# Separation and Detection of Triacylglycerols by High-Performance Liquid Chromatography

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## Contents



## I. Introduction

High-performance liquid chromatography (HPLC) is a powerful separation technique. It has opened new horizons in many areas of research because of the versatility provided by the liquid mobile phase. It is capable of high resolution of complex mixtures and can be scaled down for trace analyses as well as scaled up for preparative work. Since the early 1970s, when liquid chromatographs became commercially available, the use of HPLC in all areas of research has exploded, and liquid chromatographs are now found in most laboratories alongside gas chromatographs and spectroscopic instruments.

Because it is a nondestructive technique and large quantities of analytes can be readily isolated and purified, HPLC has become the method of choice for preparative and process work in research and industry.

The analysis of natural oils and fats, which are complex mixtures of triacylglycerols (TG), is a difficult problem. Triacylglycerols, also known as triglycerides, are the most abundant single class of lipids. They are usually chiral (*R*) and various fatty acids can be esterified to each of the three hydroxyl groups of the glycerol. As the number of different fatty acids esterified increases, the number of possible molecular species becomes substantial, resulting in extremely complex mixtures of triacylglycerols. Further, many of these TG molecular species have very similar chemical and physical properties; thus their complete separation is difficult.

Many methods have been used for the analysis of natural fat and oil triacylglycerols. Analytical techniques, such as fractional[1] and gradient crystallization[2,3] and countercurrent distribution,[4–9] were originally used. However, such methods are not practical for routine use, requiring relatively large sample sizes and long analysis times; further, they are not sufficiently reproducible.

In recent years, chromatographic techniques have been used extensively for the analysis of triacylglycerols. Thin-layer chromatography (TLC) has been applied to the separation of lipid classes, including TGs.[10–15] Absorption TLC separates mixtures of lipids according to their type and number of functional groups. Fractionations based on degree of unsaturation are obtained with argentation TLC or silver ion TLC. Thin-layer chromatography in the reversed-phase mode results in separations according to carbon number. In fact, TLC in both the liquid partition and reversed-phase modes has been used to separate triacylglycerol mixtures.[16] Two-dimensional TLC, in which a TLC plate is developed in two directions consecutively with different solvent systems, has been used to obtain additional resolution of lipid classes.[17] The separation of lipids based on two different properties of the compounds can be achieved by multimodal TLC, combining two chromatographic modes on the same TLC plate. For example, seed oils have been separated first by argentation TLC, which separates the triacylglycerols according to their degree of unsaturation, and then by TLC in the reversed-phase mode, in which separation is based on carbon number.[18,19] Silver ion TLC alone has been used to prefractionate the triacylglycerols of adipose tissue prior to positional isomer analysis.[19,20] Enzymatic reactions resulting in the stereospecific lipolysis of triacylglycerols have been successfully performed on TLC plates, shortening the time required for the entire analytical procedure and minimizing sample losses that would have otherwise resulted during sample transfer steps.[21] The development of more efficient multimodal TLC separations will result in more complete fractionations in shorter analysis times. Thin-layer methods, however, do not provide adequate separation to resolve TG molecular species that are very similar in carbon number and degree of unsaturation; they are likely to be used nearly exclusively for prefractionations of complex TG mixtures.

Kuksis and McCarthy[22] reported on the first practical separation and quantitation of natural triacylglycerols by gas–liquid chromatography (GLC). With GLC,

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Mary Jo Wojtusik received her B.S. from St. Joseph's College in 1980. While at St. Joseph's she was active in developing basic solar energy projects to be used in elementary-level classrooms. She did her graduate work at the University of Rhode Island, where her major professor was Phyllis R. Brown. She received her M.S. in chemistry in 1985. The topic of her research was "The High-Performance Liquid Chromatographic Assays for Adenosine in Porcine Plasma for Cardiac Studies". This work was done in collaboration with the Department of Cardiology at R. I. Hospital. In 1987 she received her Ph.D. degree. For her doctorate her research involved the development of HPLC analyses for fish oil derived triglycerides and the scale up of these separations to the semipreparative scale. Dr. Wojtusik's research interests are in the development of HPLC assays for biomedical research and investigations of factors affecting solute integrity in sample preparation. She has several papers published or in press and has presented papers at regional and national meetings. At present, Dr. Wojtusik is employed at Hoffmann-La Roche in Nutley, NJ, where she is a senior scientist for the Pharmaceutical Quality Control and Assurance Department.

Phyllis R. Brown was born in 1924 in Providence, RI. She received her B.S. in chemistry at George Washington University. After an educational hiatus of 18 years, she returned to school and received her Ph.D. in chemistry in 1968 from Brown University, where her graduate advisor was John O. Edwards. She did postdoctoral work in the pharmacology section at Brown for 3 years and stayed on in that section as instructor and then as an assistant professor in research. In 1973 she became an assistant professor in the Department of Chemistry at the University of Rhode Island, where she became an associate professor in 1977 and professor in 1980. In 1983 Dr. Brown was a Visiting Professor at The Hebrew University in Jerusalem, and in 1987 was awarded a Fulbright Fellowship to return to Israel to continue her research there. She was awarded the Scholarly Achievement Award for Excellence in Research at the University of Rhode Island in 1985 and also named Woman of the Year by the Business and Professional Women of South County, RI. At The International Symposium on Advances in Chromatography held in Minneapolis in August 1988, Dr. Brown was awarded the Tswett Medal in Chromatography, and she will receive The Dal Nogare Award in Chromatography at The Pittsburgh Conference in March 1989.

Joseph G. Turcotte was born in Boston, MA, in 1936. He received his B.S. (1958) degree in pharmacy and M.S. (1960) degree in chemistry from the Massachusetts College of Pharmacy and his Ph.D. (1967) in medicinal chemistry from the University of Minnesota, at which time he joined the faculty of the College of Pharmacy, University of Rhode Island; he was promoted to Professor at that institution in 1977. Dr. Turcotte is a fellow of the American Association for the Advancement of Science, has been the recipient of a number of National Institutes of Health grant awards, and presently is the principal investigator of a NIH-sponsored National Cooperative Drug Discovery Group for the Treatment of AIDS. He was the founding (1981) president of Separations Technology, Inc., Wakefield, RI, now a national and international leader in the manufacture and sales of preparative high-pressure liquid chromatography systems. Professor Turcotte's research interests have focused on the design and development of new experimental drugs based on the chemistry, physical properties, and metabolic functions of complex lipids, including anticancer liponucleotides, antihypertensive inhibitors of the renin–angiotensin system, pulmonary surfactant analogues for replacement therapy in respiratory distress of newborn infants, antithrombotic antagonists of platelet-activating factor, marine ω-3 polyunsaturates and derived analogues as nutrient, prophylactic, and therapeutic interventive agents in certain cardiovascular and inflammatory disorders, and biophysical- and chemotherapeutic-acting anti-AIDS phospholipids and vesicular forms.

triacylglycerols can be separated according to their carbon number.[23–26] Separation occurs first according to the boiling points and then according to the molecular weights of the components. Triacylglycerols have been separated on both packed and capillary columns of relatively short lengths and at temperatures >300 °C.[27–33] However, the availability of polar packings with adequate stability at the high temperatures that are required for the separation of TGs has been limited.[34] Recently, Geeraert and Sandra[35–38] have used capillary columns coated with polar liquid phases that are stable at high temperatures and provide some separation of triacylglycerols according to their degree of unsaturation. Gas chromatographic separations of simple TG mixtures may indicate the molecular composition of the various fatty acyl components but yield no information regarding positional distribution. In addition, it is difficult to distinguish species with small differences in the number or position of double bonds with polar capillary columns.

Mass spectrometry when combined with GLC (GC–MS) is a powerful technique for peak identification and quantitation in the analysis of triacylglycerols.[39–42] Murata and Takahashi[39] showed the general applicability of GC–MS for the identification of the TGs in coconut oil and animal fats. Yet, GC–MS is not satisfactory for the separation and analysis of all types of

natural fats and oils. For example, with higher molecular weight triacylglycerols, i.e., carbon numbers >54, an insufficient number of molecular ions are produced to provide sensitive detection. Gas chromatography in combination with ammonia chemical ionization MS, however, has permitted detection of some of the higher molecular weight triacylglycerols.[37] The main problem of GLC and GC–MS methods using nonpolar capillary columns—the lack of sufficient chromatographic resolution to separate positional isomers or molecular species with slight differences in unsaturation—has largely been solved by use of polar capillary GC columns.

Chromatographic techniques such as TLC and GLC do not provide adequate resolution to separate all or even most of the very similar triacylglycerols present in many natural fats and oils. As a result, TLC and GLC are often combined to provide a more complete separation of TG molecular species. This combination of techniques, however, greatly increases analysis time, preventing the routine application of TLC–GLC.

The need for better chromatographic resolution of highly complex triacylglycerol samples has largely been met by high-performance liquid chromatography (HPLC), especially when the reversed-phase mode (RPLC) is used. For example, less complex natural triacylglycerol mixtures can be resolved by RPLC according to both carbon number and the degree of unsaturation. In addition, HPLC peaks or fractions can be readily collected; fractions then can be analyzed further by chromatography and physical methods of identification, and oftentimes in quantities suitable for subsequent chemical, biochemical, and enzymatic studies.

The HPLC analysis of triacylglycerols, like HPLC analyses of other types of compounds, is hampered by the lack of a universal detector that provides sensitive detection of eluting components. The ultraviolet (UV) detector, one of the most common types of LC detectors, is not readily used for the characterization of triacylglycerols, since the only chromophores present absorb between 195 and 230 nm with low molar extinction coefficients. Many of the organic solvents used for the separation of TGs also absorb in this region of the UV spectrum. In addition, some contribution to the measured response from the methylene-interrupted double bonds of fatty acyl moieties (when present) does occur, limiting the use of the UV detector for quantitation. Refractive index (RI) detectors are often used for the analysis of triacylglycerols, mainly because of their compatibility with any type of mobile phase. In addition, since RI detectors are bulk property detectors, a response from all eluting solutes can theoretically be observed. Other detection systems, such as infrared (IR) detectors, mass or light-scattering detectors, flame ionization detectors (FID), post-column reaction detectors (PCRD), and mass spectrometers, have been investigated for use in the analysis of triacylglycerols.

## II. High-Performance Liquid Chromatography

### A. Separation

#### 1. Argentation Chromatography

Argentation or silver ion chromatography was one of the earliest chromatographic methods used for the separation of fats and oils.[43-49] With argentation chromatography, triacylglycerols are fractionated according to the number of double bonds and differences in geometric configuration. Argentation chromatography of triacylglycerols recently has been applied to HPLC. Silica gel impregnated with silver nitrate separates TG mixtures composed of the general types SSS, SSU, SUS, SUU, and UUU, where S represents a saturated esterified fatty acid and U represents an unsaturated fatty acid.[50,51] For example, Smith et al.[50] were able to distinguish between the positional isomers SUS and SSU by using a $Ag^+$-impregnated HPLC silica column. However, column stability was a major problem; silver slowly bled off the column and caused contamination in collected fractions and the detector cell.

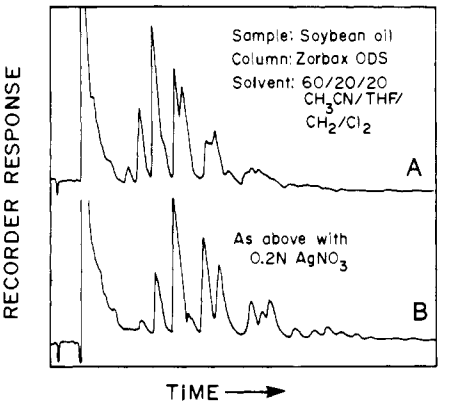


**Figure 1.** Effect of a mobile phase containing silver nitrate on the separation of soybean oil triacylglycerols: (A) acetonitrile/tetrahydrofuran/methylene chloride (60/20/20); (B) mobile phase A with 0.2 N silver nitrate. Reproduced with permission from ref 54; copyright 1981 American Oil Chemists' Society.

More recently, Takano and Kondoh[52] described a triacylglycerol analysis by argentation chromatography combined with RPLC. The triacylglycerol species first were separated according to their degree of unsaturation into various fractions of positional isomers by silver ion chromatography. The fractions then were further resolved by RPLC according to carbon number (CN) and number of double bonds (NDB). Full separation of all triacylglycerols of palm oil and cocoa butter, except the positional isomers of the trisaturated (SSS) species, was achieved with this method. However, the column used for the argentation chromatography was stable for only 1 month.[52]

A slightly different approach, which was originally used in argentation chromatographic methods, is to use a mobile phase containing silver nitrate rather than a stationary phase impregnated with $Ag^+$ ion.[49,53,54] Plattner[54] obtained a better separation of soybean oil with a silver nitrate modified mobile phase as opposed to a mobile phase without $AgNO_3$ (Figure 1). However, routine use of this method is limited, since silver ions eventually build up within the detector cell, preventing sensitive detection and requiring extensive purging of the system.

Christie[55] has recently developed a more stable silver-loaded column in which the $Ag^+$ was loaded on an ion-exchange medium. The preparation of the column is very easy, requiring no special equipment; thus, this column can be produced in any laboratory. Satisfactory resolution was obtained with the column for a 2-month period in which various lipid samples, including the triacylglycerols in palm oil, cocoa butter, and milk fat, were separated.

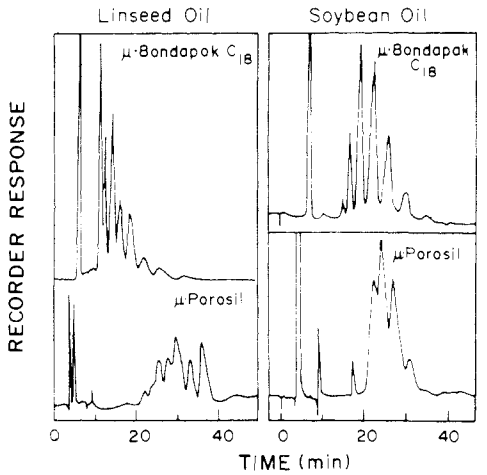


**Figure 2.** Comparison of normal-phase and reversed-phase HPLC of linseed and soybean oil triacylglycerols. Reversed-phase conditions: μ-Bondapak $C_{18}$ column (Waters Associates) eluted with acetonitrile/acetone (2/1) at 1.0 mL/min. Normal-phase conditions: μ-Porasil column eluted with isooctane/ether/acetic acid (99/1/1) at 1.0 mL/min. Reproduced with permission from ref 56; copyright 1979 American Oil Chemists' Society.

### 2. Normal-Phase HPLC

Separations of triacylglycerols by chain length and, to some extent, degree of unsaturation have been performed on silica columns. Plattner and Payne-Wahl[56] compared the separations of the triacylglycerols in soybean and linseed oil using normal-phase HPLC with separations performed with reversed-phase HPLC (Figure 2). The order of elution in the normal-phase mode is such that the longer chain and more saturated species elute first, whereas on reversed-phase columns, the more unsaturated, shorter chain triacylglycerols elute first. Poor separation was obtained in the normal-phase mode. The RPLC method yielded a more efficient separation with significantly better resolution in a shorter analysis time. However, triacylglycerols are more soluble in the solvents normally used with normal-phase columns, and higher capacities for TGs were obtained with the silica column. Generally, in view of the enhanced efficiency, shorter and more reproducible retention times, and ease in equilibration, reversed-phase columns are used often for the analysis of complex TG mixtures.

### 3. Pseudophase HPLC

Pseudophase chromatography has been defined as reversed-phase chromatography in which surface-active agents are used to modify the mobile phase.[57] These mobile phases are also called micellar mobile phases. Singleton and Pattee[58] have recently described the retention behavior of peanut oil triacylglycerols on end-capped and regular reversed-phase columns using aqueous and nonaqueous micellar mobile phases (Figure 3). They were able to reduce the elution time for triacylglycerols on an end-capped column by modifying an aqueous mobile phase, permitting improved peak integration. A reduction in capacity factors for peanut oil triacylglycerols was also obtained with nonaqueous mobile phases modified with SDS. Hexadecyltrimethylammonium bromide (HTAB), a cationic surfactant, produced reductions in capacity factors; however, the reductions were not the same magnitude as those obtained with SDS. A disadvantage of using

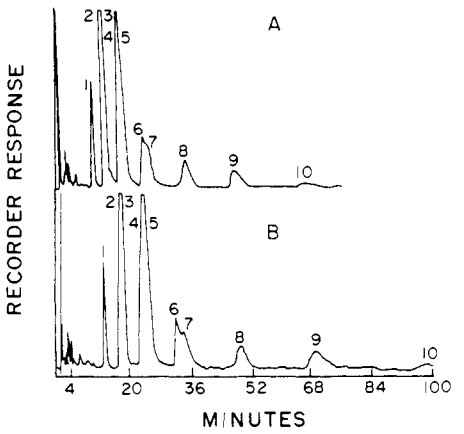


**Figure 3.** Effect of sodium dodecyl sulfate (SDS) on the analysis of peanut oil triacylglycerols using a reversed-phase column and a mobile phase of acetonitrile/ethanol/water (77/20/3): (A) SDS; (B) no SDS. Reprinted with permission from ref 58; copyright 1985 American Oil Chemists' Society.

pseudophase chromatography is the additional time required to prepare and equilibrate the column with the micellar mobile phase. Nonetheless, the application of micellar mobile phases in RPLC to the analysis of hydrophobic solutes, such as triacylglycerols, is an alternative to the more conventional chromatographic methodology.

### 4. Reversed-Phase HPLC

Reversed-phase chromatography has been shown to be relatively well suited for the analysis of complex mixtures of triacylglycerols. Even so, several difficulties still remain that limit the degree of resolution. The limited solubility of TG in the solvents commonly used in RPLC can cause precipitation during analysis, resulting in poor recoveries. Resolution of critical pairs of individual triacylglycerol molecular species that exhibit minimal differences in the numbers of double bonds and chain lengths requires highly efficient reversed-phase methodology. In addition, more complex TG mixtures can contain individual molecular species that vary widely with respect to carbon number and degree of unsaturation.

The most important features of reversed-phase columns for the analysis of complex mixtures of triacylglycerols are their efficiency or theoretical plate count and their retention of triacylglycerols. The degree of retentiveness is directly related to the amount of carbon bonded to the column. A column with high retentiveness is required for TG analysis because triacylglycerols are not completely soluble in the solvents commonly used in RPLC. In addition, the length of the bonded alkyl chain greatly affects the separation of triacylglycerols.[54,59] For example, a column containing an octadecyl bonded phase ($C_{18}$) often results in long retention times for higher molecular weight, more saturated triacylglycerols, whereas shorter retention times are obtained with octyl bonded phases ($C_8$) at the expense of a substantial reduction in resolution. Changes in the eluting solvent and the selectivity of the column can be used to discriminate between slight differences in the number of double bonds and the carbon number of triacylglycerols.

The first separations of triacylglycerols by reversed-phase HPLC were reported in the mid-1970s. Pei et

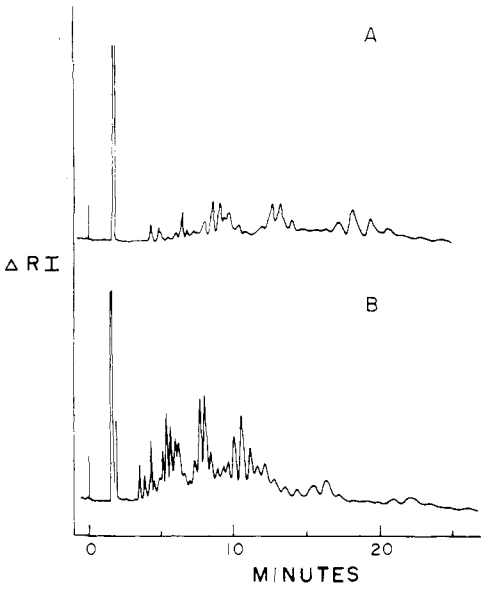


**Figure 4.** Comparison of the separation of fish oil triacylglycerols using aqueous and nonaqueous mobile phases: (A) aqueous mobile phase, water/acetone/acetonitrile (5/250/245); (B) nonaqueous mobile phase, acetone/acetonitrile (50/50).

al.[60] reported the separation of triacylglycerols using a reversed-phase column with a mobile phase of methanol–water. However, because of the lipophilicity and hydrophobicity of triacylglycerols, aqueous mobile phases often result in poor separations and sample loss on the column. For example, in a comparison of aqueous and nonaqueous mobile phases for the separation of fish oil triacylglycerols, poor resolution and peak shape, as well as sample loss, are evident with an aqueous mobile phase (Figure 4).[61] Nonaqueous, reversed-phase (NARP) chromatography is now used exclusively for the separation and analysis of natural triacylglycerols.[54,58,59,61-91]

(*a*) *Mobile-Phase Composition.* Mobile phases of different compositions have been used for the NARP chromatography of natural fats and oils. The influence of mobile-phase composition on both the resolution and retention of triacylglycerols has been studied extensively.[61-63,72,73,92] The separation of TGs is dependent on the polarity of the mobile phase and, thus, differences in the solubility of the triacylglycerols in the mobile phase. For example, El-Hamdy and Perkins[92] have shown that decreasing the polarity of the mobile phase or decreasing TG solubility resulted in shorter retention times. The extent to which the polarity of the mobile phase affects retention depends on the type of triacylglycerol molecular species being separated.

The most commonly used solvents for the NARP chromatography of natural fats and oils are acetone, tetrahydrofuran (THF), dichloromethane (DCL), chloroform, hexane, acetonitrile (ACN), propionitrile (PRN), methanol (MeOH), ethanol (EtOH), and isopropyl alcohol (*i*-PrOH). Many separations have been performed with mobile phases of acetone and ACN in various percentages, depending on the type of triacylglycerols present in the oil.[63,64,67,68,71,74,84,93,94] For example, in the separations of olive oil and sardine oil, a more polar mobile phase (50/50 acetone/ACN) is used to separate the fish oil, which contains more TGs with a higher degree of unsaturation (Figure 5).[61] Olive oil, which is composed of more saturated triacylglycerol

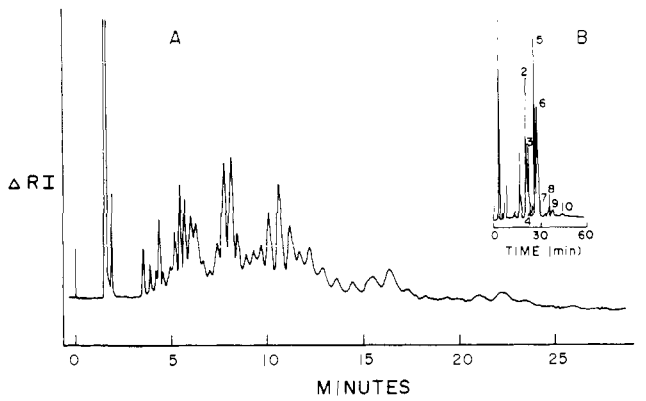


**Figure 5.** Separation of triacylglycerols of (A) fish oil and (B) olive oil: (A) acetone/acetonitrile (50/50) at 2.0 mL/min; (B) acetone/acetonitrile (63.4/36.4) at 1.0 mL/min. Chromatogram B reproduced with permission from ref 66; copyright 1981 American Oil Chemists' Society.

molecular species, is separated with a less polar mobile phase composition (63.6/36.4 acetone/ACN).

Dichloromethane, instead of acetone, with acetonitrile as the mobile phase, was used by Parris[62] in his original paper on NARP chromatography. Schulte[95,96] first proposed the use of propionitrile as an eluent in which cocoa butter triacylglycerols were separated with enhanced resolution when compared to separations obtained with mobile phases containing acetonitrile. Geeraert and De Schepper[97] separated numerous natural oils with excellent resolution using propionitrile as the mobile phase. An eluent of propionitrile was also used by Podlaha and Toregard[98] in their work on peak identification and prediction of eluting triacylglycerol molecular species. On the basis of the separations obtained, propionitrile seems to provide additional mobile-phase selectivity, resulting in better resolution of the components in complex TG mixtures.

The method used for detection of eluting components often dictates, at least partially, the solvents to be used in the mobile phase. Mobile phases containing acetone (UV cutoff, 330 nm) are not compatible with UV detection of triacylglycerols because of the absorption of the TG ester chromophore (195–230 nm). Thus, RI detection is commonly employed when mobile phases containing acetone are used. Typical lipid solvents such as dichloromethane and chloroform also are precluded with UV detection because they also absorb in the region being monitored. Mobile phases composed of THF and acetonitrile[69,72,83] or EtOH and acetonitrile[73,75] are used, therefore, when detection is by UV absorption.

Ternary mobile phases also have been investigated as a means of improving resolution in the partial separation of highly complex triacylglycerols.[65,72,99-101] Lozano[72] found enhanced resolution of certain TGs with a mobile phase of ACN/acetone/THF (58/38/4). In the separation of fish oil triacylglycerols, a mobile phase of *i*-PrOH/acetone/ACN (10/40/50) resulted in sharper peaks when compared with the separation resulting from an acetone/ACN (50/50) mobile phase (Figure 6).[61]

Solvent gradients are often necessary to resolve the more complex mixtures of triacylglycerols that occur in many natural fats and oils. Gradient elution, which is not compatible with the refractive index detector, has been used with UV,[75] IR,[61,97,101] and mass[80,84] detectors. For example, Robinson and Macrae[75] used a gradient

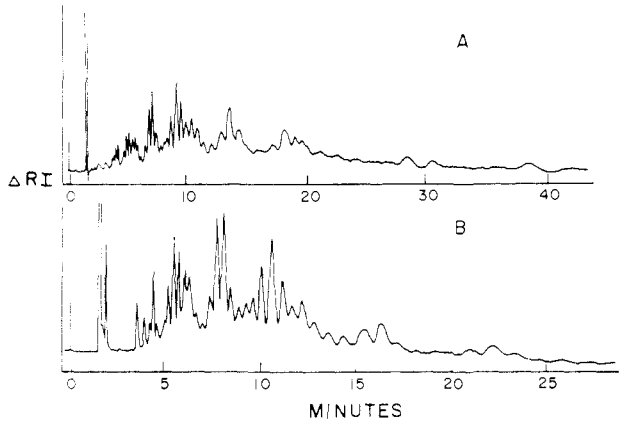


**Figure 6.** Separation of fish oil triacylglycerols: (A) *i*-PrOH/acetone/acetonitrile (10/40/50) at 2.0 mL/min; (B) acetone/acetonitrile (50/50) at 2.0 mL/min.

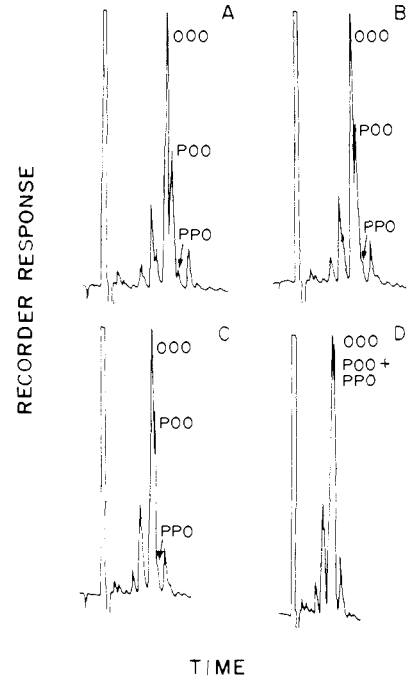


**Figure 7.** Separation of triacylglycerols in olive oil at different temperatures: (A) 14.5 °C; (B) 17.5 °C; (C) 20.5 °C; (D) 25.5 °C. Triacylglycerols eluted with acetonitrile/tetrahydrofuran/hexane (224/123.2/39.6 (w/w/w)) at 1.5 mL/min. Abbreviations: P = palmitoyl and O = oleoyl acid. Reproduced with permission from ref 65; copyright 1981 Elsevier Science Publishers B.V.

of EtOH in ACN with a UV detector for the analysis of butter fat. A gradient of THF in ACN with IR detection was used in the analysis of cod liver oil by Parris.[62] A complex gradient elution scheme was proposed by Herslof and Kindmark[83] consisting of a gradient from ACN to EtOH in a mixture of ACN/EtOH/hexane with mass detection for the analysis of various vegetable oils.

(*b*) *Temperature.* Improved separations of triacylglycerols by nonaqueous RPLC were obtained at subambient column temperatures by Jensen.[65] Temperature has the greatest influence on the retention of more saturated and higher molecular weight triacylglycerol species, for example those present in olive oil (Figure 7). Frede[85] has proposed the use of temperature *gradients* to improve the resolution of triacylglycerol mixtures that contain components of widely varying polarity. Excellent separations were achieved, including

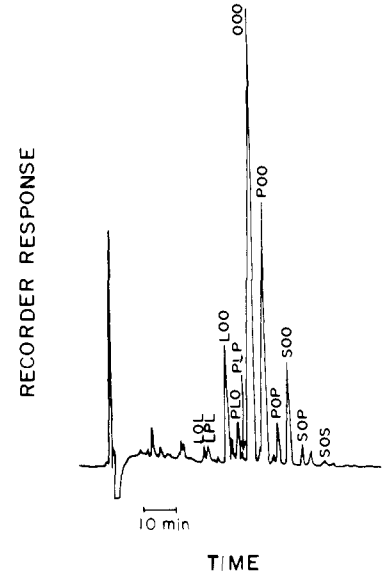


**Figure 8.** Separation of triacylglycerols in olive oil using a temperature gradient from 10 to 50 °C. Triacylglycerols eluted with propionitrile/ether (1/1). Abbreviations: P = palmitoyl, O = oleoyl, L = linoleoyl, and S = stearoyl. Reproduced with permission from ref 85; copyright 1985 Friedrich Vieweg & Sohn.

detection of minor triacylglycerol molecular species (Figure 8).

(*c*) *Sample Diluent.* Because complex mixtures of triacylglycerols often contain individual components with a wide range of polarities, the mobile phase used for their separation often do not provide adequate solubility to warrant its use as the injection solvent, or in other words, the sample diluent. In addition, the solvent used to solubilize the triacylglycerol samples has been found to affect the separation.[77] Tsimidou and Macrae[80] investigated both the theoretical and practical aspects of the influence of injection solvents and recommend using acetone or combinations of acetone and THF as sample diluent when the mobile phase does not provide sufficient solubility. Singleton and Pattee[58] also evaluated the effects of the sample diluent on TG separations and found chloroform to be the best solvent. In studies involving the RPLC separation of marine oil triacylglycerols, which contain a high percentage of polyunsaturated species, the authors[61] found no significant differences in chromatographic behavior when comparing the following solvents as sample diluents: acetone, THF, combinations of acetone and THF, chloroform, and dichloromethane.

(*d*) *Critical Pair Separations.* The analysis of natural triacylglycerol mixtures is often complicated by the occurrence of nearly identical molecular species or critical pairs, which exhibit similar chromatographic behavior on reversed-phase columns. Critical pairs can have slight differences in carbon number (CN) and the number of double bonds (NDB) and are defined as molecular species with the same equivalent carbon number (ECN).

$$ECN = CN - (2 \times NDB)$$

In addition, positional isomers of triacylglycerols, which are also known as reverse isomers, are critical pairs because the fatty acyl constituents are the same except for esterification at different positions on the glycerol moiety of the molecules.

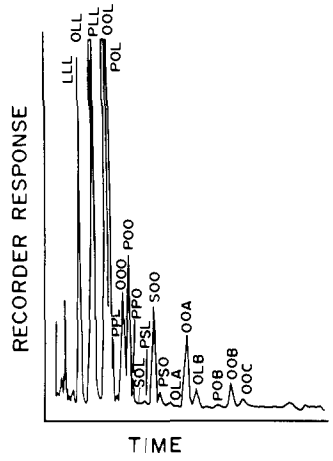


**Figure 9.** High-resolution separation of peanut oil triacylglycerols on 3-μm reversed-phase columns connected in series. Abbreviations: L, 18:2; O, 18:1, P, 16:0; S, 18:0; A, 20:0; B, 22:0; C, 24:0. Reproduced with permission from ref 91; copyright 1987 American Oil Chemists' Society.

The resolution of critical pairs has been accomplished by using highly efficient reversed-phase columns with nonaqueous mobile phases.[54,63,66,67,69,90,91,102,103] For example, an excellent separation of peanut oil triacylglycerols using 3-μm reversed-phase columns was recently reported by Singleton and Pattee[91] (Figure 9). Dong and DiCesare[71] also obtained critical pair separations with short 3-μm columns and concluded that both the selectivity and efficiency of the small particle size packing enhanced the resolution of natural oil triacylglycerols.

(e) *Peak Characterization.* As the number of molecular species in complex mixtures of triacylglycerols increases, peak identification becomes increasingly difficult. Two different approaches have been applied to the problem of peak characterization: (i) collection of separated peaks and analysis by an independent method and (ii) application of graphical or matrix methods based on elution behavior. Peak collection and subsequent analysis is time-consuming and, thus, not practical for routine analysis. A graphical method based on the relationship between retention time of the triacylglycerols and experimental or calculated ECN values has been proposed by Podlaha and Toregard.[98] However, graphical methods of triacylglycerol identification are suitable only for simple mixtures, and Takahashi and co-workers[78,104,105] have described a matrix model based on elution behavior for characterization of component TG molecular species.

## B. Detection

Detection has been a limiting factor in the development of chromatographic methods for the analysis of natural fats and oil. Recently, much work in the field of triacylglycerol analysis has focused on improvement in detection to provide more sensitive analyses as well as enhanced selectivity for peak identification.

### 1. Refractive Index Detection

The RI detector has been used extensively for the analysis of triacylglycerols because it responds to all eluting species, is relatively easy to use, and is inexpensive.[60,61,63-68,70,72,74,84,85,87,92-94,100,106] The sensitivity of the differential RI detector, which is a bulk property detector, depends on differences in refractive indices of solutes and solvents. As a result, the possibility of both positive and negative peaks can complicate the analysis of eluting components. In addition, RI detectors can be sensitive to changes in temperature and mobile compositions, which precludes the use of gradient elution. Frede and Thiele,[90] using an interferential refractometer with a thermostated HPLC system, were able to achieve a highly sensitive detection of milk fat triacylglycerols. However, the special temperature requirements of this detector limit its routine use. The main advantage of the RI detector is its application for detection of compounds that contain no or very weak chromophores for UV detection, as is the case with triacylglycerols. This detector is very useful in preparative HPLC or in analyses in which large amounts of sample are available.[107]

### 2. Ultraviolet Absorption Detection

Ultraviolet detectors have recently been used for the analysis of natural fats and oils.[65,68,72,79,96,107] The use of UV detection for triacylglycerols is difficult, since the fatty acid esters absorb only in the range of 190–230 nm with a low molar extinction coefficient. Derivatization of intact triacylglycerols is not possible without disruption of the glyceryl ester or double bonds; thus, information on the structure of the original molecule would be lost. In addition, many of the solvents that provide good separation of triacylglycerols, in particular, acetone, also absorb in the far-UV. However, UV detection does permit gradient elution, which may be necessary to resolve highly complex TG mixtures. The application of gradient elution with UV detection for the analysis of butter fat has been reported by Robinson and Macrae;[75] a more sensitive analysis was achieved with UV in contrast to RI detection. Thus, in some cases, depending on the type of triacylglycerols under study, UV detection provides better sensitivity.[71,75,99] For example, enhanced response is observed for the more unsaturated TG components that elute earlier in the reversed-phase chromatograms. These species have a higher molar absorptivity due to contributions from the methylene-interrupted double bonds. However, these contributions preclude the possibility of accurate quantitation of unsaturated triacylglycerols.

### 3. Infrared Detection

The use of the IR detector in the analysis of natural fats was first proposed by Parris,[62,101] in which the carbonyl absorbance at 5.75 μm was monitored. As with UV detection, the choice of solvents for use in the mobile phase is limited, since an appropriate solvent window must be available. Gradient elution is possible, but considerable base-line drift can occur. Parris[62,101] has suggested that base-line drift can be minimized by proper choice of the gradient conditions. The difficulties with base-line drift and poor detector properties such as high detection limits and slow response times have limited the application of IR detection for routine triacylglycerol analysis. If these problems can be overcome, the IR detector would be an attractive alternative to RI and UV detection, since it is a more selective detector. Takano and Kondoh[52] also have shown that the IR detector has molar response to triacylglycerols, allowing quantitative analysis.

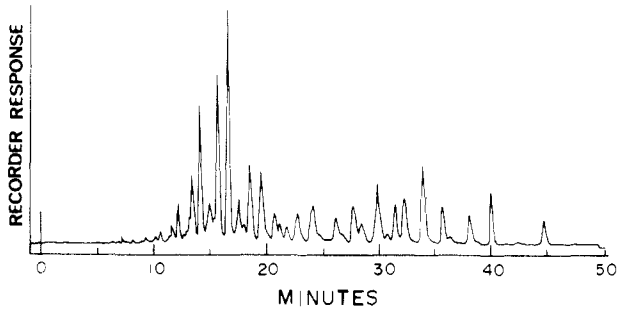


**Figure 10.** Separation of butter fat triacylglycerols with mass detection using gradient elution (acetone/acetonitrile, 80:20 to 99:1 in 30 min) at 0.8 mL/min. Two reversed-phase columns (25 cm, 5-μm particles) connected in series were used. Reproduced with permission from ref 81; copyright 1985 American Chemical Society.

#### 4. Flame Ionization Detection

The moving-wire, flame ionization detector (FID) or transport FID has been used in the analysis of triacylglycerols.[108,109] The moving-wire FID can be used with any volatile solvent and, thus, does not have the solvent limitations that the UV and IR detectors possess. Column eluents are deposited on a moving wire where the solvent is evaporated prior to solute detection by the FID. Therefore, the moving-wire FID is also compatible with gradient elution; however, it has the disadvantages of limited sensitivity[110,111] and it is mot commercially available.

#### 5. Mass Detection

The mass detector, based on the principle of light scattering, may revolutionize triacylglycerol analysis.[79,81,83,112,113] An experimental mass detector based on laser light scattering has been evaluated extensively.[114,115] Robinson and Macrae[75] evaluated the use of a commercial mass detector for TG analysis. Gradient elution is permitted since nebulization of the column eluate and removal of the solvent occur prior to detection of the separated components. With gradient elution, minimal base-line drift occurs when nonaqueous volatile solvents are used. In addition, a wide range of volatile solvents are compatible with this detector, provided the sample to be analyzed is less volatile than the solvent. Response from the mass detector is nonlinear, but highly reproducible and related to sample mass, permitting quantitation of eluting species.[81] The main advantage of the laser light scattering mass detector is its high sensitivity for all eluting triacylglycerols (Figure 10). One factor limiting the routine use of the laser-based mass detector is cost; however, as lasers become more affordable, this type of detector will become more available.

#### 6. Other Methods of Detection

The dielectric constant (DC) detector has been applied in the analysis of corn and cottonseed oils.[116] The DC detector is ideally suited for use with nonaqueous mobile phases and is generally more sensitive than RI detection. However, this detector is not commercially available, since the majority of RPLC separations performed require buffer solutions in the mobile phase and DC detectors cannot be used in separations that use this type of solvent system.

Post column reaction detectors (PCRD), which offer a high degree of specificity and molar response, have also been investigated for use in the analysis of triacylglycerols. Compton and Purdy[117] used a PCRD based on the fluorescence detection of Fluoral-P-derivatized solutes. Derivatization with acetylacetone in a PCRD was used by Kondoh and Takano[118] for the analysis of coconut oil triacylglycerols. The main disadvantage of PCRDs for TG analysis is the loss of structural information about the original molecule; hydrolysis of the triacylglycerols occurs during derivatization. In addition, the construction of a reactor can be tedious and time-consuming.

Mass spectrometers have recently been combined with HPLC for the detection and identification of triacylglycerol molecular species.[41,77,118-124] The mass spectrometer has been described as the ultimate universal LC detector[124] and is sensitive for all triacylglycerol species.[41,121] Mass spectrometers can be interfaced with narrow-bore HPLC to provide more sensitive detection.[122] The main advantage of the mass spectrometer is the wealth of structural information that is obtained and can be used for peak identification. In addition, quantitation of both resolved and partially resolved peaks can be accomplished with the appropriate computer capabilities for data processing.[77] Currently, the cost of a LC–MS instrument prohibits its use in most laboratories.

## III. Complementary Analytical Techniques

Complete characterization of naturally occurring triacylglycerols necessitates resolution of TG molecular species not only according to differences in degree of unsaturation, in overall polarity, and in molecular weight but also according to differences in positional distributions and geometric configurations of the various esterified acyl groups. No single method of analysis is capable of accomplishing this feat. Therefore, several analytical techniques for both separation and identification purposes are combined to provide a more complete picture of a TG profile.[125-129,138]

A systematic approach for the effective use of complementary techniques often includes a prefractionation step with TLC to yield fractions differing in degree of unsaturation, carbon number, or positional isomerism, followed by specific enzymatic hydrolyses and chemical modifications to facilitate separation and identification of component TGs. For example, Grignard degradation of triacylglycerols[130] results in random production of diacylglycerols, which can then be subjected to specific enzymatic hydrolysis with phospholipase C[131] and/or phospholipase $A_2$[132] after synthesis of the appropriate intermediates.[133,134] Although these transformations do not permit exact identification of all TG molecular structures, valuable information about fatty acid positional esterification is obtained.[135] Complementary instrumental techniques such as GC–MS, LC–MS, and MS–MS can then be used to identify both the triacylglycerols and the glycerolipids resulting from the chemical and enzymatic transformations.[41,42,136]

## IV. Summary

The separations or partial separations of triacylglycerols in complex mixtures is now possible with RPLC. However, GLC using polar capillary columns

is still the method of choice for analyzing complex mixtures with high resolution, especially for trace analyses, whereas RPLC is mainly used in the preparative or semipreparative work for the isolation of relatively large amounts of TGs.[137,138] With RPLC separated TG molecular species can now be detected with good sensitivity, but unequivocal peak identification is limited mainly to LC–MS; however, adequate characterization of naturally occurring triacylglycerols can be accomplished by using a combination of chemical, enzymatic, and analytical techniques. Further developments in the TG analysis field will include multidimensional chromatography, which can provide on-line methods for more sensitive detection, peak identification, and accurate quantitation.

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