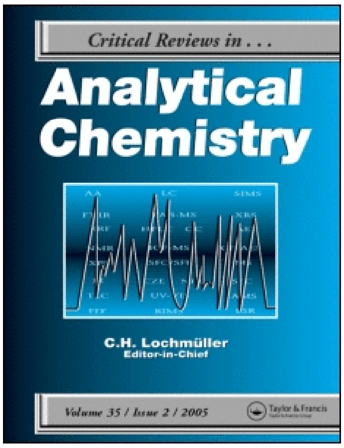

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The Separation and the Characterization of Long Chain Fatty Acids and Their Derivatives by Reversed Phase High Performance Liquid Chromatography

Phyllis R. Brown[a]; Janet M. Beebe[b]; Joseph Turcotte[c]
[a] Department of Chemistry, University of Rhode Island, Kingston [b] Chemical Process Research with Rohm and Haas Co., Spring House, Pennsylvania [c] Department of Medicinal Chemistry, University of Rhode Island, Kingston

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The Separation and the Characterization of Long Chain Fatty Acids and Their Derivatives by Reversed Phase High Performance Liquid Chromatography

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LIST OF ABBREVIATIONS

9-ADAM	diazomethylanthracene
Ag-RPLC	reversed phase argentation chromatography
APs	9-aminophenanthryl esters
AMFAs	anthrylmethyl fatty acid esters
ACs	4-bromoethyl-7-acetoxycoumarin esters
Br-Mmc	4-bromoethyl-7-methoxycoumarin esters
ECL	equivalent chain length
FAMEs	fatty acid methyl esters
FAMOAs	fatty acid p-methoxyanilide esters
FANpes	fatty acid napthacyl esters
FAMPHes	fatty acid 2-nitrophenyl hydrazide esters
FAPFBs	fatty acid pentafluorobenzyl esters
FFAs	free fatty acids
FID	flame ionization detector
GLC	gas-liquid chromatography
HPLC	high performance liquid chromatography
HPSEC	high performance size exclusion chromatography
LCFAs	long chain fatty acids
PUFAs	polyunsaturated fatty acids
RPLC	reversed phase liquid chromatography
SURES	sucrose fatty acid esters
TLC	thin layer chromatography
ACE	acetone
Solvents	
ACN	acetonitrile
MeOH	methanol
THF	tetrahydrofuran
HAc	acetic acid

I. INTRODUCTION

The potential for the application of chromatography to the analysis of fatty acids was first realized by A. J. James and A. J. P. Martin in 1952.[1] These two noted scientists successfully separated the iso- and ante-isomers of short chain free fatty acids by gas liquid chromatography (GLC). Even today, 35 years later, the method of choice for characterization of fatty acids is capillary gas chromatography with a mass spectrometer as a detector. However, high performance liquid chromatography (HPLC) is now becoming competitive in the separation of fatty acids, especially on the preparative scale.

Unlike GLC, HPLC can be applied to compounds which are thermally labile or nonvolatile. Also, nondestructive detection methods are generally used in HPLC which permit collection of analytes either for isolation on a preparative scale or for further analysis. The versatility of HPLC has led to its widespread use; consequently, numerous books and review articles dealing with its theory, instrumentation, and applications have been published.[2-8]

Any HPLC analysis is limited by the detection method. In order to enhance detectability of fatty acids, most methods require derivatization. Whereas prechomatographic derivatizations can influence the resolution, postchromatographic derivatization techniques increase the sensitivity and selectivity of detection. Reviews in the past have dealt with derivatization procedures[9] for the chromatographic separation of lipid classes (including long-chain fatty acids)[10-13] and the chromatographic characterization of fatty acids and their derivatives.[14-15]

The purpose of this review is to describe the recent advances (1975 to present) in the characterization of long chain fatty acids (LCFAs) and their derivatives by reversed phase high performance liquid chromatography (RPLC). In this review each detection technique and the derivative classes which can be utilized with that particular detector are discussed.

In order to simplify the presentation of the many separation methods of LCFAs, a short introduction describes the general retention behavior in the reversed phase chromatographic mode.

A. Retention Behavior

Naturally occurring fatty acids are even numbered carbon chain compounds with all double bonds in the "*cis*" configuration. The LCFAs have carbon chain lengths of C12 to C24 as opposed to short-chain fatty acids (<C12) and very long-chain fatty acids (>C24). They may have as many as six olefinic bonds. Complex mixtures of LCFAs derived from natural sources are difficult to separate because the LCFAs present have similar structures, differing only in the number and position of ethylenic bonds; however, LCFAs have been separated successfully by both the reversed and normal phase chromatographic modes. In normal phase a silicic acid column impregnated with silver nitrate solution is usually used. With this method the separation of the unsaturated fatty acids is based

Phyllis R. Brown earned a B.S. at George Washington University in Washington, D.C. as well as a Ph.D. at Brown University, Providence, Rhode Island. Presently, Dr. Brown is Professor in the Department of Chemistry at the University of Rhode Island, Kingston. **Joseph Turcotte** received a B.S. and a M.S. at the Massachusetts College of Pharmacy in Boston and a Ph.D. at the University of Minnesota, Minneapolis. Currently, he is Professor in the Department of Medicinal Chemistry, University of Rhode Island, Kingston. **Janet M. Beebe** earned her A.A.S. from the Thames Valley State Technical College in Norwich, Connecticut, her B.S. from the University of Connecticut, Storrs, and her Ph.D. from the University of Rhode Island, Kingston. Dr. Beebe is Senior Analytical Chemist in Chemical Process Research with Rohm and Haas Co., Spring House, Pennsylvania.

on the number of double bonds in the carbon chain. The retention is due to the formation of a reversible charge-transfer complex involving the silver ion and the pi electrons of an olefinic bond. Thus, in the normal phase mode, monosaturates always elute before diunsaturates and polyunsaturates elute last.

The retention behavior of LCFAs or their derivatives separated in a reversed phase mode is not as well understood. The LCFA retention order changes with mobile phase composition and a variety of alkyl bonded stationary phases have been used for LCFA separations. However, some general rules of retention are applicable for reversed phase separations of fatty acids.

In RPLC, LCFAs elute according to equivalent chain length values. The theory of equivalent chain length (ECL) was first defined by Miwa et al.[16] in 1960 and was used to evaluate GLC retention behavior of fatty acids when polar columns were used. For application to RPLC separations ECL was redefined by Ozcimder and Hammers[17] as

$$\mathrm{ECL} = \mathrm{N} - 2\mathrm{n}$$

where "N" is the number of carbons in the LCFA and "n" is the number of ethylenic bonds. LCFAs with the same ECL value are referred to as "critical pairs" and their separation is often difficult especially in complex sample matrices where there may be as many as four or five compounds with the same ECL.

Throughout this review the representative shorthand "CN:nωy" is used to denote the individual LCFA where "N" denotes the carbon chain length, "n" the number of methylene interrupted double bonds, and "ωy" the location of the last double bond from the methyl terminus. For example, C22:6ω3 denotes docosahexaenoic acid, a 22 carbon straight chain fatty acid with 6 methylene interrupted double bonds with the last double bond 3 carbons from the terminal methyl group, i.e., between carbons 19 and 20. In the case of geometrical isomers, "*cis*" or "*trans*" configurations are written out to avoid confusion.

II. ANALYSIS OF LONG CHAIN FATTY ACIDS USING DIFFERENTIAL REFRACTOMETRY

A differential refractometer monitors the difference in refractive index between a reference mobile phase and a column effluent. This bulk property or universal detector responds to all solutes if the refractive index of the solute is significantly different from that of the mobile phase. Because refractive index detectors respond to all solutes, these devices have excellent versatility; however they provide only modest sensitivity (0.1% concentration levels) under optimum conditions. Refractive index detectors are also limited because they are incompatible with gradient elution; it is exceedingly difficult to match the refractive indices of the sample and reference streams. Despite these limitations, refractive index detectors are used to detect LCFAs in complex sample matrices where limited sensitivity is acceptable, and derivatization of the LCFA molecule is undesirable.

A. Fatty Acid Methyl Esters

1. Analytical Scale Separations

In 1975 Pei et al.[18] reported one of the first successful applications of RPLC to the separation of saturated LCFAs, LCFA methyl esters, or polyunsaturated LCFAs. In less than 10 min a satisfactory separation of fatty acids differing in chain length and degree of unsaturation was accomplished. The separations achieved were superior to those using conventional argentation silicic acid chromatography because the RPLC method permitted the separation of LCFAs with the same degree of unsaturation. The analyses were performed using a chemically bonded VYDAC™ octadecyl stationary phase with a controlled surface porosity (35 to 45 μm) and an isocratic mobile phase of 80 to 90% (v/v) methanol (MeOH) to water. By utilizing column temperatures of 35 to 60°C, solvent viscosity was decreased and baseline resolution was achieved for mixtures of LCFAs. Chromatograms of the saturated fatty acid methyl esters (FAMEs) from C18:0 to C24:0 and the monounsaturated C18:1 fatty acids, are shown in Figure 1A.[18] Figure 1B shows the separation of the unsaturated C18 FAMES from that of the C18:3ω6. Detection was on the milligram level.

Pei et al.[18] also illustrated the separation of the biologically important 20 carbon fatty acids (eicosanoids): 5,8,11,14,17-eicosapentaenoic acid (C20:5ω3), 5,8,11,14-eicosatetraenoic acid (C20:4ω6), and 11,14,17-eicosatrienoic acid (20:3ω3) which are precursors of the prostaglandins E_1, E_2, and E_3. This work was the first indication that RPLC could be useful in monitoring biological processes such as prostaglandin synthesis.

Soon after, Scholfield[19] separated, in approximately 20 min, the FAMEs derived from linseed oil and liquid hydrogenated vegetable oil. The system employed a Corasil C_{18} (24 × 1/8 in O.D.) column and an aqueous acetontrile (ACN) mobile phase. These conditions were analogous to Scholfield's previous use of hexane and ACN in countercurrent distribution for similar separations.[20] Scholfield observed a linear relationship between carbon chain length and log k′ for the C14:0, C16:0, and C18:0 saturated esters; thus, retention of the saturated LCFAs could be predicted. In addition, ACN was compared to MeOH as an organic modifier for the separation of LCFA methyl esters. A MeOH mobile phase resulted in increased retention and broader peaks than an ACN mobile phase of comparable solvent strength. Good separations of soybean, safflower, corn, and olive oil esters were reported with geometrical isomers trans, trans-, cis, trans- and cis, cis-octadecadienoates resolved as well as methyl esters of the C12:0, C14:0, C16:0, C12:1, C14:1, and C16:1 fatty acids.

Evening primrose oil is a rich source of alpha-linolenic

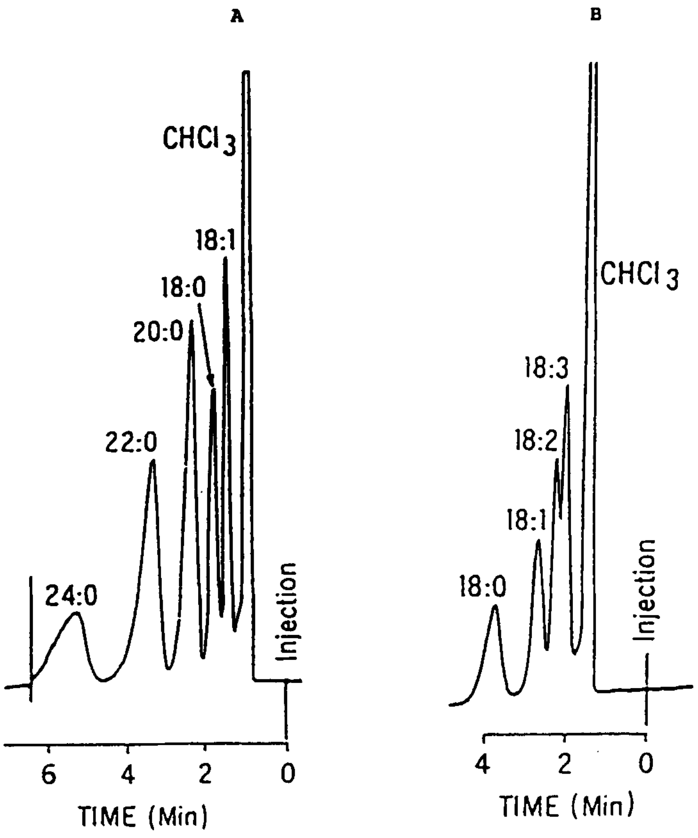

FIGURE 1. Chromatogram of fatty acid methyl esters and polyunsaturated fatty acid methyl esters. Eluting solvent: MeOH:H_2O (90:10 v/v); column VYDAC C_{18} (1 m × 2 mm I.D.); flowrate: (A) 1.7 ml/min (B) 1.0 ml/min; temperature: (A) 60°C (B) 35°C; sample injected: (A) 200 μg each component (B) 300 μg each component. (From Pei, P. T.-S., Henlyt, R. S., and Ramachandron, S., *Lipids*, 10(3), 152, 1975.)

acid (C18:3ω3). This acid is the only omega-3 fatty acid present at high levels in a vegetable source. Evening primrose oil also contains gamma-linolenic acid which differs from the alpha form only by the position of the three double bonds; as a result, their separation was difficult.

The analysis of FAMEs in evening primrose oil and soybean oils was studied by Manku.[21] Capillary and packed column GLC methods were compared to two different RPLC procedures. The first RPLC procedure utilized a μ-Bondapak C_{18} column (300 × 4.6 mm I.D.) with aqueous MeOH to elute isocratically the FAMEs. In the second RPLC procedure, attempts were unsuccessful in separating the free fatty acids (FFAs) on a μ-Bondapak Fatty Acids Analysis column with a mobile phase of a mixture of tetrahydrofuran (THF):ACN:H_2O:acetic acid (HAc) (25:40:50:0.1 by volume). Neither of the chromatographic systems resolved the C13:3ω6 positional isomers adequately. At present, capillary GLC remains the method of choice for these isomers.

In a study of column types for oleochemicals, Ottenstein et al.[22] separated in 15 min the following FAMEs (at mg levels) in a standard solution: C18:3ω6, C18:2ω6, C14:0, C18:1ω9, C16:0, and C18:0. The chromatographic system employed 3 tandem Supelcosil LC-18 (250 × 4.6 mm I.D.) columns packed with 5-μm particles and an eluent of ACE:ACN:THF (50:42:7.6). Since the nonaqueous mobile phase used produced relatively low back pressures, as a result, several columns could be used in series to enhance resolution.

In 1986 Christopoulou and Perkins[23] described a high performance size exclusion chromatographic (HPSEC) method for the separation and quantitation of FAMEs, in a mixture with mono-, di-, and triglycerides. The lipid components were separated on 2 columns (250 × 0.7 cm I.D.) in series packed with a 5-μm styrene/divinylbenzene copolymer. The eluent was toluene. The upper molecular weight limit was 5 × 10^3 Da, and the lower molecular weight limit was 100 Da. Standard mixtures of tristearin, distearin, monostearin, and methyl stearate or stearic acid (C18:0) were separated with baseline resolution in approximately 35 min. In HPSEC, elution order is in reverse order of molecular weight, i.e., larger molecules elute first followed by smaller molecules. Due to the presence of ethylenic bonds which change the "effective size" of the LCFA molecule the resolution for unsaturated FAMEs was unsatisfactory compared to their saturated counterparts.

2. Preparative Scale Separations

In the past 15 years preparative liquid chromatography has been used to separate many compounds successfully, including long-chain FAMEs, and reviews have been written concerning its theory and applications.[24-26] Some of these preparative scale separations were obtained by modification of a classical countercurrent separation[27] or by the direct application of an analytical RPLC procedure.[28] The majority of the procedures were performed in the reverse phase mode using an octadecyl stationary phase to isolate polyunsaturates in vegetable oils. Refractive index detection is well suited to preparative scale separations as low level detection is not required.

In 1975, Scholfield[28] prepared up to 200 mg samples of the fatty methyl esters derived from linseed and commercial hydrogenated vegetable oils with a C_{18} Porasil (24 × 3/8 in O.D.) stationary phase with an ACN:H_2O mobile phase. In approximately 90 min the FAMEs of C18:3ω6, C18:2ω6, C18:1ω9, C18:0, and C16:0 were separated with baseline resolution.

A variety of procedures for the purification of FAMEs from various sources was described by Bascetta, Gunstone, and Scrimgeour.[27] Using prechromatographic purification techniques such as low temperature and urea crystallizations together with repeated recycles, they successfully separated methyl linolenate with purities greater than 99% from maize, sunflower oil, and evening primrose oil. The separation of alpha-methyl linolenate (>99%) and gamma-methyl linolenate (>97%) from linseed and evening primrose oils was described.

B. Free Fatty Acids

Many researchers have analyzed by RPLC complex mixtures of free fatty acids (FFAs) with a ternary mobile phase of ACN:THF:H_2O containing small amounts of glacial acetic acid to suppress ionization of the FFA carboxylate group. Subsequently, improved peak symmetry was observed compared to the traditionally very broad, tailing peaks frequently seen in RPLC separations of carboxylic acids.

In 1982 King, Adams, and Bidlingmeyer[29] described the "fingerprinting" of FFAs from natural oils (soybean, tuna, castor, coconut, linseed) in 10 min using a μ-Bondapak Free Fatty Acid column and an ACN:THF:H_2O:HAc mobile phase. They noted FFAs were very sensitive to the aqueous content of the mobile phase and a small modification in the percentage water resulted in a large change in retention and resolution. The samples they analyzed contained various saturated and unsaturated C14 to C18 FFAs.

D'Amboise and Hanai[30] studied the relationship between retention data and hydrophobicities using test solutions containing the following FFAs: C12:0, C14:0, C16:0, C18:0, C20:0, C16:1ω7, C18:1ω9, C18:2ω6, C18:3ω6, and C20:4ω6. They suggested that the mechanisms of retention included hydrophobic interactions between the chromatographic support and water as well as interactions between the water in the mobile phase and the solute. A mathematical model was used to describe the retention behavior of free fatty acids in various aqueous mobile phases on a C_{18} stationary phase.

The FFA content in four different margarine samples was analyzed by Bailie et al.[31] using a ACN:THF:H_2O:HAc (67:3:30:1) mobile phase and a Lichrosorb RP-8 (250 × 4.6 mm I.D.) 10 μm column. The injector was proceeded by a precolumn (885 × 4. mm I.D.) packed with 37 to 35 μm Porasil. Atwood et al.[32] protected the analytical column from possible degradation due to the acetic acid in the mobile phase by their treatment of the precolumn. Seven of the C14 to C18 FFAs studied were then quantified by comparison with external standards.

Only "*cis*" configuration double bonds are present in the vegetable oils used in the manufacture of margarines. However, during the hydrogenation process in which vegetable oils are transformed into margarine, rearrangement of ethylenic bonds occurs, and geometrical isomers are formed. Bailie et al.[31] separated the oleic (C18:1 *cis* ω9) and elaidic (C18:1 *trans* ω9) acids and the linoleic (C18:2 *cis,cis* ω6) and linolelaidic (C18:*trans,trans* ω6) acids in 50 min. The remaining two positional isomers, C18:2 *cis,trans* ω6 and C18:2 *trans,cis* ω6 which could not be resolved even with recycles, coeluted between the peaks of C18:2 *cis,cis*-ω6 and C18:2 *trans,trans* ω6.

A novel industrial application of RPLC for the determination of FFAs in soap-thickened lubricating greases was introduced by Calabrese in 1987.[33] Traditionally, grease specifications are based on functional tests rather than on composition. At present the soap portion of the grease is quantified by wet chemical methods and IR spectrophotometry is used for identification. Two saturated fatty acids, stearic and 12-hydroxystearic acids, are present in greases as metallic soaps of barium, calcium, lithium, and other metals. The procedure described involved the filtration of the crystalline soap from the mineral oil base, followed by conversion of the soap to the FFAs via acid hydrolysis. The resulting FFA fraction was then analyzed by RPLC with a THF-0.1% (v/v) aqueous trifluoroacetic acid (7:3) eluent and a μ-Bondapak C_{18} (300 × 3.9 mm I.D.) column. Using this method the ratio of stearic acid to hydroxystearic acid was determined in less than 8 min.

C. Fatty Acid Sucrose Esters

Sucrose fatty acid esters (SURES) are used as emulsifiers in foods and cosmetics and are produced on an industrial scale. The synthesis of SURES results in complex mixtures because of the eight available hydroxyl groups on the sucrose molecule. For the analysis of these mixtures, TLC is used to obtain qualitative data concerning the ratio of mono-, di-, or higher substituted products. GLC has also been used; however it requires the preparation of volatile derivatives (e.g., trimethylsilyl).

In 1987, Jaspers et al.[34] reported a RPLC procedure for the quantitative analysis of sucrose fatty acid mono- and diesters. A mixture of MeOH:H_2O (85:15 v/v) was used for elution of the monoesters, while MeOH:ethyl acetate:H_2O (65:25:10 v/v/v) was used for the elution of the diesters. A Spherisorb ODS-1 or ODS-2 5 μm (300 × 4.6 mm I.D.) stationary phase was used and the analyses were performed at 65°C to prevent precipitation of the SURES onto the analytical column.

The RPLC methods described provide information concerning the structural isomers contained in the SURES mixture. A chromatogram of the monoester separation is shown in Figure 2.[34] Most importantly, the ratio of sucrose monopalmitate (C16:1ω7 SURE) and the sucrose monostearate (C18:1ω9 SURE) can be determined. This ratio is used as a hydrophyl/

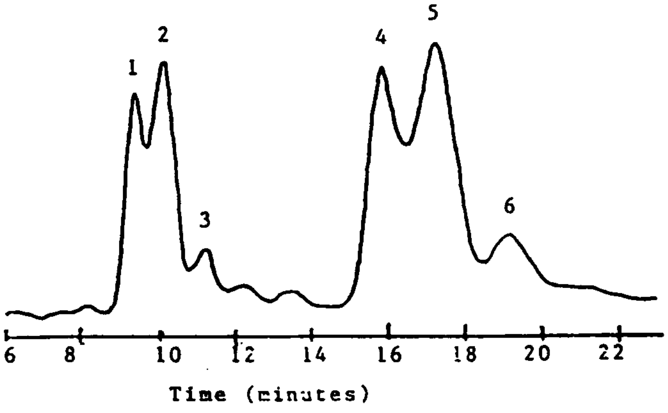

FIGURE 2. HPLC separation of SURES 70 PS 37 sucrose esters palmitoyl esters stearoyl esters = 3:7. Analytical operating conditions: stationary phase: 5 μm ODS column; mobile phase: MeOH:H_2O (85:15 v/v); flowrate: 1.2 ml/min; refractive index detection; sample concentration: 10% (ω/v); peak identification: peaks 1—3, sucrose palmitoyl monoesters; peaks 4 — 6, sucrose stearoyl monoesters. (From Jaspers, M. E. A. P., Van Leeuwen, F. F., Nieuwenhuis, H. J. W., and Vianen, G. M., *JAOCS*, 64(7), 1021, 1987.)

lipophil balance value (3:7 in Figure 2), an important indicator as to the appropriate manufacturing application of a particular SURES mixture.

D. Fatty Acid Polyhydric Alcohol Esters

1. Analytical Scale Separations

Fatty acid ethoxylates are used as emulsifiers for pharmaceuticals, colorants, and nonionic detergents. They are synthesized by two different methods: addition of ethylene oxide to the fatty acid or esterification of the fatty acid by poly(ethylene) glycols. The composition of polymer mixtures depends on the molar ratio of the reactants. Thus, in order to determine the extent of the reaction, it is imperative that reasonable separation procedures are available for the analysis of poly(ethylene) glycols and poly(oxyethylene) mono- and dialkyl fatty acid esters.

In 1980, Henke and Shubert[35] illustrated the determination of partial and fully fatty acid esters of monohydric and fully hydric alcohols by RPLC using a LiChrosorb RP-8 (250 × 4.6 mm I.D.) 5-μm stationary phase and various aqueous MeOH mobile phases. Mixtures of the C8:0 to C22:0 and C24:0 FAMES, the i-propyl, i-butyl-, n-hexyl-, and i-octyl monohydric esters of the even numbered C8 to C18 fatty acids, mono- or difatty acid esters of the bihydric alcohol, and 1,3-*bis* (2-hydroxyethyl)-5,5-dimethylhydantoin were separated in 28 min.

2. Preparative Scale

A preparative scale separation of poly(ethylene) glycols and poly(oxyethylene)mono- and dialkyl esters from LCFA ethyloxylates was described by Kudoh et al.[36] The complete separation of the compounds of interest was accomplished with an octadecyl (500 × 20 mm I.D.) column and an ACE:H_2O mobile phase. A back-flush method was used to reduce the analysis time and prevented the peak broadening which was characteristically found in chromatograms obtained with other RPLC methods.

E. Reversed Phase Argentation Chromatography for Free Fatty Acids and Fatty Acid Methyl Esters

DeJarlais et al.[37] illustrated that ACN in combination with MeOH or ACE was an effective eluent for reversed phase argentation chromatography (Ag-RPLC). With this mobile phase, rapid separation of polyunsaturated fatty acid (PUFA) methyl esters on an ion exchange column which was fully bonded with silver ions (60 × 0.045 cm I.D.) was accomplished. ACN in the mobile phase reduced the retention of PUFAs by competing with the PUFAs for the Ag sites on the column. Solvent programming from 0 to 15% ACN in MeOH permitted the separation of C18:1ω9, C18:2ω6, C18:3ω6, and C20:4ω6 methyl esters derived from safflower oil in approximately 3 h.

Up to 800 mg of safflower oil derived PUFA methyl esters were separated using a glass column (60 × 2.54 cm I.D.) by Adlof and Emken.[38] The system described was used to isolate multigram quantities of PUFAs from fish oil concentrates. Fractions of enriched (82 to 95%) combined omega-3 PUFAs or their methyl esters were obtained from a 100 mg fish oil sample which originally contained 67% combined PUFAs. Better results were observed by using isocratic elution with ACN in Ace (30, 35, or 40% v/v) rather than MeOH in Ace due to competing solute-Ag and solute-ACN interactions.

III. ANALYSIS OF FATTY ACIDS USING AN ULTRAVIOLET ADSORPTION DETECTOR

The most widely used detectors in HPLC are spectrophotomers which are based on ultraviolet (UV) or visible (vis) absorption. These detectors constitute 70% of all detection systems used at present with HPLC. UV-vis absorption detectors are solute property detectors which respond only to substances which absorb electromagnetic radiation in the UV and visible range (190 to 600 nm). The absorbing species contain chromophores such as one or more double bonds, carbonyl groups, or aromatic structures. Compounds that have extended conjugation absorb the most strongly. When radiation is absorbed by solutes in the effluent, the intensity of the light falling on the photocell is reduced and the electrical output produced is amplified and fed to a recorder. The Beer-Lambert law relates the absorbance to the solute concentration in the following way:

$$A = \epsilon c l$$

where A is the absorbance, ϵ is the molar absorptivity, c is the concentration of the absorbing species, and l is the path length. These detectors are 1000 times more sensitive than bulk property detectors and can give a signal for picograms of solutes which have high molar absorptivity. Among the types of UV detectors available are the following: fixed, single, or dual wavelength, variable wavelength, scanning wavelength, and photodiodearray detectors. Absorption detectors, which can be readily used with gradient elution, have a high sensitivity for many FFAs; however the FFA must absorb in the UV (or visible) region (e.g., 190 to 600 nm) to be detected. The detector response is affected by degree of unsaturation and the nature of the ester moiety. For the detection of FFAs, FAMEs, or other fatty acid alkyl derivatives, the UV/vis detector is most frequently used in the wavelength region between 195 and 206 nm. However, there are very few solvents that are transparent in this spectral region. As a result, most researchers have implemented a prechromatographic derivatization technique to prepare LCFA derivatives containing UV or fluorescent chromophores.

Separations of FFAs and FAMEs derived from biological fluids require detection limits in the ng range. These separations have been accomplished with low level UV detection and judicious choice of mobile phase. A wide variety of UV absorbing derivatives (Figure 3) have been successfully utilized to enhance the detectability and selectivity of LCFAs by RPLC and are discussed in the next section.

A. Free Fatty Acids and Fatty Acid Methyl Esters

The analysis of the enzyme reaction products of bovine erythrocyte glutathione peroxidase with 13-hydroperoxy linoleic acid was reported by Aoshima.[39] The separation of unsaturated fatty acids, and their hydroperoxides and hydroxy derivatives was achieved on a porous polymer gel (TSK-Gel LS-140) stationary phase with a hexane:ethanol mobile phase. The unsaturated FAMEs or FAAs were monitored at 215 nm and the hydroperoxides and hydroxides at 234 nm, with detection limits of 10 pmol. Close to baseline separation of methyl linoleate (C18:2ω6), 13-hydroxylinoleic acid, and 13-hydroperoxylinoleic acid was achieved.

Cyclopropanoic and cyclopropenoic long-chain fatty acids are present in higher plant families and have exhibited carcinogenic activity in laboratory animals. In 1982, Bianchini, Ralaimanarivo, and Gaydon[40] separated the mono-, di-, and triunsaturated C18 FAMEs as well as their cyclopropenoic (methyl stericulate) and cyclopropanoic (methyl dihydrostericulate) isomers. All separations were performed on a LiChrosorb RP-8 column in series with a LiChrosorb RP-16 column. The mobile phase was aqueous ACN (85%) and the effluent was monitored at 195 nm. The low levels of detection were 4 ng for the methyl stericulate and 125 ng for methyl dihydrostericulate. The UV response at 195 nm increases in the following order: saturated > monounsaturated > diunsaturated > cyclopropenated > triunsaturated FAMEs.

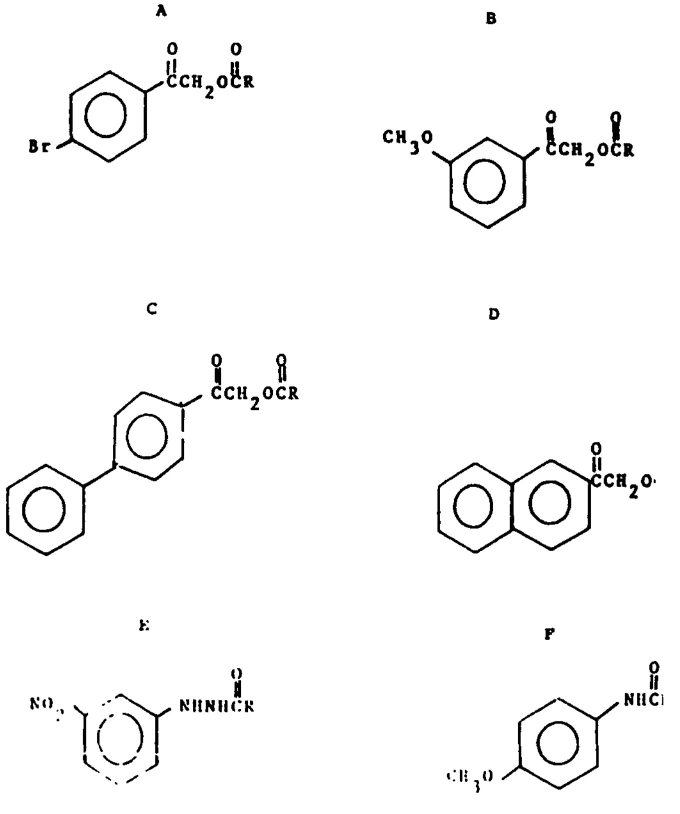

FIGURE 3. General chemical structures of UV absorbing long-chain fatty acid derivatives. (A) p-Bromophenacyl fatty acid ester; (B) p-bromo-*m*-methoxyacetophone fatty acid ester; (C) p-phenphenacyl fatty acid esters; (D) 1-bromo-2′-acetonaphthacyl fatty acid ester; (E) phenylhydrazide fatty acid ester; (F) p-methoxyanilide fatty acid ester; R = long chain fatty acid alkyl group.

The metabolic conversion of fatty acids derived from the mammalian tissue phospholipids has been investigated using RPLC. The fatty acid content of bovine brain lipid extracts was determined by Aveldano, Van Rollins, and Horrocks in 1983.[41] Rapid resolution of the C16 to C22 saturated and unsaturated FAMEs was obtained on an octyl stationary phase with an aqueous ACN eluent. The unsaturates were monitored at 192 nm and the saturates at 205 nm. An inversion in elution order was reported for many LCFA methyl esters with increasing ACN content of the mobile phase including the critical pairs: C22:4ω6 and C20:3ω6 (ECL 14), and C16:0 and C18:19ω9 (ECL 16).

To investigate arachidonic acid metabolism in blood platelets Batta et al.[42] developed a procedure for the separation of C20 saturated and unsaturated FFAs to be used in a study for the isolation of ^{14}C radiolabeled arachidonic (C20:4ω6) acid free from 8,11,14-eicosatrienoic acid. Using a Radial-Pak C_{18} cartridge (5 μm particles) and a mobile phase of MeOH:H$_2$O:HAc C20 fatty acids were analyzed. Baseline resolution of C20:5ω3, C20:4ω6, C20:2ω3, and C20:1ω11 was observed: however, the positional isomers, C20:3ω6 and C20:3ω3, were not completely resolved by this method.

Methyl, octyl, and octadecyl stationary phases have been used to separate LCFA methyl esters. The separation of unsaturated C16 to C18 FAMEs on a C_8 stationary phase was compared to that on a C_{18} stationary phase by Baker and Melhuish.[43] The mobile phase used to separate the standards was an aqueous ACN mobile phase and detection was by UV at 210 nm. The C18:3ω6, C16:1, C18:2ω6, and C18:1ω9 FAMEs were adequately resolved by both stationary phases with the same order of elution and detected at the ug level. Peak shapes were better using the C_{18} stationary phase than with the C_8 stationary phase.

Rezanka and Podojil[44] utilized a C_1 (methyl) bonded stationary phase and gradient elution with aqueous MeOH to separate saturated and unsaturated C14 to C30 FAMEs. The effluent was monitored at 210 nm and detection limits in the microgram range were observed. However, the methyl column did not provide baseline resolution for certain monoenoic FAMEs or the saturated esters that are one methylene group shorter (i.e., critical pairs) which can be separated on C_8 or C_{18} supports. Thus, the octadecyl stationary phase gives better peak shape and better resolution of FAMEs than the methyl or octyl stationary supports.

B. Fatty Acid Phenacyl Esters

1. p-Bromophenacyl Esters

Two derivatization procedures are frequently utilized to synthesize the fatty acid p-bromo phenacyl (FAPhes, Figure 3A) for subsequent analysis by HPLC.

The derivatization procedure described by Durst et al.[45] utilizes a crown ether as a solid-liquid conversion (>97%) of very small amounts of free fatty acids. The separation of the various synthetic mixtures of C2-C20 FAPhes on a Corasil II C_8 column was accomplished using both the normal phase (n-heptane:$CHCl_3$) and reversed phase (MeOH:H_2O) modes. With molar absorptivities on the order of 10^4 l mol^{-1} cm^{-1}, limits of detectability of 1 ng for the C2 FAPhe and 50 ng of the C20 FAPhe are reported.

An alternative derivatization procedure for the preparation of FAPhes reported by Borch[46] requires 8 h for completion (>905). The procedure utilizes a triethylamine catalyst and phenacyl bromide as the alkylating agent. The subsequent RPLC separation of a synthetic mixture of C12-C24 FAPhes was accomplished on a 10 μm μ-Bondapak C_{18} (90 × 0.64 cm I.D.) column using step-wise gradient elution with ACN:H_2O. These derivatization procedures permit direct quantitation of molar ratios of the FAPhes based on peak areas because the phenacyl moiety is the exclusive chromophore present at 254 nm.

Soon after these derivatization procedures were reported, Pei et al.[47] optimized the separation of saturated and unsaturated C18 fatty acid p-bromo FAPhes as well as the unsaturated positional and geometric isomers. They used a 5 μ, HI EFF Micropart C_{18} reversed phase column packing and isocratic elution with 90:10 MeOH/H_2O. Detection limits in the nanogram range were achieved with detection by UV at 254 nm for mixtures of positional oleic isomers, C18:1ω9 and C18:1ω6, the positional linolenic isomers; C18:3ω3 and C18:3ω6, and the geometric linoleic isomers; and C18:2 *cis,cis* ω9 and C18:1 *trans* ω9. The separation of these positional and geometric isomers had not previously been achieved by a liquid chromatographic technique. This work illustrates the potential of RPLC for the analysis of LCFAs in clinical applications as p-bromophenacyl derivatives.

On the basis of these results, many researchers optimized separations of LCFAs as their p-bromophenacyl esters. Engelhard and Elgass[48] optimized conditions for the separation of C4 to C22 saturated fatty acids p-bromo FAPhes. A C_8 stationary phase with a linear gradient of 70 to 100% ACN/H_2O yielded a satisfactory separation of all FAPhes. Octadecyl stationary phases were found to be unsatisfactory for the resolution of C18:1ω9 and C16:0 (ECL 16) FAPhes using this eluent although greater retention was observed. In 1980, Takayama, Jordi, and Benson[49] developed a C_{30}-bonded phase silica column and illustrated its use for the fractionation of fatty acids as their p-bromophenacyl esters. Using solvent gradients of ACN:H_2O or ACN:p-dioxane the following ester standards were resolved which have been traditionally difficult to resolve satisfactorily on a C_{18} column; C20:4ω6 (ECL 12) and C16:1ω7 (ECL 14), C18:1 *trans* ω9 and C18:1 *cis* ω9 (ECL 16), C20:0 and C22:1ω11 (ECL 20), and C22:0 and C24:1 (ECL 22). Using a convex gradient of ACN:H_2O all the C3 to C24 p-bromo FAPhes were separated satisfactorily; however, the chromatographic analysis required more than 3 h.

Roggero and Coen[50] proposed rules for the general elution order of the p-bromo FAPhes based on the effective shortening of the chain length by the double bonds. The retention behavior of 16 p-bromo FAPhes (C12-C22) was reported separated on a C_{18} Radial Pak Cartridge using two eluents of comparable solvent strength, ACN:H_2O (87:13) and MeOH:H_2O (90:10). By using a column with a low back pressure and high efficiency, a precise evaluation of the k' values of the p-bromo FAPhes was achieved. Greater k' values were obtained with the ACN:H_2O mobile phase whereas the MeOH:H_2O provided better resolution of the C14:0 and C16:1ω7 (ECL 14), and C16:0 and C18:1ω9 (ECL 16) critical pairs.

The retention behavior of isomeric monenoic and acetylenic octodecanoic FAPhes has also been studied by RPLC.[51] The isomeric octadecanoates were synthesized with triple bonds at positions from 2 to 14. These compounds were then used to prepare geometrical and positional octadecanoates by selective partial hydrogenation. The subsequent RPLC analyses were made using a 5 μm octadecyl bonded phase and an isocratic mobile phase of ACN:H_2O. The effluent was monitored at 242 nm with nanogram sensitivity. In less than 48 min the octodecanoic p-bromo FAPhes were baseline resolved with the exception of octodecanoic FAPhes with acetylene bonds at positions 2 and 3. Similar results with slightly lower retention were observed with the octadecanoate p-bromo FAPhes.

The investigation of eight of the most biologically important C14 to C20 fatty acids as their p-bromo FAPhes esters was described by Halgunset, Lund, and Sunde.[52] The p-bromo FAPhe esters studied included C14:0, C16:0, C16:1ω7, C18:1ω9, C18:2ω6, C18:3, C20:3, and C20:4ω6. A synthetic mixture was separated using a Supelcosil LC-18 5-μm column and an isocratic ternary mobile phase of ACN:MeOH:H_2O. The detection limits for the p-bromo FAPhes studied ranged from 10 to 50 pg at 254 nm.

RPLC has been successfully employed for the analysis of LCFAs as their p-bromo FAPhes in biological fluids. The determination of the C16-C20 fatty acids as their p-bromo FAPhes in human blood plasma was described by D'Amboise and Gendreau[53] and utilizes isocratic elution with a ternary mobile phase of ACN:MeOH:H_2O and an RP C8 10 μm support. The p-bromo FAPhes were monitored with UV at 254 nm. The method was applicable at the 30- to 300 ng level for the p-bromo FAPhes studied and a 30 ng detection limit was reported. Although precision was good (3% rsd), these authors reported that the extraction procedure employed was unsatisfactory and the analysis time of 3 h which includes derivatization was too long. A chromatogram of the blood plasma FAPhes analyzed by this method is shown superimposed on a standard chromatogram in Figure 4. Adequate resolution of the FAPhes of C18:3ω6 (ECL 12), C20:4ω6 (ECL 12), C16:1ω7 (ECL 14), and C18:2ω6 (ECL 14) FAPhes is shown as well as baseline resolution of C16:0, C18:1ω9, and C18:0.

The determination of the fatty acid composition of tissues and the investigation of fatty acid metabolism was reported in 1986 by Korte, Chien, and Casey.[54] The p-bromo FAPhes derived from human uterine decidua vera or human endometrial stromal cells were separated on a μ-Bondapak C_{18} bonded support with a $MeOH:H_2O$ isocratic mobile phase. The commonly occurring C10 to C22 fatty acids found in mammalian tissue were separated in 25 min. Detection limits ranged from 25 to 60 ng for the p-bromo FAPhes.

In other applications, the results obtained by RPLC and GLC were compared for the determination of the fatty acid composition of soybean oil by Jaselskis, Stemm, and Johnston.[55] With RPLC the p-bromo FAPhes of C16:0, C18:0, C18:1 *cis* ω9, C18:1 *trans* ω9, C18:2 *cis,cis* ω6, C18:2 *trans,trans* ω6, and C18:3 *cis,cis,cis* ω6 were separated on a Hibar II octyl 10 μm stationary phase with concave gradient elution from 70 to 100% $ACN:H_2O$. The low limit of detection was reported at 10 μg/ml and baseline resolution of all p-bromo FAPhes required 50 min. Quantitation was performed by comparison to standard curves (10 to 60 μg/ml) with results comparable to those obtained by GLC.

In 1987, Osterroht[56] compared p-bromo and p-phenphenacyl esters (Figure 3A to C) for the determination of 22 fatty acids from C3 to C22 including various saturated, monoolefinic, and polyunsaturates derived from particulate matter from the Baltic Sea. UV detection limits at 288 nm of 5 pmol of FAPhes were reported. The p-bromo FAPhes were separated with excellent resolution using a C_8 stationary phase and gradient elution with $ACN:H_2O$. The phenyl FAPhes were separated on a C_{18} column with a $MeOH:H_2O$ and $ACN:H_2O$ combination gradient elution. Although both analyses required approximately 90 min to complete, the resolution of the p-bromo FAPhes was better than the p-phen FAPhes.

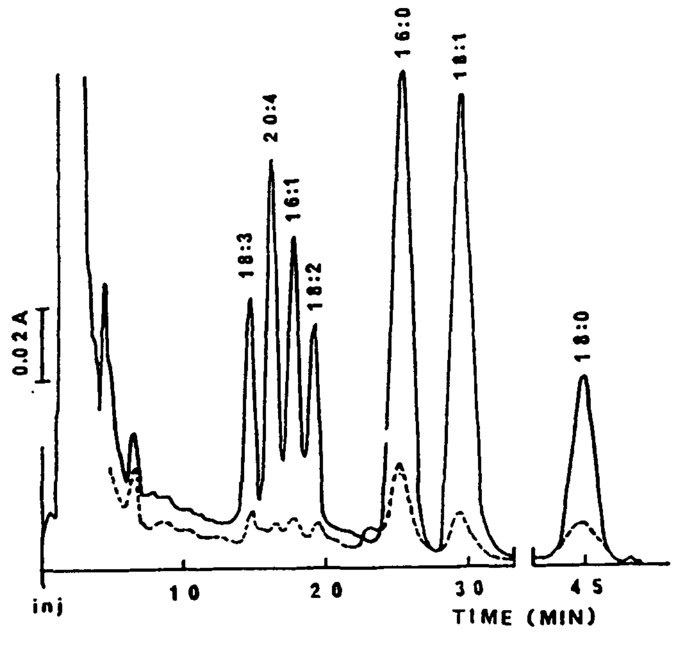

FIGURE 4. Chromatogram of fatty acid phenacyl esters. Solid line: synthetic mixtures; dashed line: human blood plasma UV detection: 254 nm. (From D'Amboise, M. and Gendreau, M., *Anal. Lett.*, 12(B4), 381, 1979.)

C. 2-Naphthacyl Esters

Cooper and Anders[57] first reported the application of HPLC to separate fatty acid 2-naphthacyl esters (FANpes, Figure 3D). A strongly absorbing UV derivative (248 nm) was desired with good chromatographic properties for the analysis of the C20 PUFAs which are prostaglandin precursors. The feasibility of the method was illustrated with synthetic mixes at nanogram sensitivity.

Jordi[58] compared the HPLC behavior of the various C2 to C24 fatty acid derivatives. The p-bromo FAPhes, p-nitrophenacyl esters, p-chlorophenacyl esters, or 2-FANpes were separated on a μ-Bondapak C_{18} column in tandem with a Fatty Acid Analysis column and eluted with $ACN:H_2O$ gradients. Elution order of all the phenacyl ester derivatives was the same; however, the retention varied. Each chromatographic analysis required approximately 3 h. Use of the 2-naphthacyl group as a chromophore gave large increases in sensitivity compared to the other esters and detection limits approached 2 pg for the FANpes.

Separation of FAPhes and naphthacyl fatty acid esters (FANpes) derived from several seed oils (coconut, corn, cottonseed, olive, and safflower) was described by Wood and Lee.[59] A C_{18} 5-μm stationary phase and an $ACN:H_2O$ linear gradient was employed over 45 min to achieve baseline resolution of 17 different C14 to C20 saturated, monoenoic, polyenoic, and geometrical FAPhes or FANpes with nanogram sensitivity. The FAPhes were found to be more suitable for routine analysis than the FANpes because they were more soluble in the mobile phase than the corresponding FANpes; thus their retention times are shorter. Also, the lower molecular weight esters (FAPhes) exhibited better resolution than their heavier counterparts (FANpes).

D. Fatty Acid Phenylhydrazine Esters

The use of both UV (230 nm) and visible detection (400 nm) for the monitoring of the 2-nitrophenylhydrazide derivatives (Figure 3E) of free fatty acids in serum was reported by Miwa et al. in 1986 and 1987.[60-62] The biologically important C14 to C22 fatty acids were derivatized to their 2-nitrophenylhydrazide esters (FANPhes) and were isocratically separated with $ACN:H_2O$ on a chemically bonded octyl stationary phase with 5-μm particles. Close to baseline resolution was observed and detection limits ranged from 400 fmol to 1 pmol for visible detection (400 nm) and from 100 to 200 fmol for UV detection (230 nm). Visible detection yielded better selectivity of the compounds. Using this method, precise quantitation of FFAs in 25 μl of serum was accomplished in 15 min. Analytical recoveries were 98.3 to 103.4%.

E. Fatty Acid Methoxyanilide Esters

The quantitative conversion of saturated and unsaturated fatty acids to their p-methoxyanilide derivatives to enhance their ultraviolet detectability was reported by Hoffman and Liao.[63] The p-methoxyanilide esters of fatty acid (FAMOAs, Figure 3F) were separated with gradient elution and a 10-μm μ-Bondapak C_{18} stationary phase. Detection limits of 5 ng were reported with the molar absorptivities of the FAMOAs to be 2.43×10^4 l mol^{-1}-cm^{-1} at 254 nm. Both ACN:H_2O and MeOH:H_2O hyberbolic gradients were used to separate FAMOAs of the even numbered saturated C14 to C24, including those of C16:1ω7, C18:1ω9, C18:2ω6, C18:3ω6, C20:4ω6, C22:1:ω11, C22:6:ω3, and C24:1. Despite the excellent detector response, many pairs of the FAMOAs were poorly resolved or not resolved at all on this system.

F. Fatty Acid Pentafluorobenzyl Esters

Netting and Duffield[64-65] reported on the development of procedures for the semipreparative separation of the pentafluorobenzyl esters (FAPFBs) of fatty acids from barley leaf extract. UV detection at 254 nm was used. Procedures have also been developed to separate FAPFBs by the number of double bonds. For this separation a silica column was used followed by separation on a semipreparative μ-Bondapak C_{18} reversed phase column. The two systems are complementary; a critical pair not resolved on one system can be completely separated on the other system. Thus, the isolation of FAPFBs derived from complex mixtures can be achieved in two sequential injections. The excellent resolution achieved with the coupled procedures permitted complete separation of *cis*-9, 10-methylene hexadecaenoate FAPFB and heptadeca-10-enoate FAPFB. The total analysis time was 40 min. Sensitivity was sacrificed with this procedure since a large enough sample must be injected to be detected in the RPLC analysis.

G. Fatty Acid M-Methoxyacetophenone Derivatives

The efficiency of HPLC for the determination of the free fatty acids on the fingertips was investigated by Bussell, Gross, and Miller.[66] The m-methoxyacetophenone derivatives (Figure 3B) of the skin fatty acids were detected at 254 nm following separation on 2 μ-Bondapak C_{18} columns in series. Elution was with a ACN:H_2O convex gradient and required 3 h.

IV. ANALYSIS OF FATTY ACID ESTERS USING A FLUORESCENCE DETECTOR

For molecules that fluoresce naturally or can be derivatized with fluorescent tags, the fluorescence detector, which is a solute property detector, affords a greatly increased sensitivity and selectivity over the absorbance detector. In this detector, radiation released by molecules, which have been excited by electromagnetic radiation, is measured. The radiation, which is emitted rapidly after excitation at a longer wavelength, is the type of photoluminescence called fluorescence. The great advantages of the fluorescence detector are its sensitivity and selectivity. The major disadvantage is that few compounds fluoresce under normal HPLC conditions. However, a large number of fluorophores can be bonded to nonfluorescent molecules, either by pre- or post-column derivatization. These derivatization procedures greatly extend the use of the fluorescence detectors, especially for fatty acids of all types. The excitation wavelength is usually lower than 400 nm and the emission wavelength is from 400 to 600 nm. The linear dynamic range and the minimum detector response varies with the solute and the derivatization reagent.

The fluorometric detector is a very sensitive and selective detector for LCFAs for which fluorescent derivatives can be made. Fluorescent derivatives of many LCFAs have been prepared and fluorescence detection with prechromatographic derivatization is particularly attractive for the HPLC analysis of LCFAs.

In many instances fluorescence detection is 100-fold more sensitive than UV detection. Because of its high sensitivity fluorescence detection is particularly suited for trace analysis of LCFAs in biological samples, foods, pharmaceuticals, and environmental samples. General chemical structures for some fluorescent LCFA derivatives are shown in Figure 5.

A. Fatty Acid Anthryl Esters

1. Fatty Acid Methyl Anthryl Esters

9-Diazomethyl anthracene (9-ADAM), which was first introduced by Barker et al.,[67] reacts with carboxyl groups to give an ester derivative which can be used as either a fluorescence or ultraviolet label for HPLC. The limit of detection by UV spectroscopy (254 nm) was 150 pg/μl and by fluorescence (360 nm excitation, 440 nm emission) approximately 15 pg/μl for C10 to C20 even numbered saturated 9-anthryl methyl fatty acid esters (AMFAs, Figure 5A). The procedure was optimized to ensure reproducible reaction times and quantitative results.

Korte[68] compared the use of 9-(chloromethyl) anthracene to that of 9-anthradiazomethane for the preparation of 9-AMFAs. The 9-chloromethylazomethane is more easily prepared than the 9-anthradiazomethane reagent and there is less chance for rearrangement of unsaturated compounds since milder reaction conditions are used. The fluorescent characteristics of the compounds were comparable.

A method for the determination of eicosapentaenoic acid (C20:5ω3) with a standard mix of C18 and C20 LCFAs acids labeled with ADAM was reported by Ichinose et al.[69] The procedure has a low limit of detection of 300 pg for C20:5ω3 and the coefficient of variation was 2.1%. The analysis conditions were similar to those of Shimomura et al.[70] except that only one Zorbax column was used at 50°C.

The LCFAs in human sera and plasma lipids have been

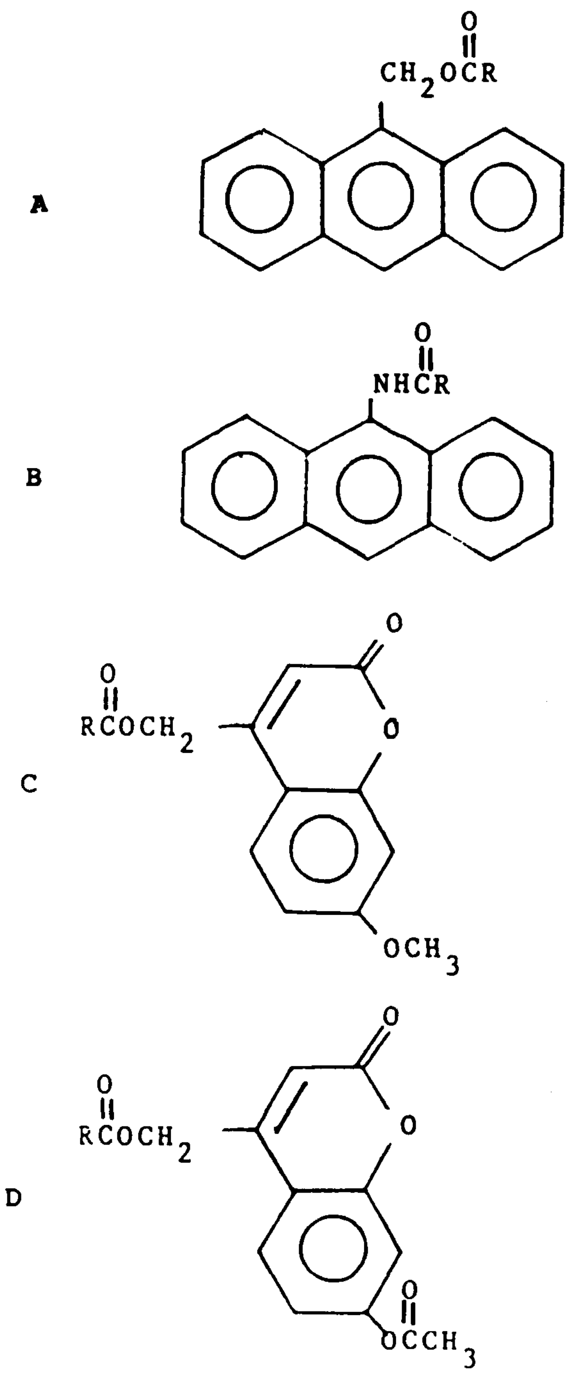

FIGURE 5. General chemical structures of long-chain fatty acid fluorescent derivatives. (A) Methylanthryl fatty acid ester; (B) 9-aminophenanthryl fatty acid ester; (C) 4-bromo-7-*m*-ethoxycourin; (D) 4-bromomethyl-7-acetoxycoumarin.

determined by many researchers as their AMFAs. Although the derivatization procedures required incubation times of more than 3 h, detection limits in the picogram range are routinely achieved.

Shimomura et al.[70] optimized the conditions for the HPLC analysis of the fatty acid composition of human serum lipids by HPLC. In the microanalysis, fatty acids of serum lipids were derivatized with 9-ADAM to the AMFAs and were detected with a fluorescence detector (365 nm excitation, 412 emission). Twelve saturated and unsaturated fatty acids C8 to C22 were determined as free and total fatty acid fractions in human serum lipids with detection limits of 5 pmol.

Prior to chromatographic analysis, the solutions of 9-ADAM and the fatty acid mixture were allowed to incubate at room temperature for 3 h to ensure a complete reaction. The resulting AMFAs were separated at 60°C on 2 Zorbax ODS (C_{18}) columns, linked in series, with an isocratic MeOH:H_2O mobile phase. All the AMFAs were baseline resolved in 60 min with the exception of C18:3ω6 (ECL 12), C14:0 (ECL 14), C22:6ω3 (ECL 10), C16:1ω7 (ECL 14), and C10:4ω6 (ECL 12) which appear as distinguishable peaks; however, there was not baseline resolution. The procedure was successfully used for the determination of serum lipids of normal subjects and diabetic patients. Diabetics showed lower levels of the individual fatty acids, C18:2ω6 and C20:4ω6. In 1986[71] this procedure was expanded to evaluate serum lipid content in patients with angina pectoris. Decreases in the C20:5ω3 fatty acid and increases in the C20:4ω6 fatty acid in serum lipids were observed for these patients.

A micro analysis of 13 fatty acids in plasma as their 9-AMFAs was reported by Hatsumi, Kimata, and Koshichiro.[72] Fluorescent detection was monitored at 418 nm with excitation at 365 nm. A 5 μm C_8 stationary phase maintained at 40°C and a step gradient quaternary mobile phase of MeOH:ACN:1,4-dioxane:H_2O gave baseline resolution of the 9-AMFAs derivatives of C20:5ω3, C18:3ω6, C14:0, C22:6ω3, C16:1ω7, C20:4ω6, C18:2ω6, C20:3, C16:0, C18:1ω9, C22:0, and C18:0. The use of an ODS minicolumn for pretreatment of plasma gave a more accurate determination of the fatty acid content than the chloroform extraction traditionally used. The method was successfully applied to plasma samples of human, dog, rabbit, guinea pig, and rat.

Free fatty acids were analyzed as their AMFA derivatives in human plasma by Baty, Pazouki, and Dolphin[73] for application to normal and diabetic subjects. Thirteen fatty acid ADAM esters were resolved on a Spherisorb 3 μm C_8 column with ACN:H_2O gradient elution. The eluent was monitored at 420 nm (excitation 360 nm) and detection limits of 50 ng were observed. Derivatives of the following 6 acids could not be resolved with the 3 different C_{18} 5 μm stationary phases tested: C12:0 (ECL 12), C18:3ω6 (ECL 12), C20:4ω6 (ECL 12), C14:0 (ECL 14), C16:1 (ECL 14), and C18:2ω6 (ECL 14). However, on a 3 μm C_8 support satisfactory resolution was achieved in 40 min. A chromatogram of plasma fatty acids as their AMFAs is shown in Figure 6 and illustrates the excellent resolution obtained with the C_8 stationary phase.

2. 9-Aminophenanthryl Esters

The use of 9-aminophenanthrene (9-AP, Figure 5B) as a

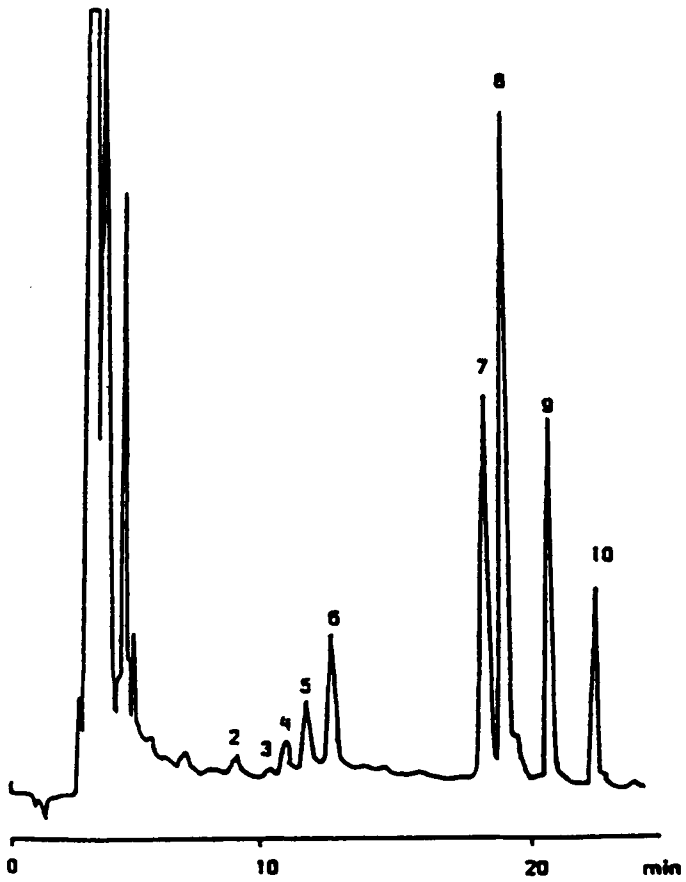

FIGURE 6. Chromatogram of plasma-free fatty acids as their methyldanthryl esters in plasma from a healthy volunteer. Stationary phase: spherisorb C_8 (250 × 4.6 mm I.D.); 3 μm particle size. Mobile phase: $ACN{:}H_2O$ step gradient. Fluorescence detection: excitation 360 nm; emission 420 nm; peak identification for AMFAs esters: (2) 18:3 (3) C20:4ω6 (4) C14:0 (5) 16:1 (6) C18:2ω6 (7) C16:0 (8) C18:1 (9) C17:0 (internal standard) (10) C18:0. (From Baty, J. D., Pazouki, S., and Dolphin, J., *J. Chromatogr.*, 395, 403, 1987.)

fluorescence labelling reagent for the determination of free fatty acids in human serum was reported by Ikeda et al.[74] The 9-AP tagged free fatty acids exhibit strong fluorescent behavior with a λ_{max} at 303 nm for excitation and 376 nm for emission. Detection limits were 10 pmol for the C14:0, C16:0, C16:1ω7, C18:1ω7, and C18:2ω6 9-AP esters, and 15 pmol for C18:0 and C20:4ω6 9-AP esters. A μ-Bondapak C_{18} column maintained at 40°C and a gradient elution with $MeOH{:}ACN{:}H_2O$ was utilized for the chromatographic analysis. This procedure was applied clinically for the quantitation of free fatty acids (as opposed to the lipid fatty acids) in serum.

B. Fatty Acid Coumarin Esters

1. Methoxycoumarin Esters

The fluorescence labeling of fatty acids with 4-bromo methyl-7-methoxycoumarin (Br-Mmc, Figure 5C) for monocarboxylic acids was introduced by Dunges in 1977.[75] All unsubstituted and most substituted aliphatic monocarboxylic acids yielded Mmc derivatives with strong blue fluorescence (excitation 328 nm, emission 360 nm).

In 1978, Zelinski and Huber[76] extended the work of Dunges to HPLC with a normal phase separation of a synthetic mix of fatty acid Mmc esters. The first reversed phase procedures were reported in 1984 by Hayashi et al.[77] They monitored the biodegradation of fatty acid calcium salts in river water by determining trace levels of fatty acid metal salts as their Mmc derivatives. Detector response of the C8 to C18 even-numbered straight chain salts was linear for 1 to 50 μg/ml of sample and the detection limit was 7 pmol at 380 nm (328 nm excitation). The Mmc esters were separated on a 3 μm C_8 stationary phase and were eluted isocratically with 80:20 $ACN{:}H_2O$. Baseline resolution of the C8 to C18 Mmc esters was observed.

A promising method for the trace analysis of FFAs was proposed by McGuffin and Zare who used laser induced fluorescence detection with micro column liquid chromatography.[78] They derivatized the FAs (C1 to C21) with 4-bromomethyl-7-methoxycoumarin. The chromatographic column, which was fabricated from fused-silica tubing, was 0.20 mm I.D. and 1.2 m in length. The mobile phase consisted of two solvent systems. System A was composed of 62.5% MeOH, 22.5% ACN, and 25% H_2O. System B contained 42.5% MeOH, 22.5% ACN, and 35% ethylacetate. Isocratic elution of A containing 10% B was carried out for 40 min followed by a linear gradient to 85% B in 170 min. The flow rate was 1.2 μl/min and the temperature 40°C. The excitation of the laser fluorescence was 325 nm and the emission wavelength was 430 nm. Using these conditions the standards of the C1 to C22 FAs were separated as well as the FAs in saponified peanut and sesame oils. The advantages of this method are high sensitivity (femtograms of analyte could be detected), broad linearity range, good resolution of the majority of the acids, and small sample volumes (0.06 nl^2 to 0.06 μl^2). The major limitations are the time of analyses and the fact that this method is limited to trace analyses and is not suitable for scale up to preparative work.

2. Acetoxycoumarin Esters

In 1982, Tsuchiya et al.[79] introduced the use of 4-bromomethyl-7-acetoxycoumarin as a fluorescent derivatizing reagent for LCFAs (ACs, Figure 5D). Increased detector sensitivity compared to analogous Mmc derivatives, was observed and detection limits were in the low femtomole range for even numbered linear saturated C6 to C20 acetoxycoumarin LCFA derivatives.

The application of their procedure to the quantitation of the saturated and unsaturated free fatty acids (C16:0-C20:4ω6) in human plasma from normal and diabetic patients was reported in 1980.[80]

Successful separations of Acs were achieved with a continuous ACN:MeOH gradient using a 10 μm C_{18} stationary phase maintained at 40°C. The fluorescence was detected at 460 nm emission with excitation at 365 nm. The low level of detection was 20 fmol.

Kelley, O'Hara, and Kelley[81] reported using the procedure of Tsuchiya et al.[79] with a mobile phase modification to determine femtomolar quantities of endogenous carboxylic acids. These carboxylic acids included arachidonic acid metabolites in plasma obtained from rats. Utilizing a convex gradient of ACN:MeOH:H_2O (35:35:30) to MeOH:H_2O (90:10) over 90 min, adequate resolution of the eicosanoid Acs with detection limits of 50 fmol were obtained.

C. Fatty Acid 5-Dimethyl-Amino-1-Naphthalene Sulfonyl Ethanolamine Derivatives

The use of 5-dimethyl-amino-1-naphthalene sulfonyl (DNS) ethanolamine derivatives for the determination of saturated and unsaturated LCFAs (C16:0 to C20:4ω6) was reported in 1984 by Ryan and Honeyman.[82] Fluorescence detection at 420 nm emission (360 nm excitation) was utilized with a C_{18} 5 μm stationary phase and a MeOH:ACN:aqueous $AgNO_3$ (45:45:10) mobile phase. Baseline resolution was achieved in 36 min.

V. MOVING WIRE DETECTORS

Transport detectors, which are solute property detectors, are versatile detectors because any solvent or mixtures of solvents can be used as the mobile phase. One requirement of this detector is that the solvent or mixtures of solvents must be volatile. On the other hand, solutes to be detected cannot be volatile or else the solutes will be lost during the evaporation of the mobile phase. The main disadvantage of this detector is its relatively low sensitivity. In addition, for highly oxygenated compounds the sensitivity is extremely low. However high boiling hydrocarbons or the long-chain fatty acids have good sensitivity. Thus lipids and oleochemicals which do not absorb significantly in the UV region can be detected using transport flame ionization detectors (FID). Flame ionization detectors are particularly useful when gradient elution is required and complicated derivatization procedures are not acceptable. The transport FID utilized by Aizetmuller[83] was operated by first pyrolyzing the organic nonvolatiles which remain on the moving carrier after the evaporation of solvent. The system then transforms all the carbon remaining on the wire into methane which is then detected by the FID. In this manner, a signal is obtained which is directly proportional to the amount of carbon in the substance deposited on the wire and quantitative results can be obtained.

Numerous applications of transport flame ionization detection of triglyceride and fatty acid analyses are described.[84] The separation of FAMEs by degree of unsaturation was illustrated with the C18 fatty acid methyl esters, methyl stearate, methyl oleate, and methyl linoleate. Also, the resolution of C18 geometric isomers, methyl oleate (C18:1 *cis* ω9), and methyl elaidate (C18:1 *trans* ω9) was shown. As an industrial application, a fingerprint chromatogram of an ethoxylated fatty acid was accomplished. These types of compounds are commonly used in the detergent and cosmetic industries as emulsifiers.

Although moving wire detectors are destructive and not as sensitive as UV detectors, they do give a similar response to a wide range of compounds. In addition, they are universal. UV detectors cannot be used for quantitation if the individual species in the mixture have different molar absorptivities.

Ozcimder and Hammers[84] examined the extent to which an HPLC fractionation using a wire detector eliminated potential critical pair problems. Cod liver oil was chosen as the sample because of its complex LCFA profile (C14-C22 saturates and unsaturates). The fish oil was prefractionated by RPLC or Ag-HPLC. The RPLC system utilized Lichrosorb RP-18 (250 × 4.6 mm I.D.) stationary phase with an ACN mobile phase. The Ag-HPLC system used 5% (ω/ω) Ag (I) on Partisil 10 (250 × 4.6 mm I.D.) with a 0.4% ACN in n-hexane mobile phase. The RPLC procedure was a "promising" prefractionation method for complex FAME mixtures. The fractions collected from the LC analysis contained most of the critical pairs which could not be resolved on polar GLC columns (e.g., 18:4ω6, 22:1ω11). Also, the isolation of geometrical isomers of monenoic LCFAs of more than 15 carbons further facilitated the GLC analysis of the RPLC fractions.

Ag-HPLC appeared to be less suitable as a prefractionation method for complex FAME mixtures due to the poor resolution of the saturated, mono-, and dienoic esters, C16:0, C18:0, C18:1, C18:2, C20:1, and C22:1 which are generally present in most vegetable and fish oil lipids. On the other hand, Ag-HPLC appears to be a good method for the fractionation of unsaturated ester species with three to six double bonds.

VI. FUTURE PROSPECTS

The detection methods discussed in this review are the primary ones used at present in the HPLC analyses of LCFAs. However descriptions of light scattering detection methods have been published by Guichon and co-workers[85-87] who developed an evaporative light scattering detector and Novotny's group[88] who described a precipitative light-scattering detector.

The major problem in the HPLC analysis of LCFAs remains the need for good, sensitive detection methods for very small amounts of LCFAs. For trace analyses, derivatization and detection by laser-induced fluorescence appears to show great promise.[78,79] An indirect fluorescence detector is also intriguing and may prove to be useful in these analyses. Another possible detection system is derivatization with an electroactive species and detection with an electrochemical detector. These detectors when originally introduced, were unstable and difficult to operate. Now, however, with improved design they are being used routinely in many laboratories with excellent results. The advantages of this detection system, as with the fluorescence detector, are the extremely high sensitivity and selectivity. However, derivatization reagents must be found which will be electroactive and which will solubilize the LCFAs in the aqueous-organic mobile phase which is required with this detector.

The most desirable solution to the problems of both detection and characterization of LCFAs, is to interface HPLC with mass spectrometry (MS). With the recent development of various ion sources in MS, HPLC-MS analyses may be realized which provide the sensitivity, selectivity, and LCFA characterization desired. Fourier Transform-infrared spectroscopy (FT-IR) may provide another detection system since IR can be used with gradient elution for the analysis of triacylglycerols which contain three LCFA moieties.[90] By monitoring the effluent at the wavelength of the carbonyl stretch of free fatty acids or their esters, the sensitivity desired for the analysis of trace amounts of LCFAs may be achieved. Recently the flame ionization detector has also shown potential in solving HPLC detection problems for the triacylglycerols.[91] Thus, these detectors may be useful for the HPLC analyses of LCFAs.

For preparative analyses, the refractive index detector is adequate since low detection limits are not a problem. However, the inability to use gradient elution is a limitation in separating large quantities of structurally close LCFAs. Therefore other methods of detection are being sought. Absorption detectors with large cell volumes are also used. However, in general either low end absorption wavelengths must be used, which limits the choice of solvents, or the LCFAs must be derivatized, which is undesirable for preparative work.

If there is enough demand, new stationary phases specifically designed to separate LCFAs will be marketed. The new graphitized carbon packings may become the packings of choice because electron donor-acceptor interactions apparently govern retention with these packings.[92] Thus for the unsaturated LCFAs the number and position of the double bonds can play an important role in retention behavior.

Although it is not stringently classified as an HPLC technique, a separation method that can be of great importance in the future for separating trace amounts of LCFAs is capillary zone electrophoresis (CZE).[93] This technique has the potential for increasing tremendously the sensitivity and resolution of closely related small molecules as well as macrobiomolecules. For example, ultramicro analyses of attomoles of amino acids has recently been reported.[94]

Another possibility for preparative work is supercritical fluid chromatography SFC. This technique is attractive because of the ease of removing the mobile phase from large quantities of purified solutes. In addition this method has potential for trace analyses. Recently, it was reported that SFC interfaced with electron impact MS was used to analyze successfully mixtures of triacylglycerols in butter.[95]

In conclusion, the main problems in the HPLC analysis of LCFAs are still in the detection systems and in the separation of critical pairs. However, in the next few years, with the development of new packing materials, computerized techniques to optimize separations and better detection systems, it is predicted that analyses will be achieved with very high sensitivity and resolution for trace amounts of LCFAs. In addition we will be able to obtain high throughput and high purity yields on the preparative scale.

Within the next decade, clinical, analytical, and industrial laboratories will increase efforts to separate LCFAs and their derivatives from natural sources and polymeric mixtures by RPLC. Improvements in column technology will yield analytical and preparative stationary supports with greater resolving power (i.e., smaller particles, increased carbon loads). Improvements in preparative scale columns will eventually lead to routine use of liquid chromatographic techniques for large scale industrial separations.

Detection and identification of LCFAs in biological fluids will be more convenient and accurate with innovations and advances in detector technology, especially reaction detectors, Fourier transform techniques and hyphenated chromatographic methods (i.e., LC-MS, LC-NMR, LC-FT-IR). Time consuming prechromatographic derivatization procedures will then not be necessary to achieve low levels of detection.

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