described in Section 20.2. The results obtained are shown in Table 19 below.

Approximately 50% of medium chain FAs, e.g. C14:0 are located in the *sn*-2 position with respect to cow milk fat. This is supported by other authors {23, 24} and similar results have been obtained for camel milk fat. Longer chained saturated FAs such as C16:0 and C18:0 are found predominantly in the *sn*-1(3) position. C18:1 predominates in the *sn*-1(3) position to a higher proportion in cow milk fat than in camel milk fat. In camel hump fat, C18:1 predominates in the *sn*-2 position; this has been previously reported {62}.

Table 19: The proportion (%) of individual FAs in the *sn*-1(3) positions in cow milk fat, camel milk fat and camel hump fat.

	Cow milk fat					Camel milk fat	Camel hump fat
FA	Expt.	Lit. {92}	Lit. {87}	Lit. {88}	Lit. {89}	Expt.	Expt.
C14:0	48.5	47.1	42.4	46.2	46.3	42.9	51.0
C14:1	44.1	-	52.0	-	-	-	-
C16:0	67.9	54.8	56.3	58.3	58.4	60.7	65.6
C16:1	72.7	50.0	52.8	64.4	64.4	36.6	47.3
C18:0	61.8	52.4	87.9	74.2	74.5	74.1	54.2
C18:1	67.4	73.7	83.0	72.1	72.2	55.5	46.4

10.2 Positional analysis of single triglycerides

Using the conditions in Section 20.2, 1-2 mg of each of the fractions isolated using RP-preparative chromatography were subjected to enzymatic splitting, isolation of the FFAs using TLC and analysis of the methyl ester derivative using gas chromatography.

Below is an example of how the structure of the individual triglycerides within a fraction e.g. camel hump fat, fraction SSM CN=55, may be determined.

The relative mole % of the individual FAs prior to enzymatic splitting was calculated *via* gas chromatographic analysis of the triglyceride fraction prior to enzymatic splitting. In all the calculations C17:0 was used as an internal standard.

FA	Mol %
16:0	28.8
16:1	1.5
18:0	37.2
18:1	32.5

Using the above data it can be deduced that the composition of the possible triglycerides are:

C16:0/C18:0/C18:1 = 95 %

C18:0/C18:0/C16:1 = 5 %

The % of the individual FAs in the *sn*-1(3) position, determined *via* enzymatic cleavage using lipase are as follows:

FA	%
16:0	56
16:1	33
18:1	42

The above results enable the elucidation of the following structures:

For triglyceride composition C18:0/C18:0/C16:1 (5 %)

33 % of C16:1 exists in the *sn*-1(3) position, thus:

C18:0/C18:0/C16:1 = 1.7 %

C18:0/C16:1/C18:0 = 3.3 %

For triglyceride composition C18:0/C16:0/C18:1 (95 %)

58 % of C18:1 exists in the *sn*-2 position, thus:

C18:0/C18:1/C16:0 = 55 %

44 % of C16:0 exists in the *sn*-2 position, thus:

C18:0/C16:0/C18:1 = 41 %

It should be mentioned that, as with the triglyceride standards, no monoglycerides or 1,3-diglycerides were detected upon separation of the reaction products using TLC.

10.3 Positional analysis of triglycerides in cow, camel milk fat and camel hump fat

Using the methods of calculation outlined in Section 10.2 above, it is possible to deduce the structures of the individual triglycerides within the respective triglyceride classes, e.g. SSS, SSM and SMM. The results for camel milk fat, cow milk fat and camel hump fat are displayed in Tables 30-32 in the Appendix, pages 112-119.

Several studies {21, 23, 24} have determined the position of short chain fatty acids in the triglycerides in cow milk fat, 100% C4:0, 93% C6:0 and 63% C8:0 have been found in the *sn*-3 position . These values were used to assist in the determination of the structure of TGs in cow milk fat.

In spite of variations in the triglyceride composition caused by alterations in feeding, Hawke and Taylor claim that the main TG species remain constant {24}. The main TG species in cow and camel milk fat are displayed in Tables 20 and 21 below.

Hawke and Taylor {24} and the results in this thesis support the conclusion that the main TG species in cow milk fat is C4:0/C16:0/C18:1. Other TG species of importance are C4:0/C16:0/C16:0 and C6:0/C16:0/C18:1.

The main triglyceride species in camel milk fat was found to be C14:0/C16:0/C16:0; another major fraction was C16:0/C18:1/C18:1, which was found to be the main fraction in human milk fat by Brühl {3}. Currie G and Kallio H {90} characterized the major triglycerides in human milk fat using ammonia negative ion tandam mass spectrometry and also found C16:0/C18:1/C18:1 to be the main TG fraction.

In Table 20 below are the five main triglycerides in the SSS and SSM fractions in cow milk fat.

Table 20: The five main TGs found in cow milk fat. * One of the major TGs in cow milk fat reported by Hawke and Taylor {24}.

TG	g/100 g fat
4:0/16:0/16:0	5.8
4:0/18:1/16:0	4.0
4:0/16:0/18:1	3.2*
4:0/18:0/16:0	2.8
16:0/16:0/18:0	2.1

Below in Table 21 are the five main triglycerides isolated from the fractions SSS, SSM and SMM in camel milk fat.

Table 21: The five main TGs in camel milk fat.

TG	g/100g fat
16:0/16:0/16:0	5.5
16:0/16:0/18:0	5.1
16:0/14:0/16:0	4.5
14:0/16:0/16:0	4.5
16:0/18:1/18:1*	2.8

* One of the major TGs in cow milk fat reported by Hawke and Taylor {24}.

In Table 22 below are the main TGs in the SSS and SSM fractions in camel hump fat. Shoeb and Osman {62} obtained similar results using different methods.

Table 22: The main TGs determined in the fractions SSS and SSM in camel hump fat.

Fraction SSS Individual TGs	Expt. g/100 g fat	Lit. {62} g/100 g fat	Fraction SSM Individual TGs	Expt. g/100 g fat	Lit{62} g/100 g fat
16:0/16:0/18:0	10.4	5.0	16:0/18:1/18:0	6.4	10.6
16:0/16:0/16:0	7.6	2.6	16:0/18:1/16:0	3.3	5.4
16:0/18:0/18:0	4.0	1.6	18:0/18:1/18:0	3.7	5.3
16:0/14:0/18:0	3.2	1.0	16:0/16:0/18:1	2.8	2.8
14:0/16:0/18:0	2.7	1.2	18:0/16:0/18:1	4.6	2.7
14:0/16:0/16:0	2.3	1.2	14:0/18:1/16:0	0.8	2.4
18:0/16:0/18:0	1.9	2.5			
Other TGs	5.9	9.3		8.1	20.3
Total fraction	38.0	24.4		29.8	49.5

11.0 Mass spectromety for the analysis of triglycerides after separation using HPLC and GC

Mass spectrometry for the analysis of triglycerides after separation using HPLC or GC has been attempted by several authors {48-50, 53, 55, 91}

Triglyceride samples were measured under the conditions given in Section 19.4 for HPLC and 18.1 for GC. Initially a 20 mmol/l triglyceride standard containing fatty acids with zero double bonds was investigated. The composition of the standard is given in Table 23 below:

Table 23: Composition of the equimolar (20 mmol/l) triglyceride standard

Triglyceride	FA composition	CN
Trimyristin	14:0/14:0/14:0	45
1,2-dimyristoyl-3-palmitoyl-rac-glycerol	14:0/14:0/16:0	47
Tripentadecanoin	15:0/15:0/15:0	48
1,2-dipalmitoyl-3-myristoyl-rac-glycerol	16:0/16:0/14:0	49
Tripalmitin	16:0/16:0/16:0	51
Triheptadecanoin	17:0/17:0/17:0	54
Tristearin	18:0/18:0/18:0	57

The results of the analysis of triglyceride standards using HPLC and GC showed that triglycerides made up of saturated fatty acids fragment almost exclusively to give diglyceride ions (see Figures 35-41 for HPLC-MS spectra in the Appendix, pages 101-104).

Triglycerides containing unsaturated fatty acids produce diglyceride and $[M+1]^+$ ions, Figures 42 and 43 (page 105) show the HPLC-MS spectra for oleic and linoleic acid. The spectra clearly demonstrate that the intensity of the $[M+1]^+$ peak increases with the degree of unsaturation.

The mass spectra showed that minimal fragmentation occurs, resulting primarily in diglyceride [M - RCOO]+ ions, and in the case of unsaturated triglycerides, additionally $[M+1]^+$ ions. Please note that the [M - RCOO]+ structures shown in Figures 35-41 are schematic, the cleavage of the RCOO species may occur in the *sn*-1, 2 or 3 position; it is not known that a preference to any particular position exists.

In the case of high temperature GC-MS, methane was used as a reagent gas for chemical ionisation, this also produced the ion species $[RCO]^+$ and $[RCO + 74]^+$ as reported in section 3.6.2, see Figure 44 (page 106) in the Appendix for an GC-MS spectra of trimyristin.

11.1 Mass spectrometry for the analysis of triglycerides in cow, camel milk fat and camel hump fat after separation using reversed phase HPLC

The main triglyceride fractions isolated using silver-ion chromatography for cow, camel milk fat and camel hump fat (see Section 8.1) were subjected to HPLC-MS (see Section 19.4 for conditions). The results complement those obtained *via* positional analysis (see Section 10.3) in the deduction of the main triglyceride components within each sub-fraction, see Tables 30-32 in the Appendix (pages 112-119).

11.2 Mass spectrometry for the analysis of triglycerides in camel milk fat after separation using high temperature GC

A sample of the camel milk fat triglyceride fraction SSS, isolated using preparative silver-ion chromatography, was subjected to high temperature GC-MS (see Section 18.1 for chromatographic conditions).

Identification of the main diglyceride ions ($[M\text{-}RCOO]^+$) in the MS spectra (figures 45-48, pages 107-108) enabled the determination of the composition of the main triglyceride components, see Table 24 below. The results are almost identical with those obtained using HPLC-MS, see Table 31 (page 115) in the Appendix.

Table 24: The main diglyceride fragments from the SSS fraction in camel milk fat.

CN of TG sub fraction	[M-RCOO]$^+$ fragment	m/z of main fragments	Main triglyceride components (see Table 31)
47	14:0/14:0 14:0/16:0 12:0/18:0	496 524	14:0/14:0/16:0 12:0/14:0/18:0
49	14:0/16:0 16:0/16:0	523 551	14:0/16:0/16:0 14:0/14:0/18:0
51	14:0/16:0 16:0/16:0 14:0/18:0 16:0/18:0	523 551 579	14:0/16:0/18:0 16:0/16:0/16:0
53	16:0/16:0 16:0/18:0	551 580	16:0/16:0/18:0

Recent developments have improved the performance of so called low cost `bench-top´ GC-MSs, especially with respect to the mass spectrometer using CI. The rapid advancement in the performance of computers and their software has greatly assisted in improving the speed, ease of use and performance of such equipment. The Perkin Elmer `TurboMass´ MS is such an example, it is now possible to determine molecular weights up to 1200 using CI or EI with improved sensitivity. Such a performance was previously restricted to large expensive equipment in combination with complex software.

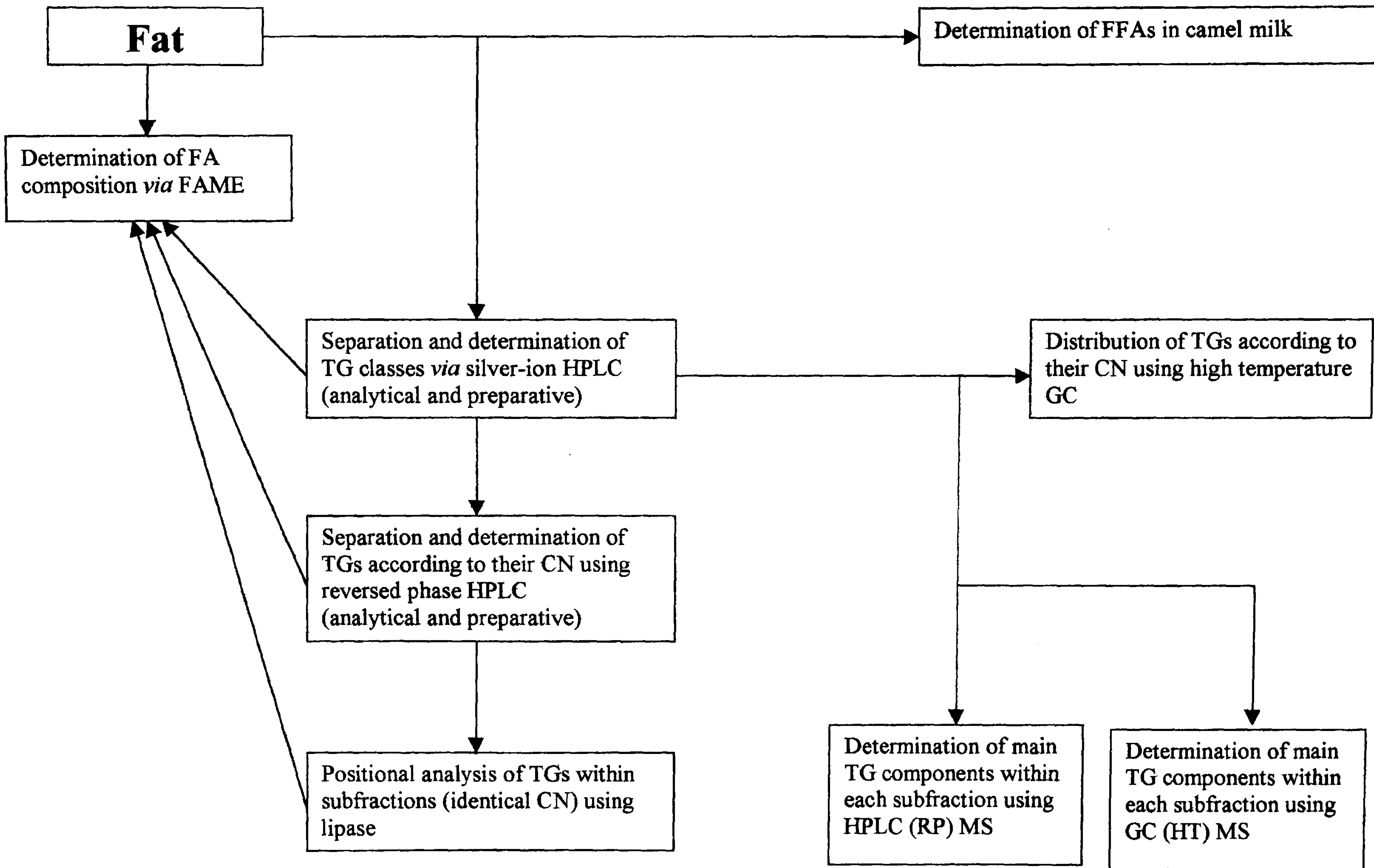
Mass spectrometry in combination with high temperature GC is a useful tool in the analysis of triglycerides. It has the following advantages over HPLC-APCI:

- A high temperature capillary column has a better resolution than an HPLC column.
- It is easier to determine the CN of a triglyceride, regardless of the level of saturation.
- With the help of an autosampler it is easier to automate the analysis and thus achieve a higher throughput of samples.
- It requires smaller amounts of sample, µg instead of mg.

12.0 Conclusions

- The use of silver-ion column chromatography for the separation of triglycerides according to their saturation class followed by reversed phase chromatography to separate triglycerides according to their CN is a powerful tool in the isolation of triglycerides in complex mixtures.
- A micro method for the positional analysis of triglycerides using lipase and approximately 2 mg of sample was successfully developed. This represents a substantial improvement on previous techniques, which require samples of 50 mg {3, 44}.
- High temperature capillary GC is a rapid and accurate method for the determination of the distribution of triglycerides according to their CN. Because no derivatisation is necessary, the method is very simple and requires small amounts of sample.
- Mass spectrometry (CI), especially with reference to high temperature GC can greatly assist in the analysis of the main triglyceride components in complex mixtures and complements results obtained using independent analytical methods, e.g. fatty acid methyl esters.
- The light scattering detector is invaluable for the detection of triglycerides in conjunction with HPLC. Detection systems employing RI or UV are strongly influenced by complex gradient systems; this is not the case with a light scattering detector.
- The above procedures were used in sequence to characterise the triglycerides of camel hump fat. Essentially the same values were found as those obtained using the much more laborious stereospecific techniques {62}.
- The procedures were also used to characterize the triglycerides of camel milk fat for the first time.
- Using a method developed by Jong and Badings {42}it was possible to determine the individual free fatty acids in camel milk for the first time. The results reflect the fatty acid composition of the triglycerides.
- The sequential procedures employed here could be applied to the analysis of a wide spectrum of fats and oils. Further optimisation of the micro deacylation method would also be possible.

A schematic of the analytical work



13.0 Chemicals

The following chemicals are of analytical grade and can be obtained from most suppliers:

Heptane (Fluka, Switzerland, No. 51745)
Sodium hydroxide (Merck, Germany, No. 1.06462.5000)
Sulphuric acid 95-97% (Merck, Germany, No. 1.00713.2500)
Formic acid 98% (Fluka, Switzerland, No. 06450)
Anhydrous sodium sulphate (Merck, Germany, No. 1539.5000)
Acetone (Merck, Germany, No. 1.00014.2500)
2-Propanol (Merck, Germany, No. 1.09634.2500)
Ethanol 96% (Merck, Germany, No. 1.00971.2500)
Anhydrous sodium sulphate (Merck, Germany, No. 106603)
Dichloromethane (Roth, Germany, No. 6053.2)
Glass wool (Assistent, Germany, No. 1408/2)
Diethyl ether (Merck, Germany, No. 1.00926)
Petroleum ether 40-60°C (Fluka, Switzerland No. 77397)
Calcium chloride (anhydrous) (Merck, Germany, No. 2388)
Ammonium acetate (Merck, Germany, No. 1.01116)
Silver nitrate (Merck, Germany, No. 1.01512)
Methanol (Merck, Germany, No. 106009)
Acetonitrile (Merck, Germany, No. 1.14291)

The following chemicals are not readily available from every supplier:

Fatty acid standards:
Butyric acid C4:0 (Fluka, Switzerland, No. 19210)
Valeric acid C5:0 (Fluka, Switzerland, No. 94530)
Caproic acid C6:0 (Fluka, Switzerland, No. 21529)
Pimelic acid C7:0 (Fluka, Switzerland, No. 80500)
Caprylic acid C8:0 (Fluka, Switzerland, No. 21639)

Pelargonic acid C9:0 (Merck, Germany, No. 818791)

Capric acid C10:0 (Fluka, Switzerland, No. 21409)

Lauric acid C12:0 (Fluka, Switzerland, No. 61609)

Tridecanoic acid C13:0 (Fluka, Switzerland, No. 91540)

Myristic acid C14:0 (Alltech, USA, No. FA0140)

Palmitic acid C16:0 (Alltech, USA, No. FA0160)

Palmitoleic acid C16:1 (Alltech, USA, No. FA0161C)

Margaric acid C17:0 (Merck, Germany, No. 398.0005)

Stearic acid C18:0 (Alltech, USA, No. FA0180)

Oleic acid C18:1 (Alltech, USA, No. FA0181C)

Linoleic acid C18:2 (Fluka, Switzerland, No. 62230)

Linolenic acid C18:3 (Fluka, Switzerland, No. 26160)

Arachidic acid C20:0 (Alltech, USA, No. FA0200)

Triglyceride standards:

Tricaproin (C 6:0/6:0/6:0) (Sigma-Aldrich, USA, No. T-0888)

Tricaprylin (C 8:0/8:0/8:0) (Sigma-Aldrich, USA, No. T-9001)

Trilaurin (C 12:0/12:0/12:0) (Sigma-Aldrich, USA, No. T-4891)

Timyristin (C 14:0/14:0/14:0) (Sigma-Aldrich, USA, No. T-5141)

Tripalmitin (C 16:0/16:0/16:0) (Sigma-Aldrich, USA, No. T-5888)

Triheptadecanion (C 17/17/17)(Sigma-Aldrich,USA, No.T-2151)

Tristearin (C 18:0/18:0/18:0) (Sigma-Aldrich, USA, No. T-5016)

Triarachidin (C 20:0/20:0/20:0) (Sigma-Aldrich, USA, No. T-9267)

Tribehenin (C 22:0/22:0/22:0) (Sigma-Aldrich, USA, No. T-7904)

1,2-dipalmitoyl-3-myristoyl-rac-glycerol(C16:0/16:0/14:0)
(Sigma-Aldrich, USA, No. D-3532)

1,2-dimyristoyl-3-palmitoyl-rac-glycerol(C14:0/16:0/16:0)
(Sigma-Aldrich, USA, No. D-3282)

Triolein (C18:1 *cis*-9) (Sigma-Aldrich, USA, No. T-7140)

Trilinolein (C18:2 *cis, cis*-9, 12) (Sigma-Aldrich, USA, No. T-9517)

Silica gel 60 (Merck, Germany, No. 1.07734)

Sea sand (Fluka, Switzerland, No. 84880)

Anhydrous methanol (Fluka, Switzerland, No. 65542)

Amberlite IRA-910, (Fluka, Switzerland No. 06457)

1,1,2-trichloro-1,2,2-trifluoroethane (TCTFE) (Aldrich, USA, No. 17,282-0)

5.4mol/l sodium methoxide solution (Fluka, Switzerland, No. 71748)

LiChrolut® extraction columns, NH_2, 500mg (Merck, Germany, No. 1.19697.0002)

Buffer pH 8.0 (Merck, Germany, No. 10027)

Lipoprotein lipase from *Pseudomonas* sporogenes (Merck, Germany, No. 056389)

14.0 Equipment

14.1 High performance liquid chromatography

The HPLC system used consisted of the following components:

- HPLC pump: Merck-Hitachi 655A-11 (Merck, Germany)
- Injector: Rheodyne 7125 (Rheodyne, USA)
- Pump controller: Merck-Hitachi L-5000 LC gradient pump controller (Merck, Germany)
- Detector: Sedex 45 light scattering detector (Sedex, France)
- Integrator: Shimadzu C-R5A chromatopac (Shimadzu, Japan)
- Sample loops: 20, 50 and 500 µl .

Columns:

Cation exchange columns

- Analytical column: NUCLEOSIL 100-10SA, 250 x 4.6 mm i.d. (Macherey -Nagel, Germany, No. 720028.46)
- 11 mm guard column (Macherey -Nagel, Germany, No. 721378)
- Preparative column: `VarioPrep´ preparative column 100-10SA, 250 x 10 mm i.d. (Macherey-Nagel, Germany)(manufactured upon request)

RP-18 columns:

- Analytical column: NUCLEOSIL 100 C18 5 µm, 250 x 4 mm i.d.
  (Bischoff, Germany: No. 25041835)
- Preparative column: NUCLEOSIL 100 C18 5 µm, 250 x 8 mm i.d.
  (CHROM, Germany)(manufactured upon request)

The column temperature was regulated by a `Desaga Frigostat´ (Desaga, Germany) connected to a self-made column water bath.

The HPLC-MS system used consisted of the following components:

- Solvent delivery system: Applied Biosystems 140b
  (Applied Biosystems, Holland)
- MS detector: Finnigan MAT TSQ 700 fitted with an APCI source
  (Finigan, USA)
- Autosampler: Triathlon (Applied Biosystems, Holland)

14.2 Gas chromatography

The gas chromatographic system for the analysis of fatty acid methyl esters comprised the following components:

- Gas chromatograph: HRGC 5160 Mega Series (Carlo Erba, Milan, Italy) fitted with a FID and autosampler. Quarz capillary column SP-2330 (nonbonded; poly (80% biscyanopropyl / 20% cyanopropylphenyl siloxane)) 30 m long, 0.32 mm i.d., 0.20 µm film thickness (Supelco inc, USA)
- Integrator: Perkin-Elmer 1020 (Perkin-Elmer, USA)
- Printer: Epson LQ-570+ (Epson, Japan)

The gas chromatographic system for the analysis of triglycerides using an FID as the detector comprised the following components:

- Gas chromatograph: Sigma 2000 with FID (Perkin-Elmer, USA)
- Quartz capillary column: DB1-HT (dimethylpolysiloxane)15 m x 0.32 mm i.d., 0.10 μm film thickness (J & W Scientific, USA)
- Integrator: Perkin-Elmer 1020 (Perkin-Elmer, USA)
- Printer: Epson LQ-570+ (Epson, Japan)

The gas chromatographic system for the analysis of triglycerides using a mass spectrometer (CI) as the detector comprised the following components:

- Gas chromatograph: AutoSystem XL with integrated autosampler (Perkin-Elmer, USA)
- Detector: TurboMass mass spectrometer (CI), (Perkin-Elmer, USA)
- Quarz capillary column: PE1-HT(dimethylpolysiloxane)15 m x 0.25 mm i.d., 0.10 μm film thickness (Perkin-Elmer, USA)
- Integrator: TurboMass software installed on a Pentium 300 MHz PC (Perkin-Elmer, USA)

The gas chromatographic system for the analysis of free fatty acids using a mass spectrometer (EI) comprised of the following components:

- Gas chromatograph: Shimadzu GC-17A (Shimadzu, Japan) attached to a QP-5000 mass spectrometer (Shimadzu, Japan).
- Quarz capillary column: FFAP (polyethyleneglycol-nitroterephthalic acid ester) 25 m x 0.32 mm i.d., 0.30 μm film thickness (Chrompack, Netherlands).
- Integrator: PC with installed QP-5000 software (Shimadzu, Japan)

14.3 Thin layer chromatography

The following equipment was used for all TLC work:
- Sample applicator: Camag Nanomat III (Camag, Switzerland)
- Capillary dispenser with 2 μl capillary tubes (Camag, Switzerland)
- TLC plates: 20 x 20 cm Silica gel 60 (No. 1.05721, Merck, Germany)

Additional equipment

- Water bath with agitator, model 1083 (GFL, Germany)
- Analytical balance, model 1712 (Sartorius, Germany)
- Evaporating block with N_2 gas stream, Reacti-Therm model 18790 (Pierce, USA)
- Rotary evaporator, Büchi RE-111 with water bath (Büchi, Switzerland)
- Vacuum pump (Vacuubrand, Germany)
- 1.5 ml Micro Test Tubes 3810 (Eppendorf, Germany)
- N11 HPLC vials (No. 11020100C, Bischoff, Germany)
- Centrifuge capable of 2000 g, for use with Micro Test Tubes
- UV lamp (254 nm)
- Rodamine 6 G (0.1% in methanol)
- Ultra Turrax, type 18/10 (Janke & Kunkel, Germany)
- TLC developing tank

15.0 Free fatty acids

The following methods are related to the determination of free fatty acids in camel milk.

15.1 Extraction of free fatty acids in camel milk

To 10 ml of milk (or reconstituted milk from milk powder), 1 ml H_2SO_4 (2.5 M), 10 ml EtOH (96%), 15 ml diethylether/heptane (1:1 v/v) and 0.5 μmol margaric acid **** were added, the latter as an internal standard to determine the recovery. The mixture was shaken at room temperature for 15 seconds and then centrifuged at 2000 rpm for two minutes, the upper layer was separated. The extraction was repeated twice using 10 ml diethylether/heptane (1:1 v/v), the pooled fractions were dried using 1 g anhydrous Na_2SO_4.

Ready made 500 mg aminopropyl columns were conditioned with 10 ml of heptane. The lipid extract was applied to the column. Lipids such as di- and triglycerides are not retained by the column, free fatty acids however are retained and subsequently eluted using 5 ml diethylether containing 2 % formic acid. The solvent was removed using a stream of

nitrogen after which 200 µl of 2-propanol/heptane (1:1 v/v) containing 2 % formic acid and 0.2 µmol of tridecanoic acid *** as an internal standard for quantification were added. 1 µl samples were then gas chromatographed . The formic acid is added to ensure that the free fatty acids are present in a protonated form for extraction and gas chromatography.

*** 40 µmol tridecanoic acid (C13:0) was dissolved in 10 ml 2-propanol/heptane (1:1 v/v), 50 µl was pipetted as internal standard.

**** 50 µmol margaric acid (C17:0) was dissolved in 10 ml 2-propanol/heptane (1:1 v/v), 100 µl was pipetted as internal standard.

15.2 Preparation of a free fatty acid standard

Amounts of individual standards, shown in Table 26 in the Appendix, page 106, were weighed out using an analytical balance and dissolved in 2-propanol/heptane (1:1 v/v). The quantities indicated were removed from each standard solution using a suitable pipette and pipetted into a 10 ml volumetric flask. The final volume was made up to 10 ml with 2-propanol /heptane (1:1 v/v).

The contents of the 10 ml volumetric flask were divided into 2.5 ml brown glass GC sample vials which were stored at -18°C. Prior to storage the bottles were gassed with helium to inhibit possible degradation of the standard whilst in storage.

Prior to use, the sample bottle was removed from the deep freeze and allowed to reach room temperature, the contents of the bottle were inspected to ensure that the fatty acids were completely dissolved. 50 µl of the standard solution were removed and pipetted into a 500 µl brown glass GC sample vial, to which 10 µl of a 10% solution of formic acid in heptane were added. 1 µl aliquots were subjected to gas chromatography.

15.3 Determination of the concentration of free fatty acids

The individual peak areas (Table 27, page 110) with reference to to the FFA standard (Section 15.2) were normalised with respect to the internal standard (C13:0). After

calculating the mean value over 5 runs, response factors of the individual fatty acids with respect to C13:0 (response factor = 1) were determined (Table 28, page 110).

The concentrations of the individual fatty acids in the samples were calculated by implementing the following formula with respect to the integrated peak areas.

$$C_{FS} = ((F_{FS} \times f_{RFS}) / (F_{IS1} \times f_{RIS})) \times (100 / W) \times C_{IS1}$$

$$W = ((F_{IS2} \times 2.5) / (F_{IS1} \times f_{RIS})) \times 100$$

C_{FS} Concentration of fatty acid (µmol)

F_{FS} Peak area of fatty acid

F_{IS1} Peak area of internal standard C17:0

F_{IS2} Peak area of internal standard C13:0

C_{IS1} Concentration of internal standard C17:0 (µmol)

f_{RFS} Response factor of fatty acid with reference to internal standard C13:0

f_{RIS} Response factor of internal standard C17:0 with reference to internal standard C13:0

W Rate of recovery (%)

15.4 Gas chromatographic conditions for the determination of free fatty acids

The conditions given below were used for the determination of free fatty acids.

Temperature programme:

Temperature (°C)	115	240
Time (min)	6.0	20.0
Temperature rate (°C/min)	10.0	-

- Carrier gas: helium
- Injector temperature: 270°C
- Interface temperature: 250°C

- Split: 1:1.
- Injection volume: 1 µl.
- Sampling time: 1 min
- Ionisation energy (EI): 70 eV

16.0 Lipid extraction

16.1 Milk samples

To 10 ml of milk (or reconstituted milk from milk powder), 10ml ethanol (96%) and 15 ml diethyl ether/heptane (1:1 v/v) were added. The mixture was shaken at room temperature for 15 seconds, centrifuged (2000 g) for two minutes and then the upper layer was separated. The extraction was repeated twice using two 10 ml portions of 10 ml diethyl ether/heptane (1:1 v/v), the pooled fractions were dried using 1g anhydrous sodium sulphate. The solution was then decanted and the solvent mixture was removed using a rotary evaporator under vacuum, leaving a lipid residue.

16.2 Fat samples

5 g of camel hump fat (dromedary) were cut into small pieces and added to 50 ml dichloromethane in a suitable vessel for the use of an ultra-turrax. Using the ultra-turrax the small fat pieces were reduced to a fine suspension which was then filtered using a medium fine filter paper in a filtering funnel, after which the dichloromethane was removed by means of a rotary evaporator to leave a lipid residue.

16.3 Isolation of triglycerides from lipid extract

The glass tube was blocked at one end using glass wool and then partially filled with a mixture of 2.5 g silica gel* suspended in 8 ml solvent mixture (petroleum ether / diethyl ether 87:13 % v/v). Care had to be taken to avoid the inclusion of air bubbles. Once the silica gel had settled 0.3 g of sea sand** was added to cover the surface of the silica gel. 250 mg of the isolated lipid extract dissolved in 2 ml of the solvent mixture was applied to

the column. The non-polar components, in this case triglycerides, were eluted using 20 ml of the same solvent mixture which was allowed to pass through the column at a rate of 1 drop per second. Polar components such as mono-, di-glycerides and phospholipids are retained on the column. The triglycerides were isolated from the solvent using a rotary evaporator, leaving a pure triglyceride extract. The extract was assessed for purity using the TLC method in Section 20.3. The above procedure was scaled up for larger quantities of fat extract.

* The silica gel is heated to 600°C for 1 hour and allowed to cool in a desiccator. After cooling 5% (w/w) water is added, the mixture is shaken and allowed to stand over night in a sealed container, the silica gel is now activated.

** Sea sand heated to 600°C over night.

17.0 Preparation and analysis of fatty acid methyl esters

17.1 Preparation of trimethylsulphonium hydroxide solution (TMSH)

A glass column (2 x 20 cm) fitted with a tap and blocked at one end with glass wool was filled with 35 ml (20 g) Amberlite IRA-910 ion-exchange material suspended in water. 150 ml NaOH solution (40 g/l) was allowed to pass over the column, followed by 150 ml water (until the eluent was neutral) and finally 100 ml methanol.

4.1 g Trimethylsulphonium iodide was dissolved in 60 ml methanol heated to 50°C in a water bath. 10 ml portions of the solution were allowed to pass over the column, which was warmed externally using a hair dryer to prevent crystallisation of the trimethysulphonium iodide in the column. Finally the column was washed with 40ml of methanol, the eluent was collected in a bottle and stored at 4°C (shelf-life 3-6 months).

17.2 Preparation of fatty acid methyl esters using TMSH

1mg fat was dissolved in 50 µl TCTFE, 25 µl TMSH solution was added and the solution was shaken and can then be injected. The method can also be applied to proportionally

larger quantaties of fat. The life of the GC column is not reduced through the use of the TMSH reagent if the reagent salts are pyrolysed at a temperature above 250°C. Thus the temperature of the injector should not be less than 250°C.

17.3 Preparation of fatty acid methyl esters using sodium methoxide

10 mg purified triglyceride extract was dissolved in 1 ml petroleum ether, to which 20-50 µl sodium methoxide solution* was added. The mixture was shaken for approximately 30 sec., after which approximately 100 mg calcium chloride was added and the mixture was centrifuged in a Micro Test Tube (2000 g). The supernatant was then ready for injection.The calcium chloride serves to hinder the formation of sodium glycerate crystals and to remove the small amount of methanol which is present in the mixture.

* 5.4 mol/l Sodium methoxide solution was diluted with dry methanol to give a 2.0 mol/l solution.

17.4 Gas chromatography of fatty acid methyl esters

The methyl esters of fat samples were prepared according to Section 17.2 or 17.3 and then analysed under the following conditions.

- Temperature programme:

Temperature (°C)	70	170	185	220
Time (min)	1.0	5.0	0	4.0
Temperature rate(°C/min)	12.0	1.5	15.0	-

- Carrier gas: 2.1 ml/min hydrogen (linear velocity 43 cm/sec.)
- Injector temperature: 260°C
- Detector temperature: 320°C
- Injection volume: 1µl
- Split injector

18.0 Analysis of triglycerides using high temperature gas chromagtography

This is a direct method and hence no derivatisation procedure is necessary: the triglyceride sample is simply dissolved in dichloromethane and injected directly under the conditions given below.

- Temperature programme:

Temperature (°C)	230	380
Time (min)	1.0	2.0
Temperature rate (°C/min)	5.0	-

- Carrier gas: 1 ml/min nitrogen (linear velocity 20cm/sec)
- Injector temperature: 380°C
- Detector temperature: 420°C
- Injection volume: 1 μl
- Split injector

18.1 Analysis of triglycerides using high temperature gas chromagtography and mass spectrometry (CI)

- Temperature programme:

Temperature (°C)	230	400
Time (min)	1.0	4.0
Temperature rate (°C/min)	10.0	-

- Carrier gas: helium with flow programme (ml/min):

Flow (ml/min)	0.4	1.5	3.0
Time (min)	-	-	20
Rate of change (ml/min)	0.1	0.2	-

- Injection volume: 0.2 μl
- Split injector
- Reagent gas for CI: methane

19.0 High performance liquid chromatography

Figure 11 below is a schematic diagram of the HPLC system used for all work, except that of HPLC-MS measurements. For analytical work the T-piece was removed.

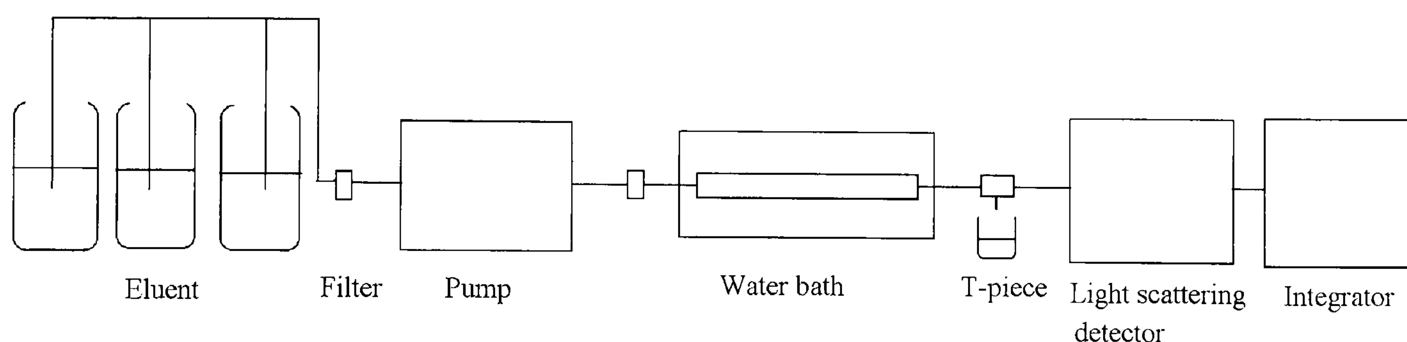


Figure 11: A diagramatic representation of the HPLC system used.

19.1 Preparation of silver-ion columns

Analytical column

An EC 250/4.6 NUCLEOSIL 100-10SA analytical column from Macherey-Nagel together with a 11 mm guard column filled with the same material were eluted with aqueous ammonium acetate solution (10 g/l) at a flow rate of 0.8 ml/min for 1h, followed by water at the same flow rate for 1h. 0.4 g silver nitrate was dissolved in 2 ml water; the solution was applied to the column via a 50 μl sample loop. The column was then rinsed with water (0.8 ml/min) until no silver could be detected (tested using dil. HCl), followed by methanol, acetone and dichloromethane (0.8 ml/min), each for 30 min. Prior to storage for longer periods the column was rinsed with hexane.

Preparative column

A `VarioPrep´ preparative column 100-10SA (25 x 1 cm i.d.) from Macherey-Nagel was subjected to an ammonium acetate solution (10 g/l) at a flow rate of 3ml/min for 1h, followed by water at the same flow rate for 30 min. 2 g silver nitrate was dissolved in 10 ml water; the solution was applied to the column via a 500 µl sample loop. The column was then rinsed with water (6 ml/min) until no silver could be detected, followed by methanol, acetone and dichloromethane (6 ml/min) each for 30 min. Prior to storage for longer periods the column was rinsed with n-hexane.

19.2 Chromatographic conditions for silver-ion chromatography

Analytical silver-ion column

The gradient conditions and other parameters for the analysis of triglycerides are given below.

Time (min)	0	5.0	20.0	45.0	45.1	55.0	55.1
% Dichloromethane	100	100	60	0	0	0	100
% Acetone	0	0	40	80	0	0	0
% Acetonitrile	0	0	0	20	100	100	0

- Sample loop: 20 µl
- Flow rate: 1 ml/min
- Column temperature: 15°C

Preparative silver-ion column

The gradient conditions and other parameters for the preparative separation of triglycerides are given below.

Time (min)	0	5.0	20.0	45.0	45.1	55.0	55.1
% Dichloromethane	100	100	60	0	0	0	100
% Acetone	0	0	40	80	0	0	0
% Acetonitrile	0	0	0	20	100	100	0

- Sample loop: 150 µl
- Flow rate: 5 ml/min
- Column temperature: 15°C

19.3 Chromatographic conditions for reversed phase chromatography

Analytical RP-18 column

The gradient conditions and other parameters for the analysis of triglycerides are given below.

Time (min)	0	16.0	16.1	20.0	20.1	24.0	24.1	28.0
% Dichloromethane	25	25	35	35	40	40	50	50
% Acetonitrile	75	75	65	65	60	60	50	50

- Sample loop: 20 µl
- Flow rate: 1 ml/min
- Column temperature: 30°C

Preparative RP-18 column

The gradient conditions and other parameters for the preparative separation of triglycerides are given below.

Time (min)	0	16.0	16.1	20.0	20.1	24.0	24.1	28.0
% Dichloromethane	25	25	35	35	40	40	50	50
% Acetonitrile	75	75	65	65	60	60	50	50

- Sample loop: 50 µl
- Flow rate: 4 ml/min
- Column temperature: 30°C

19.4 Chromatographic conditions for HPLC MS (APCI)

Analytical RP-18 column

The gradient conditions and other parameters for the analysis of triglycerides are given below.

Time (min)	0	28	35
% Dichloromethane	60	60	30
% Acetonitrile	40	40	70

- Sample loop: 20 µl
- Flow rate: 0.7 ml/min
- Column temperature: 20°C
- Sheath and auxilary gas: nitrogen @ 60 psi
- Vaporiser temperature: 450°C
- Capillary heater: 200°C
- Scan range: 10 – 1200
- Scan time: 5 seconds
- Corona voltage: 6.7 KV

20.0 Enzymatic splitting of triglycerides using lipase

20.1 Lipase solution

1 mg lipase is accurately weighed into a 10 ml volumetric flask and dissolved in buffer. Portions of the solution are then diluted 1:10 with buffer to provide a working solution. It is important to note that the activity of the solution falls by approximately 20% over two days; hence a fresh solution is necessary each day.

20.2 Method

1-3 mg of triglyceride dissolved in dichloromethane was transferred to an HPLC-vial. The solvent was removed under a stream of N_2, enabling the accurate weighing of the triglyceride. If triheptadecanoin (C17:0) was to be added as internal standard then 25% (w/w) with reference to the triglyceride was added. The triglyceride residue was redisolved in 750 µl hexane to which 750 µl lipase solution was added. The vials were then sealed and placed in a water bath set at 37°C and subjected to light agitation. After 2 hrs 2 drops of 25% HCl were injected through the septum. This reduces the pH to 2 and thus inactivates the lipase. The hexane phase was then transferred to an Micro Test Tube and the volume was reduced to 200 µl under a stream of N_2. The sample was then subjected to thin layer chromatography.

20.3 Separation of free fatty acids using TLC

Prior to use the TLC plates were cleaned using chloroform/methanol (1:1 v/v). Approximately 100 ml of the latter was placed in a TLC developing tank and allowed to equilibrate. A TLC plate was then placed in the tank for approximately 70 min, until the solvent front had reached the top of the plate. Having removed the plate it was allowed to dry (approximately 15 min) and then placed in a drying oven (set at 100°C) for 20 hrs to become activated. After cooling the plate may be stored in a dessicator filled with silica gel until required.

100 ml of the solvent mixture hexane:ether:glacial acetic acid (80:20:10 v/v) is placed in a developing tank and is allowed to equilibrate for 1hr.

2-5 µl of sample was placed on the activated TLC plate as a spot using the Linomat (not more than 5 spots per plate), approximately 2 cm from the bottom of the plate. A maximum of 2 plates may be placed in the developing tank. The plates were removed from the tank when the solvent front was approximately 1.5 cm from the top of the plate. The plates are dried under a stream of N_2 after which they were sprayed with the rhodamine solution and viewed at 254 nm. The different lipid classes were separated as shown in Figure 12 below.

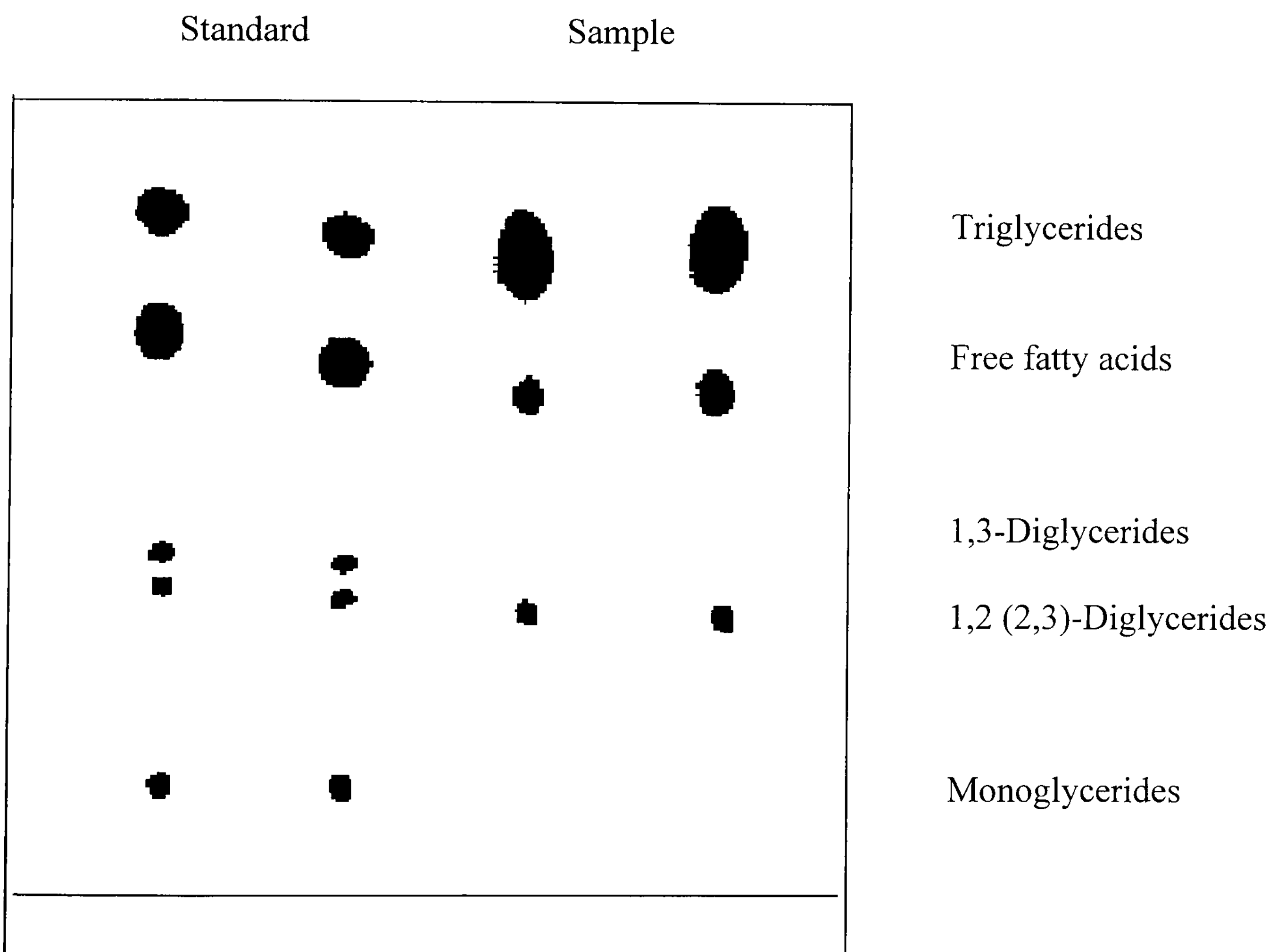


Figure 12: A TLC plate showing the separation of a standard and a triglyceride fraction from a sample which has been split enzymatically.

The separation is according to the polarity of the compounds, the non-polar components are carried with the solvent front and the polar components do not migrate so far.

Having marked the free fatty acid spots the TLC plate was placed on a specially adapted stand, enabling the scratching out of spots and the collection of the material into a Micro Test Tube. The fatty acids were then extracted using 50 µl of TCFE and then derivatised to their methyl esters by the addition of 25 µl TMSH. After centrifuging (2000 g) for 2 minutes the supernatant was removed and subjected to gas chromatography.

21.0 Camel milk samples

The data on camel milk samples in this study originated from milk samples collected on the Ol Maisor Ranch in Kenya. The Ol Maisor Ranch has approximately 500 camels and is situated roughly 100 miles north east of Nakuru (Egerton University). Between the months of April and May 1996, milk samples from the camels referred to in Table 25 below were collected twice a week. The camels were milked between 5 and 6 am, the milk was transported in a refrigerator directly to Egerton University; the duration of the journey was approximately 3 hours. Upon arrival at the university, basic parameters such as fat (Gerber), pH and dry solids were determined, simultaneously milk samples were prepared for freeze drying. After freeze drying, the samples were sealed in polythene bags and stored at -18°C until being transported to Hohenheim University. Milk samples were collected from the following camels.

Table 25: The breed and age of the camels from which milk samples were collected from the work in this thesis

Camel No.	Breed *(dromedarius)*	Age (yrs)
98	50% somali / 50% turkana	15
179	50% somali / 50% turkana	14
180	50% somali / 50% turkana	14
277	100% somali	14
325	50% somali / 50% turkana	12

2 to 4 l of mixed camel milk was also collected each time the ranch was visited and this was used for technological experiments.

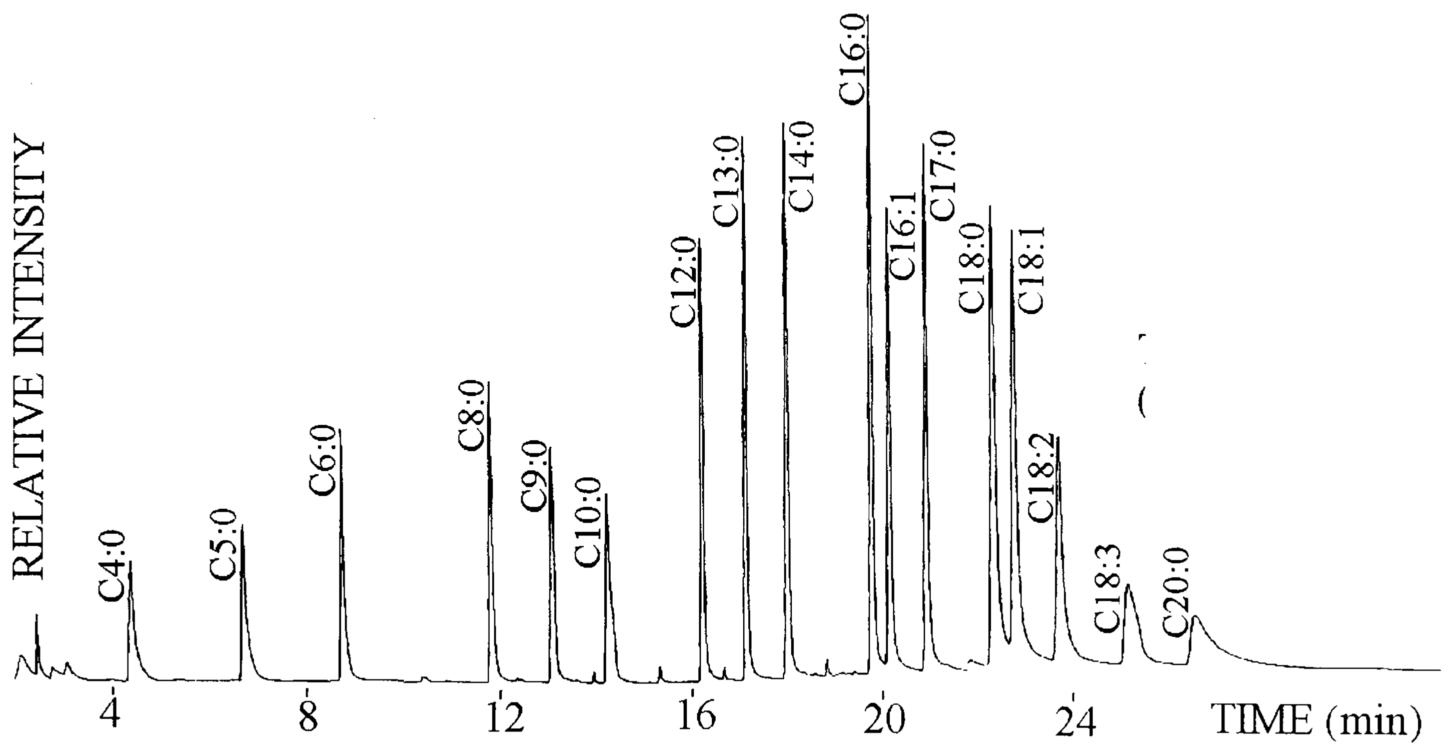


Figure 13: A GC-MS chromatogram of an equimolar free fatty acid standard. See Section 15.4 for chromatographic conditions.

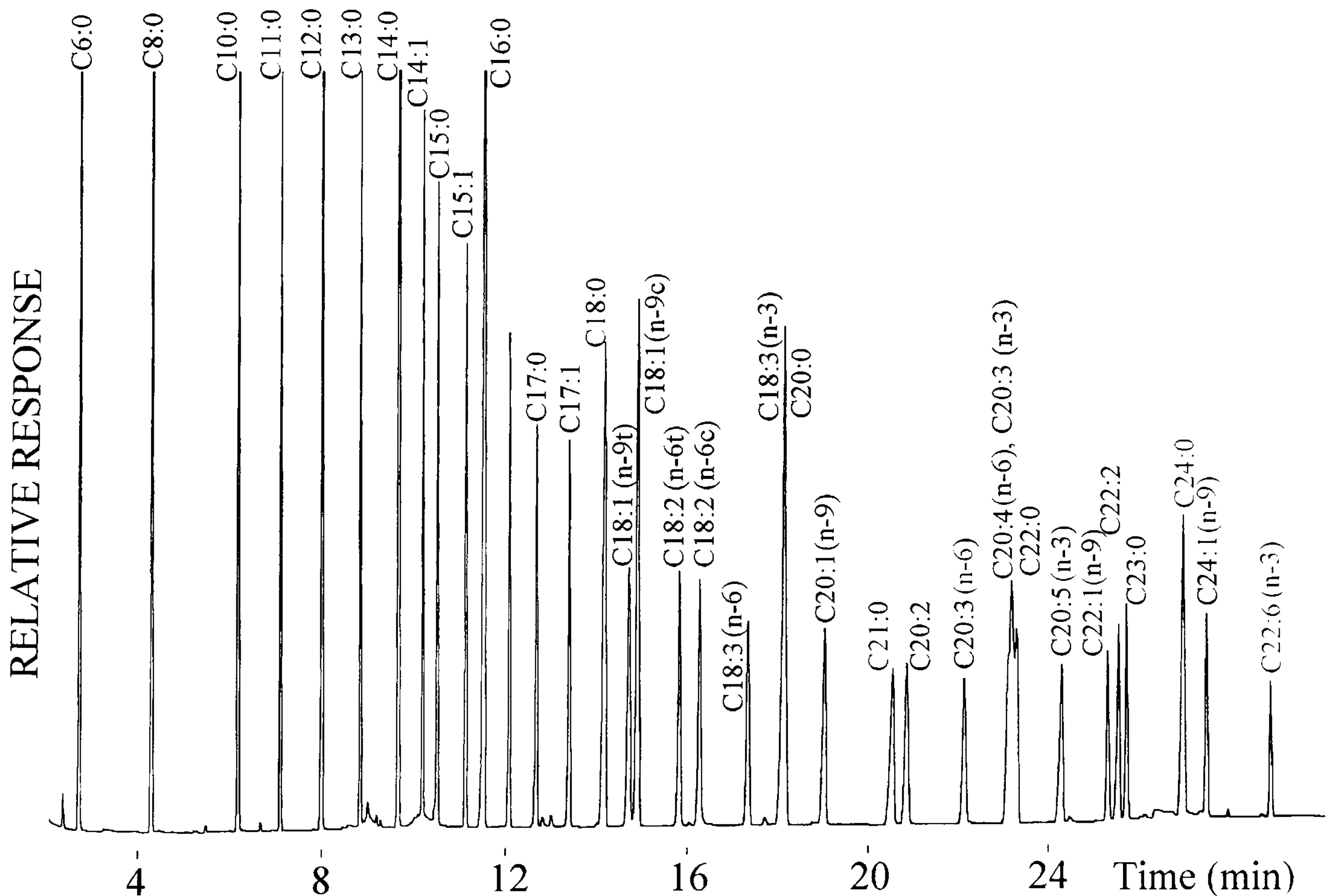


Figure 14: A GC chromatogram of the 37 component FAME standard from Supelco (No. 4-7885). See Section 17.4 for chromatographic conditions.

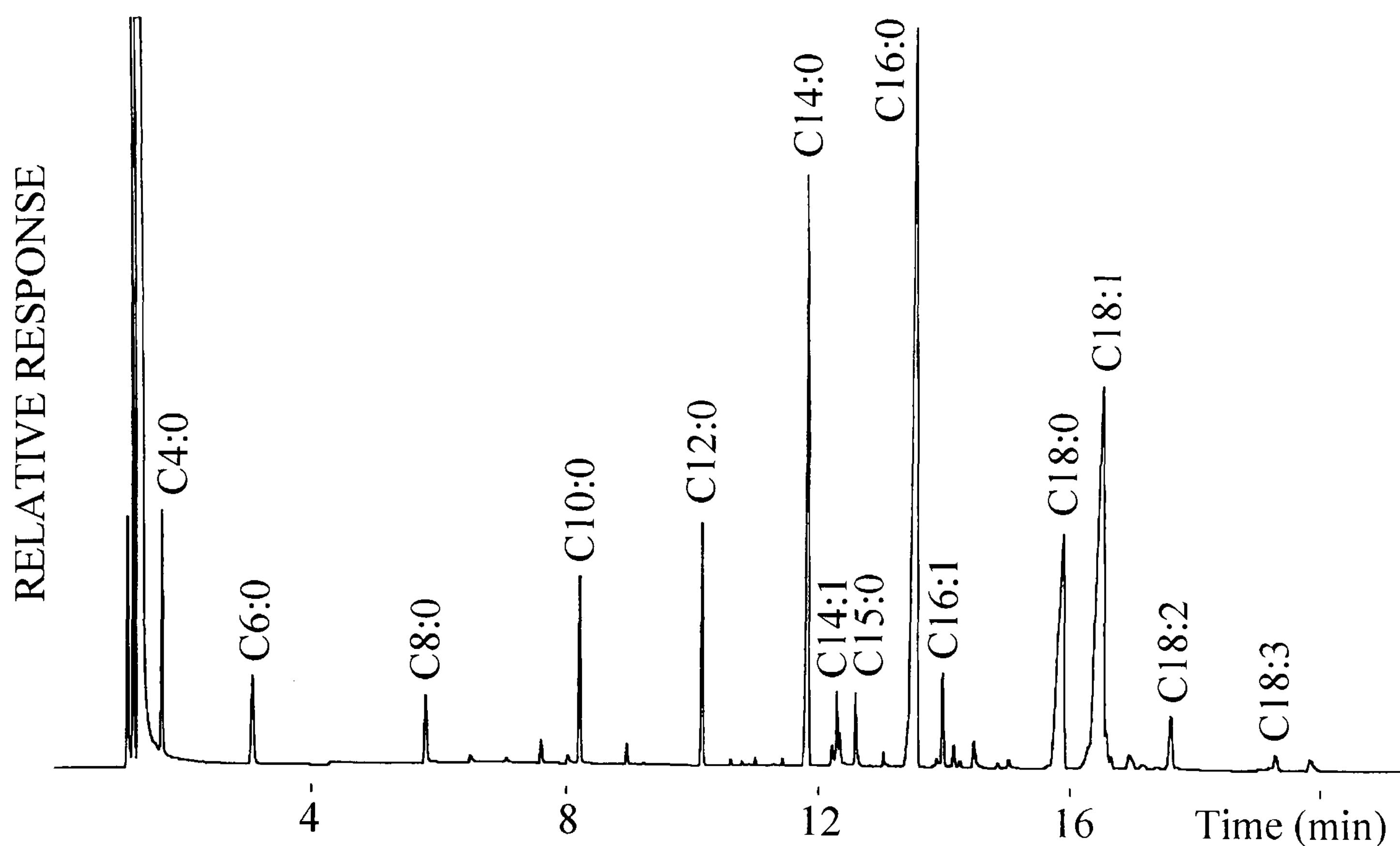


Figure 15: A gas chromatogram showing the fatty acid composition of cow milk fat. See Section 17.4 for chromatographic conditions.

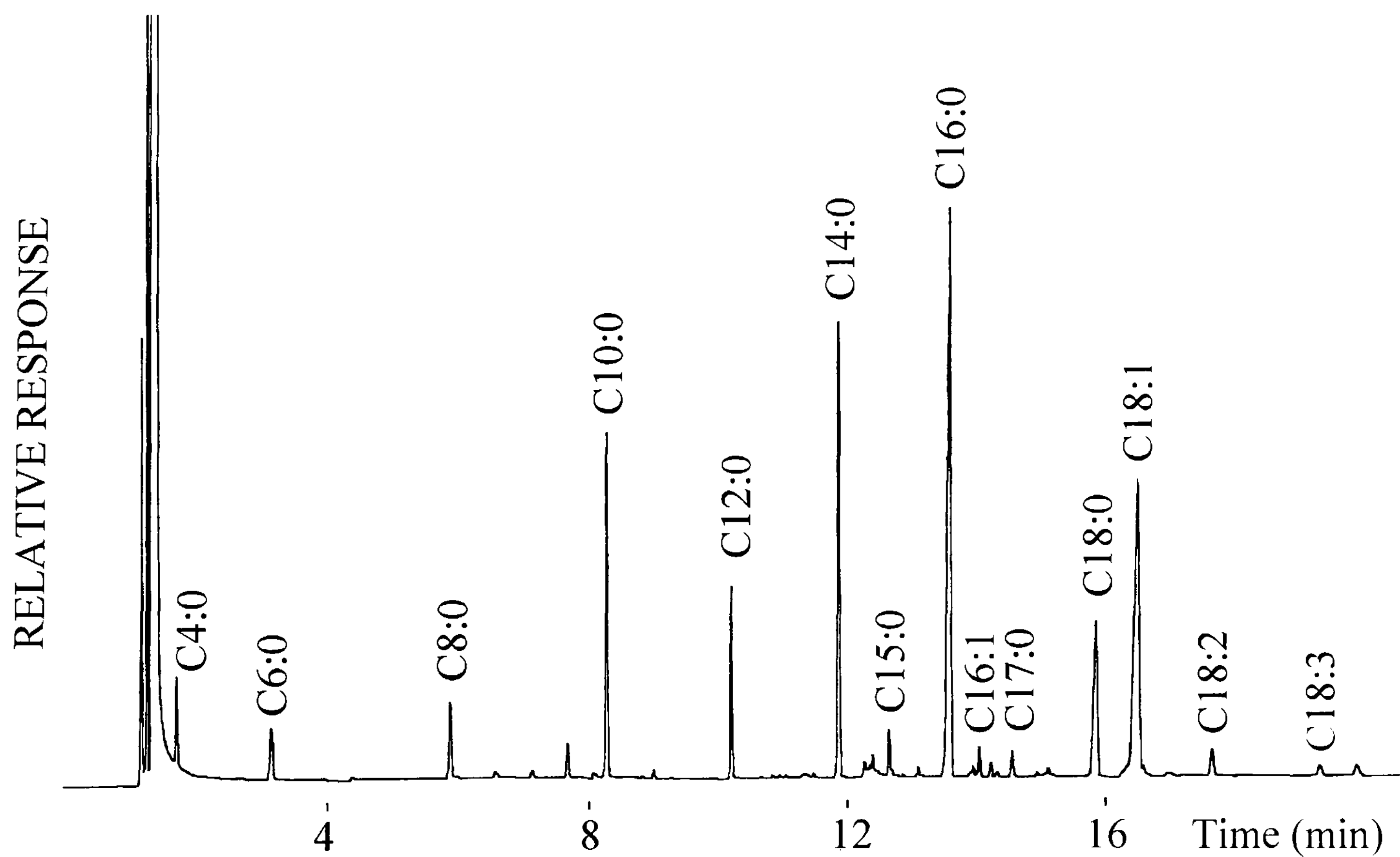


Figure 16: A gas chromatogram showing the fatty acid composition of goat milk fat. See Section 17.4 for chromatographic conditions.

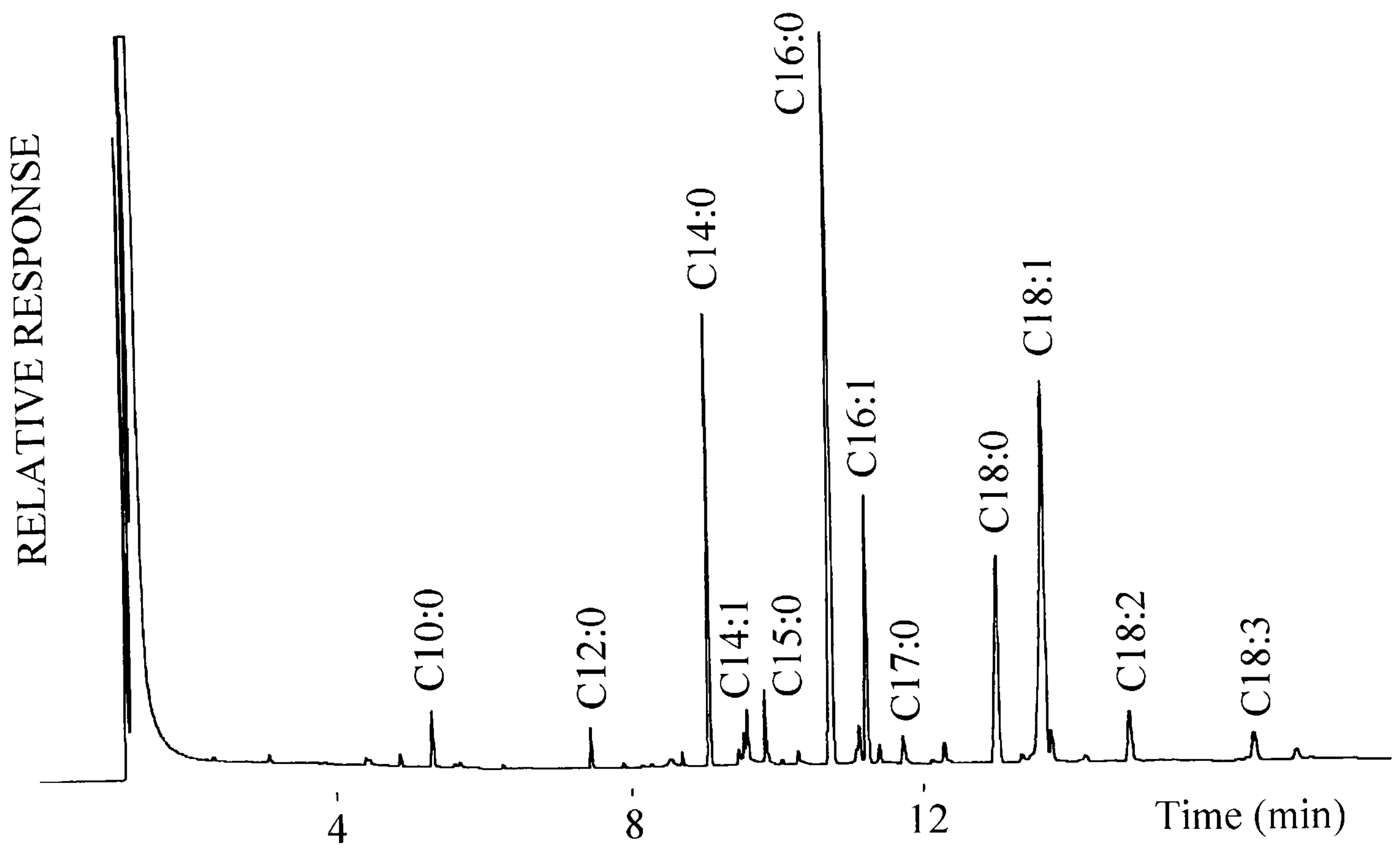


Figure 17: A gas chromatogram showing the fatty acid composition of camel milk fat. See Section 17.4 for chromatographic conditions.

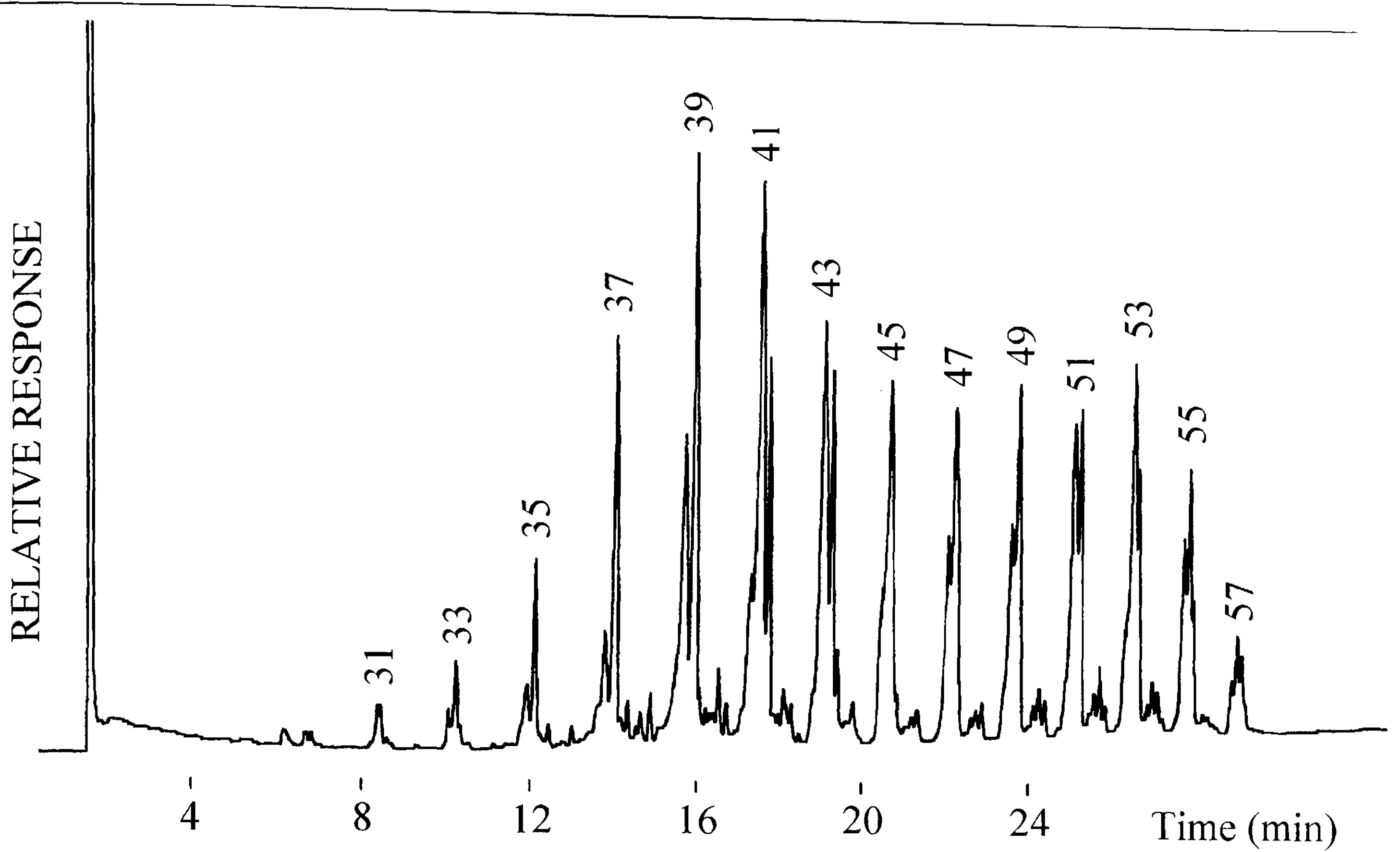


Figure 18: A high temperature GC chromatogram showing the distribution of triglycerides in cow milk fat according to their CN. See Section 18.0 for chromatographic conditions.

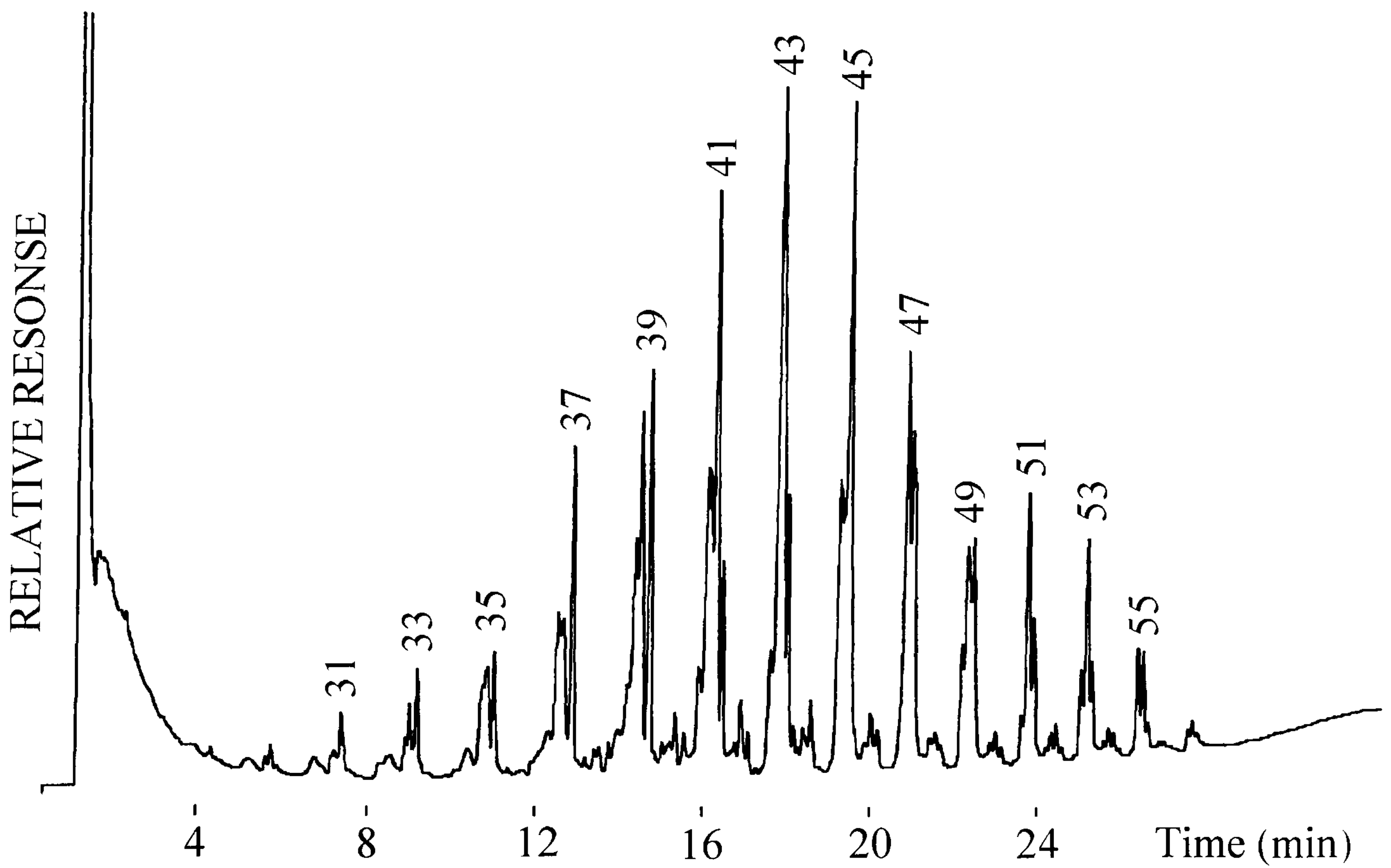


Figure 19: A high temperature GC chromatogram showing the distribution of triglycerides in goat milk fat according to their CN. See Section 18.0 for chromatographic conditions.

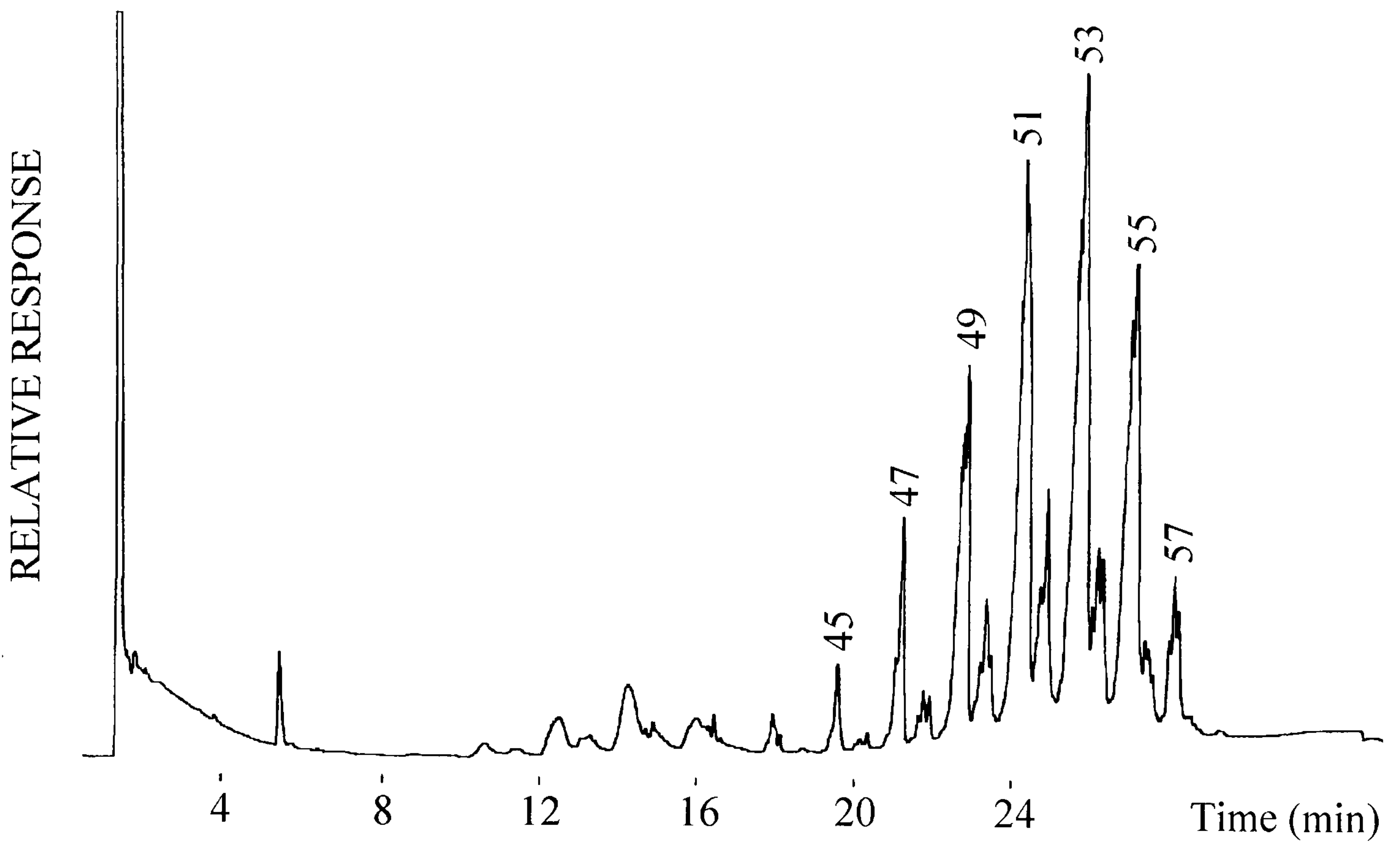


Figure 20: A high temperature GC chromatogram showing the distribution of triglycerides in camel milk fat according to their CN. See Section 18.0 for chromatographic conditions.

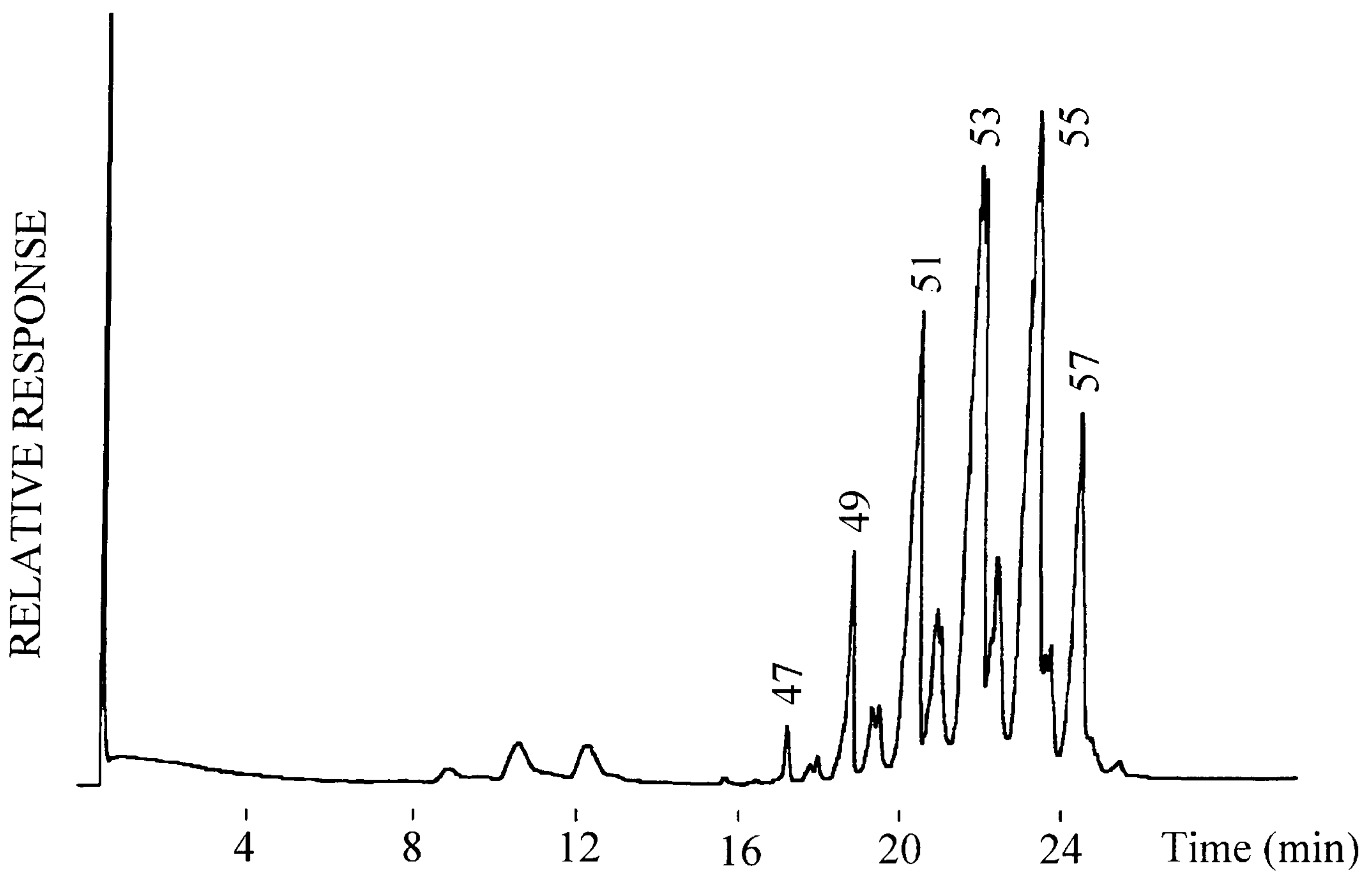


Figure 21: A high temperature GC chromatogram showing the distribution of triglycerides in camel hump fat according to their CN. See Section 18.0 for chromatographic conditions.

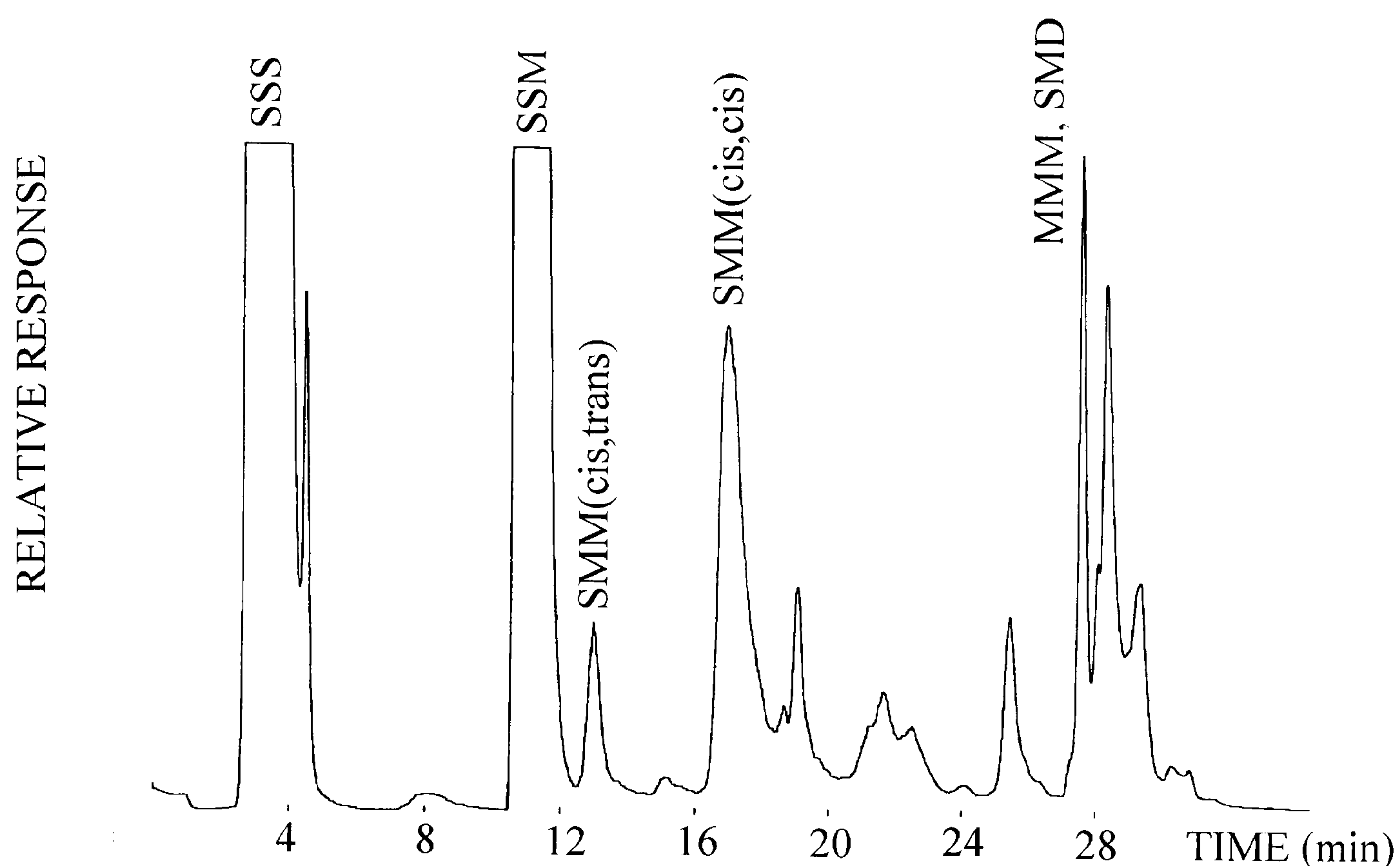


Figure 22: HPLC chromatogram of cow milk fat on an analytical silver-ion column showing the separation of triglycerides according to saturation and isomerism. See Section 19.2 for chromatographic conditions.

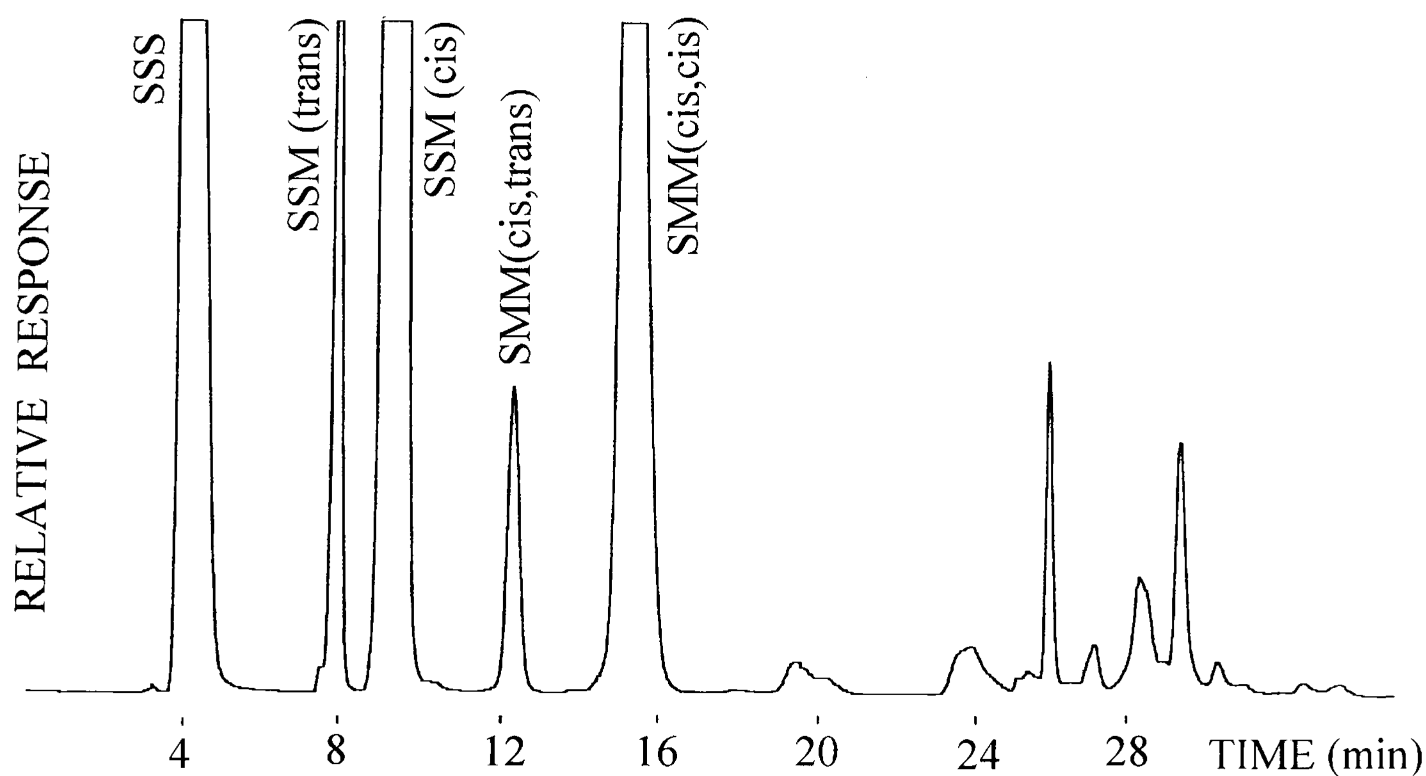


Figure 23: HPLC chromatogram of camel milk fat on an analytical silver-ion column showing the separation of triglycerides according to saturation and isomerism. See Section 19.2 for chromatographic conditions.

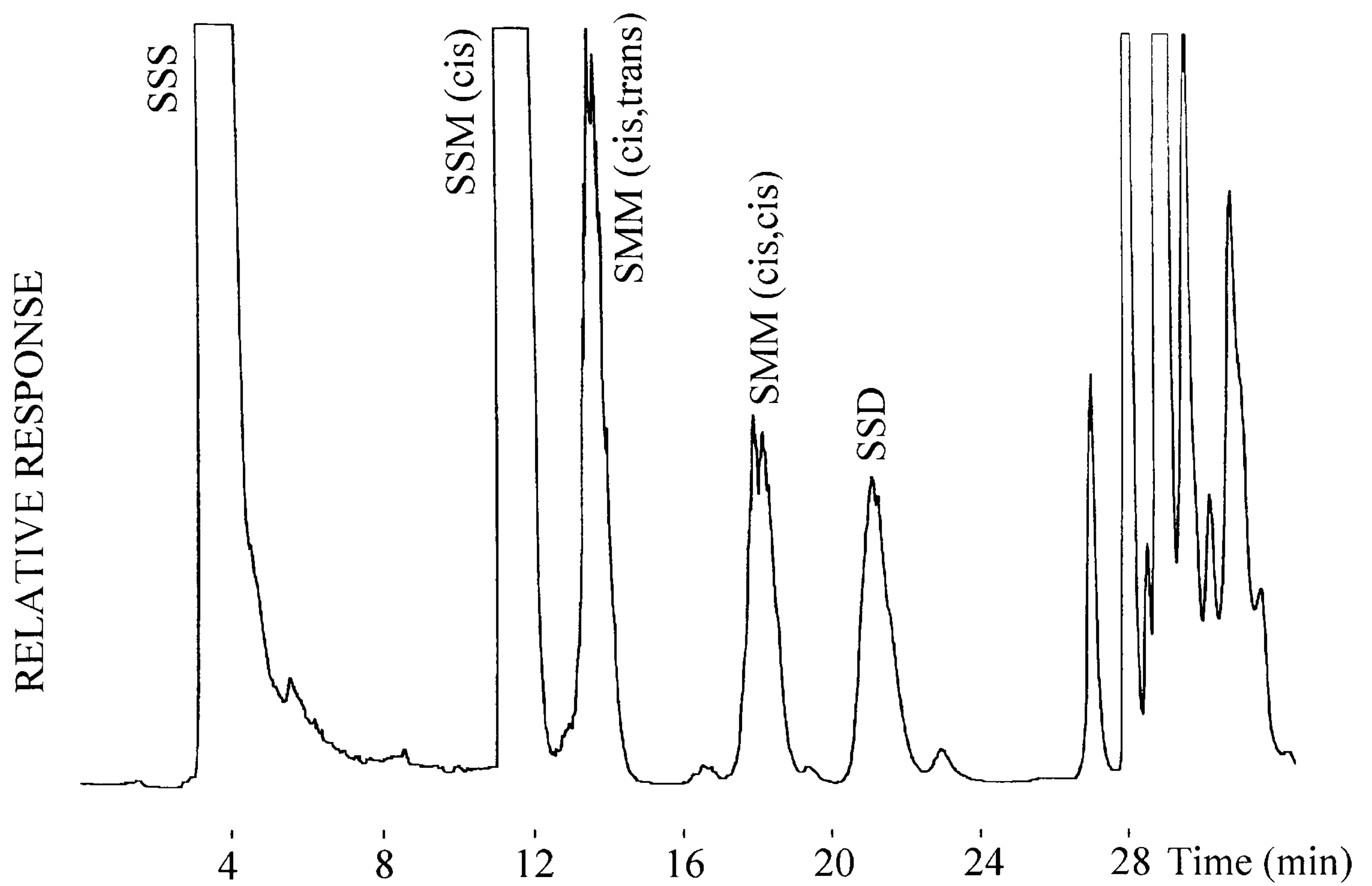


Figure 24: HPLC chromatogram of camel hump fat on an analytical silver-ion column showing the separation of triglycerides according to saturation and isomerism. See Section 19.2 for chromatographic conditions.

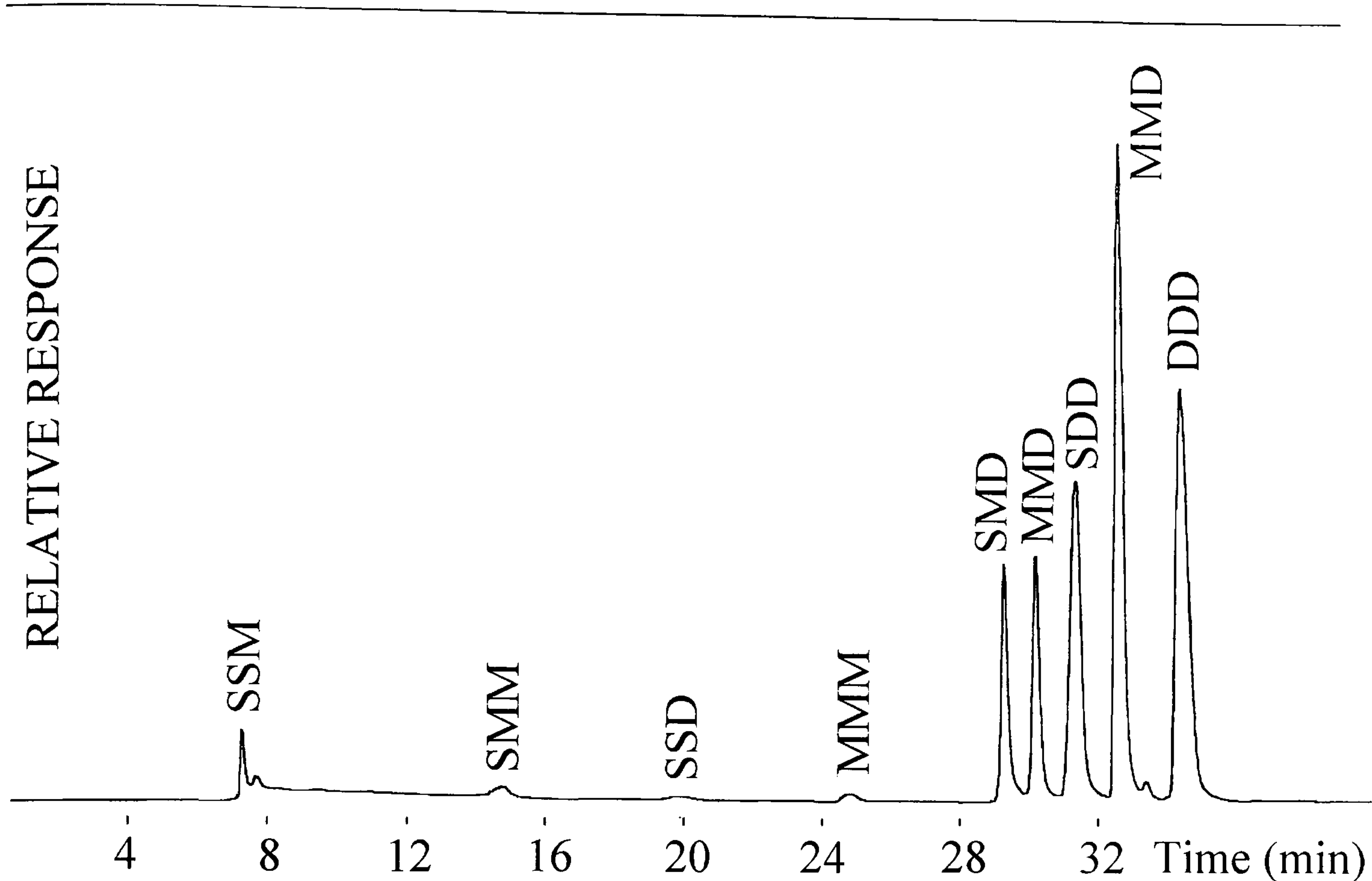


Figure 25: Separation of triglycerides from sunflower seed oil with an analytical silver-ion column. See Section 19.2 for chromatographic conditions.

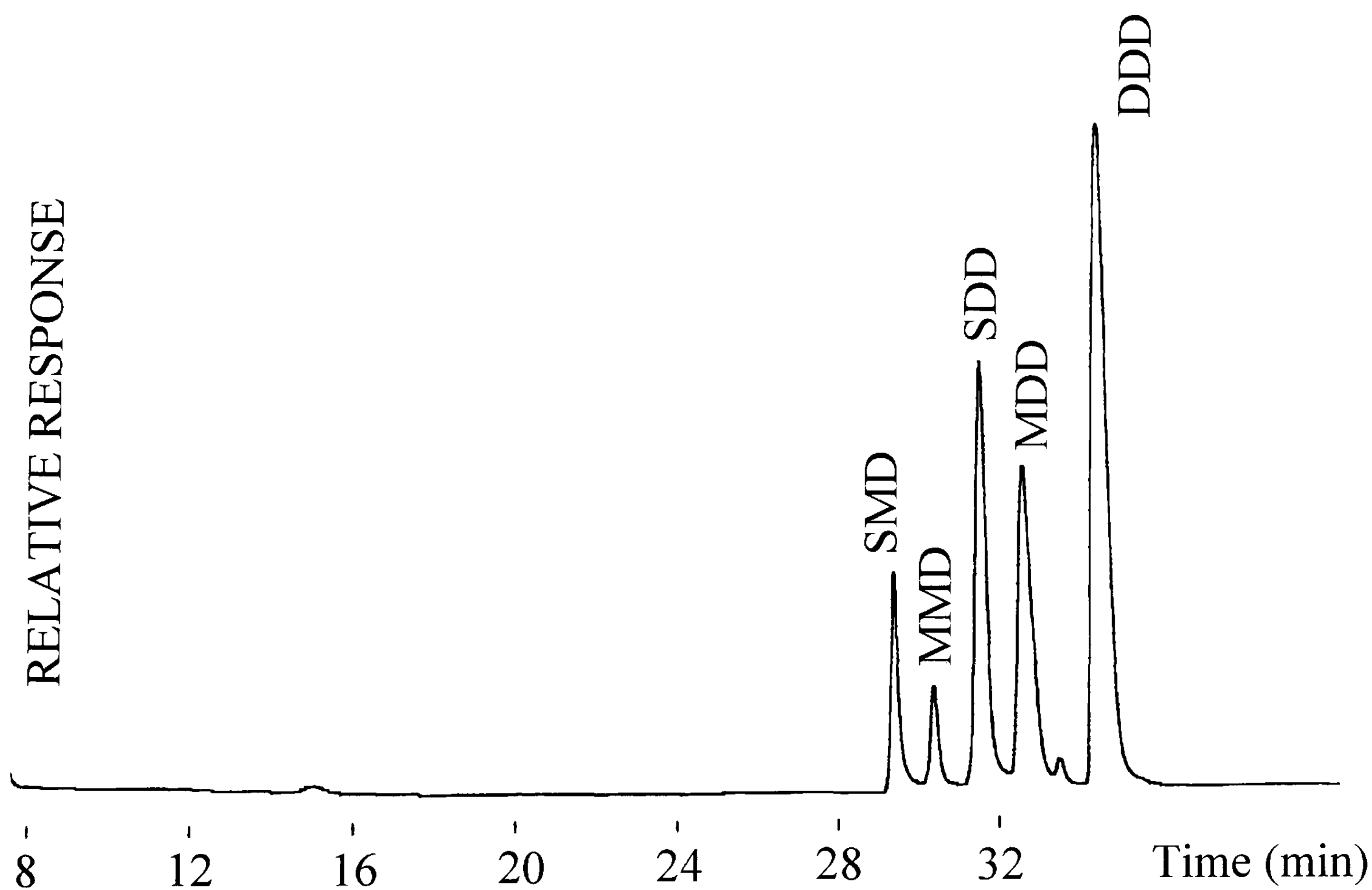


Figure 26: Separation of triglycerides from thistle seed oil with an analytical silver-ion column. See Section 19.2 for chromatographic conditions.

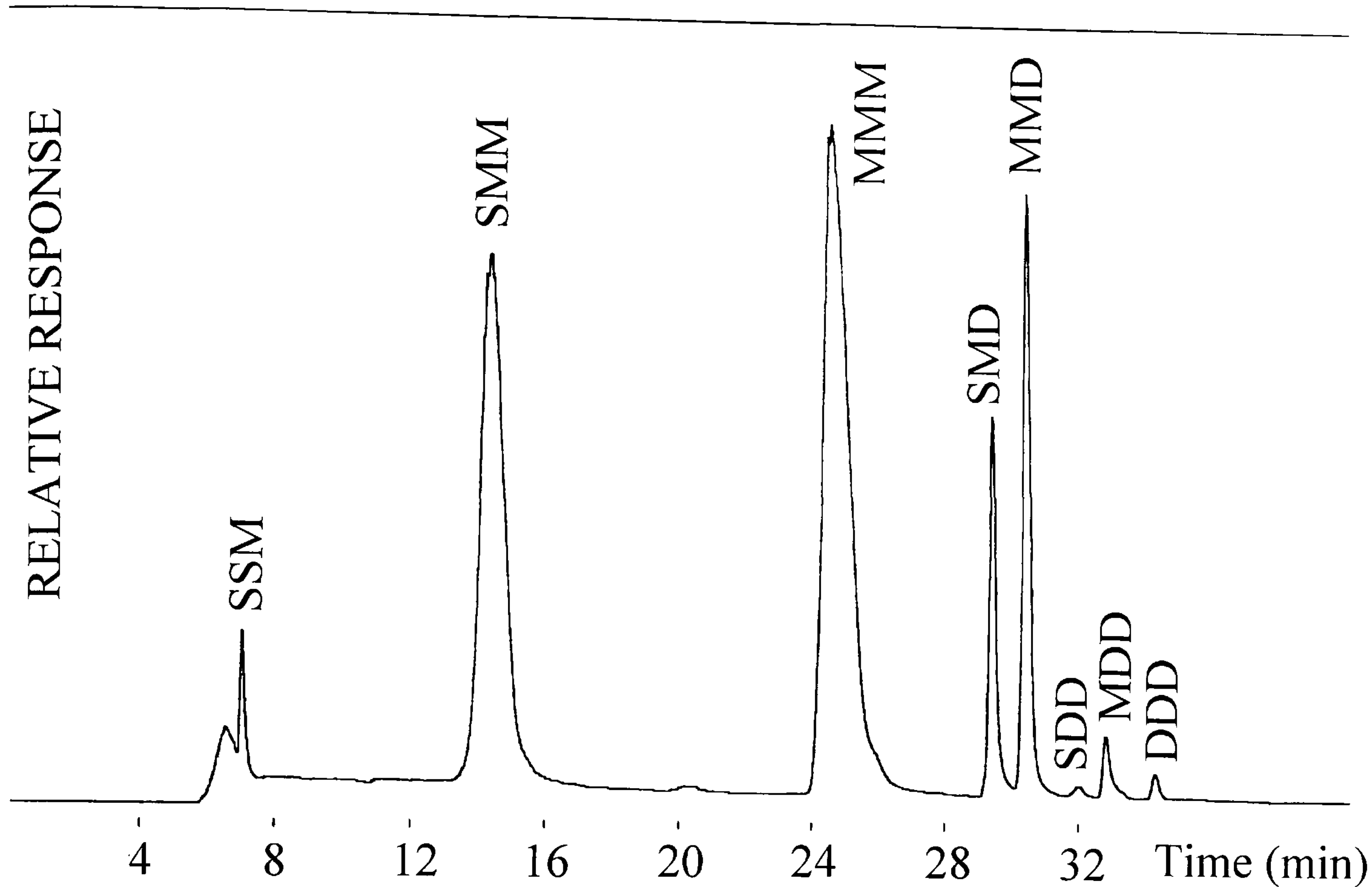


Figure 27: Separation of triglycerides from olive oil with an analytical silver-ion column. See Section 19.2 for chromatographic conditions.

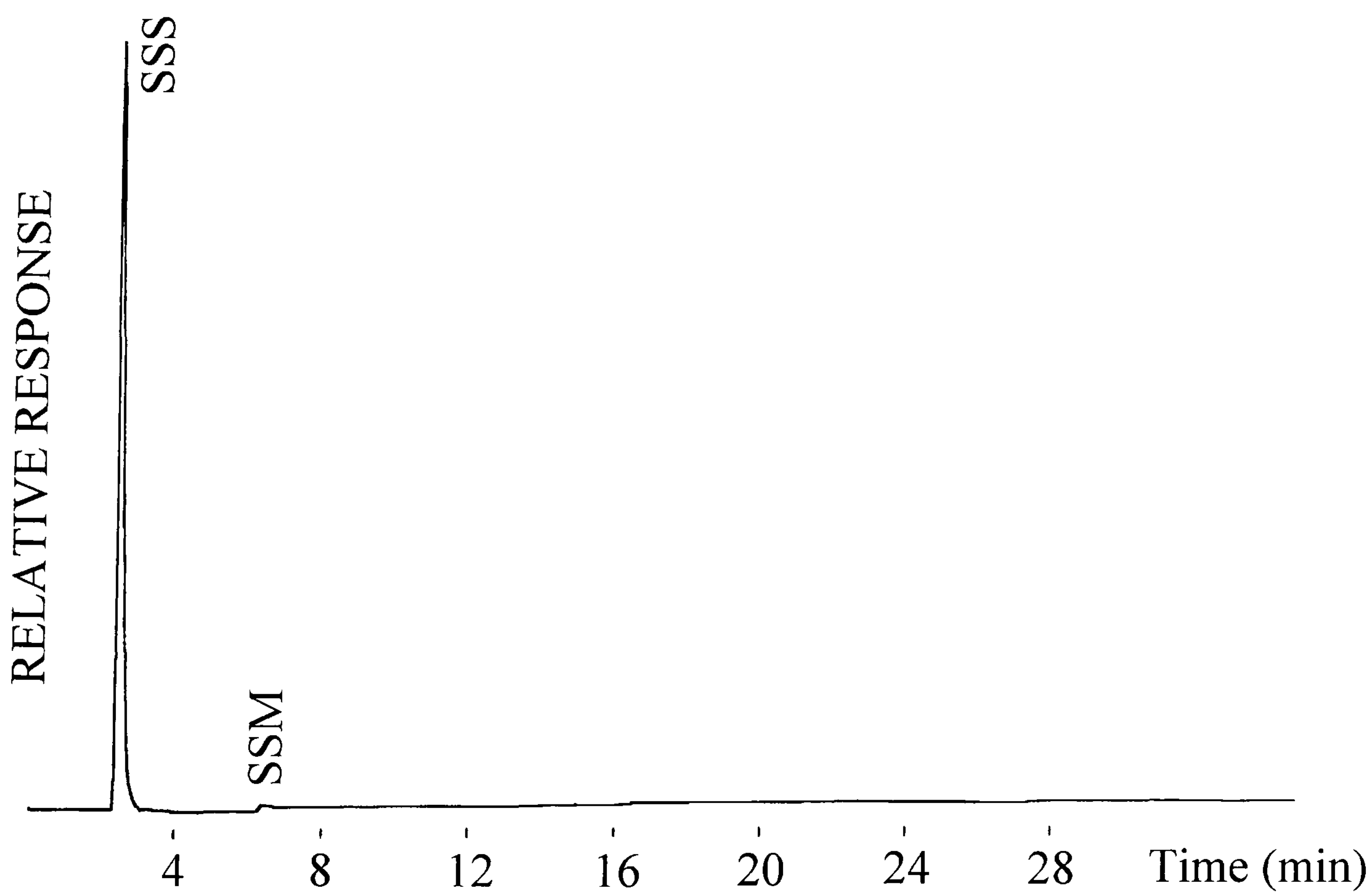


Figure 28: Separation of triglycerides from coconut fat with an analytical silver-ion column. See Section 19.2 for chromatographic conditions.

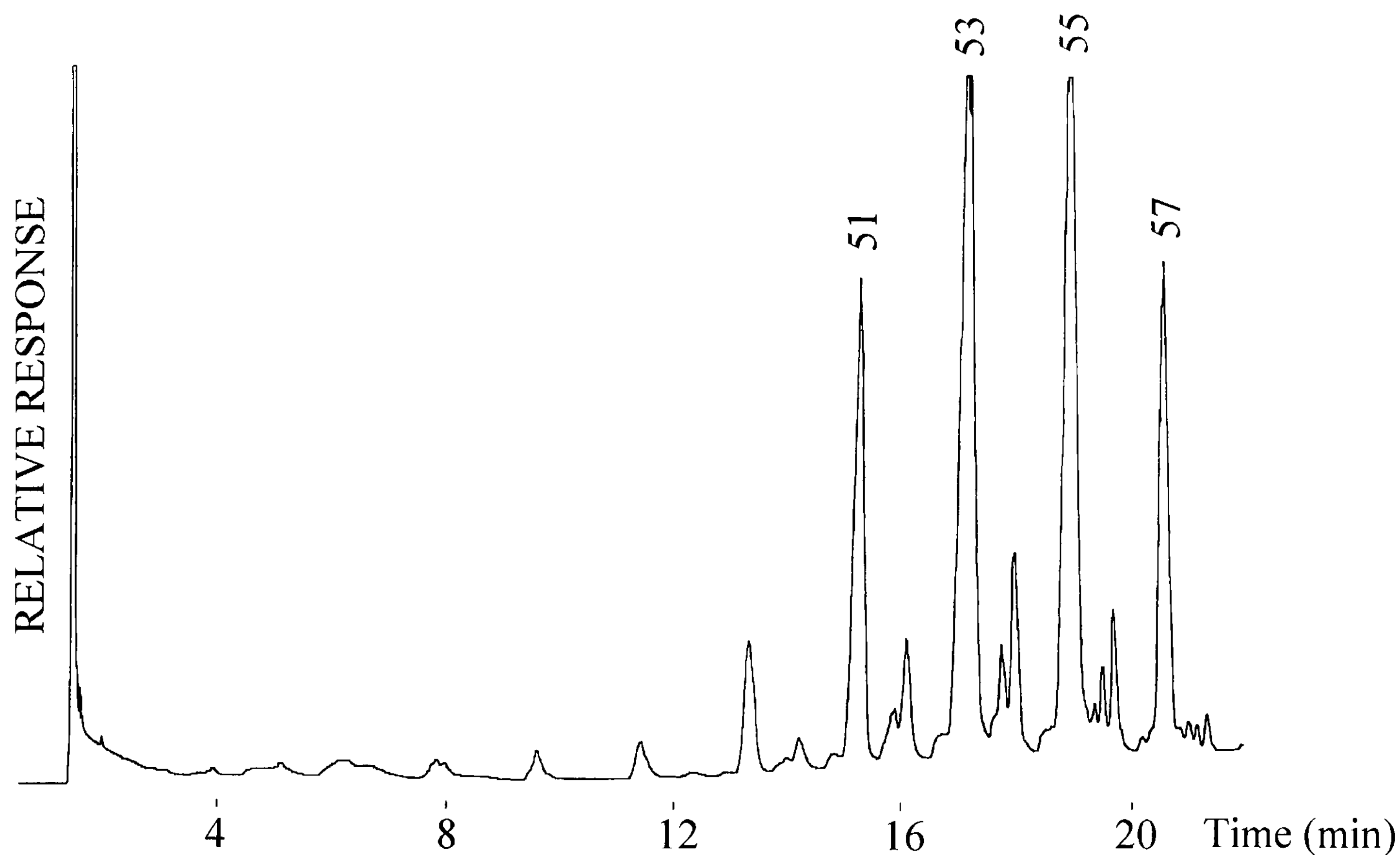


Figure 29: A high temperature GC chromatogram showing the distribution of triglycerides in the fraction SSM(cis) in camel hump fat. See Section 18.0 for chromatographic conditions.

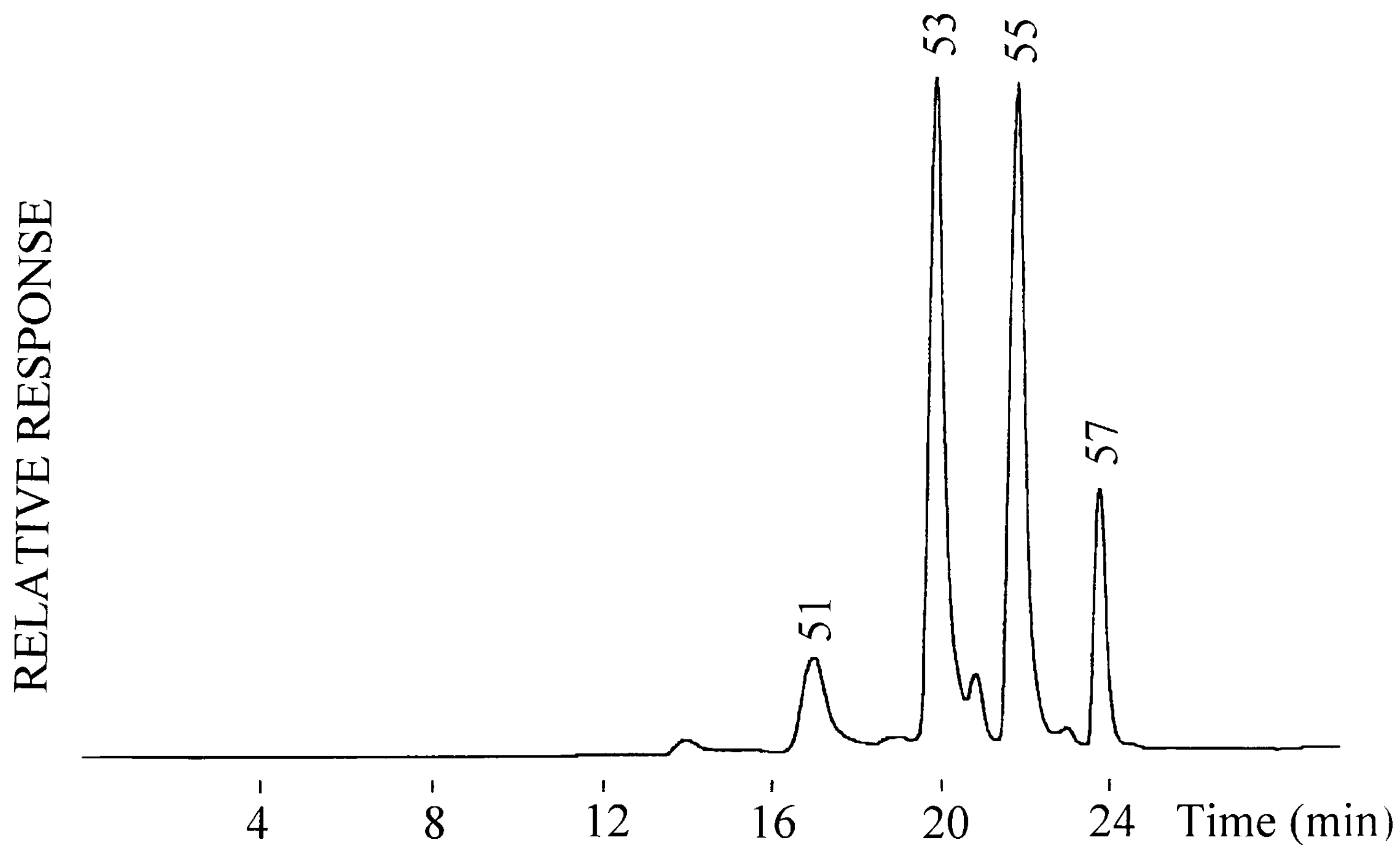


Figure 30: A HPLC chromatogram of the fraction SSM(cis) from camel hump fat on a RP-18 preparative column. See Section 19.3 for chromatographic conditions.

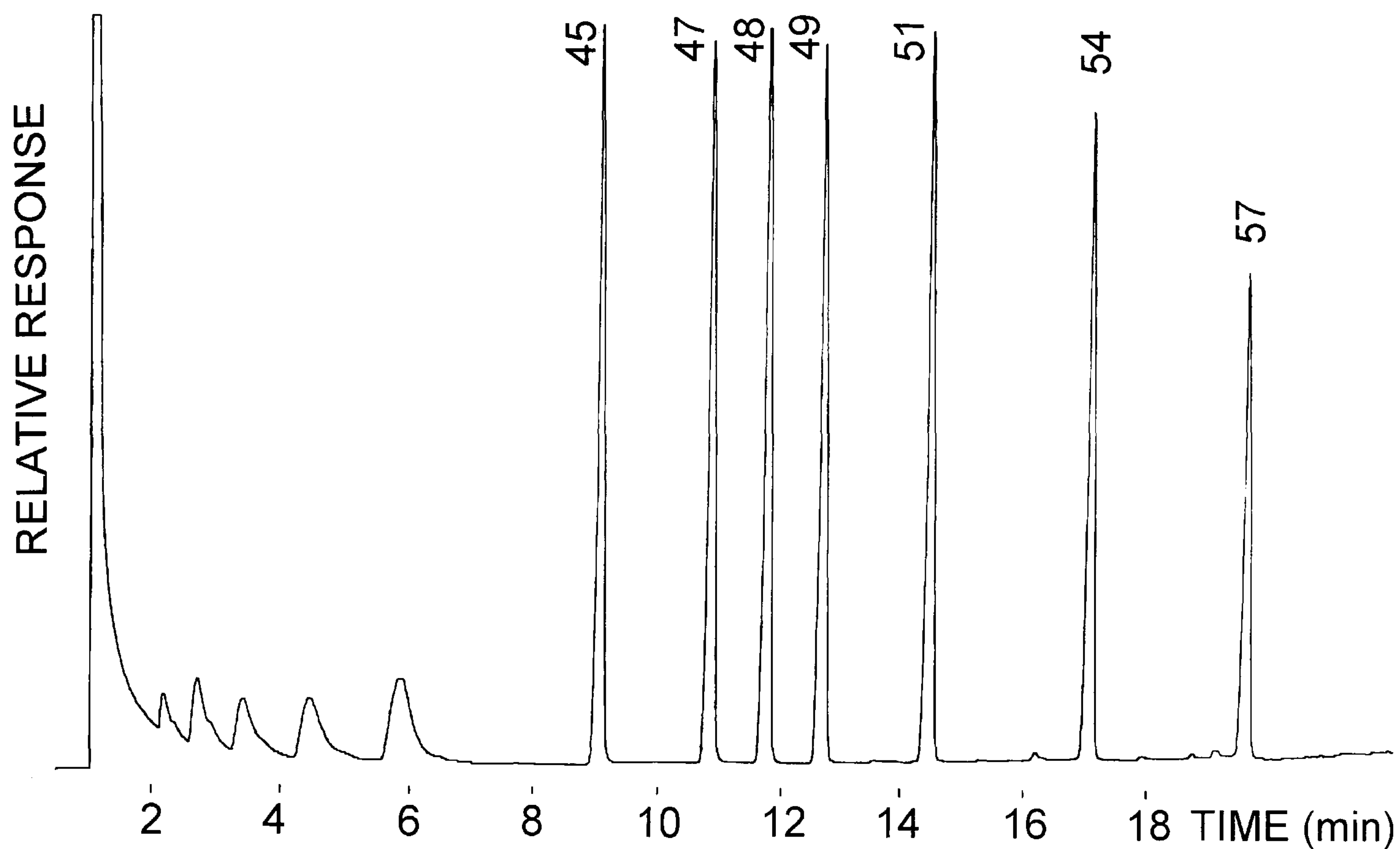


Figure 31: A high temperature GC chromatogram of an equimolar triglyceride standard (20 mmol/l). See Section 18.0 for chromatographic conditions.

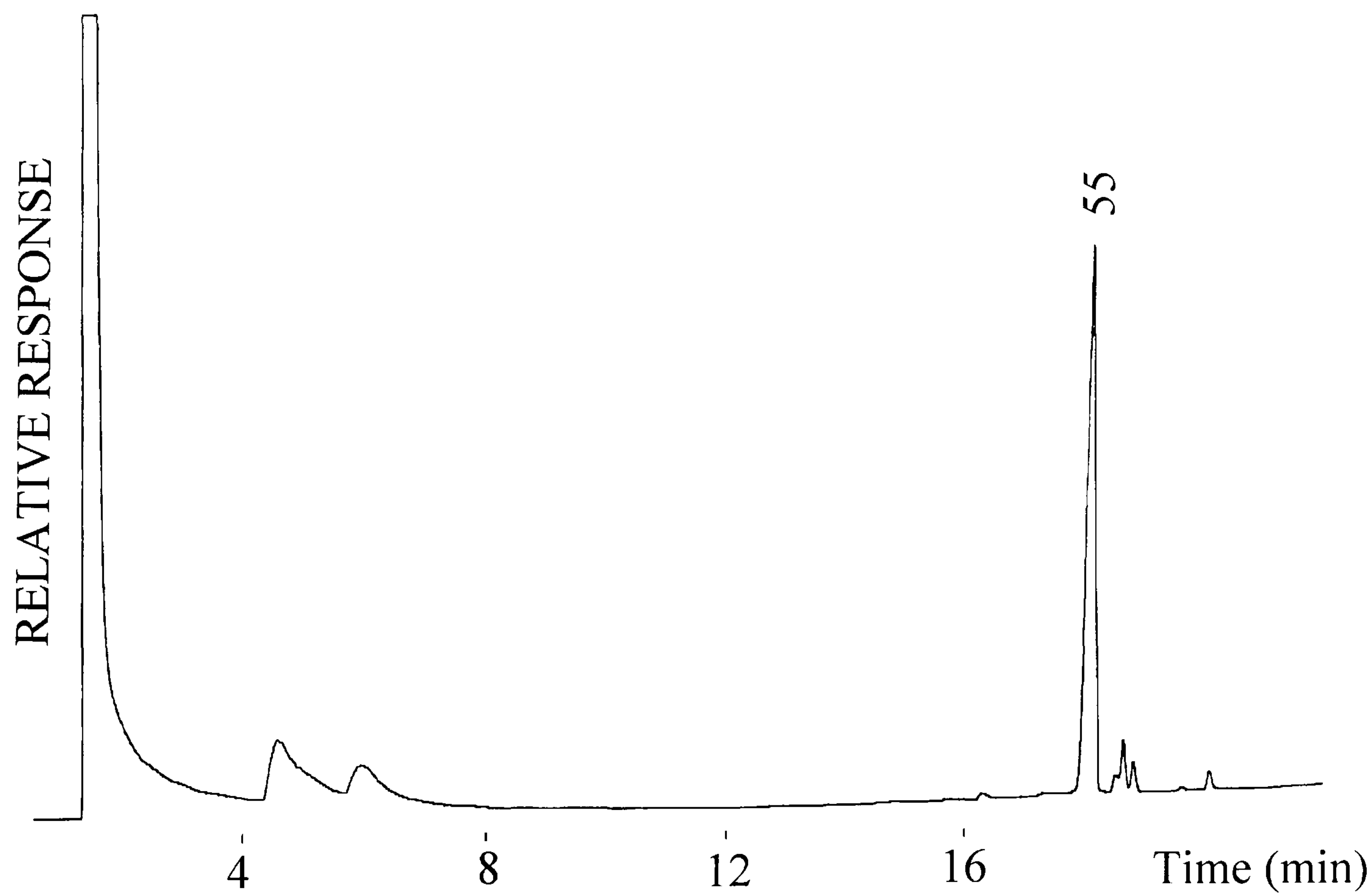


Figure 32: A high temperature GC chromatogram of the triglyceride fraction SSS, CN = 55 from camel hump fat isolated on the preparative RP-18 HPLC column, prior to enzymatic splitting with lipase. See Section 18.0 for chromatographic conditions.

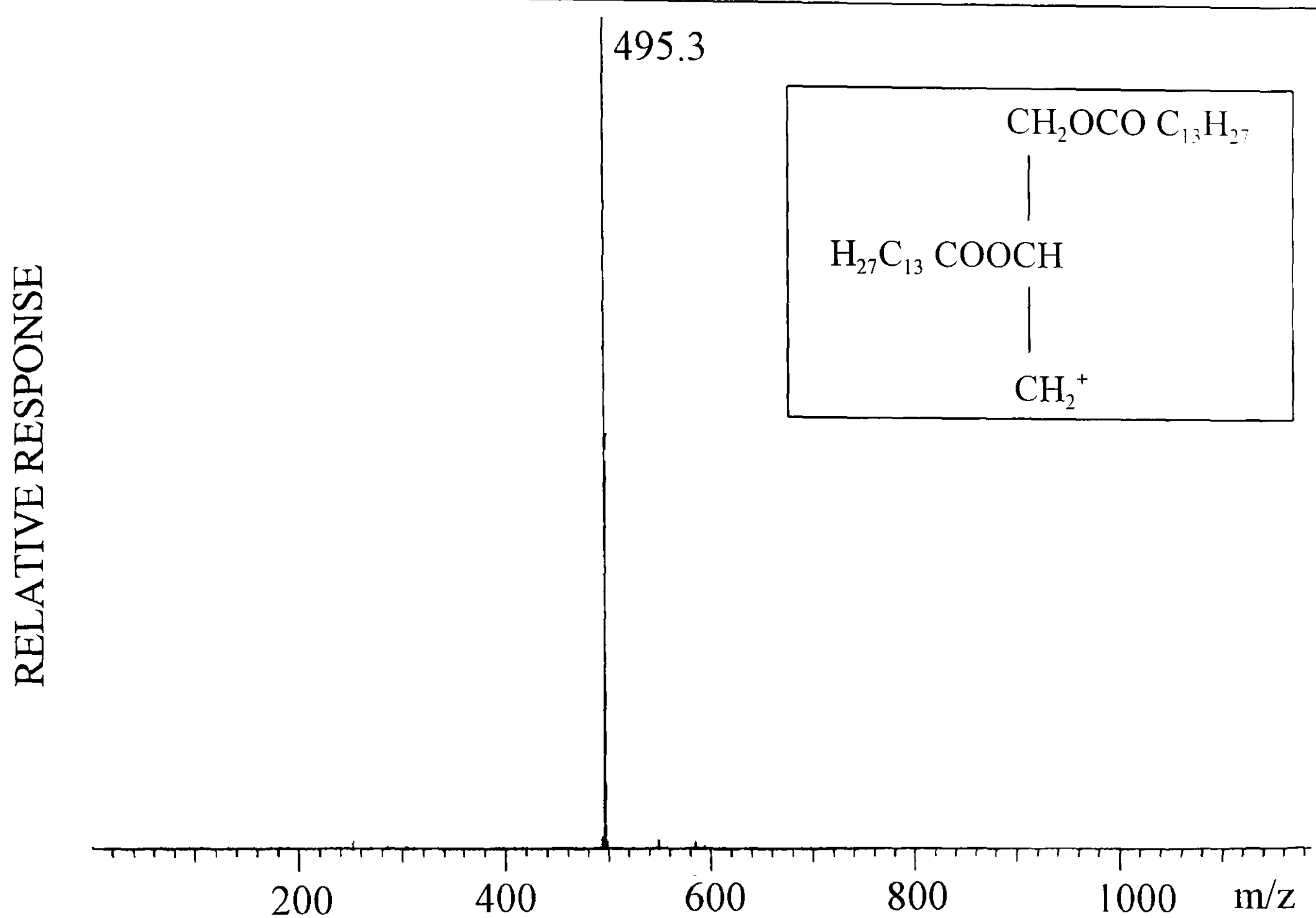


Figure 35: A HPLC-MS spectra of trimyristin (C14:0). See Section 19.4 for chromatographic conditions.

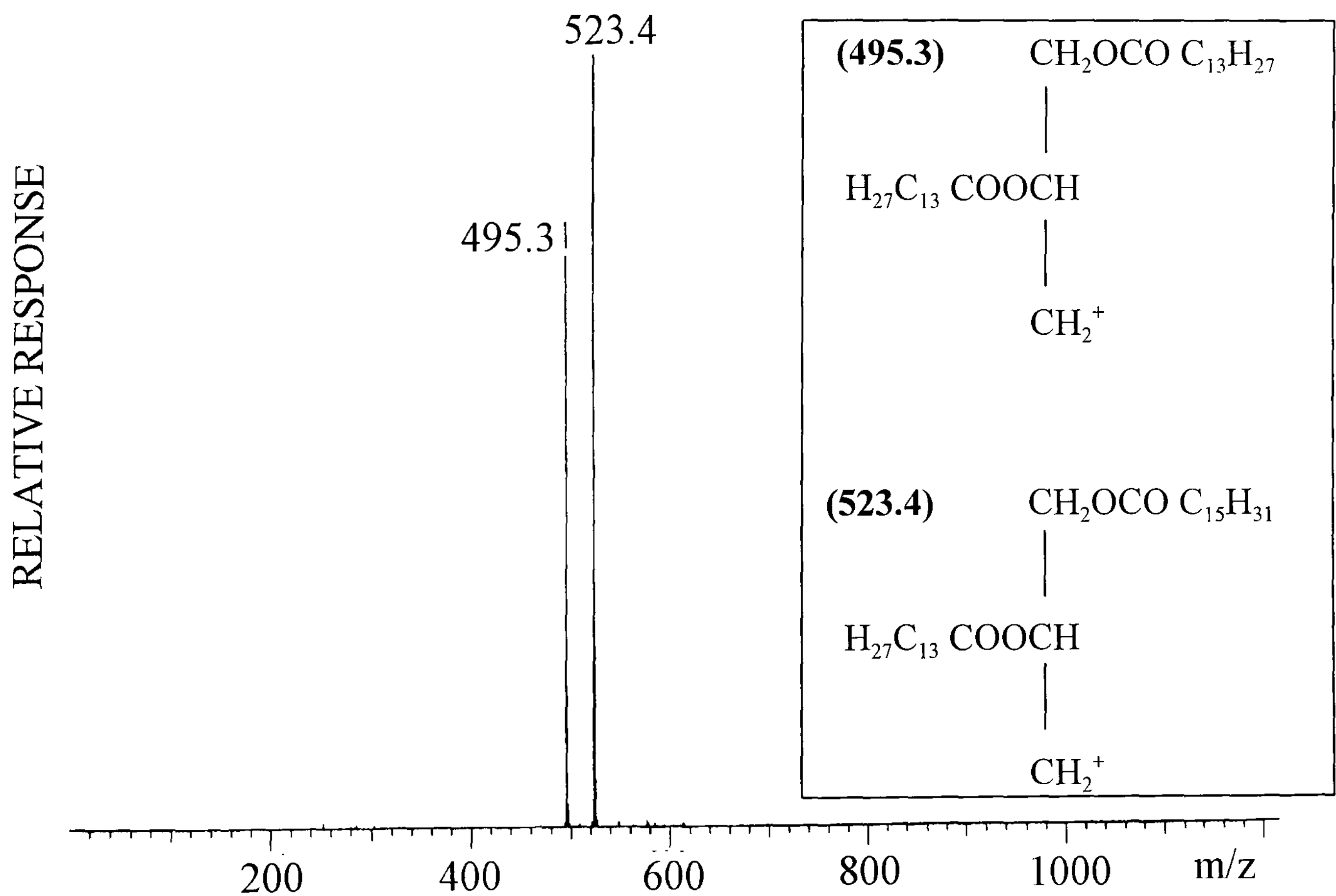


Figure 36: A HPLC-MS spectra of 1,2-dimyristoyl-3-palmitoyl-rac-glycerol (C14:0/C14:0/C16:0). See Section 19.4 for chromatographic conditions.

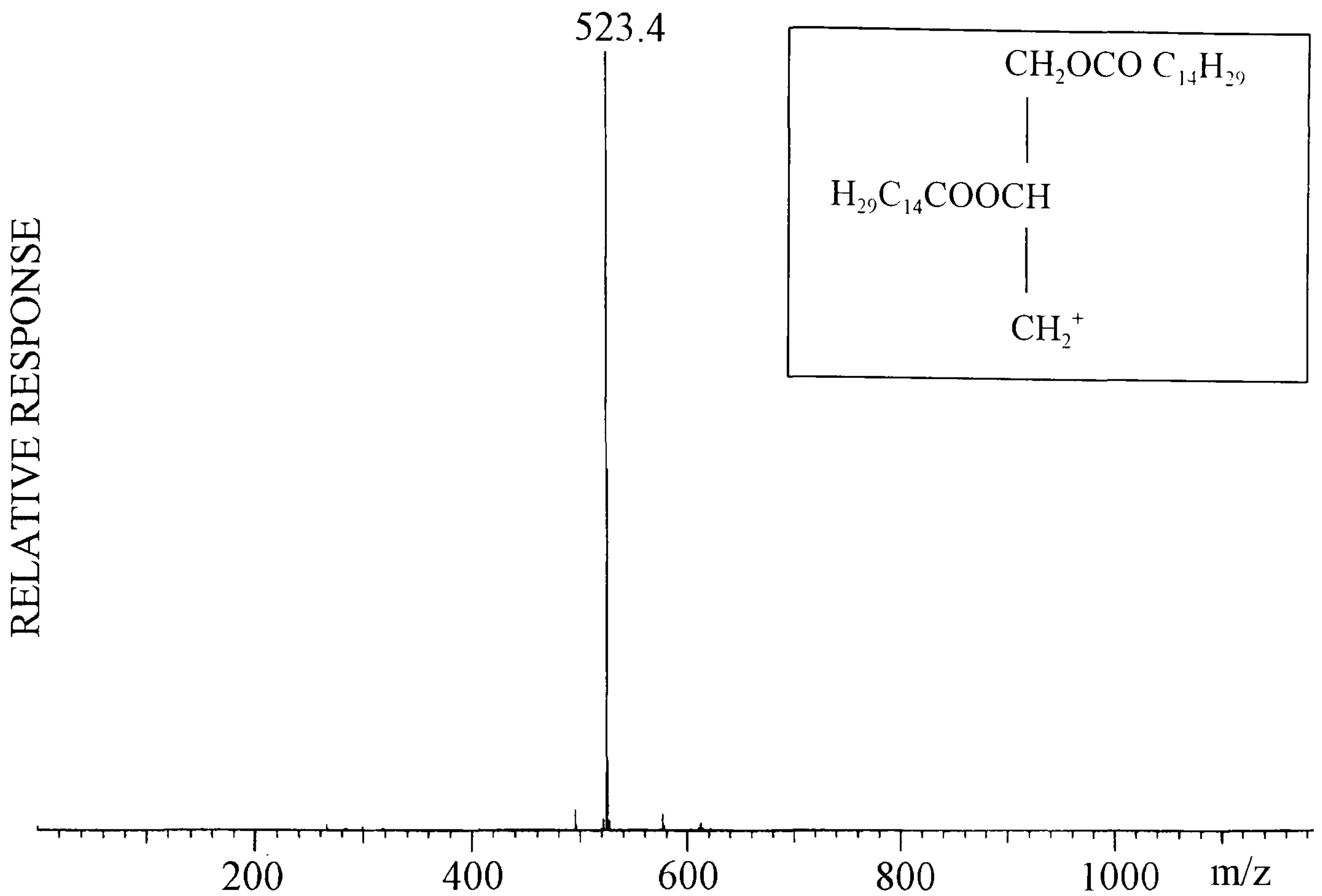


Figure 37: A HPLC-MS spectra of tripentadecanoin (C15:0). See Section 19.4 for chromatographic conditions.

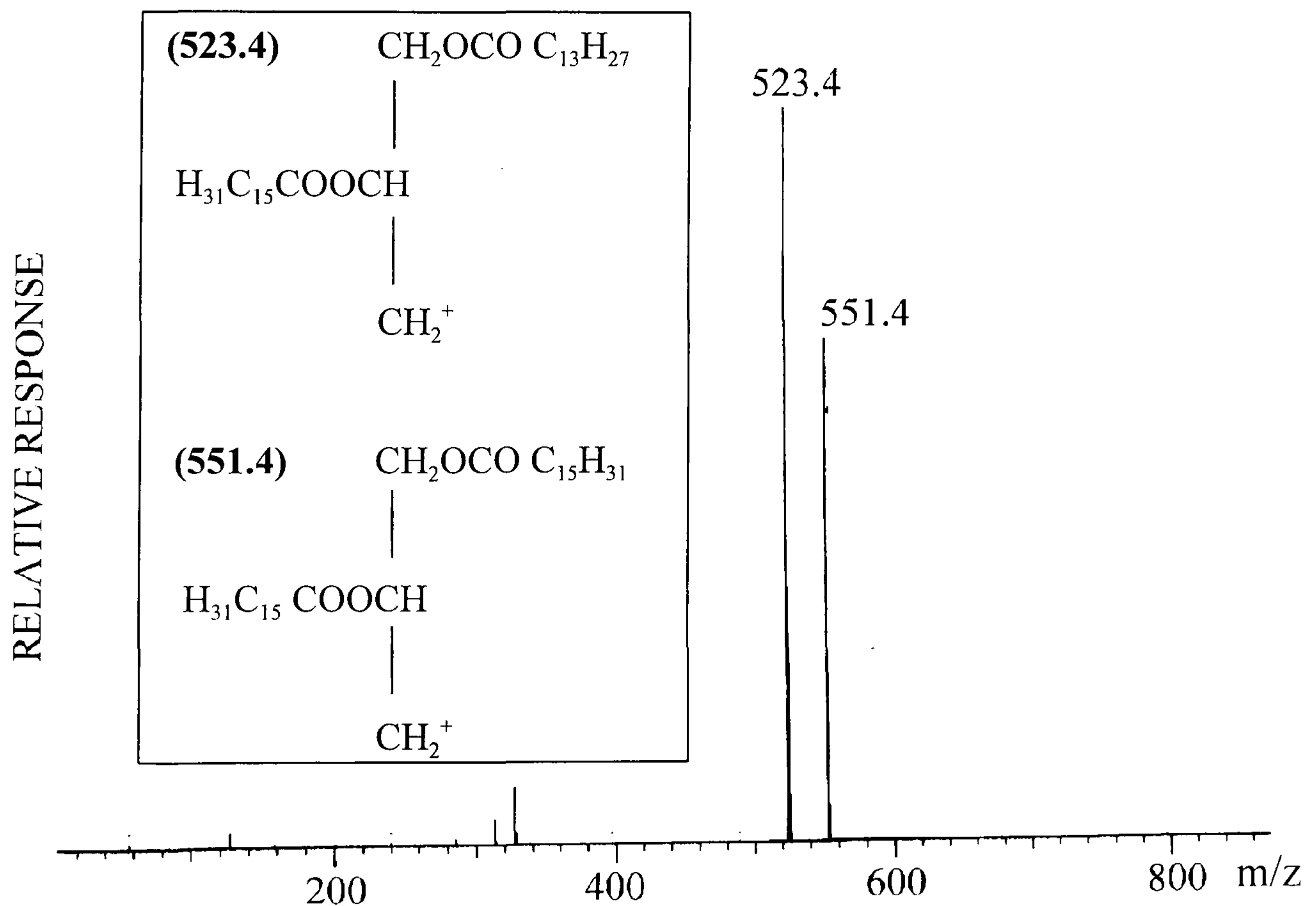


Figure 38: A HPLC-MS spectra of 1,2-dipalmitoyl-3-myristoyl-rac-glycerol (C16:0/C16:0/C14:0). See Section 19.4 for chromatographic conditions.

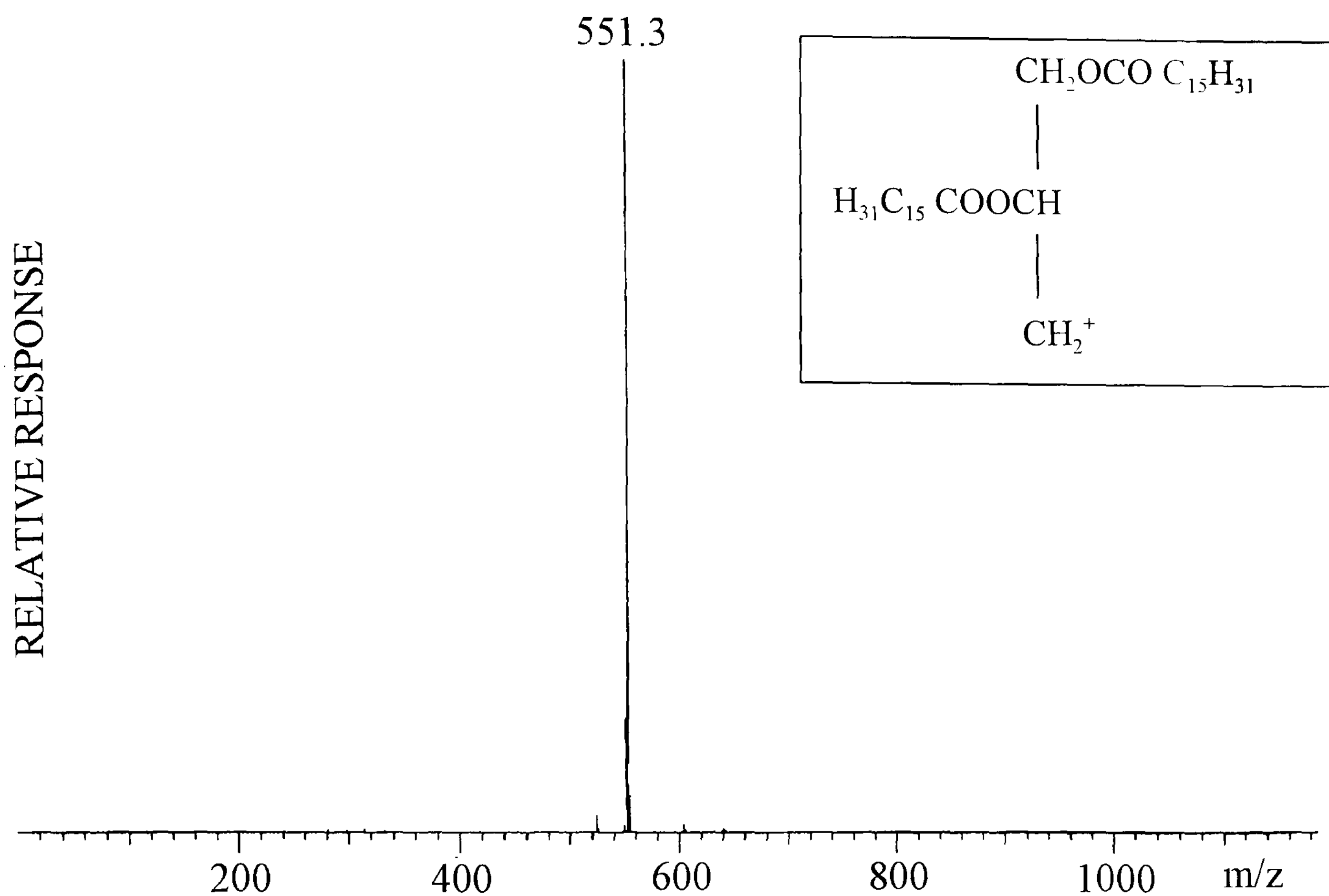


Figure 39: A HPLC-MS spectra of tripalmitin (C16:0). See Section 19.4 for chromatographic conditions.

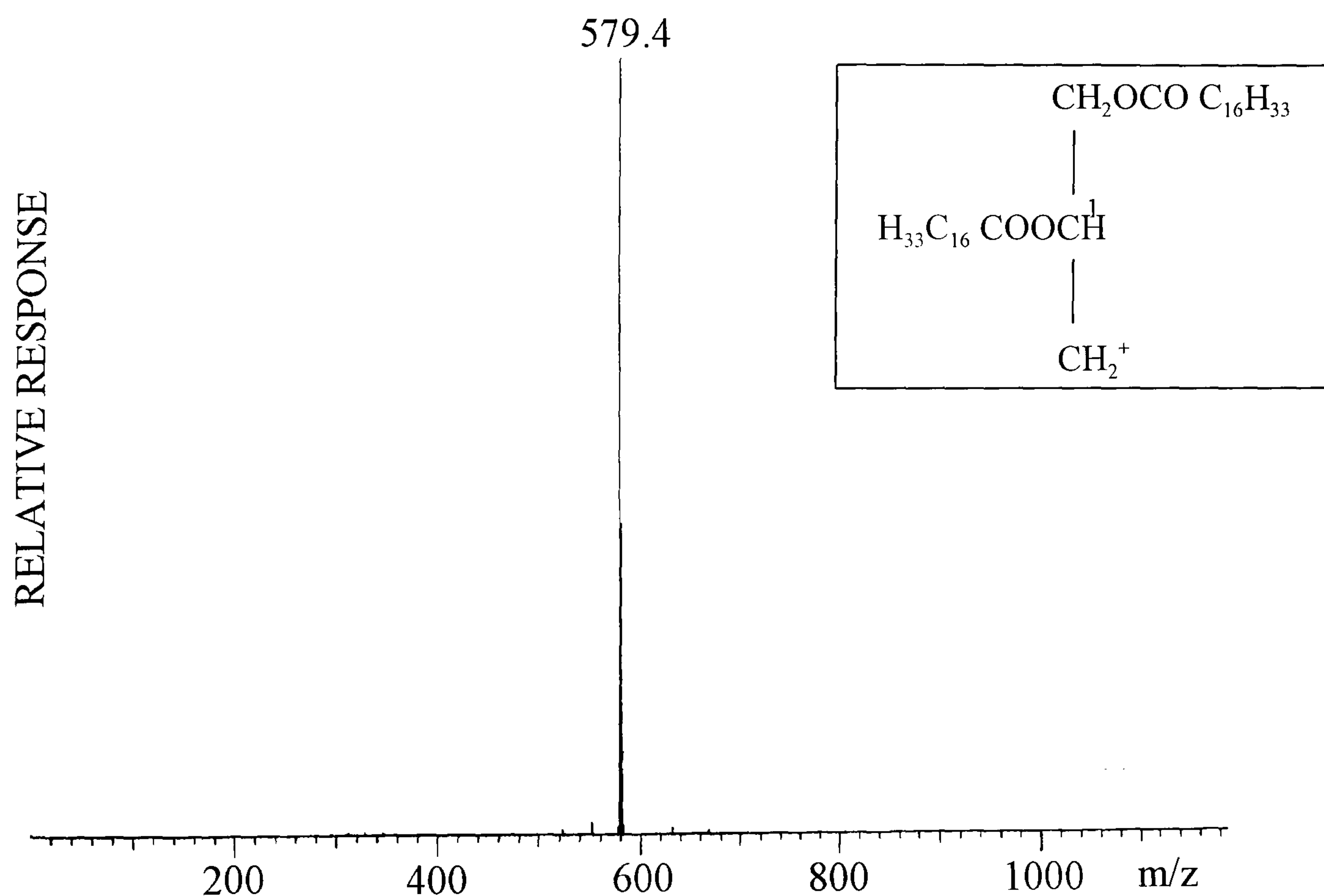


Figure 40: A HPLC-MS spectra of triheptadecanoin (C17:0). See Section 19.4 for chromatographic conditions.

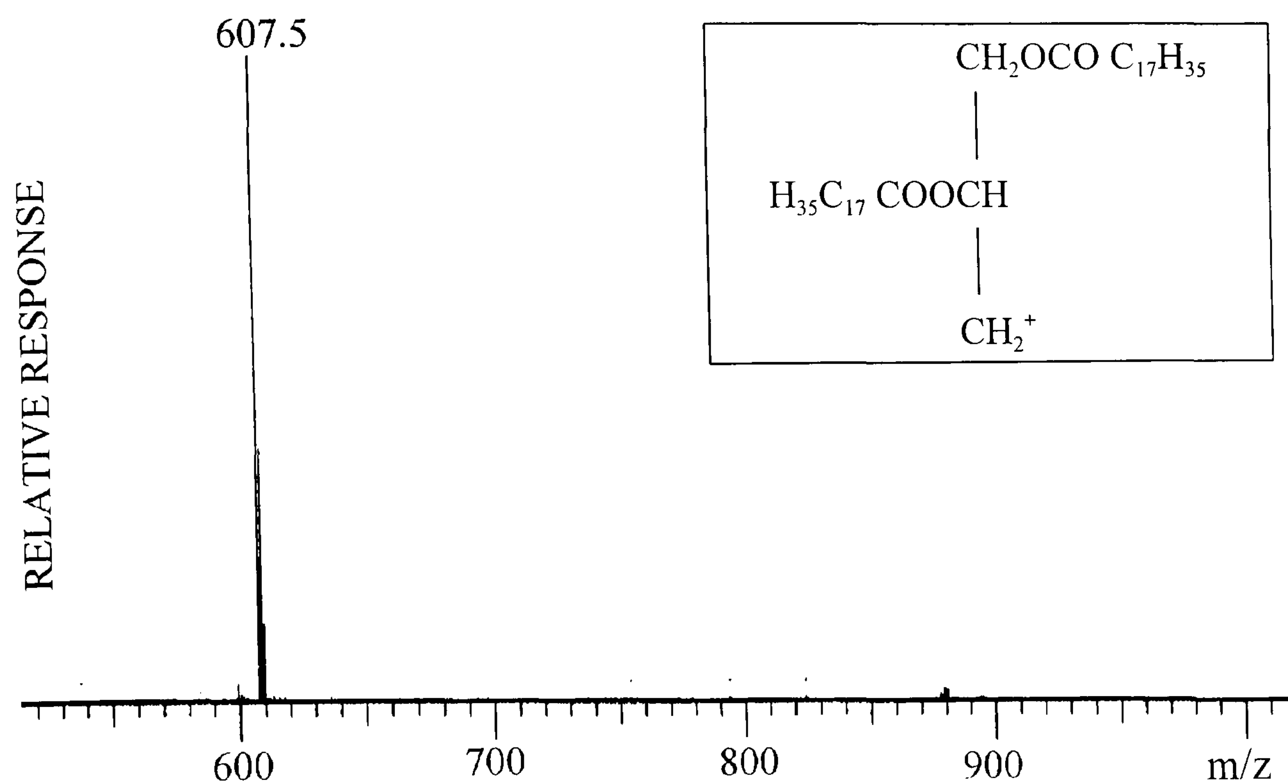


Figure 41: A HPLC-MS spectra of tristearin (C18:0). See Section 19.4 for chromatographic conditions.

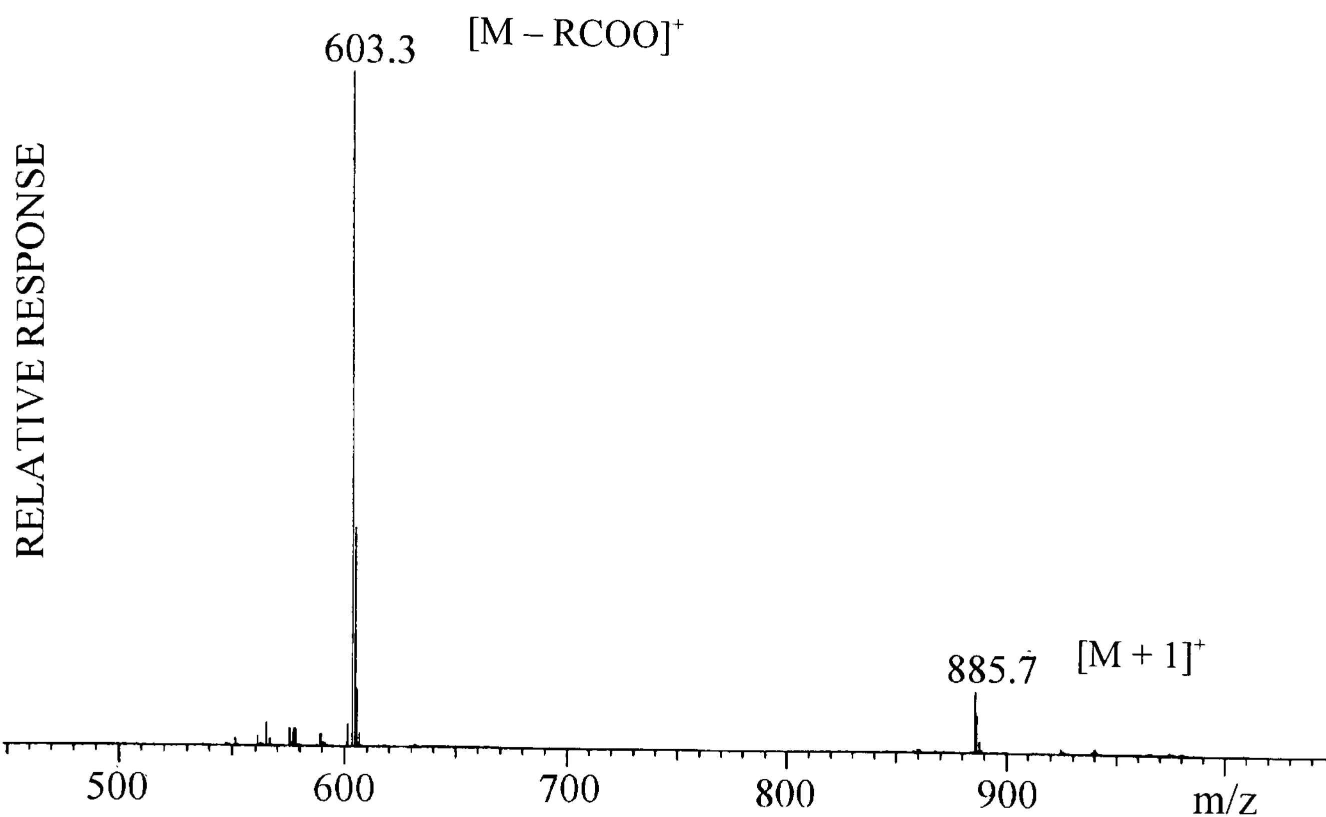


Figure 42: A HPLC-MS spectra of triolein (C18:1,[cis]-9). See Section 19.4 for chromatographic conditions

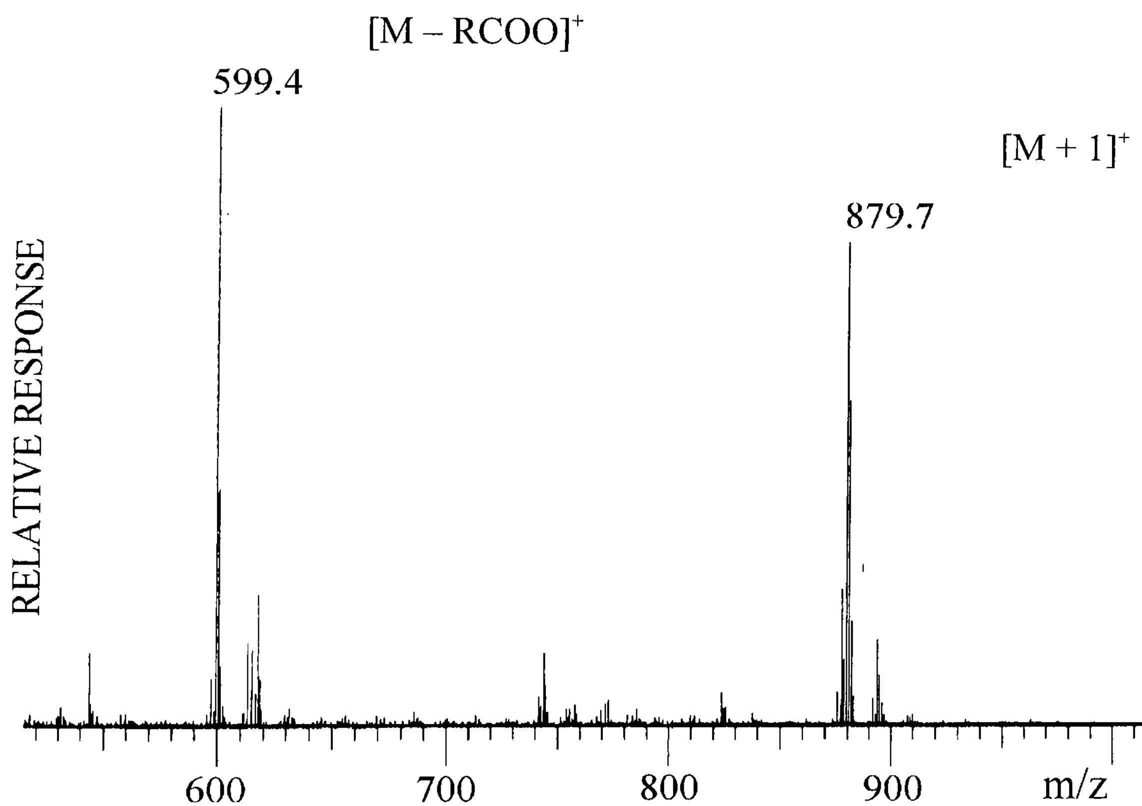


Figure 43: A HPLC-MS spectra of trilinolein (C18:2,[cis,cis]-9,12). See Section 19.4 for chromatographic conditions.

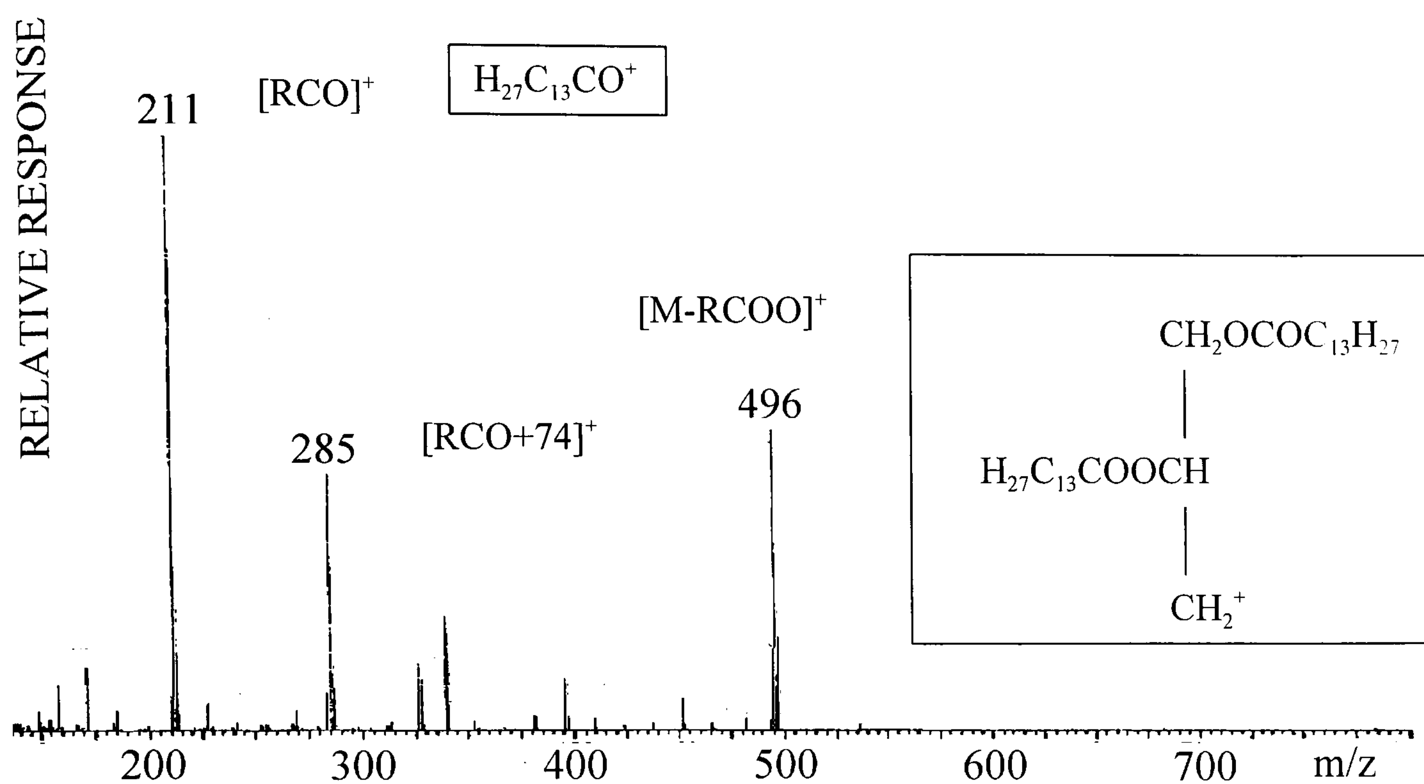


Figure 44: A high temperature GC-MS spectra of trimyristin (C14:0), using methane as a reagent gas. See Section 18.1 for chromatographic conditions.

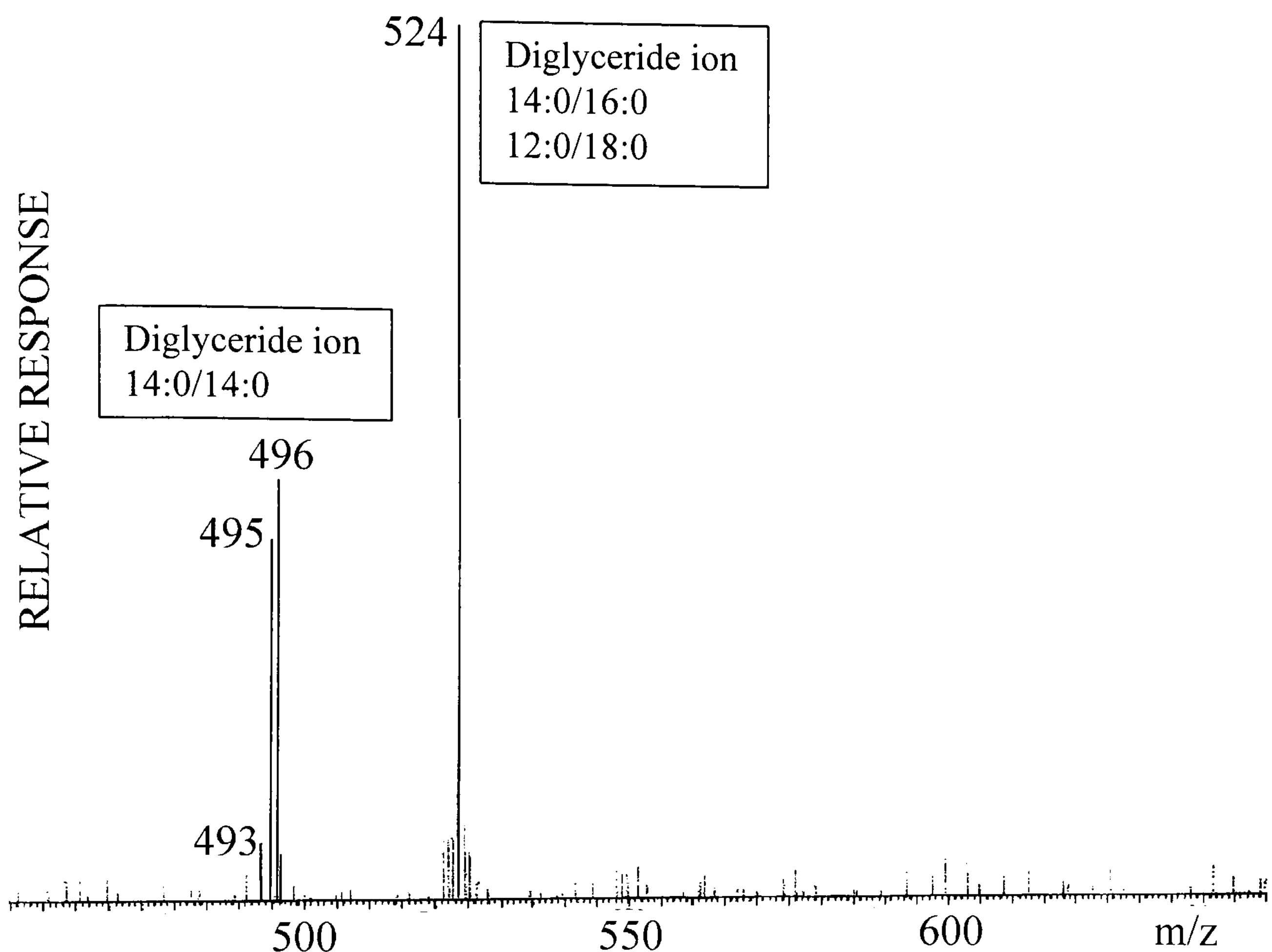


Figure 45: A high temperature GC-MS spectra of the triglyceride fraction CN=47 from the fraction SSS in camel milk fat (see Section 18.1 for chromatographic conditions).

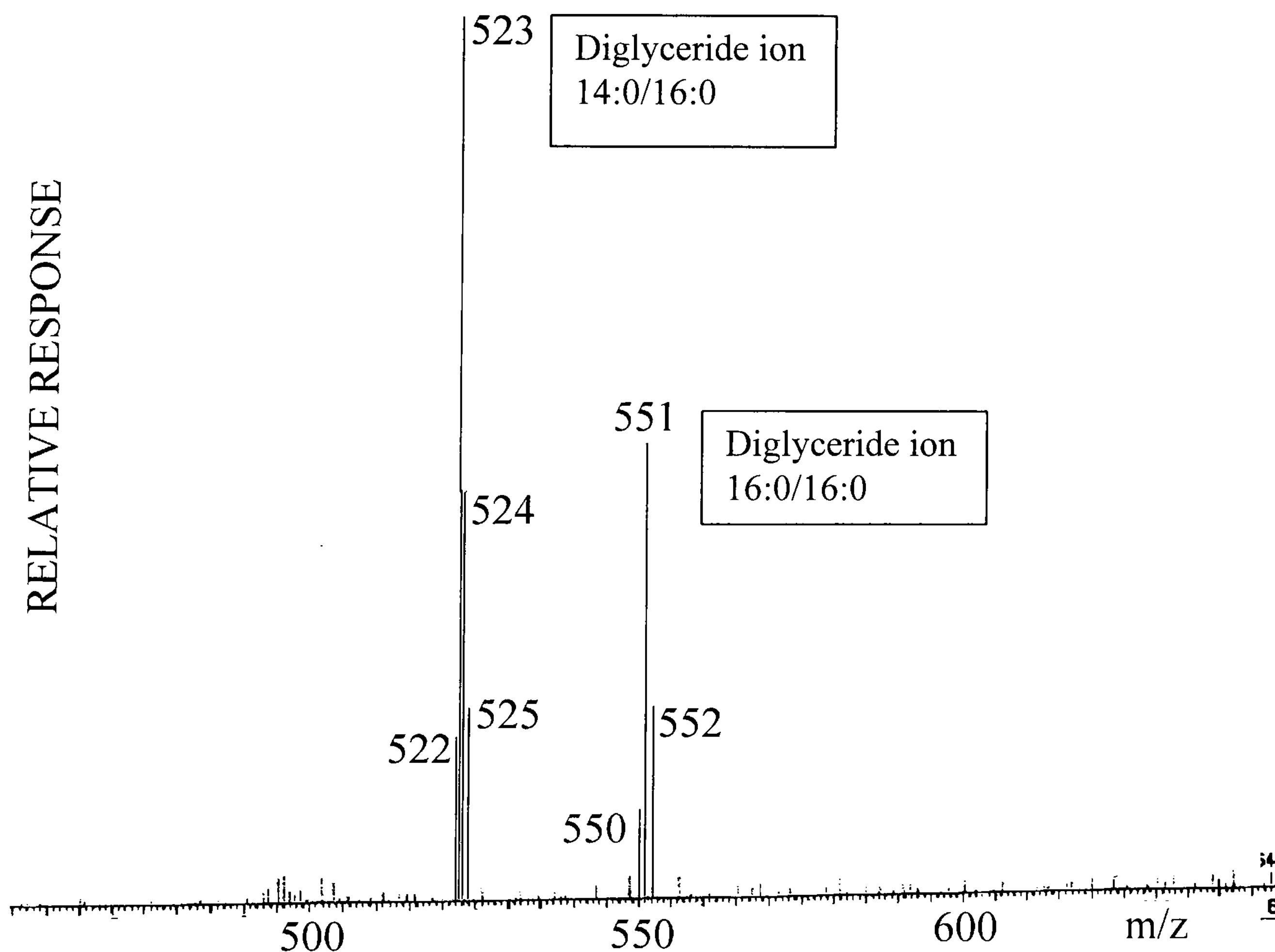


Figure 46: A high temperature GC-MS spectra of the triglyceride fraction CN=49 from the fraction SSS in camel milk fat (see Section 18.1 for chromatographic conditions).

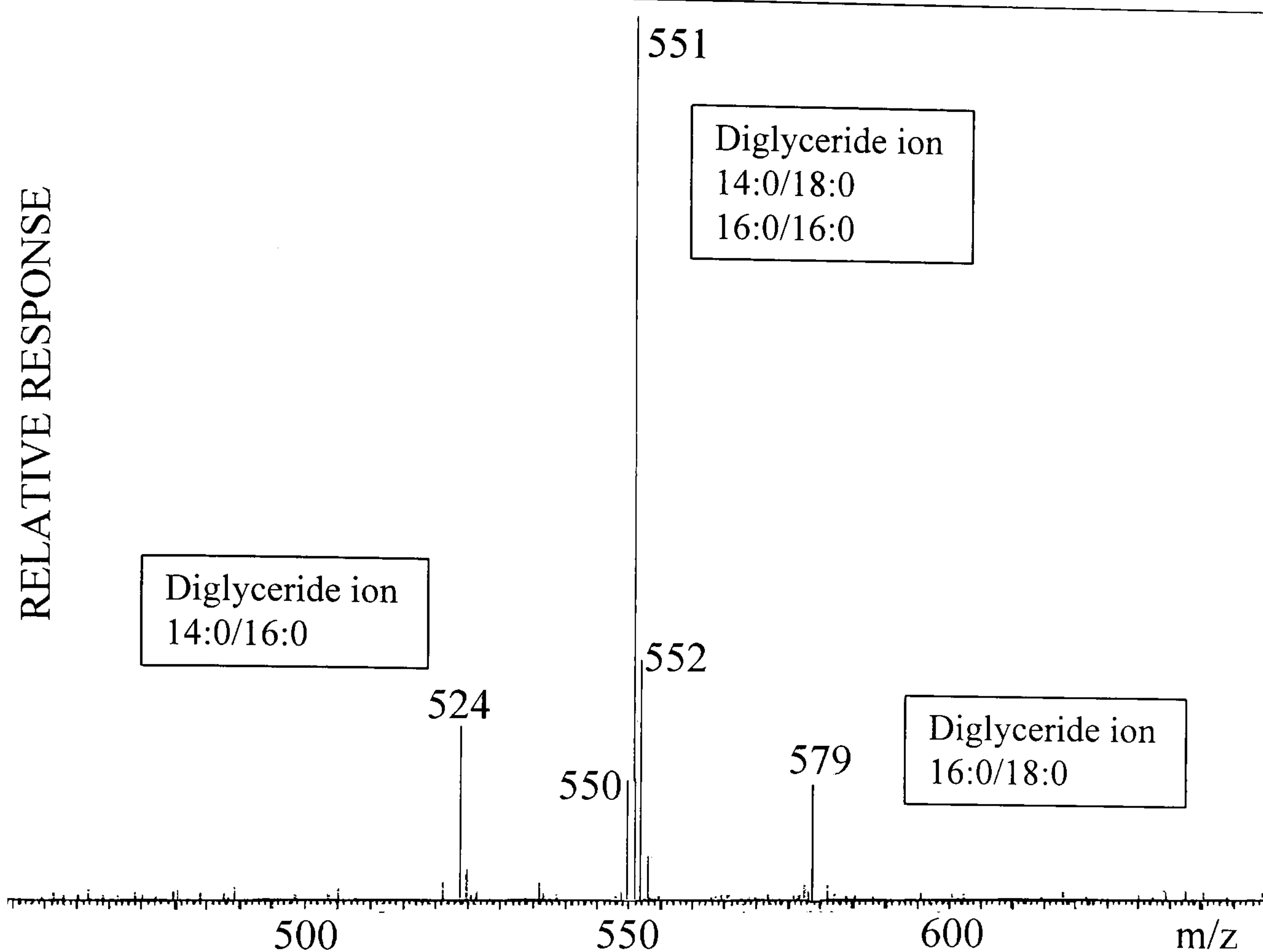


Figure 47: A high temperature GC-MS spectra of the triglyceride fraction CN=51 from the fraction SSS in camel milk fat (see Section 18.1 for chromatographic conditions).

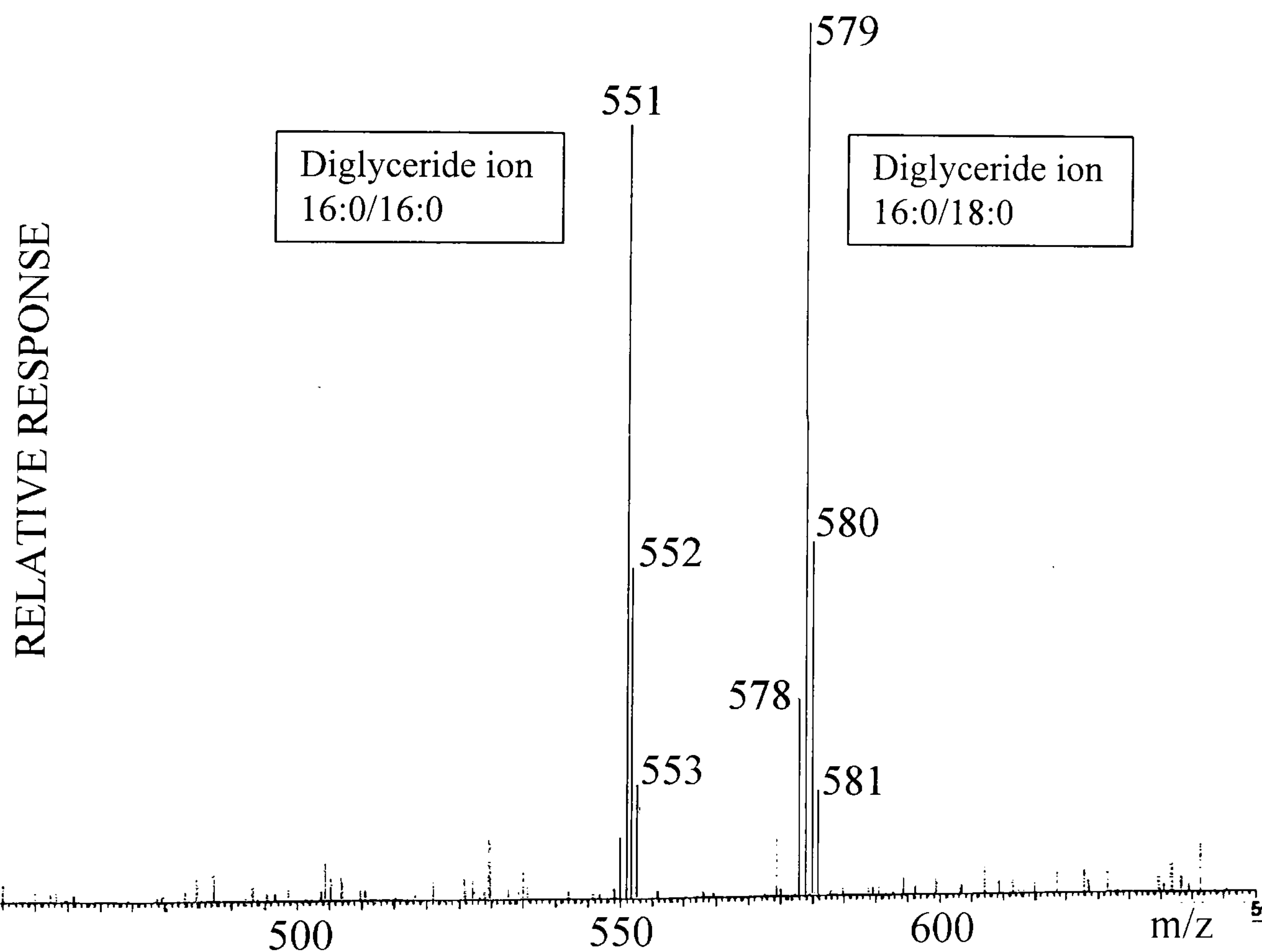


Figure 48: A high temperature GC-MS spectra of the triglyceride fraction CN=53 from the fraction SSS in camel milk fat (see Section 18.1 for chromatographic conditions).

Table 26: Composition of the free fatty acid standard used for the determination and quantification of free fatty acids in camel milk fat.

Fatty acid	Amount weighed (mg)	Molecular mass	Volume (ml)	μmol/ml	μl removed	Final concentration (nmol/ml) (standard 4)
C 4:0 Butyric acid	69.7	110.00	3.17	199.89	50	999.43
C 5:0 Valeric acid	25.5	102.14	2.50	99.86	100	998.63
C 6:0 Caproic acid	68.0	116.60	2.93	199.04	50	995.21
C 7:0 Pimelic acid	39.3	160.17	2.45	100.15	50	500.74
C 8 :0 Caprylic acid	71.0	144.20	2.46	200.15	50	1000.76
C 9:0 Perlagonic acid	31.6	158.20	2.50	79.90	125	998.74
C 10:0 Capric acid	99.5	172.27	2.89	199.86	50	999.28
C 12:0 Lauric acid	107.1	200.30	2.67	200.26	50	1001.31
C 13:0 Tridecanoic acid	42.9	214.35	2.50	80.06	125	1000.70
C 14:0 Myristic acid	22.8	228.40	2.50	39.93	250	998.25
C 16:0 Palmitic acid	25.6	256.40	2.50	39.94	250	998.44
C 16:1 Palmitoelic acid	25.4	254.40	2.50	39.94	250	998.43
C 17:0 Margaric acid	27.0	270.46	2.50	39.93	250	998.30
C 18:0 Stearic acid	28.4	284.50	2.50	39.93	250	998.24
C 18:1 Oleic acid	28.2	282.50	2.50	39.93	250	998.23
C 18:2 Linoleic acid	156.0	280.46	2.78	200.08	50	1000.41
C 18:3 Linolenic acid	160.0	278.44	2.87	200.22	50	1001.10
C 20:0 Arachidic acid	31.2	312.50	2.50	39.94	250	998.40

Table 27: The peak areas (raw data) of the individual fatty acids of the free fatty acid standard (Table 26), used for the determination of the response factors (Table 28). See Section 15.4 for chromatographic conditions.

	Peak areas (1μl samples)				
Fatty acid	1	2	3	4	5
C 4:0	1935867	2229653	2057663	2904371	2878747
C 5:0	8730009	9787231	8955495	12676810	12454028
C 6:0	8486703	9563739	8781692	12254146	11866979
C 8:0	8616225	9519221	8743038	12077671	11795430
C 9:0	6926794	8003743	7204523	10027712	9793673
C 10:0	7125440	8512388	7454525	10556856	10454802
C 12:0	3929089	4834618	4019915	5711081	5503310
C 13:0	4207186	5261902	4365819	6323510	5899669
C 14:0	4391553	5906969	4582655	6792780	6288316
C 16:0	4518976	6707208	4952122	7370065	6776645
C 16:1	7260901	10465090	7678548	11128643	10294355
C 17:0	4562916	6709049	4757613	7084731	6688620
C 18:0	4777104	7390906	5075480	7844649	7275921
C 18:1	7120305	11130153	7647911	11382970	10744866
C 18:2	4230684	6454224	4437687	6634098	5995642
C 18:3	2937766	4581058	3091154	4625219	4347189
C 20:0	3975059	6323294	4266276	6041181	5922156

Table 28: The response factors with respect to the peak areas in Table 27 for the determination of free fatty acids in camel milk, C13:0 is the internal standard. See Section 15.4 for chromatographic conditions.

	Response factors						
Fatty acid	1	2	3	4	5	Mean	± SD
C 4:0	2.17	2.36	2.12	2.18	2.05	2.18	0.115
C 5:0	0.48	0.54	0.49	0.50	0.47	0.50	0.025
C 6:0	0.50	0.55	0.50	0.52	0.50	0.52	0.023
C 8:0	0.49	0.55	0.50	0.52	0.50	0.52	0.026
C 9:0	0.61	0.66	0.61	0.63	0.60	0.62	0.023
C 10:0	0.59	0.62	0.59	0.60	0.56	0.59	0.02
C 12:0	1.07	1.09	1.09	1.11	1.07	1.09	0.015
C 13:0	1.00	1.00	1.00	1.00	1.00	1.00	0
C 14:0	0.96	0.89	0.95	0.93	0.94	0.93	0.027
C 16:0	0.93	0.78	0.88	0.86	0.87	0.85	0.053
C 16:1	0.58	0.50	0.57	0.57	0.57	0.55	0.031
C 17:0	0.92	0.78	0.92	0.89	0.88	0.87	0.056
C 18:0	0.88	0.71	0.86	0.81	0.81	0.80	0.065
C 18:1	0.59	0.47	0.57	0.56	0.55	0.54	0.045
C 18:2	0.99	0.82	0.98	0.95	0.98	0.93	0.075
C 18:3	1.43	1.15	1.41	1.37	1.36	1.32	0.113
C 20:0	1.06	0.83	1.02	1.05	1.00	0.97	0.092

Table 29: An example of the response factors obtained (with reference to C17:0) and used for the determination of fatty acids in lipid samples using the TMSH method . See Section 17.4 for chromatographic conditions.

	Injections								
Fatty acid	1	2	3	4	5	6	Mean	± SD	± %
C6:0	0.332	0.358	0.348	0.348	0.347	0.349	0.35	0.0085	2.43
C8:0	0.483	0.488	0.474	0.475	0.472	0.474	0.48	0.0064	1.34
C10:0	0.618	0.622	0.608	0.608	0.604	0.605	0.61	0.0076	1.25
C11:0	0.678	0.681	0.666	0.671	0.664	0.670	0.67	0.0064	0.96
C12:0	0.762	0.763	0.749	0.749	0.744	0.745	0.75	0.0082	1.10
C13:0	0.797	0.801	0.789	0.789	0.781	0.783	0.79	0.0078	0.99
C14:0	0.910	0.858	0.847	0.846	0.841	0.846	0.86	0.0260	3.07
C14:1	0.853	0.836	0.830	0.824	0.820	0.828	0.83	0.0116	1.41
C15:0	0.922	0.907	0.900	0.896	0.893	0.896	0.90	0.0108	1.20
C15:1	0.876	0.881	0.871	0.869	0.866	0.867	0.87	0.0059	0.68
C16:0	1.064	0.955	0.948	0.951	0.949	0.950	0.97	0.0465	4.89
C16:1	0.986	0.917	0.913	0.915	0.909	0.911	0.93	0.0298	3.26
C17:0	1.000	1.000	1.000	1.000	1.000	1.000	1.00	0.0000	0.00
C17:1	1.002	1.004	0.991	0.991	0.987	0.987	0.99	0.0075	0.75
C18:0	1.194	1.046	1.047	1.049	1.049	1.051	1.07	0.0595	5.68
C18:1(trans)	1.114	1.046	1.037	1.033	1.026	1.024	1.05	0.0340	3.29
C18:1(cis)	1.206	1.039	1.034	1.031	1.027	1.027	1.06	0.0714	6.92
C18:2(trans)	1.002	0.998	0.989	0.984	0.982	0.979	0.99	0.0092	0.94
C18:2(cis)	1.003	0.973	0.957	0.960	0.953	0.952	0.97	0.0196	2.04
C18:3n6	0.916	0.949	0.923	0.919	0.913	0.914	0.92	0.0137	1.49
C20:0	1.125	1.148	1.128	1.116	1.111	1.110	1.12	0.0141	1.26
C18:3n3	0.955	0.945	0.935	0.931	0.930	0.929	0.94	0.0103	1.11
C20:1	1.144	1.146	1.129	1.115	1.111	1.108	1.13	0.0166	1.49
C21:0	1.184	1.205	1.174	1.165	1.160	1.156	1.17	0.0185	1.58
C20:2	1.000	1.017	1.005	0.989	0.987	0.985	1.00	0.0126	1.28
C 20:3n6	1.002	1.033	1.007	0.999	0.996	0.994	1.01	0.0143	1.43
C23:0	1.304	1.324	1.277	1.263	1.259	1.245	1.28	0.0301	2.38
C20:5	0.987	0.997	0.970	0.975	0.954	0.935	0.97	0.0225	2.31
C 22:2	1.136	1.175	1.158	1.130	1.142	1.157	1.15	0.0167	1.48
C24:0	1.392	1.365	1.320	1.304	1.298	1.288	1.33	0.0414	3.18
C24:1	1.409	1.411	1.389	1.359	1.352	1.344	1.38	0.0294	2.16
C22:6	0.961	0.986	0.950	1.005	0.943	0.954	0.97	0.0240	2.39

Table 30: Positional analysis of the individual triglycerides in the SSS fraction in cow milk fat

Fraction SSS						
Carbon number	**TG-composition**	**%**	**g/100g Fat**	**FS – composition**	**%**	**Major [M-RCOO]⁺ fragments**
39	**4:0/16:0/16:0**	64 .3	5 .8	**4:0/16:0/16:0**	42.7 **	16:0/16:0
				16:0/4:0/16:0	21.6 *	16:0/4:0
	4:0/14:0/18:0	21 .3	1 .9	**4:0/14:0/18:0**	12.7 *	
				4:0/18:0/14:0	8.6 *	
	6:0/14:0/16:0	8 .4	0 .8	**6:0/14:0/16:0**	3.3 – 8.3	
				6:0/16:0/14:0	0 – 5.0 *	
				14:0/6:0/16:0	0 – 5.0 *	
	Other TGs	6 .0	0 .5			
41	**4:0/16:0/18:0**	70 .9	4 .0	**4:0/16:0/18:0**	19.4 – 28.7	
				4:0/18:0/16:0	42.2 – 51.5	
	6:0/14:0/18:0	11 .5	0 .7	**6:0/14:0/18:0**	2.2	
				6:0/18:0/14:0	9.3	
				18:0/6:0/14:0	0	
	6:0/16:0/16:0	15 .6	0 .9			
	Other TGs	2 .0	0 .1			
43	No further information					
45	No furhter information					
47	**8:0/18:0/18:0**	ca. 7 .6				18:0/16:0
	10:0/16:0/18:0	ca. 33 .7				18:0/14:0
	12:0/14:0/18:0	ca. 18 .6				16:0/16:0
	12:0/16:0/16:0	ca. 18 .6				16:0/10:0
	14:0/14:0/16:0	ca. 21 .5				14:0/12:0
49	**10:0/18:0/18:0**	8 .0	0 .1	**18:0/10:0/18:0**	8.0	18:0/16:0
						18:0/14:0
	12:0/16:0/18:0	20 .4	0 .3	**12:0/16:0/18:0**	7.0	16:0/16:0
				18:0/12:0/16:0	13.4	18:0/12:0
						16:0/14:0
	14:0/14:0/18:0	7 .8	0 .1	**14:0/14:0/18:0**	7.8	16:0/12:0
						14:0/14:0
	14:0/16:0/16:0	63 .7	0 .9	**14:0/16:0/16:0**	36.1	
				16:0/14:0/16	27.6	
51	**12:0/18:0/18:0**	5 .9	0 .1	**12:0/18:0/18:0**	2.8	18:0/16:0
				18:0/12:0/18:0	3.0	18:0/14:0
						16:0/16:0
	14:0/16:0/18:0	56 .5	1 .1	**14:0/16:0/18:0**	6.1	16:0/14:0
				16:0/18:0/14:0	21.4	
				18:0/14:0/16:0	29.0	
	16:0/16:0/16:0	37 .6	0 .8			
53	**14:0/18:0/18:0**	24 .2	1 .1	**14:0/18:0/18:0**	11.8	18:0/16:0
				18:0/14:0/18:0	12.4	16:0/16:0
	16:0/16:0/18:0	75 .8	3 .4	**16:0/16:0/18:0**	49.8	
				16:0/18:0/16:0	26.0	
55	**16:0/18:0/18:0**	100	0 .7	**16:0/18:0/18:0**	70.5	18:0/18:0
				18:0/16:0/18:0	29.5	18:0/16:0

* The TLC techniques employed did not allow the determination of short chained fatty acids and thus values were taken from the literature to determine the C 4:0 and C 6:0 in the *sn*-1(3) position {21, 23, 24}.

** It is believed that the value is incorrect as C 4:0 is only found in the 1(3)-position.

Table 30 (continued): Positional analysis of the individual triglycerides in the SSM fraction in cow milk fat

Fraction SSM						
Carbon number	**TG-composition**	**%**	**g/100g Fat**	**FA-composition**	**%**	**Major $[M-RCOO]^+$ fragments**
41	**4:0/16:0/18:1**	80 .6	7 .2	**4:0/16:0/18:1**	34.7 - 36.4 *	18:1/16:0
				4:0/18:1/16:0	44.3 - 46.0 *	18:1/4:0
						16:0/4:0
	4:0/18:0/16:1	6 .1	0 .5	**4:0/16:1/18:0**	6.1 *	
	6:0/14:0/18:1	9 .4	0 .9	**6:0/14:0/18:1**	6.2 *	
				6:0/18:1/14:0	3.2 *	
	6:0/16:0/16:1	1 .8	0 .2			
	Other TGs	ca. 2 .0	0 .2			
43	**8:0/14:0/18:1**	8 .0	0 .5	**8:0/14:0/18:1**	2.2	18:1/16:0
				14:0/8:0/18:1	0 – 5.8	18:1/6:0
				14:0/18:1/8:0	0 – 5.8	16:0/6:0
	6:0/16:0/18:1	82 .0	5 .2	**6:0/16:0/18:1**	28.6	
				16:0/6:0/18:1	23.8 – 29.7	
				6:0/18:1/16:0	23.6 – 29.5	
	Other TGs	ca.10				
45	**8:0/16:0/18:1**	29 .7	0 .7			18:1/18:0
	10:0/14:0/18:1	16 .0	0 .4			18:0/6:0
	12:0/12:0/18:1	3 .5	0 .1			16:0/6:0
	6:0/18:0/18:1	45 .8	1 .1			18:1/16:0
	Other TG´s	ca. 5 .0	0 .1			18:1/14:0
47	**8:0/18:0/18:1**	19 .6	0 .2			18:1/12:0
	10:0/16:0/18:1	42 .6	0 .5			14:1/16:0
	12:0/14:0/18:1	19 .4	0 .2			18:1/10:0
	14:0/16:0/14:1	16 .4	0 .2			14:1/14:0
	Other TGs	2 .0	0 .02			18:0/8:0
49	No further information					16:0/10:0
						14:0/12:0
51	**12:0/18:0/18:1**	11 .0		**18:0/12:0/18:1**	11.0	18:1/16:0
						18:1/14:0
	14:0/16:0/18:1	67 .0		**14:0/16:0/18:1**	8.3 – 13.0	16:0/14:0
				16:0/14:0/18:1	32.8 – 37.5	
				14:0/18:1/16:0	21.1	
	14/18/16:1	8 .0		**14:0/16:1/18:0**	2.7 - 7.4	
				18:0/14:0/16:1	0 .6 - 5 .3	
	16:0/18:0/14:1	9 .3		**16:0/14:1/18:0**	9.3	
	16:0/16:0/16:1	4 .7		**16:0/16:0/16:1**		
				16:0/16:1/16:0		

Table 30 (continued)

53	**14:0/18:0/18:1**	25 .6	0 .3	**14:0/18:0/18:1**	3.8 - 7.8	18:1/16:0
				14:0/18:1/18:0	3.2 - 7.2	16:0/16:0
				18:0/14:0/18:1	14.6	
	18:0/18:0/14:1	2 .6	0 .03	**18:0/14:1/18:0**	2.6	
	16:0/18:0/16:1	7 .8	0 .1	**16:0/18:0/16:1**	0 - 4.0	
				16:0/16:1/18:0	0 - 4.0	
				18:0/16:0/16:1	3.8	
	16:0/16:0/18:1	64 .0	0 .8	**16:0/16:0/18:1**	25.4 - 29.4	
				16:0/18:1/16:0	34.6 - 38.6	
55	**16:0/18:0/18:1**	93 .3	0 .9	**16:0/18:0/18:1**	9.6	18:1/18:0
				18:0/16:0/18:1	33.4	18:0/16:0
				16:0/18:1/18:0	50.3	18:1/16:0
	18:0/18:0/16:1	6 .7	0 .1	**18:0/18:0/16:1**	1.4	
				18:0/16:1/18:0	5.3	

Table 31: Positional analysis of the individual triglycerides in the SSS fraction in camel milk fat

Fraction SSS						
Carbon number	**TG-composition**	**%**	**g/100g Fat**	**FA-composition**	**%**	**Major [M-RCOO]⁺ fragments**
47	**10:0/16:0/18:0**	3.7	0.3			18:0/14:0
	12:0/14:0/18:0	15.8	1.0			18:0/12:0
	12:0/16:0/16:0	2.1	0.1			16:0/14:0
	14:0/14:0/16:0	44.0	2.9			14:0/14:0
49	**10:0/18:0/18:0**	2.9	0.4	**18:0/10:0/18:0**	2.9	16:0/16:0
						16:0/14:0
	12:0/16:0/18:0	5.1	0.6	**12:0/16:0/18:0**	0 – 1.9	
				16:0/18:0/12:0	0 – 1.9	
				18:0/12:0/16:0	3.2	
	14:0/14:0/18:0	19.0	2.3	**14:0/14:0/18:0**	7.6 – 9.5	
				14:0/18:0/14:0	9.5 – 11.4	
	14:0/16:0/16:0	73.0	9.0	**14:0/16:0/16:0**	33.5 – 39.3	
				16:0/14:0/16:0	33.7 – 39.4	
51	**14:0/16:0/18:0**	43.6	4.2	**14:0/16:0/18:0**	2.6	18:0/16:0
				16:0/18:0/14:0	14.4	16:0/16:0
				18:0/14:0/16:0	26.5	14:0/18:0
						16:0/14:0
	16:0/16:0/16:0	56.5	5.5			
53	**14:0/18:0/18:0**	14.7	1.0	**14:0/18:0/18:0**	6.3	18:0/16:0
				18:0/14:0/18:0	8.4	16:0/16:0
	16:0/16:0/18:0	85.3	6.1	**16:0/16:0/18:0**	71.5	
				16:0/18:0/16:0	13.8	

Table 31 (continued): Positional analysis of the individual triglycerides in the SSM fraction in camel milk fat

Fraction SSM (cis)

Carbon number	TG-composition	%	g/100g Fat	FA-composition	%	Major [M-RCOO]+ fragments
49	12:0/16:0/18:1	8 .6	0 .3	16:0/12:0/18:1	8.6	18:1/14:0 16:1/16:0
	14:0/14:0/18:1	29 .4	1 .2	14:0/14:0/18:1	22.3	16:0/14:0
				14:0/18:1/14:0	7.1	16:1/14:0 14:1/16:0
	16:0/16:0/14:1	16 .2	0 .6	16:0/16:0/14:1	3.9	14:0/14:0
				16:0/14:1/16:0	12.3	
	14:0/16:0/16:1	45 .8	1 .8	14:0/16:0/16:1	15.6	
				16:0/14:0/16:1	9.5	
				14:0/16:1/16:0	20.7	
51	12:0/18:0/18:1	1 .5	0 .1	12:0/18:1/18:0	1.5	18:1/16:0
						18:1/14:0 16:1/16:0
	14:0/16:0/18:1	52 .5	3 .1	14:0/16:0/18:1	4.0 – 9.2	
				16:0/14:0/18:1	24.2 – 29.4	16:0/14:0
				14:0/18:1/16:0	19.2	
	14:0/18:0/16:1	5 .3	0 .3	14:0/16:1/18:0	0 – 5.3	
				18:0/14:0/16:1	0 – 5.3	
	16:0/18:0/14:1	7 .9	0 .5	18:0/16:0/14:1	1.6	
				16:0/14:1/18	6.3	
	16:0/16:0/16:1	32 .8	2 .0	16:0/16:0/16:1	12.3 – 17.5	
				16:0/16:1/16:0	15.3 – 20.5	
53	14:0/18:0/18:1	24 .6	1 .0	14:0/18:0/18:1	0 – 9.9	18:1/18:0
				14:0/18:1/18:0	0 – 9.9	18:1/16:0
				18:0/14:0/18:1	14.7	18:0/14:0 16:0/16:0
	18:0/18:0/14:1	3 .4	0 .2	18:0/18:0/14:1	0.07	
				18:0/14:1/18:0	2.7	
	16:0/18:0/16:1	20 .0	0 .9	16:0/18:0/16:1	0 – 9.7	
				16:0/16:1/18:0	10.4	
				18:0/16:0/16:1	0 – 9.7	
	16:0/16:0/18:1	52 .0	2 .2	16:0/16:0/18:1	33.0 – 42.7	
				16:0/18:1/16:0	9.2 - 19.0	
55	16:0/18:0/18:1	8 .4	0 .1	16:0/18:0/18:1	0.7	18:0/18:0
				18:0/16:0/18:1	2.7	18:0/16:1
				16:0/18:1/18:0	5.0	
	18:0/18:0/16:1	91 .6	1 .4	18:0/18:0/16:1	19.0	
				18:0/16:1/18:0	72.6	

Table 31 (continued): Positional analysis of the individual triglycerides in the SMM fraction in camel milk fat

Fraction SMM (cis, cis)						
Carbon number	**TG-composition**	**%**	**g/100g Fat**	**FA-composition**	**%**	**Major [M-RCOO]+ fragments**
51	**12:0/18:1/18:1**	3 .7	0 .1	**12:0/18:1/18:1**	2.2	
				18:1/12:0/18:1	1.5	
	14:0/16:1/18:1	48 .9	1 .2	**14:0/16:1/18:1**	0 - 28.5	
				14:0/18:1/16:1	0 – 28.5	
				16:1/14:0/18:1	20.4	
	18:0/14:1/16:1	3 .7	0 .1	**18:0/14:1/16:1**	0 – 3.7	
				18:0/16:1/14:1	0 – 3.7	
	16:0/14:1/18:1	13 .5	0 .3	**16:0/14:1/18:1**	3.1 – 6.9	
				14:1/16:0/18:1	6.6 – 10.4	
				16:0/18:1/14:1	6.6 – 10.4	
	16:0/16:1/16:1	30 .2	0 .6	**16:0/16:1/16:1**	19.2 – 30.1	
				16:1/16:0/16:1	0 - 10.9	
53	**14:0/18:1/18:1**	27 .0	1 .4	**14:0/18:1/18:1**	14.9	18:1/16:0
				18:1/14:0/18:1	12.1	18:1/16:1
						18:1/14:0
	18:0/14:1/18.1	2 .2	0 .1	**18:0/14:1/18:1**	1.4	16:1/16:0
				14:1/18:0/18:1	0 – 0.8	
				18:0/18:1/14:1	0 – 0.8	
	18:0/16:1/16:1	8 .3	0 .5	**18:0/16:1/16:1**	7.0 – 7.7	
				16:1/18:0/16:1	0.5 – 1.3	
	16:0/16:1/18:1	62 .5	3 .3	**16:0/16:1/18:1**	42.1 - 42.9	
				16:1/16:0/18:1	15.4	
				16:0/18:1/16:1	4.2 - 4.9	
55	**16:0/18:1/18:1**	77 .8	4 .1	**16:0/18:1/18:1**	53.6	18:1/18:1
				18:1/16:0/18:1	24.2	18:1/16:0
	18:0/16:1/18:1	22 .2	1 .2	**18:0/16:1/18:1**	10.4	
				16:1/18:0/18:1	6.9	
				18:0/18:1/16:1	4.9	
57	**18:0/18:1/18:1**	100	0 .6	**18:0/18:1/18:1**	72.5	18:1/18:0
				18:1/18:0/18:1	27.5	18:1/18:1

Table 32: Positional analysis of the individual triglycerides in the SSS fraction in camel hump fat

Fraction SSS						
Carbon number	**TG-composition**	**%**	**g/100g Fat**	**FA-composition**	**%**	**Major [M-RCOO]⁺ fragments**
49	**10:0/18:0/18:0**	1 .9	0 .1	**18:0/10.0/18:0**	1.9	18:0/14:0 16:0/16:0
	12:0/16:0/18:0	7 .6	0 .5	**12:0/16:0/18:0** **16:0/18:0/12:0**	0 – 3.2 0 – 3.2	18:0/12:0 16:0/14:0
	14:0/14:0/18:0	22 .0	1 .3	**14:0/14:0/18:0** **14:0/18:0/14:0**	4.1 – 7.3 3.6 - 6.8	
	14:0/16:0/16:0	68 .5	4 .2	**14:0/16:0/16:0** **16:0/14:0/16:0**	35.6 - 38.8 29.7 - 32.9	
51	**12:0/18:0/18:0**	1 .1	0 .2	**12:0/18:0/18:0** **18:0/12:0/18:0**	0.3 0.8	18:0/16:0 18:0/14:0 16:0/16:0
	14:0/16:0/18:0	43 .7	6 .0	**14:0/16:0/18:0** **16:0/18:0/14:0** **18:0/14:0/16:0**	19.7 0.6 23.4	16:0/14:0
	16:0/16:0/16:0	55 .2	7 .6			
53	**14:0/18:0/18:0**	14 .6	1 .9	**14:0/18:0/18:0** **18:0/14:0/18:0**	4.5 10.0	18:0/16:0 16:0/16:0
	16:0/16:0/18:0	85 .4	11 .2	**16:0/16:0/18:0** **16:0/18:0/16:0**	79.0 6.4	
55	**16:0/18:0/18:0**	100 .0	5 .9	**16:0/18:0/18:0** **18:0/16:0/18:0**	68.3 31.7	18:0/18:0 18:0/16:0

Applicability

Determination of fatty acids or fatty acid components in triglycerides or other lipid compounds e.g. phospholipids.

2 Principle

The determination of fatty acids *via* their methyl esters is carried out using a gas chromatograph. The fatty acid methyl ester is prepared using trimethylsulphonium hydroxide (TMSH) in methanol at room temperature. After a short reaction time the solution is ready for injection without the necessity to remove excess reagent.

In the injection block of the gas chromatograph, excess reagent is pyrolised above 250°C into dimethyl sulphide and methanol.

The methyl esters are detected using a flame ionization detector (FID). The concentration and identification of the respective fatty acids is determined using a suitable standard mixture as a reference. An internal standard is required for quantification, e.g. triheptadecanoin (C17:0).

3 Interference

The fatty acid residues in all lipid compounds will be derivatised and determined as their methyl esters. Thus if fatty acids are to be determined in a particular class of compounds within a mixture, isolation prior to derivatisation (e.g. TLC, column chromatography) is necessary. Such a procedure also serves as a cleaning up process.

Apparatus

4.1	Gas chromatograph	GC Auto System (No. B050-6120) in conjunction with \`Turbochrome´ software for control and integration. Perkin Elmer, USA
4.2	Capillary column	SP 2330 (30 m x 0.32 mm i.d.) SUPELCO, USA

Further Apparatus — Analytical balance (No. 1712 MP8 Sartorius, Germany)
Micropipette (10-100 µl)
Volumetric cylinder (100, 250 ml)
Glass beakers (100, 250 ml)
Magnetic stirrer/hot plate
Glass column with fritt and tap (20 x 2 cm i.d.)
pH paper
N11 HPLC vials with caps (No.11020100C Bischoff, Germany)
Micro inserts for N11 vials

Table 32 (continued): Positional analysis of the individual triglycerides in the SSM fraction in camel hump fat.

Fraction SSM (cis)						
Carbon number	**TG-composition**	**%**	**g/100g Fat**	**FA-composition**	**%**	**Major $[M-RCOO]^+$ fragments**
51	12:0/18:0/18:1	2 .9	0 .1	12:0/18:1/18:0	0 - 1.0	18:1/16:0
				12:0/18:0/18:1	0 - 1.0	18:1/14:0
				18:0/12:0/18:1	1.9	16:1/16:0
						14:1/18:0
	14:0/16:0/18:1	62 .1	2 .1	14:0/16:0/18:1	12.5	16:0/14:0
				16:0/14:0/18:1	24.9	
				14:0/18:1/16:0	24.7	
	14:0/18:0/16:1	0	0	14:0/16:1/18:0	0	
				18:0/14:0/16:1	0	
	16:0/18:0/14:1	9 .8	0 .3	18:0/16:0/14:1	0 – 6.4	
				16:0/14:1/18:0	3.4	
				16:0/18:0/14:1	0 – 6.4	
	16:0/16:0/16:1	25 .2	0 .8	16:0/16:0/16:1	14.4	
				16:0/16:1/16:0	10.8	
53	14:0/18:0/18:1	15 .2	1 .3	14:0/18:0/18:1	0 – 8.6	18:1/16:0
				14:0/18:1/18:0	0 – 8.6	18:0/14:0
				18:0/14:0/18:1	6.6	16:0/16:0
	18:0/18:0/14:1	4 .2	0 .4	18:0/18:0/14:1	1.2	
				18:0/14:1/18:0	3.0	
	16:0/18:0/16:1	10 .2	0 .9	16:0/18:0/16:1	0 – 4.8	
				16:0/16:1/18:0	5.4	
				18:0/16:0/16:1	0 – 4.8	
	16:0/16:0/18:1	70 .4	6 .3	16:0/16:0/18:1	32.3	
				16:0/18:1/16:0	38.1	
55	16:0/18:0/18:1	95 .3	11 .1	16:0/18:0/18:1	39.1	18:1/18:0
				18:0/16:0/18:1	1.2	18:1/16:0
				16:0/18:1/18:0	55.1	
	18:0/18:0/16:1	4 .7	0 .6	18:0/18:0/16:1	1.5	
				18:0/16:1/18:0	3.2	
57	18:0/18:0/18:1	100 .0	5 .9	18:0/18:0/18:1	36.4	18:0/18:0
				18:0/18:1/18:0	63.6	18:1/18:0

5 Chemicals Dichloromethane (No. 6053.2 Roth, Germany)
Dry methanol (No. 65542 Fluka, Switzerland)
Sodium hydroxide (No. 71692 Fluka, Switzerland)
Trimethylsulphonium iodide (No. 818119 Merck, Germany)
Amberlite IRA-910 (No. 06457 Fluka, Switzerland)
Tricaproin (C 6/6/6) (No. T-0888 Sigma-Aldrich, USA)
Tricaprylin (C 8/8/8) (No. T-9001 Sigma-Aldrich, USA)
Trilaurin (C 12/12/12) (No. T-4891 Sigma-Aldrich, USA)
Timyristin (C 14/14/14) (No. T-5141 Sigma-Aldrich, USA)
Tripalmitin (C 16/16/16) (No. T-5888 Sigma-Aldrich, USA)
Triheptadecanoin (C 17/17/17)(No.T-2151 Sigma-Aldrich,USA)
Tristearin (C 18/18/18) (No. T-5016 Sigma-Aldrich, USA)
Triarachidin (C 20/20/20) (No. T-9267 Sigma-Aldrich, USA)
Tribehenin (C 22/22/22) (No. T-7904 Sigma-Aldrich, USA)

6 Preparation of solutions

6.1 TMSH-solution (0.2 mol/l in methanol)

35 ml (20 g) Amberlite is added to ca. 100 ml water, the glass column is then filled with the resulting suspension. 150 ml NaOH solution (4 g/100 ml) is then passed over the column, after which the ion exchange column is washed with ca. 150 ml water, until neutral (test with pH paper). Finally the column is rinsed with methanol, ca. 100 ml.

4.1 g Trimethylsulphonium iodide is dissolved in methanol at 50°C using the magnetic stirrer/hot plate. 5-10 ml portions of the solution are allowed to pass over the column. The solution is maintained at 50°C to prevent crystallisation of the trimethylsulphonium iodide. Finally the column is rinsed with 40 ml methanol.The collected eluant (trimethylsuphonium hydroxide 0.2 mol/l) may be stored in a sealed glass bottle at 4°C for 3-6 months.

Triglyceride standard

The amounts as shown in the table below of individual triglycerides were weighed and dissolved in dichloromethane to give a final volume of 10 ml.

Triglyceride	Molecular wt.	Sample mg	Conc. (mmol/l)
C6:0	386.5	14.82	3.835
C8:0	470.7	14.45	3.070
C12:0	639.0	7.18	1.123
C14:0	723.2	8.74	1.208
C16:0	807.3	9.53	1.181
C17:0	849.4	10.29	1.211
C18:0	891.5	10.16	1.140
C20:0	975.7	11.09	1.137
C22:0	1059.8	11.98	1.130

Calibration solutions

Portions of the stock solution are then diluted to give solutions of a suitable concentration to cover the range in which measurements are to be made. As the volumes of the final solutions are low (10ml), accurate dilutions are carried out by means of an analytical balance.

Triglyceride	Solution 1 (mmol/l)	Solution 2 (mmol/l)	Solution 3 (mmol/l)	Solution 4 (mmol/l)
C6:0	11.505	6.375	3.411	0.579
C8:0	9.210	5.103	2.730	0.465
C12:0	3.369	1.866	0.999	0.171
C14:0	3.624	2.007	1.074	0.183
C16:0	3.543	1.962	1.050	0.177
C17:0	3.633	2.013	1.077	0.183
C18:0	3.420	1.896	1.014	0.171
C20:0	3.411	1.890	1.011	0.171
C22:0	3.390	1.878	1.005	0.171

7 Derivatization and analysis

100 µl of the respective standard solution is pipetted into a GC sample vial, followed by 50 µl TMSH solution. The vial is shaken to ensure that the solution is homogeneous, the mixture is now ready for injection (1µl).

The samples are measured under the following conditions:

Injector temperature: 260°C
Detector temperature: 280°C

Temperature gradient:

Time	Temperature (°C)	Heating rate (°C/min)
0.5	70	12
1.0	170	2.6
6.5	191	7
10	225	

8 Evaluation of results

In the table below the integrated peaks areas for the respective fatty acids and concentrations are shown.

Concentration (mmol/l)	Fatty acid	Peak areas (raw data)									
		1	2	3	4	5	6	7	8	9	10
0.579	C6:0	35652	48884	44059	34362	35765	36398	32661	31632	36049	36284
0.465	C8:0	35653	49315	47978	35852	41473	40590	36961	37473	42205	41557
0.171	C12:0	20371	24286	24841	19586	22141	22554	17993	21184	24303	22913
0.183	C14:0	21375	27868	26892	21281	25074	25800	18875	23262	28093	27385
0.177	C16:0	21905	29068	29631	23493	26948	28853	20752	26355	30202	29926
0.183	C17:0	21529	29459	28391	22498	25855	26377	19544	24047	28239	28943
0.171	C18:0	19883	31340	29718	23447	27130	29055	19857	23458	29860	29887
0.171	C20:0	20084	27860	27615	21301	24612	26982	16252	20455	27065	26178
0.171	C22:0	17308	29829	27895	20507	21347	26336	17023	22746	28433	28100
3.411	C6:0	277406	206540	214256	193502	213447	211391	287277	248818		
2.730	C8:0	237566	234164	236095	212783	247163	244450	330747	271534		
0.999	C12:0	129340	126772	133901	105974	133067	133839	174666	143306		
1.074	C14:0	162042	157860	176504	131739	168244	172949	234695	192404		
1.050	C16:0	154651	153571	180178	129886	162629	160298	225319	186186		
1.077	C17:0	155906	156661	183800	136591	167756	166833	238862	196502		
1.014	C18:0	155923	156167	181932	136466	167255	163870	239783	201384		
1.011	C20:0	166499	164085	195713	144274	179742	179042	253016	215711		
1.005	C22:0	157983	158957	184903	133500	171312	171404	254134	213688		
6.375	C6:0	463681	475322	449791	531950	262863	539202	474712	458712		
5.103	C8:0	526907	531434	506803	604345	276475	589642	535784	522874		
1.866	C12:0	280236	287398	267951	302347	114134	326386	278727	277446		
2.007	C14:0	323234	359347	334414	364317	124890	407299	350542	333044		
1.962	C16:0	340475	344991	328859	357283	110821	413877	346100	336820		
2.013	C17:0	345436	367433	342540	358520	114493	433427	349425	346971		
1.896	C18:0	348382	365082	345706	354091	111083	441347	353268	355812		
1.890	C20:0	377779	395406	373467	387495	117168	479992	375172	384078		
1.878	C22:0	379150	399445	375648	381845	114858	476813	381734	383668		
11.505	C6:0	724107	690373	721644	853000	674218	700817	704282	1171628	1136286	602987
9.210	C8:0	817360	783870	833608	829281	756767	789584	797043	1237820	1272234	688853
3.369	C12:0	419438	406949	436114	444564	407529	425488	427746	683867	732071	346244
3.624	C14:0	507775	492551	528889	536954	489637	505329	511836	846913	902247	415465
3.543	C16:0	502948	495434	538793	550762	501148	527293	527796	895886	963980	420877
3.633	C17:0	530657	521519	559461	574442	518514	546992	551020	928781	998162	433365
3.420	C18:0	530833	522999	560176	578340	520835	549950	551493	942792	1019017	434341
3.411	C20:0	591672	592699	639184	653232	564289	620414	610842	1052511	1089124	467097
3.390	C22:0	596438	593645	657584	702086	585801	619721	622582	1053969	1058905	452568

Taking into account the concentrations of the individual fatty acids as well as their corresponding peak areas, response factors with reference to C17:0 can be calculated as shown in the table below.

Conc. (mmol/l)/ Fatty acid	Response factors											
C6:0	1	2	3	4	5	6	7	8	9	10	Mean	± SD
0.579	1.91	1.91	2.04	2.07	2.29	2.29	1.89	2.41	2.48	2.52	2.21	0.24
3.411		2.40	2.71	2.23	2.49	2.50	2.63	2.50			2.49	0.15
6.375	2.36	2.45	2.41	2.13		2.54	2.33	2.39			2.37	0.13
11.505	2.32	2.39	2.45	2.13	2.43	2.47	2.48	2.51	2.78	2.27	2.42	0.17
											2.37	**0.20**
C8:0												
0.465	1.53	1.51	1.50	1.59	1.58	1.65	1.34	1.63	1.70	1.76	1.58	0.12
2.730	1.66	1.70	1.97	1.63	1.72	1.73	1.83	1.83			1.76	0.11
5.103	1.66	1.75	1.71	1.50	1.05	1.86	1.65	1.68			1.61	0.25
9.210	1.65	1.69	1.70	1.76	1.74	1.76	1.75	1.90		1.59	1.73	0.09
											1.66	**0.16**
C12:0												
0.171	0.98	1.12	1.06	1.06	1.08	1.08	1.01	1.05	1.08	1.17	1.07	0.05
0.999	1.12	1.15	1.27	1.19	1.17	1.16	1.27	1.27			1.20	0.06
1.866	1.14	1.19	1.18	1.10	0.93	1.23	1.16	1.16			1.14	0.09
3.369	1.17	1.19	1.19	1.20	1.18	1.19	1.19	1.26	1.26	1.16	1.20	0.03
											1.15	**0.08**
C14:0												
0.183	1.00	1.05	1.05	1.05	1.03	1.02	1.03	1.03	1.00	1.05	1.03	0.02
1.074	0.96	0.99	1.04	1.03	0.99	0.96	1.01	1.02			1.00	0.03
2.007	1.06	1.02	1.02	0.98	0.91	1.06	0.99	1.04			1.01	0.05
3.624	1.04	1.05	1.05	1.06	1.05	1.08	1.07	1.09	1.10	1.04	1.06	0.02
											1.03	**0.04**
C16:0												
0.177	0.96	0.99	0.93	0.93	0.93	0.89	0.92	0.89	0.91	0.94	0.93	0.03
1.050	0.98	0.99	0.99	1.02	1.00	1.01	1.03	1.03			1.01	0.02
1.962	0.99	1.04	1.01	0.98	1.01	1.02	0.98	1.00			1.00	0.02
3.543	1.03	1.03	1.01	1.02	1.01	1.01	1.02	1.01	1.01	1.00	1.02	0.01
											0.99	**0.04**
C17:0												
0.183	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	
1.077	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00			1.00	
2.013	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00			1.00	
3.633	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	
C18:0												
0.171	1.02	0.89	0.90	0.90	0.90	0.86	0.93	0.97	0.89	0.91	0.92	0.05
1.014	0.94	0.95	0.95	0.94	0.95	0.96	0.94	0.92			0.94	0.01
1.896	0.93	0.95	0.93	0.95	0.97	0.93	0.93	0.92			0.94	0.02
3.420	0.94	0.94	0.94	0.94	0.94	0.94	0.94	0.93	0.92	0.94	0.94	0.01
											0.93	**0.03**
C20:0												
0.171	1.01	0.99	0.97	0.99	0.99	0.92	1.13	1.10	0.98	1.04	1.01	0.06
1.011	0.88	0.90	0.88	0.89	0.88	0.88	0.89	0.86			0.88	0.01
1.890	0.86	0.87	0.86	0.87	0.92	0.85	0.88	0.85			0.87	0.02
3.411	0.84	0.83	0.82	0.83	0.86	0.83	0.85	0.85	0.86	0.87	0.84	0.02
											0.90	**0.08**
C22:0												
0.171	1.16	0.92	0.95	1.02	1.13	0.93	1.07	0.99	0.93	0.96	1.01	0.09
1.005	0.92	0.92	0.93	0.95	0.91	0.91	0.88	0.86			0.91	0.03
1.878	0.85	0.86	0.85	0.88	0.93	0.85	0.85	0.84			0.86	0.03
3.390	0.83	0.82	0.79	0.76	0.83	0.82	0.83	0.82	0.88	0.89	0.83	0.04
											0.90	**0.09**

Linear function

For the equation: $y = b\,x + a$

y = area for corresponding x
b = gradient
x = concentration
a = intercept on the x axis

R = coefficient of regression

The results are summarised in the table below, where R is the coefficient of regression.

Fatty acid	b	a	R^2	R
C6:0	211080	-7923	0.9996	0.9998
C8:0	291093	-4365	0.9996	0.9998
C12:0	425931	-2891	0.9999	0.9999
C14:0	481247	282	0.9995	0.9997
C16:0	459932	-7894	0.9994	0.9997
C17:0	513835	-8356	0.9998	0.9999
C18:0	499119	-10244	0.9992	0.9996
C20:0	559637	-18844	0.9984	0.9992
C22:0	565891	-22133	0.9977	0.9988

With the exception of C22:0 all the fatty acids display a coefficient of regression better than 0.999. The concentrations of the fatty acids measured ranged from 0.057 – 3.832 mmol/l.

Response factors

The response factors, standard deviation and % deviation are summarised below.

Fatty acid	Response factor	± SD	± %
C6:0	2.37	0.20	8.4
C8:0	1.66	0.16	9.6
C12:0	1.15	0.08	7.0
C14:0	1.03	0.04	3.9
C16:0	0.99	0.04	4.0
C17:0	1.00	-	-
C18:0	0.93	0.03	3.2
C20:0	0.90	0.08	8.9
C22:0	0.90	0.09	10.0

Quality assurance of results

Regular injection of a suitable standard enables monitoring of the retention times of individual fatty acids and their response factors. Duplicate measurements should be carried out where possible. Alternating between sample and solvent (dichloromethane) samples helps to prevent carryover and assures consistency in the retention times.

Maintenance

The following should be controlled on a daily basis:

Condition of the septum
System should be gas tight, especially gas fittings
Rinse solvents for autosampler system
Condition of the syringe
Performance of the column, *via* chromatogram of the standard

Periodically the following should be carried out:

Glass insert in the injector should be removed and cleaned
Column should be heated to its maximum temperature to remove accumulated components from the column and thus obtain an improved baseline

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