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Journal of Chromatography B, 671 (1995) 133–168

JOURNAL OF CHROMATOGRAPHY B: BIOMEDICAL APPLICATIONS

Review

Methods for the analysis of triacylglycerols

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Abstract

This article discusses the methods most commonly employed in the analysis of the triacylglycerols (TAGs) in natural fats and considers the main advantages and disadvantages of each and the techniques for optimising analytical conditions. Complete analysis of the composition of a natural fat calls for a method of extracting and purifying the triglyceride fraction, normally by preparatory thin-layer and column chromatography. Determination of the individual components of triglyceride mixtures still entails certain difficulties, namely, the separation and identification of the TAGs in natural fats. High-performance liquid chromatography (HPLC) offers significant advantages over gas and thin-layer chromatography. Many workers have developed non-aqueous, reversed-phase HPLC systems capable of successfully resolving complex mixtures of TAGs, and combining reversed-phase (RP) HPLC and argentation chromatography may improve the results. Identification of the TAGs separated by HPLC becomes an extremely complex task if many different fatty acids are involved and if the *sn*-stereoscopic positions on the glycerol are to be determined. Enzymatic analysis and chiral-phase chromatography are capable of localising fatty acids on the TAG molecule. In closing, some of the most interesting biomedical applications of TAG analysis are summarised.

Contents

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0378-4347/95/$29.00
SSDI 0378-4347(95)00093-3

List of abbreviations

Abr.		IUPAC	Trivial
Bu	C4:0	Butanoic	Butyric
La	C12:0	Dodecanoic	Lauric
P	C16:0	Hexadecenoic	Palmitic
Po	C16:1,*cis*-9	*cis*-9-Hexadecenoic	Palmitoleic
Pot	*trans*-9	*trans*-9-Hexadecenoic	Palmitelaidic
Mg	C17:0	Heptadecenoic	Margaric
St	C18:0	Octadecenoic	Stearic
Pet	C18:1,*cis*-6	*cis*-6-Octadecenoic	Petroselinic
O	*cis*-9	*cis*-9-Octadecenoic	Oleic
Ot	*trans*-9	*trans*-9-Octadecenoic	Elaidic
Va	*cis*-11	*cis*-11-Octadecenoic	*cis*-Vaccenic
Vat	*trans*-11	*trans*-11-Octadecenoic	*trans*-Vaccenic
L	C18:2,*cis*-9,12	*cis*-9,12-Octadecadienoic	Linoleic
Lt	*trans*-9,12	*trans*-9,12-Octadecadienoic	Linoelaidic
γLn	C18:3,*cis*-6,9,12	*cis*-6,9,12-Octadecatrienoic	γ-Linolenic
Ln	*cis*-9,12,15	*cis*-9,12,15-Octadecatrienoic	Linolenic
A	C20:0	Eicosenoic	Arachidic
Ga	C20:1,*cis*-11	*cis*-11-Eicosenoic	Gadoleic
	C20:1,*cis*-13	*cis*-13-Eicosenoic	
Eid.	C20:2,*cis*-11,14	*cis*-11,14-Eicosadienoic	
Eitr	C20:3,*cis*-5,8,11	*cis*-5,8,11-Eicosatrienoic	
Ar.	C20:4,*cis*-5,8,11,14	*cis*-5,8,11,14-Eicosatetraenoic	Arachidonic
Eip.	C20:5,*cis*-5,8,11,14,17	*cis*-5,8,11,14,17-Eicosapentaenoic	
Er	C22:1,*cis*-13	*cis*-13-Docosenoic	Erucic
Bra	*trans*-13	*trans*-13-Docosenoic	Brassidic
Dot	C22:4,*cis*-7,10,13,16	*cis*-7,10,13,16-Docosatetraenoic	
Doh	C22:6,*cis*-4,7,10,13,16,19	*cis*-4,7,10,13,16,19-Docosahexaenoic	
Ner	C24:1,*cis*-15	*cis*-15-Tetracosenoic	Nervonic
Be	C22:0	Docosanoic	Behenic
Tric	C23:0	Tricosanoic	
Lig	C24:0	Tetracosanoic	Lignoceric

AT	Adipose tissue
BHA	Butylated hydroxyanisole
BHT	Butylated hydroxytoluene
CE	Cholesteryl ester
CIMS	Chemical ionisation mass spectrometry
CL	Chain length
CN	Carbon number, number of carbon atoms in the fatty acid
CHOL	Cholesterol
DB	Double bond
DG	Diglyceride
DNPU	3,5-Dinitrophenylurethane derivative
ECN	Equivalent carbon number
ELSD	Evaporative light-scattering detector
FA	Fatty acids (unesterified)
FID	Flame ionisation detector
GC	Gas chromatography
HDL	High-density lipoprotein
HPLC	High-performance liquid chromatography
IDL	Intermediate density lipoprotein
L	Equivalent chain length
LDL	Low-density lipoprotein
LPC	Lipoprotein lipase moieties of a triglyceride; CN for SSS, SOO, SLO . . . . 54.
MG	Monoglyceride
MS	Mass spectrometry
MUFA	Monounsaturated fatty acid with 18 carbon atoms (O, L, Ln)
ND	Number of double bonds
NUFA	Number of unsaturated fatty acids in the triglyceride; NUFA for PPS
ODS	Octadecylsilane
PCRD	Post-column reaction derivatisation
PL	Phospholipid
PLO	Triglyceride glycerol-palmitate-linoleate, oleate, etc.
PN	Partition number
PPP	Triglyceride glycerol-tripalmitate
PUFA	Polyunsaturated fatty acid
RI	Refractive index detector
RP-HPLC	Reversed-phase high-performance liquid chromatography
S	Saturated
TCN	Theoretical carbon number
TAG	Triacylglycerol
TLC	Thin-layer chromatography
U	Unsaturated
UV	Ultraviolet detector
VLC	Very-long-chain polyunsaturated fatty acid
VLDL	Very-low-density lipoproteins

1. Introduction

While in plants fats are synthesised from carbohydrates by the plant itself, animal lipids come from two different sources: exogenous lipids ingested in food and endogenous lipids synthesised by animal tissues. The former are nearly all triacylglycerols (TAGs), whereas the latter include a high proportion of polar lipids, chiefly phospholipids. The main fat deposits in animals are located in the subcutaneous tissue and in the abdominal cavity. TAGs make up over 90% of adipose tissue (AT). The distribution of fatty acids (FAs) in fats from animal tissues differs appreciably according to the animal species and the location of the AT in the body [1]. In most species, saturated FAs tend to occupy mainly position *sn*-1, though substantial amounts of oleic acid can also be found at that position. Unsaturated acids and certain medium-chain saturated acids (C_{14}) are mainly concentrated at position *sn*-2. The distribution in porcine AT differs from that in most species, with palmitic acid concentrated at the middle position. Long-chain saturated acids, such as stearic acid, are primarily located at position *sn*-1, and unsaturated acids, e.g. oleic acid and linoleic acid, at position *sn*-3. Very-long-chain, polyunsaturated acids mainly occupy the middle position in fish oils, with monoenoic acids concentrated in the outer positions.

Different vertebrates exhibit important variations in the composition of their fat reserves, though such changes are not as large or as specific as in plants and to a certain extent are related to the composition of the ingested fats.

Animals hydrolyse ingested fats in order to redistribute the FAs and manufacture their own TAGs for storage or mobilisation purposes.

However, when animals are fed special diets, for example, containing highly unsaturated fats or uncommon fats (like erucic acid), the composition of the TAGs in the body is altered to some extent. Studies of the TAG metabolism in higher animals have considered a variety of initial problems, such as digestion, absorption and transport of the ingested fats. For that reason, systematic analytical methodology for TAGs will help ensure optimal results in metabolic studies.

Just as the chromatography of FAs represented a great stride forward in the study of animal and vegetable TAGs in the 1960s, today analysis of intact TAGs and their behaviour is unquestionably one of the most important issues in the study of fats in general. Accordingly, research into the TAGs in the fats in margarines, chocolate, etc., could harbour major advances for those manufacturing industries. Its application to lipoproteins, AT, maternalised milks, growth, and so forth is of great importance in determining the condition and behaviour of the body at any given time.

In recent years advances in chromatography have enabled the analysis of TAG metabolism. Thin-layer chromatography (TLC) has been used to separate different categories of lipids according to their functional groups. Argentation TLC (with $AgNO_3$) separates TAGs according to the degree of unsaturation. RP-TLC sorts according to carbon number (CN). Gas chromatography (GC) involves the degree of unsaturation and CN, while GC with mass spectrometry (MS) is used for peak identification and quantification. HPLC is perhaps the pre-eminent method for separating TAGs. This article presents a discussion of the different techniques used in TAG analysis.

2. Nomenclature

The convention originally proposed by Hirschmann [2] has now been universally adopted for numbering the three hydroxyl groups on the glycerol molecule [3]. A "*sn*"- (for stereospecifically numbered) prefix is included in the names of all glycerols [4]. A single molecular species is identified by listing the *sn*-1, *sn*-2 and *sn*-3 positions in order. A "rac" prefix indicates that the middle fatty acid in the abbreviation is attached at the *sn*-2 position, while the remaining two acids are equally divided between the *sn*-1 and *sn*-3 positions, yielding a racemic mixture of two enantiomers. A "β" prefix indicates that the middle fatty acid in the abbreviation esterifies the β- or *sn*-2 position. For example:

sn-POL = *sn*-1-palmito-2-oleo-3-linolein
rac-POL = equal proportions of *sn*-POL + *sn*-LPO
β-POL = *sn*-POL + *sn*-LOP in any proportion

3. Extraction and isolation of lipids, fatty acid analysis of extracts

3.1. Isolation of lipids

Quantitative extraction of liposoluble components from biological samples involves certain difficulties stemming from their high water content (ranging from around 50% to up to 90% for serum). In principle, extraction should be carried out using weakly polar solvents such as petroleum ether, ethyl ether or chloroform, but those solvents are not miscible in water and therefore do not achieve good penetration of tissues, and extraction is incomplete. Although methyl and ethyl alcohol are water-miscible, they likewise do not penetrate deeply into tissues. Furthermore, extraction methods normally involve comminution, which brings the glycerides into close contact with lipolytic enzymes, with the resulting risk of hydrolysis when the contact time is protracted, especially if the temperature rises during treatment. For that reason extraction should always be carried out under cold conditions using an antioxidant, normally BHA or BHT.

Lipid extraction is quite simple for certain samples of biological material, e.g. red blood corpuscles, serum and plasma. On the other hand, the homogenisation step needed for lipid extraction from various other animal and human tissues may complicate the procedure considerably.

Chloroform–methanol (2:1) [5] (or 7:1 satu-

rated with ammonia) or chloroform–isopropanol [6] mixtures are normally used for extraction. Folch's method [5] is nearly always employed in practice. Once the lipids have been extracted, isolation may be carried out on silica-gel plates, and a simple procedure using hexane–diethyl ether–acetic acid (80:20:1) suffices to separate out the polar lipids (phospholipids), which remain close to the origin (cholesterol), and the TAGs which migrate to the middle of the plates. The said TAGs can be scraped off and used for subsequent study, applying the methods reviewed below. Tissues that contain large amounts of lipids, such as AT, can be extracted using diethyl ether or chloroform, with isolation then completed using the above procedure [7].

3.2. Fatty acid analysis

In GC, the retention time is reflected by the distance on the chromatogram between the air peak and the position of the band peak. Working with an H_2 flame detector, the air outflow is not detectable, and in that case elution of the solvent can be taken as a starting point. However, relative retention times are normally used, calculated by dividing the retention time for each peak by the time recorded for the peak for an ester taken as a standard.

Quantitations are based on the principle that the masses of each of the separate constituents in the mixture are proportional to the area under each peak. The latter is calculated from the triangle formed by drawing tangents to the inflexion point.

The methods used to prepare methyl esters (MEs) are normally based on saponification of the glycerides and isolation of the FAs, followed by conversion to methyl esters by reaction with diazomethane [8], methyl sulphate [9] or methyl hydrochloride [10] using C17:0 as the internal standard [11,12]. The choice of procedure for preparing MEs should be based on the composition of the FAs making up the TAGs and the type of GC analysis to be performed [13,14].

Packed or capillary columns may be employed. Packed columns range from 1.5 to 3 m in length, with an internal diameter of 2–4 mm and diatomaceous earth or other support and a narrow range of particle sizes (between 125 and 200 μm). The stationary phase is normally a polyester-type polar liquid, e.g. diethylene glycol succinate, cyano silicone, methyl silicone, etc. [15]. The working temperature is from 175° to 200°C, depending upon the column type.

Capillary columns may be made of glass or fused-silica gel. Internal diameter ranges from 0.2 to 0.8 mm, and column length from 25 to 100 m, depending upon the type of acid to be separated. The stationary phase is a polyethylene glycol-type chemically bonded one. The bonded phase thickness is from 0.1 to 0.2 μm.

Studies of FA composition mainly cover the analysis of $n-7$ [16,17] and *trans* [18,19] isomers. Turnover of *trans*-FAs in the tissues appears not to be permanent but to vary according to intake levels, with their levels in tissue lipid fractions varying accordingly [20–22].

For instance, in rats levels of *trans*-FAs are practically nil in the tissues (with the sole exception of AT) 8 weeks after changing from a diet rich in *trans* acids to a diet free of them [23]. Similarly, the *trans* acid content of human milk is very low a few days after following a diet free of such acids [24]. Also, the brain tissues of both young and adult rats have been shown to metabolise elaidic acid and oleic acid at the same rate [25].

When the diet contains *trans*-FAs, such acids are found in most body tissues [26–28]. The highest proportions in human tissues are in the liver and AT, where they may account for up to 14% of the total FA content. The most abundant forms in human tissues are *trans*-9-hexadecenoic and *trans*-9-octadecenoic acids [29,30]. Studies in rats have yielded still lower levels (of between 1 and 2%) of *trans* acids in the lipids present in the tissues [31]. The ultimate metabolic fate and physiological effects of *trans*-FAs have not been fully characterised. However, in light of recent findings [32–35], it has become increasingly clear that the physiological properties of *trans*-FAs may differ substantially from those of their *cis* monounsaturated counterparts [19]. *Trans* acids occur in all lipid fractions though, as might be expected, they are most common in the TAGs, the main lipid fraction in the body [26]. They tend to occupy positions 1 and 3 on TAG

molecules [36,37]. In such tissues as the brain, heart and liver, phospholipids contain a substantial proportion of *trans* acids, which then behave like saturated acids: they mainly occur at position 1 on the phospholipid molecules, whereas *cis* acids exhibit no preference for any particular position [31].

4. Assaying of TAGs by enzymatic methods

Analysis of TAGs has been simplified by the introduction of enzymatic methods, which have been automated to provide analysts with quick, easy and direct procedures. Enzymatic methods are based on determinations of the glycerol portion of the TAG molecules after hydrolysis to remove the FAs. Dosing takes place in the following three stages: TAG emulsion, complete hydrolysis by a lipase and a stearase, and indirect spectrophotometric measurement of the glycerol at 340 or 500 nm [38].

There are two alternative pathways for TAG determination, depending upon whether a dehydrogenase or the oxidase/peroxidase system is used as the indicator enzyme.

(1) $\mathrm{TAG} + 3\mathrm{H_2O} \xrightarrow{\text{lipase + stearase}} \text{glycerol} + 3\ \mathrm{FFA}$

(2) $\text{Glycerol} + \mathrm{ATP} \xrightarrow{\text{glycerol kinase}} \mathrm{G3P} + \mathrm{ADP}$

(3) $\mathrm{ATP} + \mathrm{PEP} \xrightarrow{\text{pyruvate kinase}} \mathrm{ATP} + \text{pyruvate}$

(4) $\text{Pyruvate} + \mathrm{NADH} \xrightarrow{\text{lactate dehydroganase}} \text{lactate} + \mathrm{NAD}$

and measuring the decrease in A_{340} produced by oxidation of the NADH.

Using the oxidase/peroxidase system, the first two steps are the same, followed by: glycerophosphate oxidase

(3) $\mathrm{G3P} + \mathrm{O_2} \xrightarrow{\text{glycerophosphate oxide}} \mathrm{DHAP} + \mathrm{H_2O_2}$

(4) 4-chlorophenol + 4aminophenazone + $2\mathrm{H_2O_2} \xrightarrow{\text{peroxidase}}$ 4-(4-benzoquinonemonoimino)-phenazone + HCl + $2\mathrm{H_2O}$

and measuring blue colour formation at 500 nm [39,40].

Using enzymes for analysing TAGs brings three main advantages. (a) It enables certain measurements that would otherwise be impossible using traditional chemical reagents or instrumental methods, e.g. determination of FAs at TAG position 2. (b) Enzymes are very well suited to micromethods for working with direct samples such as serum and lymph and also facilitate quantification using TLC. (c) Enzymes are useful in developing fast, reproducible methods appropriate for large autoanalysers. Nevertheless, the usefulness of the method is limited to measuring serum TAG levels only, and it is comparatively difficult to determine those levels from the lipid extracts. Organic solvents used as dissolvents interfere with enzymatic reactions [41–55].

Recently, Danno et al. [51] developed a simple enzymatic quantitative analysis for TAGs from tissues. The method starts with 0.5 g of liver, which is extracted with 15 ml of chloroform–methanol (2:1 by volume) using Folch's method [5]. An aliquot of known working standards and the hepatic lipid extracts in benzene are transferred to test tubes. The solvents are evaporated using a centrifugal concentrator, and the standards and liver extracts are redissolved in 30 μl of *tert.*-butyl alcohol and 20 μl of a Triton X-100–methanol mixture (1:1) and stirred. A 1-ml aliquot of enzymatic reagent is added to each tube and carefully mixed. The standard, extracts and a blank are incubated at 37°C for 18 min, and absorbance is then measured at 505 nm in the visible region of the spectrum. Fig. 1 illustrates this method.

5. Thin-layer and conventional column chromatography

The most commonly employed methods are TLC with $AgNO_3$ (Ag-TLC) [56–58] and RP-TLC [59].

5.1. Argentation TLC

This method was developed in 1962 [60], mostly as a result of work by Morris [61], and is based on the ability of olefinic bonds to form π-complexes with silver ions, enabling the separation of substances with differing degrees of unsaturation: it has been shown to be particularly efficient at separating mixtures of TAGs [62]. The thin layer is a silica-gel slurry containing 5–25% silver nitrate applied to a plate. The solution of glycerides is applied to the plates in the form of a streak, and the plate is developed using ether–benzene or chloroform–methanol mixtures. Under these conditions, saturated TAGs migrate virtually together with the front, followed by the various groups of TAGs in order of increasing unsaturation [63,64]. A fat that contains saturated oleic and linoleic acids may have TAGs with up to six double bonds, and separation with silver nitrate is so effective that TAGs with the same degree of total unsaturation but different acid radicals (e.g. dioleostearate and linoleodistearate) can be separated. When linoleic acid is present, the system becomes more complex, but the following migration order has been established empirically (the numbers 3, 2, 1 and 0, respectively, stand for the linolenic, linoleic, oleic and saturated-acid chains) (Fig. 2):

Origin, 333, 332, 331, 330, 322, 321, 320, 311, 222, 310, 221, 300, 220, 211, 210, 111, 200, 110, 100, 000, front.

In other words, linoleic chains form stronger complexes than two oleic chains on the same TAG, and linolenic chains form stronger complexes than two linoleic chains. These relationships are applicable to polyunsaturated acids with the same chain length with *cis* bonds separated by a methylene group, although the chain length does not greatly affect the location of the TAG on the plate. On the other hand, *trans* double bonds, and particularly conjugated double bonds, can substantially alter the order given above.

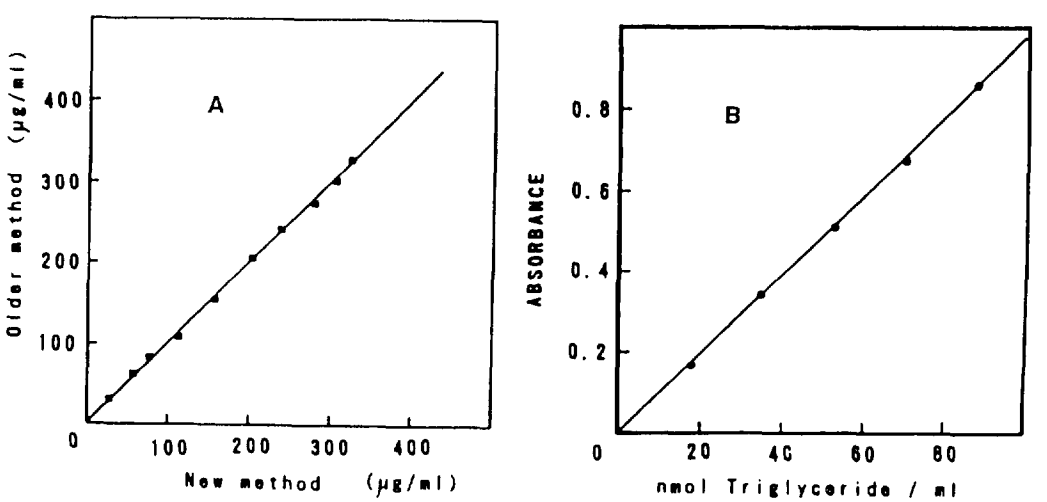

Fig. 1. A simple enzymatic quantitative analysis of TAG in tissues. (A) Results obtained with the older method (chloroform–methanol 2:1, v/v) and the present method on ten different samples from liver tissue. (B) TAG concentration and absorbance at 550 nm. Curve was obtained with triolein standard alone without tissue influences or extraction losses. The reaction mixture is composed of 30 µl of *tert.*-butyl alcohol, 20 µl of equivolume Triton X-100–methyl alcohol mixture and 0.995 ml of the "enzymatic kid". (Reproduced by kind permission from Ref. [51].)

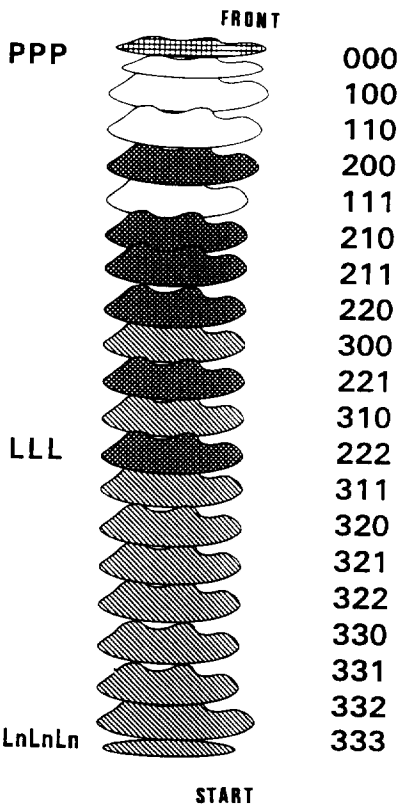

Fig. 2. Analytical separation of TAG by Ag^+ adsorption thin-layer chromatography. Horizontal development with benzene–diethyl ether (85:15, v/v). Bands are visualised by charring with 50% sulphuric acid.

After separation the TAGs are located by ultraviolet light and may be quantitatively recovered by elution of the respective bands. Each band is usually composed of over 80% of the primary TAG, whose composition can be determined by its location on the chromatogram and analysis of its FAs by applying conventional methods of conversion to methyl esters and GC.

5.2. RP-TLC

Perhaps the best work published on RP-TLC is that by Nikolova-Damyanova and Amidzhin [59]. RP-TLC is one of the simplest and easiest modifications of partition chromatography to perform. First introduced into TAG analysis by Kaufman et al. [65], it has been successfully used to resolve model and natural TAG mixtures according to their partition number (PN; PN = CN − 2m, where CN is the carbon number and m the number of double bonds in the TAG molecule). The best resolutions have been achieved using stationary phases consisting of long-chain hydrocarbons or liquid paraffin [66,67]. However, the use of chromogenic reagents for the detection and quantitation of TAGs with these highly effective phases is quite difficult. Quantitation usually proceeds by indirect GC or spectrophotometric methods [68] and is laborious and time-consuming. Today, densitometry is the method of choice for quantification of the compounds separated by TLC. Since charring is most often used for the direct densitometric determination of TAGs, long-chain hydrocarbons and liquid paraffin are unsuitable as the stationary phase. RP-chromatographic systems must therefore combine good selectivity with the possibility of direct densitometric quantitation. Plates are cooled and stored until silanisation in a desiccator over phosphorus pentoxide. Silanisation of the layer is achieved by placing the plates in a closed chamber over dimethylchlorosilane (DMCS) vapours for a certain length of time. The plates are washed by elution with methanol and dried at 110°C for 1 h and then stored in closed glass boxes without any special precautions. Densitograms were carried out on a Shimadzu CS-930 densitometer by zigzag scanning in transmission mode at 450 nm using a 1.2-mm slit. Resolution is strongly dependent upon the shape of the chromatographic chamber, saturation of the atmosphere with mobile-phase vapours, and volume of the mobile phase.

To ascertain which systems are optimal for TAG analysis, various layer types, mobile phases and detection methods suggested in the literature have been tested under identical conditions using a variety of TAG standards [70]. Along with the standards, Masterson et al. [69] performed analyses to identify the TAG components of three actual samples. They compared three different types of layers using chromatographic standards and hen's egg yolk, lard and snail extracts on: plain (unimpregnated) silica-gel TLC; silica-gel

layers impregnated with $AgNO_3$ by the manufacturer or in the laboratory; and silica-gel layers with chemically bonded C_{18} groups.

Baker-Flex (20 × 30 cm) IB2 silica-gel sheets and Whatman (20 × 10 cm) LHP-KDF channeled high-performance silica-gel plates with a preadsorbent spotting area were used unimpregnated for silica-gel TLC and impregnated with $AgNO_3$ for Ag-TLC. Argentation plates were prepared by hand-dipping the silica-gel plates into a Desaga glass dipping chamber containing a 2.5% solution of $AgNO_3$ in methanol followed by activation in an oven at 110°C for 1 h just before use. Commercially prepared Uniplates (20 × 30 cm) impregnated with 10% $AgNO_3$ were also used.

Standards were chosen based on both saturation class (S3, S2M, M3 and D3) and PN. These two classifications and the TAG group designations (St = stearate, P = palmitate, O = oleate) appear in parentheses after each compound below. All standard solutions were prepared at a concentration of 1.0 μg/μl in chloroform.

Tripalmitin (S3, 48), trilinolein (D3, 42), triolein (M3, 48), tristearin (S3, 54), rac-glyceryl-1,3-palmitate-2-oleate (POP-S2M, 48), rac-glyceryl-1-palmitate-2-oleate-3-stearate (PO-St-S2M, 50), rac-glyceryl-1,2-oleate-3-stearate (OOSt-S2M, 50), rac-glyceryl-2,3-stearate-1-oleate (OStSt-S2M, 52) and rac-glyceryl-1-palmitate-2-stearate-3-oleate (PStO-S2M, 50) standards were obtained commercially.

The results are summarised in Table 1 and show that the best separations were achieved using isopropyl alcohol–chloroform with plates impregnated with $AgNO_3$ and mixtures of isopropyl alcohol–chloroform (1.5:98.5) with plain silica-gel TLC and acetonitrile–methyl ethyl ketone–chloroform (50:35:15) [57–59, 61, 63, 64, 71, 73].

5.3. Separation of lipid classes of conventional column chromatography

The separation and isolation of neutral and

Table 1
Solvent systems for TLC of TAG [69]

Solvent number	System components	Volume ratio of components	Ref.	Rank of separation[a]
Argentation TLC (silica-gel plate impregnated with 2.5% methanolic $AgNO_3$)				
1	Hexane–diethyl ether	80:20	[58]	P
2	Chloroform–methanol	96:4	[58]	P
3	Isopropyl alcohol–chloroform	1.5:98.5	[60]	E
4	Light petroleum ether–acetone	10:4.5	[59]	G
5	Toluene–chloroform	50:50	[63]	P
6	Petroleum ether–diethyl ether–acetic acid	80:20:2	[71]	G
Plain silica-gel TLC				
7	Light petroleum ether–acetone	10:4.5	[59]	G
8	Isopropyl alcohol–chloroform	1.5:98.5	[60]	E
9	Toluene–chloroform	50:50	[63]	P
10	Petroleum ether–diethyl ether–acetic acid	80:20:2	[71]	P
Reversed-phase TLC				
11	Acetonitrile–methyl ethyl ketone–chloroform	50:35:15	[64]	E
12	Acetone–acetonitrile–water	70:30:12	[72]	G
13	THF–acetonitrile	3:7	[58]	P

[a] E = excellent; G = good; P = poor.
Detection was made with PMA for reversed-phase and silica gel without impregnated silver, and 50% aqueous sulphuric acid for silica gel with silver.

polar lipid classes for subsequent use or analysis have been the subject of many reports in the literature [73]. Of the different techniques available, chromatographic mode sequencing is the one most commonly employed. This technique involves creating selectivity in the isolation of compounds by serially altering the solid/support solvent system, so that a unique interaction exists between the compound to be isolated and the functional group of the solid phase. Compounds that are very diverse in chemical structure may differ greatly in their interactions with the solid-phase surface moieties, whereas compounds that are chemically similar may exhibit only subtle though still exploitable differences. Thus, by varying the solvent environment (e.g. polarity) around the solid phase, lipids can be selectively isolated with a high degree of purity and recovery. The most widely used columns are aminopropyl bonded phase (Bond Elut) columns that may or may not be placed in a vacuum curve to enhance the separation rate [73]. When ordering, it is important to specify columns with stainless steel frits which contain a plasticiser elutable with organic solvents. The mixtures used with this type of column to elute TAGs are 1% diethylether and 10% methylene chloride in hexane, and the quantity used to elute the TAGs is 8 ml [74].

This technique yields TAG fractions of higher purity than those obtained with traditional silica columns, especially if the vacuum apparatus recommended by Kaluzny et al. [73] is used. Up to ten lipid extracts can be processed in 1 h by the Bond Elut method. These techniques have been routinely employed by certain workers to separate blood plasma lipid classes before analysis and are reported to be adaptable for the separation of lipids from AT and very-low-density lipoproteins (VLDLs), which contain large amounts of TAGs. Some separations have been accomplished with recoveries and purities comparable to those for the standard lipid mixture used to develop the separation methodologies. Sample quantities permitting, these columns are very useful in the separation of plasma samples [75,76].

6. TLC with flame ionisation detection

TLC with flame ionisation detection (FID) is a simple, easy-to-use analytical method that is sufficiently sensitive to produce good separations and is suitable for the quantification of complex lipids. In TLC–FID the sample is spotted on a quartz rod coated with silica gel, developed chromatographically, and finally subjected to detection by FID as the rod is passed through a hydrogen flame. Using this method sample handling is minimal, but it gives rise to a new set of variables, and further study is required if it is to be used to its full potential [7]. Several investigators have examined different aspects of lipid analysis using TLC–FID [77–81]. In particular, it has been reported that response factors for various lipids differ and depend upon such variables as sample volatility, amount of material analysed, FA composition and rate of passage of the Chromarod through the flame [82–86]. Fraser et al. [83] quantified the neutral and polar lipids of the eggs and larvae of marine fishes using a composite standard of similar composition to marine fish. The impetus for this was that the response factor (the ratio of analyte to internal standard) of some lipids depends upon the amount of sample present [87]. For that reason, it is important for the analytes to be present in the calibration solution in the same proportion as in the biological sample. The results for this method were comparable to the more commonly employed gravimetric, colourimetric and densitometric methods for most analytes, with the exception of TAGs.

On reviewing Whitsett and Kennish [87], the data analysis of the TAGs suggests that the variability in the slopes (C.V. = 17.5%) is too great to rely on a single TAG standard for accurate quantitation of the whole class. The slope for the TAG composite was 0.1380. The relative percentage difference between this slope and that for triarachidonin, the TAG closest to the composite, was 12.0%. For trimyristin and triolein, the relative percentage composite to standard difference was 46.8% and 26.2%, respectively. The differences in chain length and

unsaturation of the TAGs are such that none of their responses is comparable. Sample volatility and ion formation were probably responsible, rather than the non-carbonyl content, as in FAs.

Quantification of lipids by FID after separation by TLC on Chromarods has been greatly improved by the use of internal standards that reduce rod-to-rod variation. However, the relative response of different lipid classes to an internal standard has not been found to be constant: it varies with the amount of sample applied, the ratio of sample to internal standard, and scanning speed. Kramer et al. [84] and Mares et al. [88], among other investigators, observed larger coefficients of variation for biological samples than for comparable amounts of model synthetic mixtures, which they attributed to the presence of unsaturated and unstable lipids in the former.

Accordingly, we consider it appropriate to summarise the method of TAG quantification using this technique [87,91–94].

Since Iatroscan FID sensitivity varies depending upon the substances to be detected, it is necessary to obtain a suitable calibration curve prepared on the basis of the peak area measurements and the amounts of the components [89].

A chromatogram is recorded for a mixed sample in which a known amount of internal standard has been added to a known amount of the pure components to be detected, and the respective peak areas are measured [90].

Many workers have determined FID correction factors for different classes of lipids using a single molecular species and have applied those factors in analyses of biological samples [92,95–99].

To ensure a good response, the rod manufacturer's recommendations must always be followed: reactivation of each rod should be constant; the degree of solvent vapour saturation in the development tank should be constant; the solvent volume in the development tank should also be constant; in many cases it is effective to humidify the Chromarod.

The Iatroscan technique is very useful for quantification in separations of the TAGs in

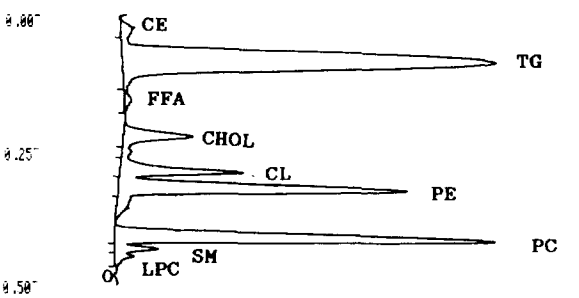

Fig. 3. Iatroscan TLC–FID chromatograms showing the separation of rat heart total lipids on Chromarods S. CE = cholesteryl esters; TG = triacylglycerols; FFA = free fatty acids; CHOL = free cholesterol; CL = cardiolipin; PE = phosphatidylethanolamine; PC = phosphatidylcholine; SM = sphingomyelin; LPC = lysophosphatidylcholine; O = origin.

other polar lipids [100–103] and of plasma lipids or rat heart lipids (Fig. 3) and has proven highly effective in separating phospholipids, cholesterol, TAGs and FAs [104]. However, the system may not always be so effective in separating TAGs [85]. Separations have been performed on mixtures of even TAGs to which an internal standard was added. Type S Chromarods have been used with mixtures of 1,2-dichloroethane–chloroform–formic acid (98:8:0.1) for development, which enabled pure TAGs to be separated according to the degree of unsaturation. However, separation using this technique is always much more effective for phospholipids than for TAGs [105].

All the TAGs and TAG mixtures have been sufficiently well-resolved from the ME internal standard on the Chromarods to permit calculation of the relative peak area responses of the TAGs to ME. Among the TAGs, the R_F values declined with decreasing unsaturation and chain length. Sufficient data were collected from each Chromarod (four analyses per rod for each pure TAG) to determine the effect of individual rods, a factor considered necessary to improve precision by a number of researchers [78,80,106–108].

In recent work, Parrish et al. [109] separated saturated and polyunsaturated TAGs in an attempt to ascertain whether TLC–FID may provide a rapid screening method to evaluate the potential toxicity of marine samples due to the

presence of polyunsaturated lipid classes. However, the method has succeeded only in separating saturated and polyunsaturated TAGs; it did not separate the different molecular species of the TAGs [110–112].

7. Stereospecific analysis by enzymatic and HPLC methods

When determining the composition of a natural fat, it is necessary not only to identify the acid constituents but to determine the distribution of those acids in the TAG molecule as well. The physical and chemical properties of fats are related both to FA composition and FA distribution in the TAGs. Indeed, that distribution characterises fats, since there may be several different patterns of distribution on the glyceride molecule for a given FA composition, thereby producing fats with entirely different properties, particularly physical ones.

It is therefore not surprising that for many years continuous efforts have been expended on developing analytical methods for determining the distribution of the acyl groups in the TAGs of natural fats. Nevertheless, qualitative and quantitative analysis of mixtures of the pure species making up the TAGs in natural fats is an extremely difficult task because of the close resemblance in their physical and chemical properties, which is heightened in the case of positional isomers, that is, TAGs consisting of the same acids esterifying different positions on the glycerine molecule. To get an idea of the complexity of the problem, let us consider the case of a fat composed of only two FAs. Taking positions 1 and 3 on the glycerine molecule to be identical and interchangeable, the two fatty acids, A and B, can form six different TAGs, four if no distinction is made between isomers:

AAA (A_3); AAB and ABA (A_2B); ABB and BAB (AB_2); BBB (B_3).

With three FA constituents, A, B and C, the number of possible TAGs rises to ten (excluding isomers): A_3, B_3, C_3, A_2B, AB_2, A_2C, AC_2, B_2C, BC_2, ABC; or eighteen if isomers are included. The number of possible TAGs climbs rapidly with the number of FA constituents, making truly complete analysis of a fat normally composed of 3–5 main FAs and a large number of minor FA constituents practically impossible.

Consequently, the problem is customarily simplified in various ways, perhaps the two most common being: (a) many studies do not contemplate isomers; (b) determinations are not normally made for pure TAGs but rather for closely related groups of TAGs. The categories or groups established at any given time will depend upon available analytical techniques. Accordingly, in the beginning only four categories of TAGs were determined, based on the number of saturated (S) and unsaturated (U) acyl groups they contained. These groups were designated S_3, S_2U, SU_2 and U_3. With currently existing methods it is possible to identify additional, more specific categories besides these original four.

In addition to the preceding simplifications, it is nearly always necessary to establish specific cases depending upon the analytical method employed to enable the TAG composition to be calculated from the data obtained.

The goal of analysing FA distribution is to determine the TAG composition of fats and to develop models of FA distribution on natural glycerides in order to elucidate biosynthetic pathways and thus predict the approximate glyceride composition of a given fat. With advances in analytical techniques, new theories explaining the distribution of acyl remnants have been put forward. Currently, such theories are much more consistent for vegetable fats, synthesised entirely in situ by the plant, than for animal fats, which are partly endogenous and partly exogenous.

The first hypothesis explaining the distribution of FAs in natural TAGs was the theory of the formation of simple TAGs, that is, each chemical species consisting of a single FA only: triolein, tripalmitin, etc. There followed several more theories, the main ones being: the theory of minimal distribution or formation of the smallest possible number of pure TAGs; the rule of even distribution, developed by Hilditch and

his group [113], which postulates that FAs are distributed as widely as possible among all TAGs (namely, an acid accounting for 33.3% of the total will be present in all TAGs only once; an acid accounting for between 33.3% and 66.6% will be present between one and two times; and simple TAGs composed of that acid will be formed only when the share contributed by that acid exceeds 66.6%); the random distribution theory, which holds that FAs are statistically distributed among all the hydroxyl groups on all the glycerol molecules; the random distribution or restricted statistical distribution theory, a modified version of the former, developed by Kartha [114], to keep the content of the completely saturated glycerides calculated to within the bounds of the experimental range; and lastly, the theories of random distribution at position 2 and random distribution at positions 1 and 3.

This last-mentioned theory was developed independently by Vander Wal [115] and Coleman and Fulton [116] from new data obtained by hydrolysis using pancreatic lipase and is based on the following assumptions: (a) positions 1 and 3 are equivalent; (b) the FA composition at position 2 and positions 1 and 3 is different and independent; (c) the acids occupying position 2 as well as the those occupying positions 1 and 3 are randomly distributed.

They demonstrated that it was possible, based on these premises, to compute the TAG composition of natural fats from the data on the FA composition at different positions obtained by lipolysis. The computation method is as follows. If the total concentration of an acid, A, in mole% in the TAGs is known and the concentration for that same acid at position 2, A_2, is known, the concentration at positions 1 and 3 will be:

$$A_1 = A_3 = 3/2A - 1/2A_2$$

and so on for the remaining acids. Thus, the molar concentration of the different TAGs will be: $AAA = (A_1)(A_2)(A_3) \cdot 10^{-4}$; $ABA = (A_1)(B_2)(A_3) \cdot 10^{-4}$; $AAB = 2 \cdot (A_1)(A_2)(B_3) \cdot 10^{-4}$; $ABC = 2 \cdot (A_1)(B_2)(C_3) \cdot 10^{-4}$; $ACB = 2 \cdot (A_1)(C_2)(B_3) \cdot 10^{-4}$; $BAC = 2 \cdot (B_1)(A_2)(C_3) \cdot 10^{-4}$.

In the years since, the results obtained using this procedure have been shown to be in good agreement with the direct determinations made for various animal and vegetable fats by other methods such as silica gel and argentation TLC and HPLC.

Application of lipolysis to a number of vegetable and animal fats has shown that unsaturated C_{18} acids (oleic, linoleic and linolenic acids) occupy nearly all available position 2 sites, whereas saturated and unsaturated C_{20} and C_{22} acids are present only at positions 1 and 3. For that reason certain workers have suggested that in vegetable fats position 2 is preferentially acylated by unsaturated acids with 18 carbon atoms and positions 1 and 3 are acylated by the remaining acids and those unsaturated C_{18} acids that are unable to find a free position 2 site. Within those limitations, acyl groups follow a pattern of statistical or random distribution. Calculating the composition of the basic glyceridic groups, S_3, S_2U, SU_2 and U_3 (including unsaturated acids with 20 and 22 carbon atoms), shows that the results obtained using the random distributions at position 2 and positions 1 and 3 are in better agreement with lipolysis data than the results for the statistical and even distribution theories.

7.1. Hydrolysis of TAGs by pancreatic lipase

In the years from 1950 to 1965, a number of laboratories in France [117] and the USA [118] reported that porcine pancreatic lipase catalysed stepwise hydrolysis of TAGs to glycerol and FAs via the intermediate di- and monoglyceridic stages [117,119,120].

TAGs → 1,2-diglycerides + 2,3-diglycerides + (FAs) → 2-monoglycerides + (FAs) → glycerol + (FAs)

7.2. Stereospecific analysis of triacyl-sn-glycerols by HPLC

HPLC determination of the *sn* positions occupied by FAs on the triacyl-*sn*-glycerol molecule is rather complicated, since most analyses

require, first, careful sample preparation and, second, GC analysis of the FAs obtained from the enantiomeric fractions separated by preparatory HPLC.

Sample preparation entails prior partial hydrolysis of the triacyl-*sn*-glycerols to obtain mono- and/or diacyl-*sn*-glycerols, normally by means of the Grignard reaction with ethylmagnesium bromide [121], followed by formation of derivatives of the mono- and diacyl-*sn*-glycerols and isolation by solid-phase extraction or TLC. HPLC analysis of the mono- and diacyl-*sn*-glycerol derivatives, and in some cases of the derivatives of the triacyl-*sn*-glycerols themselves, has been performed using normal-phase adsorption chromatography, RP-chromatography and chiral-phase chromatography.

Normal-phase adsorption chromatography has been employed to analyse 1,3-, 1,2- and 2,3-diacyl-*sn*-glycerols by forming diastereoisomeric (*S*)-(+)-1-(naphthyl)ethyl urethane derivatives [122,123]. Laakso and Christie [124] achieved good separations of the diastereoisomeric derivatives of diacyl-*sn*-glycerols using silica-gel columns which were connected in series and an isocratic mobile phase of hexane–isopropanol (99.5:0.5, v/v). Under those conditions, the diacyl-*sn*-glycerols derivatised with (*S*)-(+)-1-(naphthyl)ethyl isocyanate eluted in ascending order of the 1,3-, 1,2- and 2,3-enantiomers. Various researchers have reported that the magnitude of the steric effects of the substituents on the chiral carbon of the urethane group and the primary carbon on the glycerol are the main factors affecting the order of elution of diastereoisomers [125]. Using the (*R*)-form of the derivatising agent reverses the order of elution of the 1,2- and 2,3-diacyl-*sn*-glycerols, though resolution has been reported to be much lower [58].

Laakso and Christie [124] also observed that diacyl-*sn*-glycerols formed by a single FA eluted in the order 18:1 (oleic) < 18:0 (stearic) < 18:2 (linolenic) < 16:0 (palmitic), unlike the expected order in normal and RP-HPLC. This may be attributable to the effect of the spatial conformation of the FAs on the interactions between the substrates and the stationary phase.

As pointed out above, the *sn*-stereospecific composition of FAs in the triacyl-*sn*-glycerols of a natural fat may be calculated from data from GC analysis of the FAs for the total triacyl-*sn*-glycerol fraction and data on the 1,2- and 2,3-diacyl-*sn*-glycerol fractions from HPLC.

RP-chromatography has been used much less frequently than adsorption chromatography for stereospecific analysis of triacyl-*sn*-glycerols. Analyses using this technique have been carried out by forming diacyl-*sn*-glycerol derivatives or derivatives of the triacyl-*sn*-glycerols themselves. Semporé and Bézard [126] carried out separations of the 3,5-dinitrophenyl urethane (DNPU) derivatives of the 1,2- and 2,3-diacyl-*sn*-glycerols on an octadecylsilane column using an acetonitrile–acetone mobile phase and a mass detector. DNPU derivatives have normally been used to analyse the mono- and diacyl-*sn*-glycerols by chiral-phase chromatography. Deffense [127] developed an RP-HPLC method of analysing the direct derivatives of triacyl-*sn*-glycerols obtained by epoxidation of triacyl-*sn*-glycerols containing a single monounsaturated FA. Epoxidation of the double bond was achieved by reaction with *m*-chloroperbenzoic acid in a dichloromethane medium. Separation of the epoxidated derivatives was performed by gradient elution using dichloromethane–acetonitrile–acetone, with detection by laser light scattering. Under the conditions employed, it was possible to separate isomers with the unsaturated acid at position *sn*-2 from the *sn*-1 and *sn*-3 isomers, which were not resolved.

Chiral-phase chromatography has also been applied successfully in the stereospecific analysis of TAGs. In this chromatographic method the stationary phase presents chiral molecules that have been chemically bonded to a silica-gel support medium, thereby avoiding the need to prepare diastereoisomeric derivatives of the partially hydrolysed di- and monoacyl-*sn*-glycerols, as is necessary in normal-phase adsorption chromatography. The forces involved in the separation are complex and depend upon the nature of the bonded chiral molecules and the solutes [128].

Itabashi and Takagi [129] achieved good separations of DNPU derivatives of mono- and

diacyl-*sn*-glycerols obtained by partial prior hydrolysis of triacyl-*sn*-glycerols on a column with a chiral (*S*)-2-(4-chlorophenyl)isovaleroyl-D-phenylglycine stationary phase chemically bonded to a silanised aminopropyl silica support. Using a hexane–1,2-dichloroethane–ethanol mobile phase (40:12:3, v/v/v), monoacyl-*sn*-1-glycerols were separated from monoacyl-*sn*-3-glycerols [130]. Similar results have been obtained using D-naphthylamine chiral phases [131]. While DNPU derivatives of the mono- and diacyl-*sn*-glycerols have generally been used, benzoylisopropylidene glycerol derivatives have also produced good results [132].

Since the above-mentioned research, Takagi and Ando [133,134] substantially improved the separation of DNPU-monoacyl-*sn*-glycerols using an N-(*R*)-1-(1-naphthyl)ethylaminocarbonyl-(*S*)-valine chiral phase. Employing a long (500 × 4 mm I.D.) column, a slow flow-rate (0.5 ml/min) and a weakly polar ternary mobile phase like that mentioned above, they were able to separate the enantiomers with 18 carbon atoms and from zero to three double bonds, though elution times were excessively long (6 h or more), and certain isomers such as palmitic and oleic acid were not resolved.

Besides analysing monoacyl-*sn*-glycerols, chiral-phase chromatography has also been used to separate the DNPU derivatives of isolated diacyl-*sn*-glycerols [135,136]. On the whole, the method has yielded good results, for instance for DNPU derivatives of monoacyl-*sn*-glycerols, using long chiral-phase N-(*R*)-1-(1-naphthyl)ethylaminocarbonyl-(*S*)-valine columns, slow flow-rates and weakly polar mobile phases [136,137]. Complete separation of saturated diacyl-*sn*-glycerol homologues with total chain lengths differing by more than six carbon atoms is feasible, though various enantiomers may remain unresolved.

The development of new chiral stationary phases is a major factor in improving the resolution of DNPU-diacyl-*sn*-glycerol enantiomers. Chiral columns using (*R*)-(+)-1-(1-naphthyl)ethylamine chemically bonded to a silica-gel support and a hexane–dichloroethane–ethanol (40:10:1, v/v/v) mobile phase have achieved good separations of enantiomers. In addition, analysis times have been shortened appreciably by the use of short columns (250 × 4.6 mm I.D.) and higher flow-rates (1 ml/min). This technique holds great promise for future application in the stereospecific analysis of the triacyl-*sn*-glycerols in natural fats.

Finally, combinations of several different analytical techniques such as argentation TLC, HPLC–GC, HPLC–MS and enzymatic analysis can furnish valuable information for use in determining the stereospecific composition of the triacyl-*sn*-glycerols in natural fats [138].

8. High-performance liquid chromatography of TAGs

8.1. RP-HPLC

RP-HPLC is the most commonly employed chromatographic technique for separating mixtures of TAGs. The mechanism for separating TAGs in RP-HPLC involves chain length and the degree of unsaturation of the FAs. Wada et al. [139] were the first to establish a parameter, termed the partition number (PN; PN = CN − 2ND, where CN is the total number of carbons and ND the total number of double bonds in the FAs) for characterising TAG molecules and determining their elution according to their ascending order of PN. Following their lead, other workers have developed other parameters analogous to PN for molecularly discriminating between TAGs with the same PN value, mainly to improve identification of chromatogram peaks [140–142].

Since then, considerable progress has been made in improving RP-HPLC methodology, far outstripping adsorption chromatography, and achieving notable successes in analysing complex mixtures of TAGs. Optimisation of the conditions of analysis has made it possible to overcome, with greater or lesser ease, a series of difficulties encountered in separating the TAGs in natural fats. The first difficulty is the separation of mixtures of TAGs composed of FAs differing only minimally in chain length and

degree of unsaturation, that is, compounds with the same PN value. Despite the improvements in efficiency achieved using (ODS) columns with a particle size of 3 μm connected in series and elution gradients with acetone as the organic modifier, poorly resolved or even unresolved TAG pairs and groups still exist, especially in highly complex mixtures of natural fats such as milk fat and fish oils [143].

The second difficulty involves the simultaneous analysis of TAGs differing markedly in molecular structure, that is, short-, medium- and long-chain TAGs with differing degrees of unsaturation spanning a broad range of PN values. The problem lies in achieving, simultaneously, both good resolution of the least strongly retained TAGs and reasonable analysis times and elution in narrow chromatographic bands for the most strongly retained TAGs; in other words, achieving an efficient chromatographic system [144].

The third difficulty involves the detection of TAGs at the column outlet; refractive index and ultraviolet detectors have been most commonly used in HPLC analyses for fat up to now [145,146]. This situation has made it necessary to develop new systems such as light-scattering detectors and combined HPLC–MS, considered below.

The last major difficulty is peak identification on the chromatograms, because pure standards are relatively unavailable for many natural mixed TAGs and because many critical pairs or groups of TAGs have not been resolved, particularly for complex mixtures [147]. Combining HPLC and MS may furnish valuable information on the structure of TAGs [58].

Octadecylsilane (ODS) supported on particles of silica has provided the stationary phases yielding the best separations. On the whole, most silica packings consist of spherical particles, with particle diameter being the factor exerting the greatest effect on resolution. While good separations have been obtained using a particle diameter of 5 μm, reducing the diameter to 4 and 3 μm improved selectivity between chromatogram peaks for columns of similar size [148–150].

ODS columns are normally 15 to 30 cm long, with an internal diameter of 4–5 mm, and two and three columns have been connected in series to improve separations of the TAGs from natural fats [151–154]. Increases in column length are limited by the rise in column head pressure and the substantially longer analysis times. Fig. 4 presents the separation of the TAGs in avocado oil using two ODS columns in series (250 × 4.6 mm I.D.) and a particle diameter of 3 μm.

8.1.1. Mobile phase

Mobile-phase composition is the most important chromatographic factor affecting the separation of TAGs in RP-HPLC because of the small variability in the type of stationary phase employed. Most investigators have elected to use a mobile phase composed of a combination of an organic base solvent and an organic modifier. Acetonitrile is the most widely used organic base solvent, though *n*-propionitrile has sometimes been preferred [155,156]. However, this latter solvent has certain shortcomings, e.g. high cost and extreme toxicity.

Organic modifiers are added to improve the solubility of the TAGs in the base solvent of the

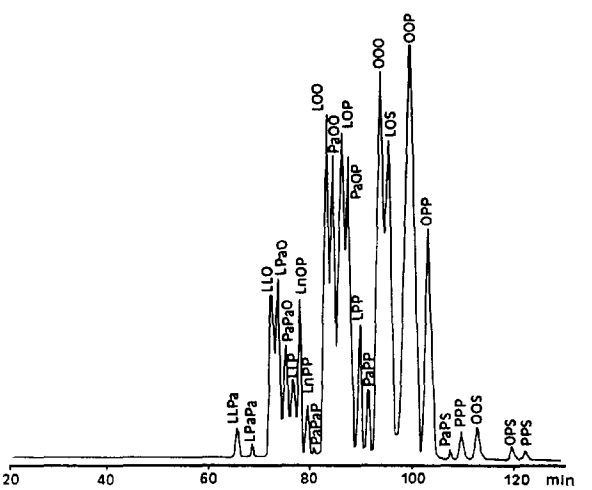

Fig. 4. HPLC separation of avocado oil (cv. Hass) TAGs using two 3-μm Spherisorb ODS-2 columns (250 mm × 4.6 mm I.D.) connected in series. Column temperature: 30°C; mobile phase: gradient from 30% to 70% (v/v) acetone in acetonitrile for 75 min at a flow-rate of 0.8 ml/min and a pressure of 147 bar; mass detection (from Ref. [176]).

mobile phase, bring about changes in mobile-phase polarity, and increase peak selectivity [141,157]. When choosing an organic modifier, the following factors should be taken into account, in addition to the nature of the solvent: whether the mixture consists of single or multiple components; the percentage of organic modifier in the mobile phase; and whether that percentage is constant or varies over the course of elution.

A number of investigators have studied the effect of the percentage of organic modifier in the mobile phase, and their work has shown that under both isocratic and gradient elution conditions, within the solubility range for the TAGs in the mobile phase, an increase in mobile-phase polarity leads to an increase in retention times and in the selectivity of pairs or groups of TAGs with the same PN [140,157,158]. Furthermore, work has also focused on explaining the causes of the preceding effects, and some investigators have established linear relationships between the logs of the capacity factor (k) and TAG selectivity and the percentage organic modifier in the mobile phase, irrespective of solvent type and elution conditions [154,157,159,160].

The choice of organic modifier, single or multiple component composition of the modifier, proportion of modifier to base solvent, and possible variations in that proportion during elution depend mainly upon the type of TAGs to be separated and the detection system employed. Although many different solvents have been used as the organic modifier, acetone is most commonly employed because of its effect in improving the selectivity of TAG pairs or groups with the same PN [161,162]. However, acetone cannot be the modifier with UV detectors, because of its strong absorbance of the wavelengths absorbed by the TAGs (200–237 nm) [163]. In such cases, hexane, *n*-propanol, ethanol and methyl-*tert.*-butylether have been proposed as organic modifiers and have produced good separations of complex mixtures [160,164–166].

For a number of years, mainly in the early years of fat analysis by RP-HPLC, TAG analysis was carried out using a constant proportion of organic modifier in the mobile phase with refractive index (RI) detection [142,158,167,168]. Those conditions of analysis yielded relatively satisfactory separations of simple mixtures of TAGs, such as most vegetable oils, which have a mean composition of 5–7 FAs and 15–20 TAG species. Although the resolution of most such TAGs was good, certain critical pairs remained unresolved, e.g. LLL–LnLO and LnLO–LnLP for PN 42; LLO–LnOO, LnOO–LnLSt and LLP–LnOP for PN 44; LLSt–LOP for PN 46; and OOO–LOSt and OOP–LStP for PN 48 [141,158,159].

Varying the proportion of organic modifier during elution has been proposed as a solution when analysing more complex natural mixtures [153,164,166,169–171]. The composition of complex fats includes a larger number of FAs than vegetable oils and usually a much higher number of TAG types, and as a result there is a much greater likelihood of encountering molecules with the same PN value [147]. Other causes of complexity are the presence of a larger number of highly saturated TAGs, as in the case of animal fats [152]; an FA composition spanning a broad range of unsaturations, from saturated FAs to five or six double bonds, as in the case of fish oils [172]; and the presence of TAGs encompassing a broad range of PN values, as in the case of milk fat [143].

Gradient elution systems are intended to ensure good solubility of all the TAGs present in the mixture in the mobile phase throughout the run, reduce retention times for highly saturated TAGs, and at the same time achieve good resolution of critical pairs with the same PN. Resolution of such unresolved critical pairs improves appreciably under isocratic conditions, irrespective of the type and proportion of organic modifier in the isocratic mobile phase [173]. Perrin and Prevot [174] achieved excellent separation of the TAGs in vegetable oils and animal fats from ATs by employing two 5-μm ODS columns, a linear gradient of from 20% to 80% (v/v) of dichloromethane–acetone (3:1, v/v) in acetonitrile, and light-scattering detection (Fig. 5).

Despite the enhancement in resolution brought about by elution gradients, complex

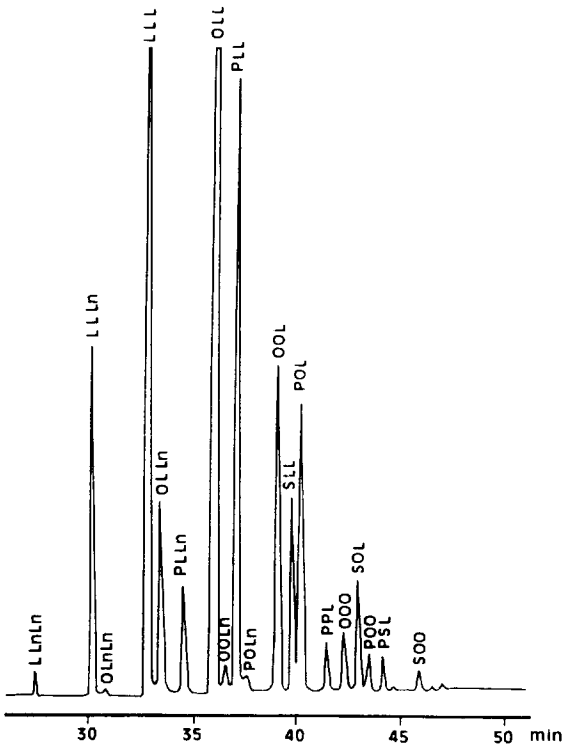

Fig. 5. HPLC separation of soybean oil TAGs using two 5-μm Lichrospher 100 CH-100 SUPER columns (250 mm × 4.0 mm I.D.) connected in series. Column temperature: ambient; mobile phase: linear gradient of from 20% to 80% (v/v) dichloromethane–acetone (3:1, v/v) in acetonitrile for 60 min at a flow-rate of 1.0 ml/min; mass detection (with permission from Ref. [174]).

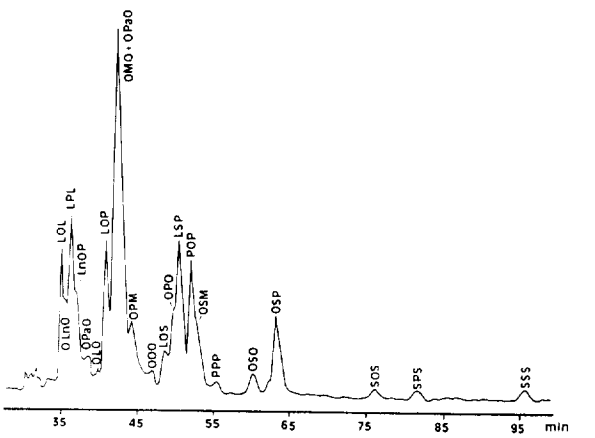

Fig. 6. HPLC separation of pork lard TAGs using two 5-μm Spherisorb ODS columns (200 mm × 4.6 mm I.D.) connected in series. Column temperature: 30°C; mobile phase: linear gradient of from 23% to 30% (v/v) methyl-*tert.*-butylether in acetonitrile for 25 min at a flow-rate of 1.0 ml/min; UV detection at 220 nm (with permission from Ref. [153]).

mixtures of natural fats still contain many unresolved TAG pairs or groups with the same PN, and this is one of the most important unsolved analytical challenges remaining today [143,175,176]. The selection of gradient elution type depends chiefly upon the complexity of the TAG mixture and the type of detector employed. The gradient type for both simple and complex mixtures has in the main comprised linear variation of the proportion of organic modifier with elution time [153,170,174]. However, non-linear gradients and specific step gradients have been used, chiefly with a view to improving the separation of critical pairs [150,154,177]. Figs. 6 and 7 present the chromatograms for two gradient elution analyses, the former for pork lard TAGs and the latter for ewe's-milk cheese fat.

The influence of mobile-phase flow-rate, which ranges from 0.5 to 2.0 ml/min, on TAG res-

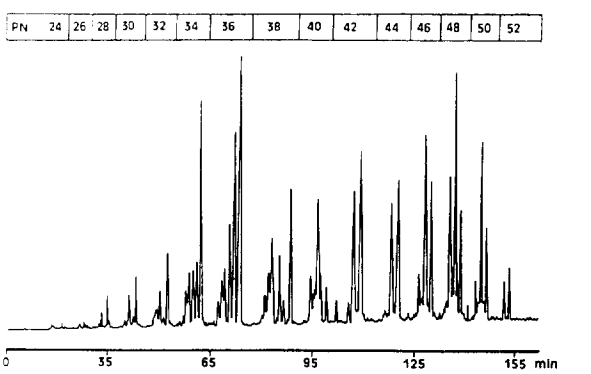

Fig. 7. HPLC separation of ewe's-milk cheese TAGs using two 3-μm Nucleosil ODS-2 columns (200 mm × 4.6 mm I.D.) connected in series. Column temperature: 30°C; mobile phase: step gradient of acetone in acetonitrile; step one: linear gradient of from 0% to 35% acetone (v/v) for 50 min; step two: isocratic elution using 35% acetone over 75 min; step three: linear gradient of from 35% to 80% acetone over 140 min; step four: isocratic elution using 80% acetone for a further 175 min; flow-rate 1.0 ml/min; mass detection. PN, partition number (unpublished data).

olution has not been studied systematically; however, certain workers have, as might be expected, reported a decrease in TAG capacity factors along with a decrease in the number of plates for the chromatographic system with increasing flow-rate [157]. On the other hand, other researchers have reported that, under isocratic elution conditions, small increases in flow-rate enhanced the resolution of certain critical pairs [158].

8.1.2. Sample solvent and column temperature

The sample solvent may exert a major effect on the resolution of a chromatographic system. Investigations into various solvents have revealed substantial differences according to the nature of the injection solvent and the mobile phase [160,178,179]. The effect of the solvent on the chromatographic resolution can be explained in terms of the theory put forward by Horvath and Melander [180], whereby in order for a dissolved solute molecule to be carried by the mobile phase, it must create room and give rise to a reduction in free volume. TAGs can interact with the mobile phase and with the hydrocarbon ligand group on the stationary phase. Tsimidou and Macrae [179] proposed the mobile phase as the ideal sample solvent. However, other investigators have suggested chloroform as the sample solvent when working with mobile phases in which solubility of TAGs, particularly highly saturated TAGs, was poor.

Most RP-HPLC analyses of TAGs have been done with the column at ambient temperature, i.e. unthermostatted. However, various workers have shown that changes in column temperature resulted in changes in chromatographic resolution [157,178,181]. The results revealed that higher column temperatures decreased retention times and selectivity for the TAGs, especially for critical pairs with the same PN [157]. In addition, various linear relationships between the logs of the capacity factor and the selectivity factor for TAGs and various column temperature functions could be used to establish such variations [144,157,181,182].

Although lower temperatures result in better separation of the TAGs, the most highly saturated TAGs may precipitate out of the mobile phase, and consequently many workers have preferred working temperatures of at least 30°C [149,151]. Furthermore, Singleton and Pattee [178] noted that the number of theoretical plates increased with temperature, enhancing the elution of highly saturated TAGs immensely and yielding narrow, well-defined peaks [157].

The choice of column temperature represents a compromise designed to ensure good solubility of highly saturated TAGs concomitantly with good selectivity of critical pairs with the same PN. Various investigators have proposed using temperature gradients to analyse complex mixtures of TAGs spanning a broad range of PN values [147,175]. Fig. 8 illustrates the separation of butterfat TAGs using a temperature gradient of from 10° to 55°C with a 5-μm ODS column (250 × 4.0 mm I.D.) and a mobile phase of acetonitrile–acetone (50:50, v/v) [147].

8.1.3. Detectors

Many researchers have focused on the detection of TAGs, particularly since the development of the mass detector and of the combination of HPLC and MS.

Refractive index (RI) detectors have been used in most analyses of simple mixtures of TAGs under isocratic conditions of elution [142,167,178]. They have certain drawbacks compared with other types of detector, namely,

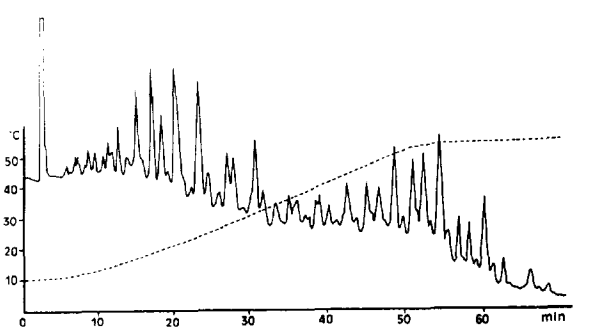

Fig. 8. HPLC separation of butterfat TAGs using a single 4-μm Lichrospher 100 RP-18 column (250 mm × 4.0 mm I.D.). Column temperature: programmed (dashed line) from 10° to 55°C at a rate of 1°C/min; mobile phase 50% (v/v) acetone in acetonitrile at a flow-rate of 1.0 ml/min; mass detection (from Ref. [147]).

low sensitivity, low stability on account of their sensitivity to changes in temperature and pressure, and unsuitability for gradient elution because they are affected by the composition of the mobile phase. Despite these problems, TAGs have response factors very similar to the RI [158,183].

Infrared (IR) detectors have occasionally been used at 5.75 μm, especially for qualitative purposes [184–186]. Although this type of detector is suitable for gradient elution, substantial baseline drift may occur.

Absorption in the UV region of 200–237 nm provides for highly sensitive detection of TAGs and good detector stability and can be used with gradient elution [160,171,178,187]. Considerable baseline drift may occur when the gradient composition of the mobile phase varies over a broad range [188]. UV detector response in that region of the spectrum is mainly due to the existence of weakly chromophoric ester bonds with a lower molar extinction coefficient. However, UV absorption can be considerably enhanced by interactions between the carbonyl groups and the double bonds [162,165]. Fig. 9 compares the RP-HPLC analysis of a sample of fish oil using RI detection and UV detection at 225 nm.

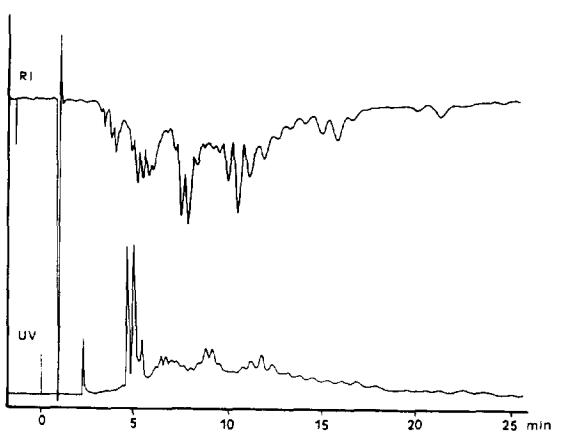

Fig. 9. HPLC separation of fish-oil-derived TAGs using a single 5-μm Supelcosil LC 18 column (250 mm × 4.0 mm I.D.). Column temperature: ambient; mobile phase: 20% (v/v) tetrahydrofuran in acetonitrile at a flow-rate of 2.0 ml/min. Top: response with RI detection. Bottom: response with UV detection (with permission from Ref. [162]).

Other workers have used moving-fire FIDs and attained good sensitivity and baseline stability when using elution gradients [173,189,190]. However, this type of detector is not widely used or available commercially for HPLC analysis and may be subject to shortcomings involving low sensitivity, because only a small portion of the solvent eluting from the column can be used for FID detection [191].

In recent years a number of researchers have performed RP-HPLC analyses of TAGs using mass detectors [150,152,154,166,177,192,193]. Although this detection system was described at the end of the 1970s [194,195], its use in TAG analysis did not become widespread until the mid-1980s.

Mass detection is based on light scattering by solute molecules following nebulisation of the column eluate and evaporation of the solvent [195,196]. Mass detection can be used with gradient elution and non-aqueous solvents and does not undergo baseline drift [169]. Considerable work has focused on optimising detection conditions. Composition of the mobile phase, elution rate, nebuliser gas (normally compressed air) flow-rate and solvent volatilisation temperature all have an important influence on TAG response during mass detection [188,196–199]. In addition, several workers have established that for a broad range of concentrations the mass detector response to solute concentration follows an exponential curve and the detection threshold is of the order of 1 μg, showing that mass detection is as sensitive or more so than other methods [58,200,201]. Figs. 4, 5 and 7 provide examples of mass detection of TAGS.

Chemical detection by means of post-column reaction derivatisation (PCRD) has seldom been used for TAG detection in HPLC. Kondoh and Takano [202] described a method in which the glycerol produced by hydrolysis of TAGs with potassium hydroxide was oxidised and derivatised to 3,5-diacetyl-1,4-dihydrolutidine and then the absorbance of that derivative was measured at 410 nm. This detection system yielded high sensitivity and detection thresholds of around 0.1 nmoles.

Lastly, HPLC–MS has been successfully em-

ployed as a detection system and affords an advantage over other detectors in that MS also provides valuable information on TAG structure [203,204]. Chemical ionisation mass spectra (CIMS) have successfully identified all the major TAGs together with the sufficiently resolved minor TAGs in various natural fats and oils [203,205].

Although HPLC–MS detection can be used with gradient elution and affords high sensitivity (with detection thresholds of around 50 μg), it still has certain drawbacks. First, only one one-hundredth of the column eluate can be transferred to the mass spectrometer through the interface, and hence the amount injected into the HPLC system must be on the order of 100 times greater than the amount of a given solute to be detected [205]. Second, the quasi-molecular ion and ion fragments in the TAG mass spectrum vary according to molecular mass, degree of unsaturation and *sn*-stereospecific position of the FAs esterifying the glycerol, making calibration necessary for quantitative analysis. In view of the unavailability of pure standards for many mixed TAGs and their structural isomers, response factors can be estimated by comparison with the peak area measurements in separations of fats and oils of known composition [155].

8.1.4. Identification of molecular species

One of the most challenging research areas in RP-HPLC of natural samples of TAGs is the identification of the molecular species in the peaks on the chromatogram. Since the early work carried out by Wada et al. [139] establishing a linear relationship between the capacity factor (k) values of chromatographic peaks and the PN values of the TAGs, aspects of the theoretical prediction and identification of the TAGs in the peaks have been examined.

Herslöf et al. [140] estimated theoretically the equivalent carbon number (ECN) for unsaturated TAGs on the basis of their relative retention times using an experimental linear relationship between relative retention time and the total number of carbons (CN) in saturated TAGs. The ECN ($ECN = CN + a'ND$) was defined in the same way as the PN, although for the former the value of the constant a' is dependent upon each chromatographic system [160]. However, a' may take on values approaching $|2|$; when $a' = 2$, the PN and ECN values are the same.

Takahashi et al. [206] calculated the value of a' from the linear relationship between log k, CN and ND ($\log k = q' + b'\ CN + c'\ ND$). The value of a' is equal to c'/b'.

The parameters PN and ECN have generally been used in RP-HPLC to characterise the TAG molecules. However, TAGs with the same PN can be differentiated by ECN.

The preceding relationships were calculated for isocratic elution, and consequently a number of workers have shown that the aforementioned estimation of the ECN is not always appropriate for gradient elution, as TAGs of very similar molecular characteristics may elute in descending order of ECN [150,176]. In such cases, it has been proposed to estimate the ECN by means of a second-degree function using CN and ND ($ECN = CN + d'\ CN^2 + e'\ ND + f'\ ND^2$) [176].

Other researchers have established a theoretical carbon number (TCN), analogous to the PN and ECN, calculated from the CN and a function depending upon the degree of unsaturation of the FAs [141,207].

Goiffon et al. [142] carried out an extremely interesting study of TAG identification in RP-HPLC. On analysing pure standards of simple TAGs and mixed TAGs obtained by transesterification of the former, those investigators reported a linear relationship between what they termed equivalent chain length (L) and selectivity of the TAGs with respect to triolein (α_0) ($L = g' + h' \log \alpha_0$). They calculated the value of L for the TAGs in the chromatogram based on the experimental value of α_0 for the peaks and then used it to estimate the molar FA composition of the peaks for a natural fat.

Several years later Perrin and Naudet [183] used the L value to estimate the TAG composition of several vegetable oils.

The procedure for predicting the TAGs in RP-HPLC peaks for a natural fat based on the ECN is tremendously complicated for large numbers of FA constituents (e.g. more than seven

FAs), since the variety of possible molecular species, even taking *sn*-stereospecific positions to be equivalent, is extremely high [143]. Therefore, different workers have proposed using the equations developed by Takahashi et al. [206,208] as a second stage in the prediction procedure based on the ECN. Those investigators developed a matrix model using six variables, for the chain length (CL) and number of double bonds (DB) for each of the three acids esterifying the glycerol, taking *sn*-stereospecific positions as equivalent ($\log k' = i' + j'_1CL_1 + j'_2CL_2 + j'_3CL_3 + z'_1DB_1 + z'_2DB_2 + z'_3DB_3$).

Certain researchers have estimated the TAG composition of different types of natural fats based on their total FA composition, the ECN value and the above-mentioned matrix model [168,176,209].

Applying partition chromatography theory [210], Takahashi et al. [209] found a linear relationship between the log of the relative retention time for TAGs and potential relative retention indices for each constituent FA. Those findings were subsequently used to estimate the composition of the major TAGs in a fish oil based on the relative retention times for pure simple TAGs [211].

Theoretical prediction of the TAGs in RP-HPLC peaks for a fat furnishes very useful information for analytical identification of those TAGs, particularly for complex mixtures. The prediction provides a number of possible TAGs for each chromatographic peak. That number will be larger or smaller according to the complexity of the fat, and the error will depend upon the mathematical equations employed.

Identification of RP-HPLC peaks has been performed using a variety of analytical techniques. The combination of HPLC and MS unquestionably furnishes the most structural information [156,212]. Other workers have combined RP-HPLC with GC to analyse the FAs in RP-HPLC peaks collected at the column outlet [143,147,164,213]. Still, identification is quite problematical when TAGs with the same or very close ECNs remain unresolved; in such cases, theoretical estimates can be of great assistance.

Lastly, identification of the TAGs in a fat may be carried out by combining HPLC with other chromatographic techniques, such as argentation TLC, or with enzymatic methods to analyse *sn*-stereoscopic positions [214–216].

8.2. Argentation chromatography

Despite its potential for providing structural information, argentation chromatography in TAG analysis has been used much less often than RP-HPLC, chiefly as a result of the technical difficulties encountered in applying it in HPLC systems [58]. It separates TAGs according to their degree of unsaturation, unlike RP-HPLC in which the separation is more complex, depending on the chain length of the constituent FAs in addition to the degree of unsaturation. The basis for TAG separation is the ability of the π-electrons in the double bonds on the FA chains to interact with the silver ions on the stationary phase and form stable polar complexes. As the number of double bonds in the molecule increases, so does the complex-formation effect and hence retention [217].

Development of a stable, reproducible chromatographic system has been the main technical stumbling-block encountered in applying argentation techniques in HPLC. To date, it has not been possible to achieve a completely reproducible system suitable for ready application of this technique in any laboratory. Christie and his team have made the greatest progress in developing silver-ion chromatographic systems in general and for HPLC in particular [58,218–220].

Of all the factors influencing HPLC techniques, the stationary phase is unquestionably the main factor in argentation chromatographic separations. The silver ions may be added to the stationary phase in one of two ways: by impregnating the silica-gel support with a silver salt, normally silver nitrate, or by bonding silver ions to the phase by means of an ion-exchange phase.

Silica-gel columns impregnated with silver nitrate were the first to be developed and perhaps for that reason have been employed in most analytical separations of TAGs [78,217,221–223]. Percentage impregnation with silver nitrate has varied from 5% to 10%, since at higher values chromatographic resolution is not im-

proved, and at lower ones the silica-gel adsorption activity increases considerably, resulting in peak tailing [221]. Columns impregnated with silver ions are not available commercially and require careful preparation in order to achieve reproducible results [224,225]. The main difficulty with this type of column is the release of silver ions by the stationary phase, which results, on the one hand, in short column-working lifetimes and, on the other, in possible damage to detection systems due to the frequent formation of corrosive silver nitrate precipitates. However, stationary phases using cation-exchange supports achieve good silver-ion retention levels, thereby avoiding these difficulties. Cation-exchange supports may consist of macroreticular sulphonic acid resins or silica-gel supports with chemically bonded methylsulphonic acid groups [58, 217,220].

Separation of TAGs on macroreticular resins calls for highly polar mobile phases to achieve elution of the most extensively unsaturated constituents, giving rise to insolubility of the most highly saturated constituents and hydrolysis and transesterification reactions [226]. Christie [218] successfully obviated these difficulties by including aprotic solvents such as 1,2-dichloroethane and acetone in the mobile phase.

Stationary phases consisting of phenylsulphonic groups chemically bonded to the silica gel appear to be the most advantageous ones. Such columns are available in acid or sodium salt form and must first be neutralised by washing with sodium or ammonium hydroxide before treatment with aqueous sodium nitrate. The silver-ion content depends upon the number of bonded sulphonic acid groups and the degree of substitution of the initial cation by silver ions [218].

Although the stationary phase was earlier held to be the most important factor affecting argentation chromatography, composition of the mobile phase exerts a decisive influence on TAG separations. The nature of the interaction between the silver ions, unsaturated solutes and solvents in the mobile phase on silver-ion cation-exchange columns has not been fully elucidated. Several researchers have ascribed solute retention on such columns to a mechanism of mixed interaction with the more highly polar TAG groups involving the formation of complexes with the silver ions and adsorption by the unbonded polar groups in the support [221,227]. Thus, such solvents as benzene, toluene and acetonitrile appear to interact preferentially with the silver ions, thereby interfering with the interactions with the double bonds, whereas methanol, isopropanol and acetone may block interactions with the unbonded polar groups on the support [228,229]. Other workers have suggested using elution gradients combining chlorinated hydrocarbons with acetone and acetonitrile to separate a broad range of mixtures of TAGs [193,218,219,230,231].

Fig. 10 illustrates the separation of linseed oil TAGs on a cation-exchange column with silver ions chemically bonded to sulphonic groups under conditions of gradient elution [219]. Elution was in ascending order according to degree of unsaturation, with stronger retention of dienoic residues than of two monoenoic residues on the same molecule and equal retention of a trienoic residue as of two dienoic residues. On the whole, TAGs have been shown to be more

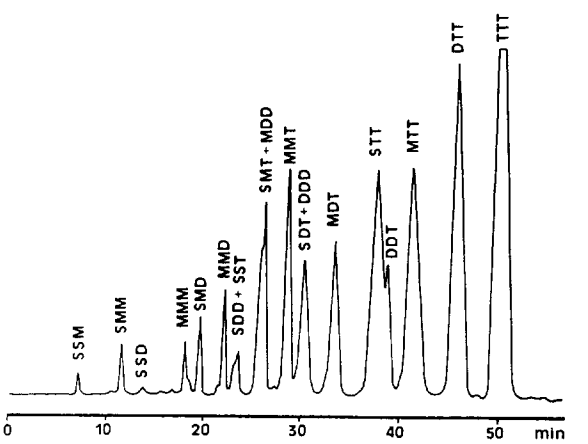

Fig. 10. HPLC separation of linseed oil TAGs using a single silica-gel-based argentation exchanger Nucleosil column (250 mm × 4.6 mm I.D.). Column temperature: ambient; mobile phase composition: (A) 1,2-dichloroethane–dichloromethane (1:1, v/v). (B) acetone, (C) acetone–acetonitrile (4:1, v/v); flow-rate: 1 ml/min; mass detection. Linear step gradient: step one, from 100% A to 50% A–50% B in 10 min; step two, from 50% A–50% B to 70% B–30% C in 20 min; step three, from 70% B–30% C to 100% C in 30 min. S = saturated; M = monoenoic; D = dienoic; T = trienoic FAs (from Ref. [219]).

strongly retained when the double bonds are concentrated in a small number of FA molecules [219,231].

Certain researchers have achieved good separations of position isomers and isomers with varying configurations of double bonds in the TAG molecules based on the differing effects of mobile-phase solvents on the retention mechanism [232,233].

Mass or light-scattering (ELSD) detectors and moving-flame FID have been the most successful ones used in TAG analysis by argentation HPLC because they do not limit the choice of solvents for the mobile phase, as is the case for UV detectors and acetone. They also afford good stability and sensitivity when using complex elution gradients with mixtures of three or more solvents [193,218,222,231].

Lastly, although argentation HPLC affords high resolving power for TAGs according to the degree of unsaturation, it is not able to separate TAGs that differ only in respect of the chain length of their constituent FAs. In view of the results obtained by different workers to date, RP-HPLC can be regarded as providing better separation of individual TAG molecules, because it operates on the principle of both chain length and degree of unsaturation of the FAs, thereby characterising peaks on the basis of such molecular variables as ECN. However, as was noted in the preceding subsection, RP-HPLC can hardly separate and identify TAGs with the same ECN or isomers with the same degree of FA unsaturation. Thus, combining these two HPLC techniques, first separating TAG fractions with the same degree of unsaturation and then using RP-HPLC to analyse the different fractions, may furnish more useful information on the molecular make-up of natural mixtures of TAGs [220,233].

9. TAG analysis by gas chromatography

The initial studies on separations using gaseous-phase chromatography were carried out by Fryer et al. [234] and Hubber [235], but the first chromatograms devoid of signs of decomposition were published by Kuksis and McCarthy [236], who improved the separation technique by employing direct injection onto the column. Up to the end of the 1960s, TAG analysis was carried out on columns packed with dimethyl polysilene (OV-1, OV-101, JXR, S-30) [3,237] or phenyl methyl polysiloxane (OV-17, SE-52). The internal diameter was 2.5–3.5 mm [238,239]. The column length depended upon the type of separation. For TAGs above C_{40}, 0.5–0.7-m columns were used [236,240]. Columns longer than 2.0 m are impractical for separating TAGs above C_{48}. Litchfield [3] published an excellent review on the subject.

Since the 1970s, capillary GC using apolar columns (OV-1, SE-52, SE-54) has become a routine tool for characterising the TAG profile of fats [241]. TAG separation on apolar silicone phases is based mainly on molecular mass differences (CN determinations), whereas unsaturation barely contributes to peak retention [242]. When a mixture of TAGs is injected under a given set of experimental conditions, the detector response turns out not to be optimal for all the mixture components, thus misrepresenting the exact quantitative composition. For that reason, working conditions are established for each mixture separately; this necessitates a prior study of FAs to select the most appropriate conditions according to the composition of the mixture being analysed.

On employing isothermal chromatographic conditions to analyse a mixture of TAGs with different vapour tensions or long retention times at high temperature, the amount of the stationary phase and column efficiency (length) are selected so as to achieve good separation of the most volatile components. With TAGs, the main problem is always to reduce the analysis time; for isothermal chromatography, however, longer columns are needed to achieve separation of the low-molecular-mass components, while retention times for high-molecular-mass TAGs are very extended, and certain ones may not elute. When programmed-temperature chromatography is used, the retention times for a series of compounds with the same CN generally increases linearly with the number of carbon atoms rather

than exponentially, as in the case of isothermal chromatography.

Programming the temperature has enabled elution of TAGs of up to 60 carbon atoms in reasonably short times. However, the use of short columns is affected by a number of different factors: injection technique, carrier gas flow-rate, column type and, above all, the type of substance being analysed. Thus, problems are always encountered in the case of, for instance, neutral fats from nervous tissue and fish fats, in which the thermal decomposition undergone by the TAGs during vaporisation may be an important factor affecting the assessment of the TAG composition of those polyunsaturated fats.

9.1. General aspects

All these factors caused a number of workers to begin studying quantitative TAG analysis in 1979–1980 using fused-silica capillaries with a chemically bonded, non-polar stationary phase (replacing the former method of capillary GC on apolar columns) (OV-1, SE-52, SE-54) [243–247]. Resolution on these columns depends on the number of unsaturated FAs on the TAG molecule; with a "polarisable" methylphenylsilicone stationary phase, the CN sequence is retained, but unsaturation now contributes to the fine structure of each CN fraction [248–251]. Thus, for example, the order of elution becomes tripalmitin (PPP), palmitoil-oleil-palmitin (POP) and palmitoil-linolenoil-palmitin (PLP), all of which have saturated acids on the molecule, and separation by triplets takes place according to the unsaturation of the middle acid [252]. A highly illustrative example of this type of separation was presented by Ruiz-Gutiérrez and Cert [13]. Moreover, certain workers have described analyses of vegetable and animal fats using this type of column [253,254] and succeeded in separating substances with the same number of carbon atoms, differing by only a single double bond [241,255–259].

As already stated above, the columns used today have chemically bonded, polarisable stationary phases (Crompac, Supelco, etc.); their length varies from 5 to 25 m, with an internal diameter ranging between 0.2 and 0.32 mm. Column length increases as the molecular mass of the TAGs to be separated increases. The stationary phase used has an upper temperature limit of 360°C. Hydrogen is the most commonly used carrier gas with this type of column, and as Mares [260] noted, the hydrogen flow-rate decreases significantly with increasing temperature. Using hydrogen as the carrier gas affords several advantages over nitrogen, used in packed columns and in the earlier capillary columns [243,245–247,261–267]. One such advantage is a lower elution temperature as compared with nitrogen and helium, leading to shorter analysis times and less thermal decomposition.

9.2. Operating conditions

In recent years capillary columns filled with high-temperature polarisable phenylmethylsilicone have been shown to be as effective as HPLC, provided the analysis results are corrected by a response factor for the different molecular species [268]. Separation of saturated and monounsaturated TAGs from samples of plasma lipids [269] is nearly as effective as with HPLC, except for quantification of long-chain FAs. A further advantage of GC is that several types of lipids can be separated at the same time, and in a recent paper Kuksis et al. [270] presented a useful method for improving the analytical conditions for simultaneous separation of different lipids, based on dephosphorylation of plasma lipids with phospholipase C, converting them to their trimethylsilyl ethers. The molecular species of plasma lipids were identified by comparing the relative retention times to reference standards. The method employed on-column injection. Analyses by CN were performed on an HP 5880 using a flexible quartz capillary (8 m × 0.32 mm I.D.) coated with a permanently bonded SE-54 liquid phase. The sample was injected on-column at 40°C, and the column temperature was programmed as follows: 30°C/min to 150°C followed by 20°C/min to 230°C and finally 10°C/min to 340°C. The carrier gas was hydrogen at 1 bar column head pressure. Recovery of the lipid classes was determined by

summing the molecular species for each CN and comparing the proportions of CNs obtained on polar and non-polar columns. These workers concluded that high-temperature polarisable capillary columns were suitable for qualitative and quantitative assessment of plasma lipids and furnish more information per man-hour, instrument time and unit cost than any other existing analytical method.

Although considerable progress has been made in the quantification of TAGs by GC, problems with highly saturated TAGs have not been completely resolved [269–272]. The possibility of calculating the relative FID response for substances not available in pure form on the basis of the number of effective carbons in the molecule has been considered [273]. However, this approach is not suitable for larger, more highly unsaturated TAGs. Although this is a drawback for fish fats, it does not affect studies on fats from mammals or human, in which most FAs are saturated or monounsaturated and the polyunsaturated FA content is only 17–18% [14].

Unquestionably, GC analysis is incomplete unless it includes MS analysis. That work has been carried out by Mares et al. [88], who identified and quantified over 50 molecular species of TAGs from pooled human plasma. The TAGs were resolved by silver-ion TLC into seven fractions which were separately analysed by polarisable GC and desorption chemical ionisation MS (DCI-MS, commonly employed in TAG analysis) in the presence of an internal standard. The ion source temperature was 200°C, the heating rate 1°C/s, and the reaction gas ammonia. In the view of those researchers, the two methods were equally good at estimating the major and minor constituents of saturated TAGs but not for estimating polyenoic species, that is, species containing more than three double bonds (which are degraded by high temperatures). The two methods produced similar values for the estimates of oligoenoic species, but the former method underestimated polyenes, while the latter method overestimated saturation. For that reason, those authors recommended TAG analysis by GC in association with DCI-MS.

10. Perspectives for biomedical applications

10.1. Determination of the TAG composition of human lipids

Cardiovascular diseases are responsible for roughly one-half of all deaths worldwide and for up to 20% of all disabilities in the Western world. Arteriosclerosis is a progressive disease that normally starts in infancy and expresses clinical symptoms in the adult from middle age. The pathological causes are varied, but like other inflammatory processes and immune system disorders, its incidence is low in regions where the diet is rich in vegetable or fish oils.

A series of events that culminate in generally irreversible damage to the cells of the smooth muscle and endothelium of the arterial wall leads to the onset [274] and progression of cardiovascular diseases. The end result is a severe inflammatory-fibroproliferative response. The beneficial effect of a diet containing appropriate proportions of oils with high MUFA and PUFA is the enhancement of the ability of specific receptors located on the cell surface to capture LDL-cholesterol and take it out of the circulation. But is a diet with a different TAG structure equally effective? Thus, evaluating the impact of diets rich in different types of oils on the atherogenic index has become an important research topic. Moreover, responses from the time of gestation and infancy are now considered to be fundamental from various standpoints. The human brain completes its morphological development over the first six to eight months after birth. Therefore, proper nutrition in premature and normal infants is of vital importance. Fats supply infants with between 40% and 50% of the total calories contained in mother's milk, and a newborn baby consumes around 7.5 g of fat/kg of body weight daily [275]. Such fats, which supply the FAs necessary for brain development, are an integral part of all cell membranes and are the sole vehicle for fat-soluble vitamins and hormones in milk.

In addition to an appropriate TAG composition, fat digestion in newborns requires adequate lipase activity and bile salt levels, the former to

break down the TAGs, the latter to emulsify the fat before and during lipolysis. In newborn and especially in premature infants, the pancreatic lipase and bile acid levels (the major factors in intestinal fat digestion) are low, and they depend upon an alternative mechanism for the digestion of dietary fat. The lipases, which make their appearance before the 26th week of gestation and have attained high activity levels at birth, compensate for low levels of pancreatic lipase [276].

Studies of the lactic components in maternalised and mother's milk may be one of the most important applications in the study of TAGs. Breast milk is regarded as an ideal food for meeting the nutritional needs of newborn infants [277], because it contains essential FAs (linoleic acid, α-linoleic acid and arachidonic acid) necessary for development of the skeletal and nervous systems [278,279]. Some paediatricians recommend the addition of PUFAs to maternalised milks [280,281]. The structure of the TAGs used in commercial mixtures appears to affect the bioavailability of their constituent FAs. A number of studies have examined the adaptation of pancreatic enzymes to their primary substrates in the diet. A higher fat content in the diet brings about a concomitant increase in lipase activity in rat pancreas homogenates [282]. The results of other studies suggest that the degree of TAG unsaturation does not seem to affect the pancreatic lipase levels [283], whereas the chain length of the constituent FAs in dietary TAGs does appear to exert an effect. According to the review by Martin et al. [284], the presence of very-long-chain polyunsaturated FAs (VLC-PUFAs) such as 20:4($n-6$), 20:5($n-3$) and 22:6($n-3$) at the outer positions on the TAGs induces resistance to pancreatic lipase hydrolysis in vitro [285,286]. Furthermore, the distribution of FAs between the outer and the *sn*-2 positions on the glycerol molecule regulates the luminal partition between the free and 2-monoacyl-*sn*-glycerol forms [287–289] and subsequently modifies the rate of intestinal FA uptake. Absorption of palmitic acid increases when it is located at the *sn*-2 position on the TAG as compared with the *sn*-1,3 positions [290–294]. A similar difference in absorption has been reported for stearic acid [295].

Consequently, there is considerable interest in understanding the TAG structure in milk during its maturation within the mammary gland. Some work has been carried out on the FA composition of colostrum during maturation. Table 2 presents results for the stereospecific analysis of human colostrum [284].

That study showed that PUFAs in human milk, particularly 20:4($n-6$) and 22:6($n-3$), were esterified at positions *sn*-2 and *sn*-3. It concluded that the highly specific positional distribution of VLC-PUFAs in human milk TAGs is likely to be of biological significance. These findings could prove valuable in designing efficient lipid sources for infant formulas [285,286].

10.2. Determination of the TAG composition of human VLDL

Very-low-density lipoproteins constitute a family of lipoproteins with a significant level of variability in respect of size, density and chemical composition. Generally, the diameter is between 25 and 70 nm, density between 0.95 and 1.006 g/ml, and molecular mass 5 to 10×10^6. VLDLs consist of a non-polar core composed mainly of cholesterol esters and TAGs ($>70\%$). Following synthesis in the liver, TAGs predominate in the core [296], but as catabolism progresses and their diameter decreases, cholesterol esters become the principal components of this hydrophobic nucleus [297,298]. VLDLs are formed in the liver from cholesterol and FAs, both exogenous ones and those synthesised in the liver. Once in the plasma, the particle undergoes a process of maturation consisting of a gain of apolipoprotein C from the HDL. Its structure is thus altered so that it can interact with lipoprotein lipase (LPL) [299], the enzyme responsible for the degradation of that compound. While some of its phospholipids are degraded by LPL and other lipases, apo-B undergoes conformational changes that make the VLDL particle capable of capturing apo-E [300,301]. Hydrolysis of the TAGs and transfer

Table 2
Results (mol%; mean ± S.D.) of the stereospecific analysis of colostrum triacylglycerols (TAG) ($n = 11$) [284]

Fatty acid	*sn*-1 (%)	*sn*-2 (%)	*sn*-3 (%)	Calculated TAG (%)[a]	Original TAG (%)
Saturated					
10:0	0.00	0.36 ± 0.34	0.84 ± 0.41	0.40 ± 0.15	0.56 ± 0.13
12:0	1.41 ± 1.1	3.81 ± 1.32	9.11 ± 3.22	4.78 ± 1.41	4.54 ± 1.05
14:0	4.80 ± 1.90	11.08 ± 2.20	9.73 ± 2.91	8.54 ± 2.11	7.41 ± 1.63
15:0	0.34 ± 0.22	0.69 ± 0.22	0.39 ± 0.12	0.47 ± 0.10	0.40 ± 0.08
16:0	12.58 ± 3.38	53.50 ± 3.21	11.21 ± 3.33	25.76 ± 2.33	26.22 ± 1.70
17:0	0.49 ± 0.13	0.33 ± 0.04	0.31 ± 0.13	0.38 ± 0.09	0.37 ± 0.05
18:0	11.35 ± 1.34	1.65 ± 0.42	4.67 ± 1.08	5.89 ± 0.67	6.65 ± 0.63
20:0	0.14 ± 0.08	1.15 ± 0.05	0.17 ± 0.08	0.15 ± 0.04	0.17 ± 0.04
Monounsaturated					
14:1($n-5$)	0.22 ± 0.22	0.19 ± 0.13	0.39 ± 0.14	0.27 ± 0.08	0.18 ± 0.06
16:1[b,d]	2.93 ± 1.05	3.15 ± 0.64	3.52 ± 0.83	3.20 ± 0.62	2.80 ± 0.51
18:1[b,d]	48.43 ± 5.54	13.84 ± 1.58	36.62 ± 3.35	32.62 ± 2.20	34.40 ± 3.07
20:1[c]	0.74 ± 0.37	0.42 ± 0.21	0.72 ± 0.32	0.63 ± 0.25	0.70 ± 0.20
22:1($n-9$)	0.44 ± 0.38	0.12 ± 0.05	0.45 ± 0.33	0.34 ± 0.23	0.14 ± 0.04
24:1($n-9$)	0.00	0.00	0.11 ± 0.15	0.00	0.07 ± 0.05
n − 6 Polyunsaturated					
18:2($n-6$)[d]	14.04 ± 3.65	8.42 ± 2.22	17.25 ± 3.89	13.24 ± 3.18	13.13 ± 3.11
20:2($n-6$)	0.60 ± 0.19	0.28 ± 0.11	0.73 ± 0.22	0.54 ± 0.14	0.67 ± 0.13
22:2($n-6$)	0.00	0.12 ± 0.05	0.00	0.00	0.10 ± 0.08
20:3($n-6$)	0.37 ± 0.16	0.25 ± 0.08	0.50 ± 0.24	0.37 ± 0.14	0.39 ± 0.12
20:4($n-6$)	0.17 ± 0.17	0.74 ± 0.16	0.74 ± 0.22	0.55 ± 0.17	0.59 ± 0.11
22:4($n-6$)	0.00	0.34 ± 0.08	0.15 ± 0.10	0.16 ± 0.12	0.19 ± 0.07
n − 3 Polyunsaturated					
18:3($n-3$)	0.66 ± 0.15	0.41 ± 0.10	1.03 ± 0.21	0.70 ± 0.25	0.68 ± 0.13
20:3($n-3$)	0.05 ± 0.09	0.00	0.05 ± 0.09	0.00	0.00
18:4($n-3$)	0.12 ± 0.30	0.06 ± 0.09	0.20 ± 0.29	0.12 ± 0.21	0.23 ± 0.11
20:4($n-3$)	0.00	0.00	0.05 ± 0.09	0.00	0.00
20:5($n-3$)	0.00	0.06 ± 0.07	0.11 ± 0.20	0.06 ± 0.10	0.06 ± 0.12
22:5($n-3$)	0.00	0.34 ± 0.18	0.15 ± 0.14	0.16 ± 0.10	0.19 ± 0.11
22:6($n-3$)	0.10 ± 0.12	0.74 ± 0.65	0.62 ± 0.55	0.49 ± 0.40	0.52 ± 0.35

[a] (*sn*-1 + *sn*-2 + *sn*-3)/3 was used for the calculated triacylglycerols.
[b] $n-7 + n-9$ isomers.
[c] $n-9 + n-11$ isomers.
[d] Includes *trans* isomers.

of non-esterified cholesterol esters and apolipoproteins from HDL to VLDL [302] leads to the transformation of the VLDL first into IDL (with apo-E) and then into LDL by the loss of apo-E, probably with the participation of hepatic lipase [303].

There is considerable variation in the prevalence of coronary heart disease in different populations around the world. The Mediterranean region, in particular, exhibits a rather low incidence of cardiovascular disease. Of the different hypotheses that have been put forward to account for this situation, the most appealing is that the typical Mediterranean diet contains a high proportion of monounsaturated FAs. This is supported by data from the Seven Countries Study [304].

Blood lipids and lipoproteins are the most

important mediators in the relationship between dietary fat and coronary heart disease [305]. Ruiz-Gutiérrez et al. [306] studied dietary TAGs in an Andalusian population (Table 3). On capillary columns, TAGs are separated according to chain length (CN separation). Each CN is further subdivided according to differences in TAG polarity. Polarity increases with the degree of FA unsaturation (L > O > S) and with the total number of double bonds in the TAG (OOO > SOO > SOS > SSS). Accordingly, retention is the strongest for unsaturated FAs. Fig. 12 illustrates the VLDL-TAGs on the capillary column.

Table 3 presents the average TAG composition of VLDLs. The major TAG constituent is POO (glycerol-palmitate-oleate-oleate), followed by PLO (glycerol-palmitate-linoleate-oleate) and OOO (glycerol-trioleate). Together, these three TAGs account for 67% of the total. This is the only study dealing specifically with the VLDL fraction only. However, it is comparable with the results for studies of whole plasma and

Table 3
Average triacylglycerol composition of VLDL in man [306]

Triacylglycerol	Composition (%, w/w)
MMP	0.65 ± 0.04
MMPo	0.23 ± 0.06
MPP	0.25 ± 0.10
MOM	0.20 ± 0.01
MLM	0.82 ± 0.01
PPP	0.52 ± 0.09
MOP	0.23 ± 0.06
MLPo	0.19 ± 0.02
POP	5.95 ± 1.7
PLP + PPoO	5.49 ± 1.9
PLPo–MLO	1.22 ± 0.2
MLL	0.27 ± 0.01
POS	0.99 ± 0.07
POO	32.83 ± 3.29
PLO	22.30 ± 1.32
PLL	4.63 ± 0.6
SOS	0.25 ± 0.04
SOO	0.86 ± 0.08
OOO	12.20 ± 1.6
SOL	1.09 ± 0.2
OLO	7.53 ± 0.7
OLL	1.23 ± 0.21
Σ *n*	99.93

Results are given as mean ± S.D.
Nomenclature: Fatty acids: M = Myristic acid, tetradecenoic acid, $C_{14:0}$; P = Palmitic acid, hexadecenoic acid, $C_{16:0}$; S = Stearic acid, octadecenoic acid, $C_{18:0}$; Po = Palmitoleic acid, hexadecenoic acid, $C_{16:1}$; O = Oleic acid, *cis*-9 octadecenoic acid, $C_{18:1}$; L = Linoleic acid, *cis.cis*-9,12 octadecenoic acid, $C_{18:2}$. Glycerides: PPP = triglyceride glycerol–tripalmitate; MLP = triglyceride glycerol–myristate–linoleate–palmitate; PLO = triglyceride glycerol–palmitate–linoleate–oleate, etc.

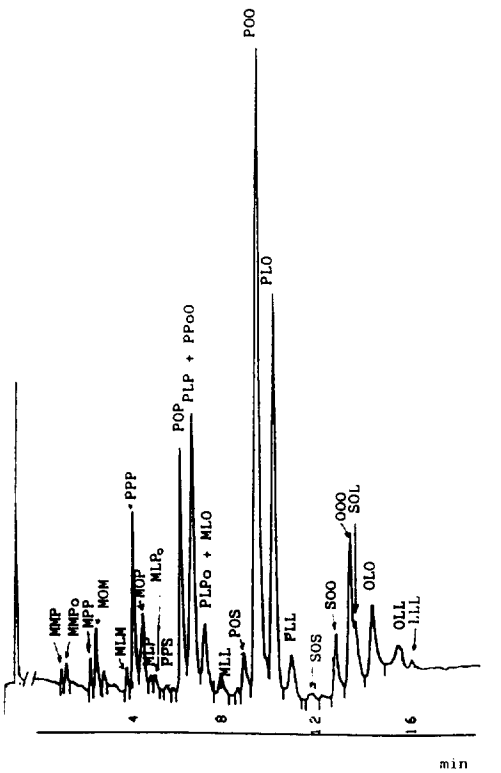

Fig. 11. Triacylglycerol capillary GC analysis of human tissue fats. The analysis was carried out using a Chrompack CP 9000 GC (Chrompack International, Middelburg, Netherlands) fitted with a split injector and a FID. Samples (1 μl) of triglycerides in hexane (0.1%) were injected into the GC equipped with 400 65 HT, 25 mm × 0.25 mm I.D., aluminium-clad, silica capillary column coated with 65% phenylmethylsilicone (Quadrex, New Haven, CT, USA) and operated under the following conditions: injector and detector temperatures 360°C; initial temperature 350°C for 1 min and then raised to 360°C (6 min) at 0.5°C/min. Helium was used as the carrier gas at a column head pressure of 130 kPa; split ratio 60:1; the detector auxiliary flow-rate 30 ml/min. The triacylglycerols were identified by their elution times, because the retention is affected not only by the number of carbon atoms but also by the number of double bonds (from Ref. [307]).

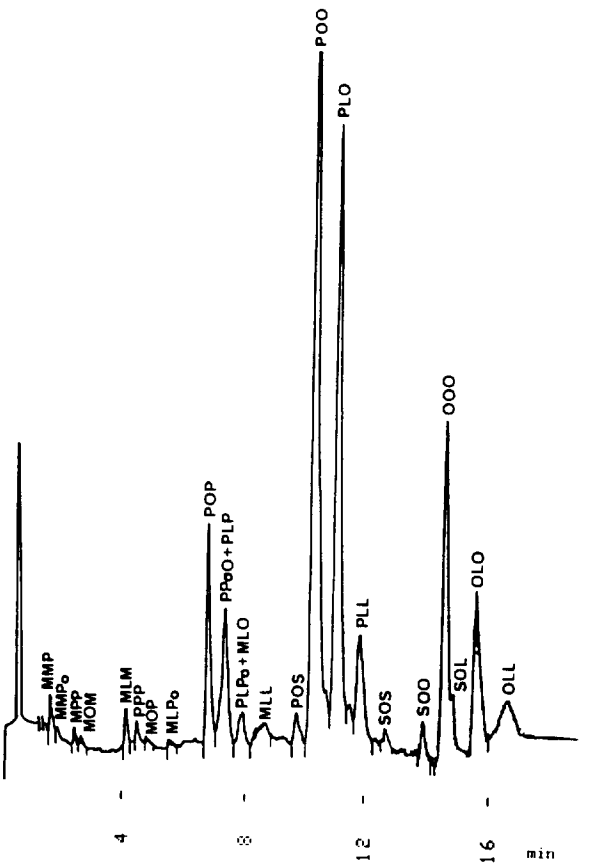

Fig. 12. Triacylglycerol capillary GC analysis of human VLDL lipids. Triacylglycerols are separated according to chain length (CN separation). Polarity increases with increasing degree of unsaturation. Capillary GC conditions and instrumentation as in Fig. 11. Peak identification as in Table 2 (from Ref. [308]).

populations that consume drastically different diets. Studies on Canadian populations [269,270] found substantially different TAG compositions. The percentages of POO and PLO in the Andalusian population considered were significantly higher than in the Canadian population [269]. But the most important finding from comparing the two studies is the substantial difference in triolein concentration, 34% in the Andalusian population and 12.2% in the Canadian population. This difference can definitely be attributed to the fact that triolein is one of the major TAGs in olive oil, the primary oil consumed by the Andalusian population [307].

Diet [308–311], the presence of free radicals [312,313] and certain surgical operations [314,315] are known to affect the TAG composition of tissues in different populations.

AT consists of adipose cells, fibroblasts, blood vessels and nerve fibres. It is almost entirely fatty, basically in the form of TAGs, whose FA composition varies from individual to individual. Human AT is made up mainly of neutral lipids ($<95\%$), primarily TAGs. Traditionally, determinations of free and total FAs have been carried out on packed columns using GC [316,317]. The use of capillary GC with FID has made it possible to study AT composition more systematically, focussing on the major and minor FA constituents and their positional and geometric isomers [318,319].

Studies of the FA composition of AT have

Table 4
Average triacylglycerol composition of adipose tissue in man [307]

Triacylglycerol	Composition (%, w/w)
MMP	0.14 ± 0.03
MMPo	0.20 ± 0.06
MPP	0.40 ± 0.10
MOM	0.45 ± 0.12
MLM	0.44 ± 0.12
PPP	3.64 ± 2.41
MOP	1.72 ± 0.27
MLP	0.30 ± 0.20
MLPo	0.22 ± 0.02
PPS	0.14 ± 0.02
POP	5.88 ± 1.75
PLP + PPoO	8.86 ± 2.63
PLPo + MLO	2.13 ± 1.24
MLL	0.29 ± 0.19
POS	1.75 ± 0.17
POO	32.17 ± 3.29
PLO	16.28 ± 1.32
PLL	1.50 ± 0.50
SOS	0.25 ± 0.04
SOO	3.20 ± 0.38
OOO	11.80 ± 1.69
SOL	2.34 ± 0.62
OLO	4.68 ± 0.65
OLL	1.05 ± 0.23
LLL	0.20 ± 0.01

Results are given as mean ± S.D. For nomenclature see Table 3.

demonstrated that in most mammals (including man but excepting marine mammals), the AT contains saturated and monounsaturated medium-chain FAs, namely, palmitic, stearic and oleic acids [319–324]. These acids are present in considerable amounts and thus may be detected by routine chromatography [325–327].

Table 4 shows the average TAG content. POO (*sn*-glycerol-palmitate-oleate-oleate), followed by PLO (*sn*-glycerol-palmitate-linoleate-oleate) and OOO (*sn*-glycerol-trioleate) together make up 60% of the total. The major TAGs contain oleic acid in the molecules, normally esterifying the *sn*-2 position. In fact, oleic acid esterified the *sn*-2 position in some 50% of the cases, followed by linoleic acid, which esterified around 20% of the *sn*-2 sites. The *sn*-2 position on TAGs is usually occupied by saturated acids, i.e. palmitic and stearic acids.

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