5. A chelate of the trace metal is adsorbed and coprecipitated with a water-insoluble organic compound. Several metal dithizonates can be coprecipitated with phenolphthalein.
6. The metal ions are coprecipitated by means of colloidal–chemical sorption on a mixture of insoluble organic reagents.

Typical examples of the coprecipitation of trace metals with organic collectors are listed in **Table 2**.

See also: **II/Extraction:** Analytical Extractions; Analytical Inorganic Extractions.

Further Reading

Alfassi ZB (ed.) (1994) *Determination of Trace Elements*. Weinheim: VCH Verlagsgesellschaft.

Alfassi ZB and Wai CM (eds) (1992) *Preconcentration Techniques for Trace Elements*. Boca Raton, FL: CRC Press.

Bonner NA and Kahn M (1951) *Radioactivity Applied to Chemistry*. New York: John Wiley.

Kolthoff IM, Sandell EB and Meehan EJ (1969) *Quantitative Chemical Analysis*, 4th edn. New York: Macmillan.

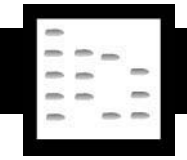
Minczewski J, Chwastowska J and Dybczynski R (1982) *Separation and Preconcentration Methods in Inorganic Trace Analysis*. Chichester: Ellis Horwood.

Mizuike A (1983) *Enrichment Techniques for Inorganic Trace Analysis*. Berlin: Springer-Verlag.

Walton AG (1967) *The Formation and Properties of Precipitates*. New York: Interscience.

Zolotov YA and Kuz'min NM (1990) *Preconcentration of Trace Elements*. Amsterdam: Elsevier.

TRIGLYCERIDES



Liquid Chromatography

V. Ruiz-Gutierrez and J. S. Perona,
Instituto de la Grasa (CSIC), Seville, Spain

Synopsis

High performance liquid chromatography (HPLC) has become a useful tool for the analysis of triglycerides from all sources. This article reviews developments for the analysis of molecular species of triglycerides, including stationary phases, mobile phases, sample solvents, detection and identification. It also points out the advantages of silver-ion HPLC and emphasizes the need for stereospecific analysis in the complete determination of triglyceride molecular species because currently this is not possible by reversed-phase HPLC. Finally, the application of HPLC to triglycerides from fats and oils is described.

Introduction

The goal of chromatographic analyses of lipids is the resolution of all classes and molecular species for the purpose of a complete identification and characterization of all the components of a fat or an oil. This characterization is not complete without the determination of their triglyceride (TG) molecular species profile. Once the fatty acid composition of a determined fat or oil is clear, the knowledge of how these fatty acids are distributed within the glycerol molecule is of major interest.

Fractionation of TGs has been carried out by different chromatographic techniques. Argentation thin-layer chromatography (Ag-TLC) has been employed to separate TG fractions, with subsequent analysis of their fatty acid methyl esters. Direct gas chromatography, using fused-silica capillary columns coated with high-temperature polar stationary phases has also been used for this purpose with rather poor results.

The introduction of chemically bonded phases and high performance liquid chromatography (HPLC) increased the usefulness of liquid chromatography for the separation of TGs. The first paper dealing with the HPLC of triacylglycerols (TGs) was published in 1975 by Pei *et al*. Simple TGs of medium-chain length were separated on a reversed-phase column. Other workers then began to use HPLC for the analysis of long-chain TGs, on silicic acid columns, reversed-phase columns, or both. The first fractionation of natural TGs by HPLC on reversed-phase columns was performed independently in 1977 by Plattner *et al*. and Wada *et al*. The later authors 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 is the number of double bonds in the fatty acids constituting the TG molecule) for

characterizing TG molecules. They found that TGs on reversed-phase columns eluted in increasing order of PN. Today, reversed-phase high performance liquid chromatography (RP-HPLC) is the most frequently employed technique for separating complex mixtures of TGs, as it allows a good resolution of mixtures into molecular species, based on properties such as molecular weight, degree of unsaturation, polarity and molecular configuration. Nevertheless, despite notable success, the progress in RP-HPLC has not been easy, due to difficulties encountered in the process of separation, detection and identification.

One of the main difficulties in the HPLC analysis of TGs is the formation of the so-called 'critical pairs', that is, molecules found to have close behaviour on reversed-phase columns in spite of the difference in chain lengths, number of double bonds and geometrical configuration. Critical pairs, therefore, have been defined as those structures, with the same PN. This problem has not been solved in natural fat analysis yet. However, a long time ago standards of critical pairs of TGs were separated. El-Hamdy and Perkins were able to separate two geometrical isomers: triolein (54 : 3 ccc) from trielaidin (54 : 3 ttt), which differ only by the configuration of the double bonds.

The second difficulty is the establishment of a chromatographic system capable of simultaneously resolving TGs with large differences in carbon chain lengths. The separation of short-chain, medium-chain and long-chain TGs in the same chromatogram, involves the utilization of elution gradients and sometimes yields different responses in different parts of the chromatogram.

The third difficulty is the detection of molecules at the column outlet. Refractive index and ultraviolet detectors have been employed, but the analysis of complex mixtures of TGs requires specific detectors. The emergence of the evaporative light-scattering detector (ELSD) and the application of mass spectrometry (MS) to HPLC has been decisive for the analysis of TGs.

The last major problem is the identification of chromatographic peaks. As very few pure standards are commercially available and as many critical pairs remain unresolved, this is one of the most difficult aims to attain. Again, HPLC–MS looks like a useful tool for this purpose, although several authors have developed other systems for TG identification.

Nomenclature

The proposal of Hirshmann has now been universally adopted for structural assignments. An '*sn*-' prefix is included in the names of all glycerols. Each fatty acid in the glycerol molecule is identified by listing the *sn*-1, *sn*-2 and *sn*-3 position 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 esterifies the *β*- or *sn*-2 position.

Mobile Phase

The selection of the mobile phase is one of the most important factors regarding TG liquid chromatographic analysis. Plattner *et al*. briefly examined the effect of solvent composition upon triglyceride separations. Later, Pauls compared seven binary solvent mixtures for the analysis of olive oil triglycerides. They achieved the best critical pair separation with the use of acetonitrile as weak solvent. *n*-Propionitrile has also been proposed as an eluent but disadvantages include high cost and toxicity. Recently, Hirano and Takahasi have established three factors for the selection of mobile phase solvents in order to obtain optimum column efficiency. Solvents should be low in molecular weight and viscosity but high in solubility of TGs. These factors must be balanced to ensure high column efficiency.

The function of the organic modifier is to improve the solubility of the compounds in the mobile phase, so as to provide changes in their polarity, and thus increase peak selectivity. An increase in the solvent strength of the mobile phase is directly related to an increase in both retention time and resolution of TGs, including critical pairs. Among the organic modifiers tried, Pauls *et al*. showed that chloroform and tetrahydrofuran had the greatest solvent strength for the elution of the critical pair POO–OOO (52 : 2–54 : 3, PN = 48), while the best resolution for the pair LOO–LPO (54 : 4–52 : 3, PN = 46) was achieved with dichloromethane. The dependence of resolution upon solvent composition is a function of the extent to which a solvent can shift retention per double bond compared to the extent to which it shifts retention per carbon unit. The most commonly employed binary solvent mixture for TG analysis is acetone in acetonitrile as the weak solvent. However, acetone is incompatible with UV detectors as it absorbs at the same wavelengths as TGs.

The analysis of TGs by RP-HPLC has been performed for a long time with isocratic elution, due to the general use of refractive index (RI) detection. This system has provided good results for simple oils, but the analysis of complex fat mixtures, i.e. animal fats, requires gradient elution conditions. The goal is to achieve a good resolution for poorly retained TGs (saturated molecular species with short-chain fatty acids) and, at the same time, to elute, in a reasonable

separation time, the most retained TGs (saturated molecular species with long-chain fatty acids). This permits the resolution of complex mixtures of TGs, such as those from fish oils, containing long-chain polyunsaturated fatty acids, and from milk fats, with a broad range of PN values.

Acetone, *n*-propanol, methyl tert-butyl-ether or dichloromethane, give good results when used in gradient conditions with acetonitrile. The gradient systems can be linear or nonlinear. Nonlinear gradients, and step gradients have shown better separations of critical pairs.

Sample Solvent

The sample solvent is of great importance when the sample is a complex mixture of TGs with a wide range of polarity, because it is enormously difficult to find an appropriate solvent for all the TGs. Moreover, the selected solvent must permit an appropriate contact between the solute and the stationary phase for chromatographic separations. Tsimidou and McRae studied the influence of the injection solvent on the RP-HPLC of TGs. They found that chloroform produced inferior resolution under all conditions, which was accentuated by the injection of large volumes. Acetone was recommended, but it is not suitable for high-molecular-weight saturated TGs. Mobile phase has also been suggested as an ideal solvent, but others have employed hexane obtaining better results.

Stationary Phase

Reversed-phase columns are used for separating homologous series of compounds, such as TGs. Previous studies have shown that octadecylsilane (ODS) stationary phases on spherical particles have the best selectivity for TGs, with little variation among the columns of different manufacturers. Columns with a particle size of 3 μm have the highest intrinsic efficiency; however, until recently, their use was restricted because of the high operating pressure needed.

Most RP-HPLC analyses are carried out without column thermostating. However, various workers have shown that an increase in temperature affects retention and selectivity, yielding poorer separations. Although lower temperatures give better separations, elution times are increased significantly. Moreover, highly saturated TGs may precipitate out of the mobile phase. For these reasons, the choice of column temperature must represent a compromise

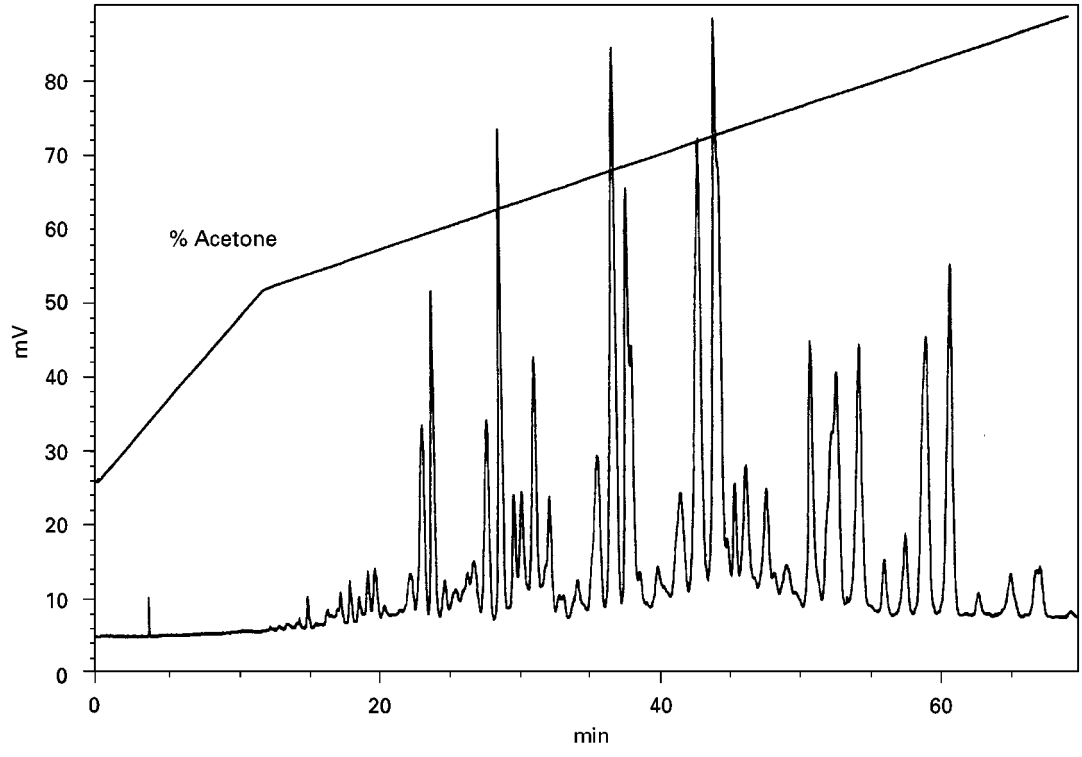


Figure 1 RP-HPLC of fish oil triglycerides. HPLC conditions: Waters 2690 liquid chromatograph equipped with a Spherisorb ODS-2 column (250 × 4.6 mm), coupled to a Eurosep DDL-31 light-scattering detector; solvent, a two-step gradient of 20–80% acetone in acetonitrile at flow rate 1 mL min^{-1}.

between good solubility of saturated TGs concomitant with good selectivity of critical pairs.

In 1996, Hirano and Takahashi discussed the theoretical aspects of improving resolution of TG molecular species via RP-HPLC when working at low temperatures. They analysed fish oils (**Figure 1**), with a low melting point, establishing a critical temperature ($-15^{\circ}C$), below which there is no improvement in resolution. Similar results had been obtained before, through lowering temperature only to 15°C.

Detectors

When most separations were made by isocratic elution systems. Refraction index (RI) detection was extensively employed, but complex mixtures of TGs require gradient elution, making RI detection impossible. Moreover, it had low sensitivity and different responses for saturated and highly unsaturated TGs.

The UV detector is compatible with gradient elution and has been used for HPLC analyses of TGs. The absorption region from 200 to 230 nm (ester bond) is used to detect TGs. However, many solvents also absorb at these wavelengths, causing baseline drift with gradient elution systems. In addition, different TGs have nonuniform molar extinction coefficients, and consequently calculation of their response factors with standards is needed for quantitative analysis. Other workers have used flame ionization detection (FID) and attained good sensitivity and baseline stability with elution gradient. Nurmela and Satama tested FID for TGs. They found a variable response for different TGs, although the variation was smaller than with UV detectors. In addition, a nonlinear response of the detector was observed for injections <5 μg. This may be a shortcoming, because only a small portion of the solvent eluted from the column can be introduced into the FID.

The introduction of the mass or evaporative light-scattering detector (ELSD) has brought a major advance in the detection of lipid classes upon HPLC separation. ELSD, being sensitive only to the mass of vaporized analyte, is not limited by the absorption characteristics of the individual components and/or the nature of the eluents. For this reason, it is compatible with gradient elution and volatile solvents do not give baseline drift, as they are removed before detection of the analyte by evaporation. The only requirement is that the compounds to be detected must be much less volatile than the solvent.

ELSD was described for the first time at the end of the 1970s. In 1984, Robinson and Macrae, compared ELSD with UV and RI detectors for the analysis of butter TGs (**Figure 2**). ELSD provided better chromatograms, and unlike UV, allowed utilization of acetone. Subsequently, the influence of nebulizer gas pressure, temperature, mobile phase composition and flow rate on the response of the detector was investigated. Regardless of the exponential response of the detector, which depends on solute concentration, nowadays ELSD is the most commonly

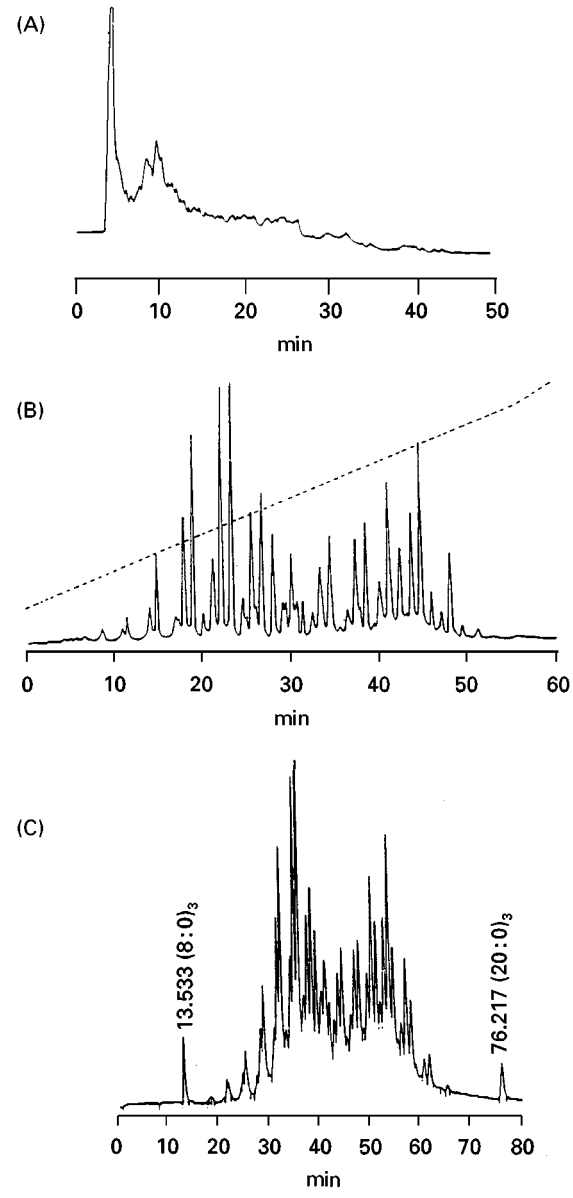


Figure 2 RP-HPLC of butter triglycerides. (A) Refractive index detection; Spherisorb-5-ODS-2 and isocratic elution of acetone in acetonitrile. (B) Light-scattering detection: same conditions as (A). (Reproduced with permission form Robinson JL, Tsimidou M and Macrae R (1984) *Journal of Chromatography* 303: 386. Copyright Elsevier Science). (C) Ultraviolet detection; two Lichrospher 100 CH-18/2 columns and isocratic elution of acetone in acetonitrile; tricaprylin $(8:0)_3$, and triarachidin (20 : 0) are internal standards. (Reproduced with permission from Nurmela KVV and Satama LT (1988) *Journal of Chromatography* 435: 139. Copyright Elsevier Science.)

employed detector for TG molecular species determination.

Identification of Molecular Species

In spite of their usefulness, all detectors described above have the shortcoming of poor limited structural identification. Mass spectrometry (MS) has become necessary for a complete identification of TG species.

Several methods for mass spectrometry of TGs have been proposed but some drawbacks have been found. Electron impact ionization methods generally result in spectra containing low molecular weight fragments, with no quasimolecular ions present. Electrospray ionization (ESI) provides only quasimolecular ions with no fragmentation. Unfortunately, this lack of fragmentation can result in ambiguity in structural assignments for TGs with identical molecular weight. Information on both the molecular weights and the fatty acyl residues of TGs have been achieved by combination of RP-HPLC and atmospheric pressure chemical ionization MS. Desorption chemical ionization (DCI) and positive ion chemical ionization (PICI) have also been successfully used for TG structural characterization.

When MS is not possible, some authors have used the equivalent carbon number (ECN) for the tentative identification of TGs. The ECN of each TG in the sample is the ECN of the hypothetical saturated TG having the same retention time. When carbon numbers (CNs) are plotted against ECN, straight parallel lines are found for different unsaturated TGs. Thus a theoretical prediction can be achieved, which has become a useful tool for TG identification.

The linear relationship between the retention factor (k) and PN values of the TG, was first established by Wada *et al.* in 1977. Then Herslöf *et al.* estimated theoretically the ECN for unsaturated TGs, on the basis of their relative retention times, from an experimental linear relationship between relative retention time and carbon number. This ECN is analogous to PN (ECN = CN $-$ a'ND), with the difference that in this case the value of a' depends on the chromatographic system used for measurements. However, a' generally takes values close to 2 and when $a' = 2$, the values for the ECN and NP are equal. Takahashi *et al.* calculated the value for a' from the relationship between log k', CN and ND ($\log a' = q + b'\text{CN} + c'\text{ND}$). The value of a' is the quotient between the constants b' and c'. These equations are calculated under isocratic conditions, and are not appropriate for gradient-elution systems. For this reason, some workers have developed new relationships based on the same parameters, as the equivalent chain length (L) or the theoretical carbon number (TCN).

The chromatographic behaviour of TG molecules in RP-HPLC depends not only on CN and ND but also on the number of unsaturated fatty acids within the molecules (NUFA), because TGs with the same ECN are eluted in the order of the increasing constituent saturated fatty acids. This leads to the equation for ECN (ECN = CN + a_1ND + a_2NUFA).

The TG prediction process becomes increasingly complex when the fat contains a great number of different fatty acids, since the number of possible combinations can be extremely high. Therefore, and as a second part of the prediction process from the ECN, some authors have proposed the application of the equations developed by Takahashi *et al.* These workers developed a matrix model with CN and ND as variables for each fatty acid esterifying the glycerol molecule.

Silver-ion Chromatography

Silver-ion HPLC can be performed on a reversed-phase column (silver ions in the mobile phase), on a silver-loaded, cation-exchange column, or on a silver-loaded silica column. Silver-ion chromatography separates TGs according to their degree of unsaturation, the distribution of double bonds between the fatty acyl residues within a single molecule, the configuration and position of double bonds within each fatty acid and the stereospecific position in which fatty acids are esterified. The mechanism of separation is based on the ability of the π-electrons in the double bonds of the fatty acids to interact with the silver ions of the stationary phase.

Silver ions are incorporated into columns in two different ways: by impregnating the silica-gel support with a silver salt or by bonding silver ions to the phase by means of an ion-exchange phase. The impregnation of columns with silver ions is generally made with silver nitrate in concentrations from 5% to 10%. The problem of short column life is avoided with cation-exchange supports, such as macroreticular sulfonic acid resins or silica-gel supports with chemically bonded methylsulfonic acid groups.

The mobile phase is an important factor affecting the separation of TGs by silver-ion HPLC. However, the nature of the interactions between the silver ions, unsaturated solutes and solvents in the mobile phase has not been fully elucidated. Some workers have suggested using elution gradients combining chlorinated hydrocarbons with acetone and acetonitrile.

Components separated by silver-ion HPLC are commonly detected by evaporative light-scattering detectors (ELSD) or FID, because they place fewer

limitations on the choice of solvents for the mobile phase, but these detectors do not provide structural information on molecular composition. For this reason, mass spectrometry has recently been employed for this purpose.

Christie *et al*. have made the greatest progress in developing silver-ion chromatographic systems. Subsequently, other authors have applied their method for separation of TGs from different natural sources. More useful information of the TG composition of natural fats may be achieved by combining this technique with RP-HPLC. Silver-ion HPLC allows separation of TGs with the same degree of unsaturation; the fractions obtained can then be analysed by RP-HPLC with chain length as a factor for separation.

Stereospecific Analysis

For the complete TG characterization of a fat it is necessary to know not only the fatty acids that constitute a TG molecule but also the positions of attachment. This is of importance because physicochemical properties change depending on the position in which a fatty acid is attached.

However, the stereospecific analysis of a fat is one of the most difficult tasks to undertake, since these molecules are similar in physical and chemical properties. When positions *sn*-1 and *sn*-3 are occupied by distinct acyl groups, the TG molecule will be asymmetric and will have optical activity. However, when the same fatty acid is allocated at both positions, diastereomer forms are outlined. This is not rare, since the main biosynthetic route in animal and plant tissues is the *sn*-glycerol-3-phosphate pathway, and enzymatic systems in this pathway can be specific to certain fatty acids or to certain fatty acid combinations.

Vander Wall and Coleman and Fulton independently developed a theory of fatty acid distribution in the glycerol molecule. They postulated that fatty acids are distributed randomly in the *sn*-2 position and randomly, but independently from *sn*-2, in the *sn*-1 and *sn*-3 positions. They demonstrated that it is possible to know the fatty acid distribution from data obtained on the stereospecific fatty acid composition of the distinct fractions collected after hydrolysis. However, hydrolysis has revealed that fatty acids do not follow a random distribution in TG molecules. In fact, vegetable oils have C_{18} polyunsaturated fatty acids at the *sn*-2 position, with saturated and C_{20} and C_{22} polyunsaturated fatty acids at *sn*-1 and *sn*-3. Oleic acid ($C_{18:1}$) is distributed at the three positions. Among animal tissues, ample differences can be found. The majority of animal fats have saturated fatty acids at the *sn*-1 position; however, there are fats like pig adipose tissue, with palmitic acid at *sn*-2, or milk fat, with long-chain saturated fatty acids at the *sn*-1 and *sn*-2 positions.

HPLC analysis, which gives the stereospecific distribution of TGs, uses as substrate mono- and diacylglycerols, obtained after hydrolysis of TGs in the first step of the process. This hydrolysis is usually made through a Grignard reaction with magnesium ethyl bromide. Mono- and diacylglycerols are separated by thin-layer chromatography (TLC) or by solid-phase extraction (SPE). The products obtained may be analysed by liquid–solid chromatography, reversed-phase liquid chromatography or chiral-phase liquid chromatography. By liquid–solid chromatography 1,2-, 1,3- and 2,3-diacylglycerols are separated through formation of (*S*)-(+)-1-(1-naphthyl)ethyl urethane diastereoisomeric derivatives. The combination of the total fatty acid composition obtained by gas chromatography and liquid–solid chromatography permits calculation of the stereospecific composition of the fatty acids in the TGs of a natural fat.

Reversed-phase chromatography (RP-HPLC) has been less widely employed for this purpose. Semporé and Bezard achieved separations of 3,5-dinitrophenyl urethane (DNFU) derivatives of 1,2- and 2,3-diacylglycerols with a octadecylsiloxane-bonded silica (ODS) column and a mobile phase composed of acetonitrile and acetone. By RP-HPLC Redden *et al*. separated fractions containing all the molecular species of 1,2-, 1,3- and 2,3-diacylglycerol.

Finally, greater success has been achieved using chiral-phase HPLC. Acceptable separations have been obtained for both DNFU derivatives of monoacylglycerols and diacylglycerols employing chiral phases of (*S*)-2(4-chlorophenyl)isovaleroyl-D-phenylglycine or *N*-(*R*)-1-(1-naphthyl)ethylaminocarbonyl-(*S*)-valine chemically attached to an aminopropylsilane support. However, drawbacks include high retention times and poor resolution. Recently, new chiral stationary phases have been proposed, with (*R*)-(+)-1-(naphthyl)ethylamine. These phases provide improved resolution and reduction of separation times by using shorter columns. By this method 1,2- and 2,3-diacylglycerols are separated into fractions, which are subsequently analysed by gas chromatography in order to determine their fatty acid composition.

Applications of Triglyceride Analyses by HPLC

Knowledge of the TG profile could be a more appropriate tool to characterize oils and fats, avoiding the

use of saponification and formation of methyl esters. HPLC has become as routine as gas chromatography (GC), providing more complete information about TG composition of fats and oils.

At a research and development level, detailed TG structural information might facilitate understanding of TG biosynthesis in plant and animal cell metabolism, where the activity of acyltransferases are involved. In this regard, knowledge of the TG molecular species of a dietary fat, as well as the TG composition of organs and tissues, can provide significant information for nutritional purposes.

Vegetable Oils

Virgin olive oil presents a characteristic and unique pattern of TGs, which may be used to determine origin and to detect adulteration. Due to its relative simplicity in TG composition and its relevance in human nutrition, HPLC was soon employed in the study of olive oil. In this work, isocratic mobile phases and refractive index detectors were employed. With these conditions, up to 10 TG molecular species could be detected. Triolein (OOO) was found to be the main TG, with the important presence of dioleoyl-linoleoyl-glycerol (LOO) and dioleoyl-palmitoyl-glycerol (POO). Later studies, carried out by RP-HPLC with ELSD, showed that approximately one-half of the total content of TGs corresponds to OOO, while the corresponding percentage of POO is close to 20% and LOO close to 10%. In spite of the improvement achieved, with the utilization of gradient elution systems and ELSD, some critical pairs still remain unresolved.

TG analysis has been extensively employed for the characterization of edible oils. El-Hamdy and Perkins determined the TG composition of olive and soybean oils. The latter oil contained mainly trilinolein (LLL) and dilinoleoyl-oleoyl-glycerol (LLO). Similar results were reported by other authors using isocratic conditions. In the first analysis of soybean oil using gradient elution and ELSD, 19 chromatographic peaks were detected, but could not be identified. A similar number of chromatographic peaks were resolved by Barron *et al.* and Hierro *et al.* using gradients and ELSD. Unexpectedly, LLO was not as abundant as was originally determined; in both studies, LLL was the main TG, followed by LnLO and then LLO. However, Rezanka *et al.* found significant amounts of LLO and low amounts of LLnO by RP-HPLC with MS. These differences might be due to different gradients and mobile phases.

Several other oils of interest have been characterized by HPLC. Perrin and Prevot determined the TG composition of various vegetable oils by gradient elution RP-HPLC with a light-scattering detector (**Figure 3**). They analysed oils rich in oleic acid, such as olive and rapeseed oils, oils rich in linoleic acid (soybean and sunflower oils), oils rich in both oleic and linoleic acids (peanut oil) and oil rich in saturated fatty acids (palm oil). They also developed analyses of lard and tallow with great success, identifying more

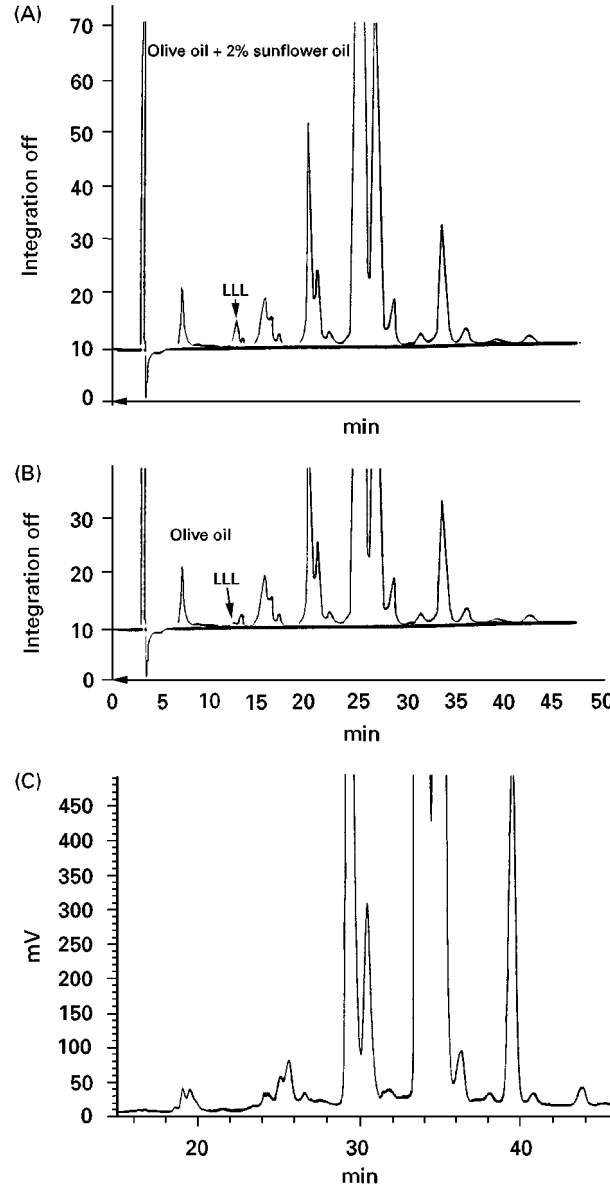


Figure 3 RP-HPLC of virgin olive oil triglycerides. (A) Refractive index detection. Hewlett-Packard HP-1050 liquid chromatograph equipped with a RP-18 column (250 × 4.6 mm), coupled to a Hewlett-Packard HP-1047A refractive index detector; solvent, 50 : 50% acetone in acetonitrile at a flow rate of 0.9 mL min^{-1}. (B) Virgin olive oil with 2% sunflower oil. Same conditions as (A). (C) Light-scattering detection. Waters 2690 liquid chromatograph equipped with a Novapak column (150 × 3.9 mm), coupled to a Eurosep DDL-31 light scattering detector; solvent, linear gradient of 50 : 50% acetone in acetonitrile at flow rate 1 mL min^{-1}.

than 11 chromatographic peaks for each oil or fat. More recently, a newly introduced oleic-rich oil, high oleic sunflower oil, as well as two oils with similar fatty acid composition, borage and primrose oil, have been analysed, each showing a different TG distribution.

Animal Fats

Characterization of animal fats Animal fats are more complex than vegetable oils. The great difference in the fatty acids contained in these fats causes two basic problems. The first one is the difference in chain length and degree of unsaturation, which makes it difficult to achieve a good resolution for all TGs. The second problem is that there is a great number of different fatty acids in animal fats, thus a greater number of TGs appear in the chromatograms, and there are more critical pairs.

Animal fats are employed for industrial purposes. The prediction of the melting behaviour of a fat is difficult due to the complexity of the constituent TGs. Although the amount of stearic or linoleic acids has been proposed as a good predictor of the consistency of a fat, determination of the TG species provides more information, since not only the fatty acid composition but also the positions in which those fatty acids are esterified, are responsible for its physical behaviour.

Lard is the cheapest animal fat, and commercial shortenings, providing improved physical properties, are usually prepared by its interesterification. Al-Rashood *et al.* analysed pig lard by RP-HPLC with RI detection to characterize it before and after randomization. Lard has been analysed many times before. Other interest has been focused on pig fat TG characterization to determine the conditions used in pig husbandry.

Because of its complexity, the structural elucidation of milk and butter fat TGs is a formidable task. The large number of fatty acids it contains has made milk fat a particular challenge in terms of TG separation and identification. Until the introduction of ELSD, no satisfactory results had been obtained. The

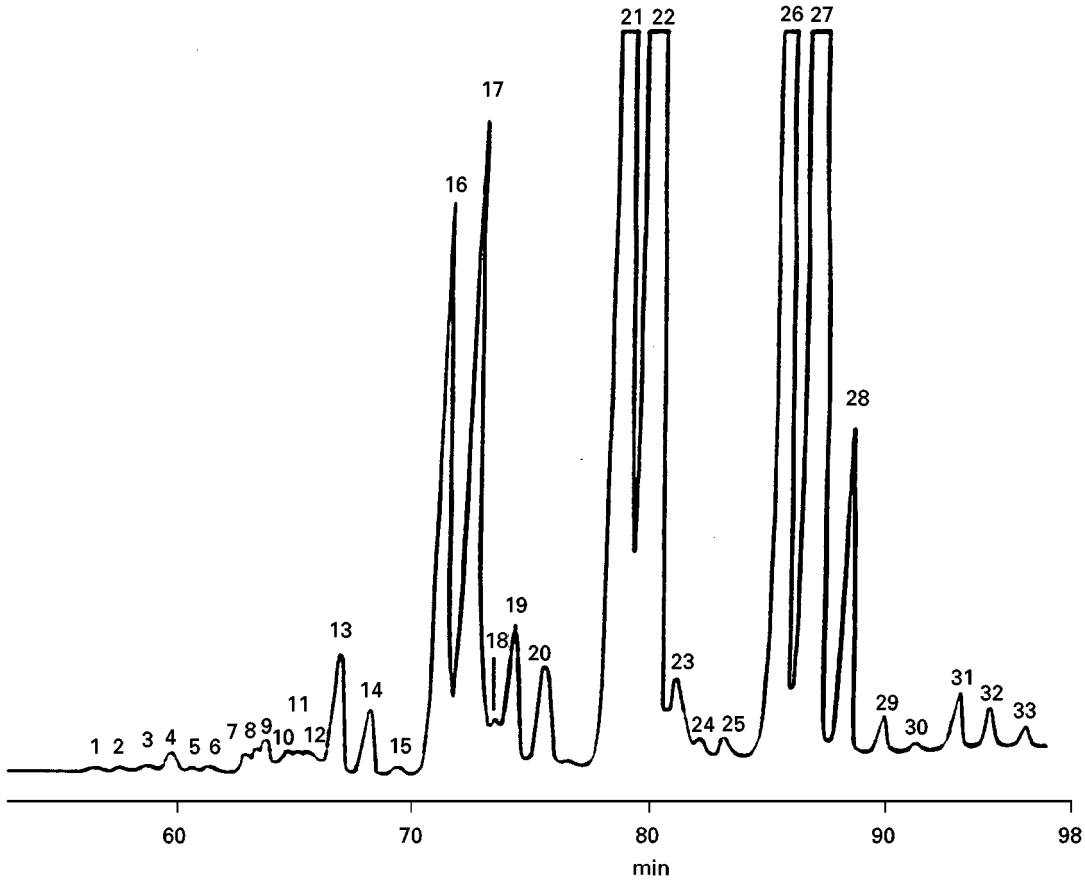


Figure 4 RP-HPLC of rat liver triglycerides. Two columns (Spherisorb ODS-2 3 μm) connected in series with a nonlinear elution gradient of 20–100% (v/v) acetone in acetonitrile were developed at a rate flow of 1.0 mL min^{-1}. (Reproduced with permission of Perona JS and Ruiz-Gutierrez V. *Journal of Liquid Chromatography and Related Technologies*, in press. Copyright Marcel Dekker.)

first to analyse butter fat by HPLC with ELSD were Robinson and Macrae in 1984; they also compared the chromatograms obtained with those of other detectors, such as UV and RI. As milk fat needs gradient-elution systems, the latter detectors offered poor resolution. FID, with linear and non-linear gradients of acetone in acetonitrile has also been used to give 62 peaks. Resolution was enhanced when ELSD was applied, almost always with acetone in the elution system. Using this method, up to 111 peaks were separated with a nonlinear gradient of acetone–acetonitrile as mobile phase.

Nutritional interest Not only industry is interested in the evaluation of the TG content of foods. Medical and nutritional benefits can be achieved through determination of molecular species of TGs.

Human milk, as well as cow or ewe milk fat, have been subjected to analysis for both industrial and nutritional purposes. The objective is to achieve the substitution of the oils employed at present in milk formulas for infants (coconut oil, corn oil) with others closer in composition to human milk. The TG structure seems to be an important factor for the bioavailability and absorption process of fats in the first weeks of life. It has been suggested that unsaturation of TG fatty acids does not affect pancreatic lipase levels, whereas the chain length of the constituent fatty acids does appear to exert an effect. The distribution of fatty acids within the glycerol molecule might also effect absorption, as it has been shown to regulate TG hydrolysis to 2-monoacylglycerol and fatty acids.

The physiological effects of TG structure and composition of the diet are more relevant in the intestine and liver, the most actively involved tissues in TG synthesis and secretion. The specificity of lipolytic enzymes for fatty acids acylated at the *sn*-1 position of the glycerol molecule affects the re-synthesis of TGs either in enterocytes or hepatocytes. After this re-synthesis, TGs are transported via lipoproteins, to peripheral tissues so that their constituent fatty acids are incorporated into the cellular lipid metabolism. However, until recently little has been done on TG from these tissues or lipoproteins.

Thirty-one molecular species of TG from rat liver have been identified by RP-HPLC with an ELSD by Perona *et al.* (**Figure 4**). Oleic, linoleic or palmitic acids formed the main TGs in the rat liver. Rat liver had also been investigated for TG molecular species by other workers using ELSD, resolving a lower number of TGs. Yang *et al.* observed that the fatty acid composition and the major molecular species of TG in the rat liver were very similar to those of TG in very low density lipoproteins (**Figure 5**). Parreño *et al.* studied plasma TG composition of a Catalonian population by HPLC.

Adipose tissue is the most important extrahepatic tissue in regulating lipid metabolism. Although it contains up to 97% of TGs, little work has been done to study its composition of TG molecular species. Huang *et al.* have reported 18 molecular species of TGs in rat adipose tissue using HPLC with UV detection (**Figure 6**).

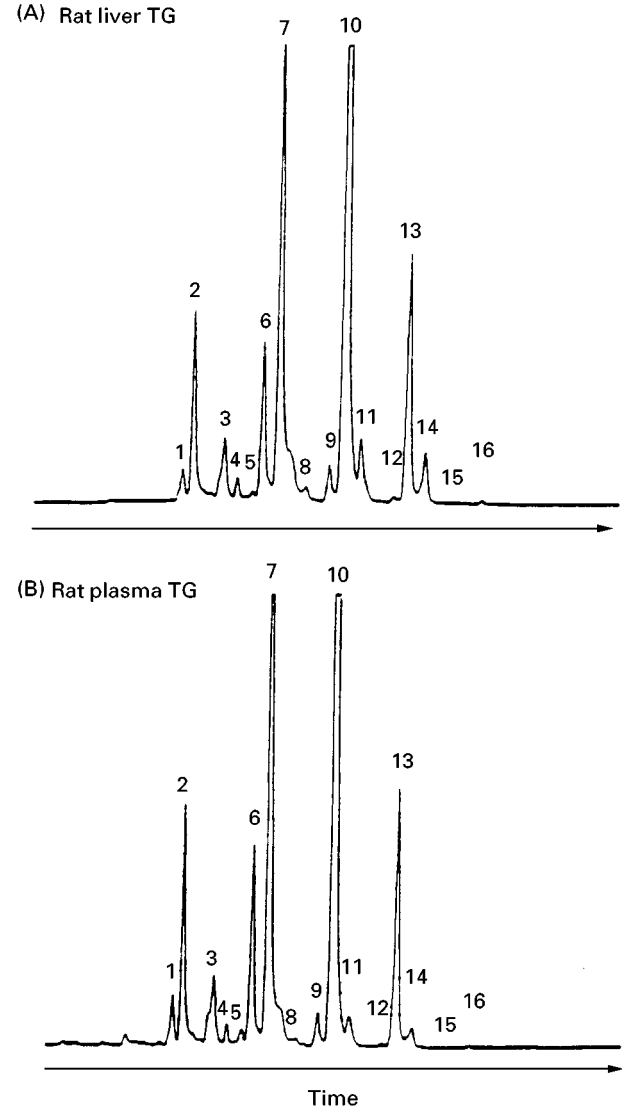


Figure 5 RP-HPLC of rat liver (A) and very low density lipoprotein (B) triglycerides. Supelcosil LC-18 column (250 × 4.6 mm), coupled to a Varex ELSD II light-scattering detector; solvent, linear gradient of 10–90% isopropanol in acetonitrile at flow rate 1 mL min^{-1}. (Reproduced with permission from Yang LY, Kuksis A, Myher JJ and Steiner G (1995) *Journal of Lipid Research* 36: 125. Copyright Journal of Lipid Research.)

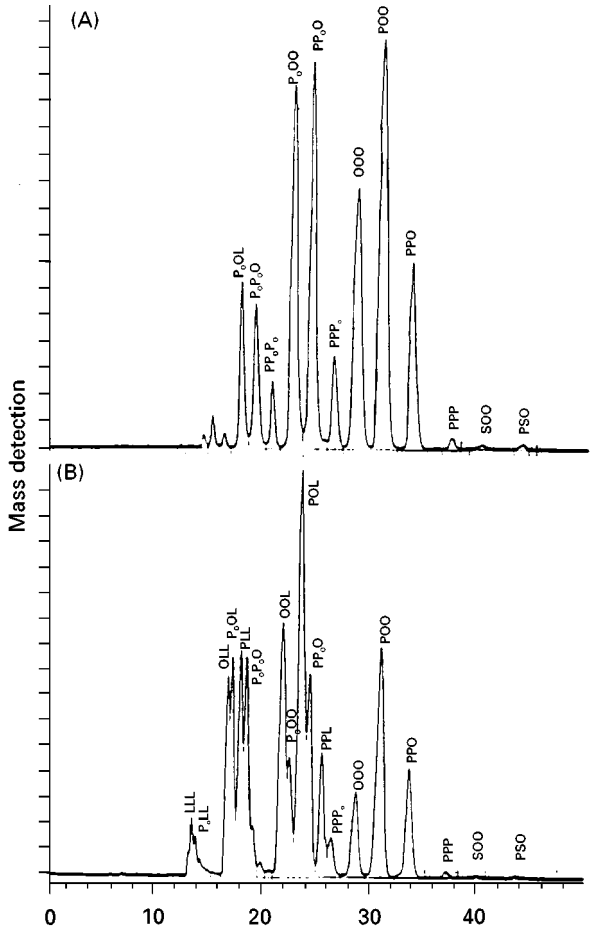


Figure 6 RP-HPLC of rat adipose tissue triglycerides. Two Supelcosil LC-18 (250 × 4.6 mm) columns in series, with UV detector; solvent, 35 : 65% isopropanol in acetonitrile at a flow rate of 2 mL min^{-1}. Rats were fed a diet containing linoleic acid at a level of (a) 0.01 g kg^{-1}, (b) 24 g kg^{-1}. (Reproduced with permission from Huang YS, Lin X, Sminth RS *et al.* (1992) *Lipids* 27: 711. Copyright AOCS Press.)

Conclusion

The chromatographic analysis of TGs has undergone great advances in the last few years. Among other factors, the advent of reversed-phase HPLC and the emergence of the evaporative light-scattering detector (ELSD) allow the resolution of many TG applications in vegetable and animal samples. Nevertheless, the shortcomings of TG identification need to be improved. Mass spectrometry (MS) is a helpful tool for this purpose. However, it is still difficult for many researchers to incorporate such a technique in their laboratories. The drawback of the incomplete resolution of critical pairs of TGs with the same ECN and the stereo specific analysis of TGs are the two challenges which investigations will have to address in the near future.

See Colour Plate 123.

See also: **II/Chromatography: Liquid:** Detectors: Ultraviolet and Visible Detection; Mechanisms: Normal Phase; Mechanisms: Reversed Phases. **III/Lipids:** Gas Chromatography; Thin-Layer (Planar) Chromatography. **Oils, Fats and Waxes: Supercritical Fluid Chromatography. Silver Ion:** Liquid Chromatography; Thin-Layer (Planar) Chromatography.

Further Reading

Aitzetmuller K (1997) Recent developments in the analysis of food lipids and other lipids. *Ol. Corps Gras*, *Lipides* 4(1): 8–19.

Beare-Rogers JL (1983) *Advances in Nutrition Research*, vol. 5. London: Plenum Press.

Breckenridge WC (1978) In: Kuksis A (ed.) *Handbook of Lipid Research*, vol. I. New York: Plenum Press.

Christie WW (1987) *High-Performance Liquid Chromatography and Lipids: A Practical Guide*. Oxford: Pergamon Press.

Geeraert E and Sandra P (1987) In: Kuksis A (ed.) *Chromatography of Lipids in Biomedical Research and Clinical Diagnosis*. Amsterdam: Elsevier Science Publishers.

Hammond EW (1982) In: Macrae R (ed.) *HPLC in Food Analysis*. London: Academic Press.

Hammond EW (1993) In: Hammond EW (ed.) *Chromatography for the Analysis of Lipids*. Florida: CRC.

Litchfield C (1972) *Analysis of Triglycerides*. New York: Academic Press.

Marini D (1992) In: Nollet LML (ed.) *Food Analysis by HPLC*. New York: Marcel Dekker.

Nikolova-Damyanova B (1997) In: Christie WW (ed.) *Advances in Lipid Methodology*. 4. Dundee: The Oily Press.

Ruiz-Gutiérrez V and Barrón LJR (1995). Methods for the analysis of triacylglycerols. *Journal of Chromatography B* 671: 133–168.