*papers and notes on methodology*

# Separation and quantitation of free fatty acids and fatty acid methyl esters by reverse phase high pressure liquid chromatography

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**Abstract** Reverse phase high pressure liquid chromatography (HPLC) on octadecylsilyl columns separates mixtures of either free fatty acids or fatty acid methyl esters prepared from mammalian tissue phospholipids. Acetonitrile–water mixtures are used for the elution of esters. Aqueous phosphoric acid is substituted for water for the separation of the free acids. Unsaturated compounds are detected and quantitated by their absorption at 192 nm. Saturates are detected better at 205 nm. The order of elution of fatty acids in complex mixtures varies as a function of acetonitrile concentration. At any given concentration, some compounds overlap. However, by varying the solvent strength, any fatty acid of interest can be resolved including many geometrical and positional isomers. Methyl esters prefractionated according to unsaturation by argentation thin-layer chromatography (TLC) are rapidly and completely separated by elution with $CH_3CN$ alone. Argentation TLC–reverse phase HPLC can be used as an analytical as well as a preparative procedure. Octylsilyl columns are used for rapid resolution and improved detection of minor or low ultraviolet-absorbing components in the fractions. For example, monoenoic fatty acids with up to 32 carbons have been detected in bovine brain glycerophospholipids. Specific radioactivities of $^3H$- and $^{14}C$-labeled fatty acids and the distribution of radioactivity among acyl groups from complex lipids are measured. The method is not recommended for complete compositional analysis, but is useful for determinations of specific radioactivities during studies on turnover and metabolic conversions of labeled fatty acids.—**Aveldano, M. I., M. VanRollins, and L. A. Horrocks.** Separation and quantitation of free fatty acids and fatty acid methyl esters by reverse phase high pressure liquid chromatography. *J. Lipid Res.* 1983. **24:** 83–93.

**Supplementary key words** fatty acid positional isomers • fatty acid geometrical isomers • polyunsaturated fatty acids • very long chain fatty acids

Gas–liquid chromatography (GLC) coupled to a flame ionization detector is the technique of choice for the routine quantitation of fatty acids, usually after their conversion to methyl esters. Fatty acid analyses, including positional and geometrical isomers, can be done with high resolution and sensitivity on capillary columns. The determination of specific radioactivities of labeled fatty acids by radio–gas–liquid chromatography has problems of low sample capacities of GLC packings, low counting efficiencies, and/or variable recoveries of radioactive methyl esters from the gas phase. These factors affect the accuracy and precision of radioactivity measurements, especially for compounds with low specific radioactivities. Moreover, although GLC can be used preparatively, thermal degradation or structural modifications of highly unsaturated fatty acids are always a possibility.

Simple fatty acids or methyl ester mixtures have been resolved by reverse phase high pressure liquid chromatography (HPLC) using acetonitrile–water (1) or methanol–water (2, 3). The inherently low absorptivity of fatty acids in the ultraviolet has led to the use of aromatic derivatives such as p-methoxyanilides (4), or

Abbreviations: GLC, gas–liquid chromatography; HPLC, high pressure liquid chromatography; TLC, thin-layer chromatography; FFA, free fatty acids; FAME, fatty acid methyl esters. Fatty acids are abbreviated by the convention, number of carbon atoms:number of double bonds.

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2-naphthacyl- (5), phenacyl- (6, 7), p-bromophenacyl- (8), and methoxyphenacyl- (9) esters to enhance detectability. However, mixtures containing long-chain saturated to poiyenoic fatty acids as they occur in lipids from mammalian tissues have not been studied. In this report underivatized fatty acids or methyl esters from brain phospholipids are separated by reverse phase HPLC. Unsaturated fatty acids are quantitated by their absorbance at 192 nm, which has proven useful for the detection of prostaglandins and their metabolites (10, 11). Specific radioactivities of biologically labeled fatty acids are determined. Argentation thin-layer chromatography (TLC)–reverse phase HPLC is used for complete resolution of methyl esters, including some positional and geometrical isomers.

## MATERIALS AND METHODS

### Materials and instruments

All organic solvents and phosphoric acid were of HPLC quality and were purchased from Burdick and Jackson Laboratories, Muskegon, MI, MCB Manufacturing Chemists, Cincinnati, OH, or Fisher Scientific Co., Pittsburgh, PA. Water was distilled and further purified by adsorption, deionization, and filtration using a Milli-Q system plus an Organex-Q cartridge (Millipore Co., Bedford, MA). Any remaining UV absorbing materials were removed by photooxidation using an Organic-Pure water purifier (Barnstead, Boston, MA). Aqueous and organic solvents were filtered through 0.2 and 0.5 $\mu$m Millipore filters, respectively, and degassed prior to use. Fatty acid standards were from Nu-Chek-Prep, Elysian, MN. [5,6,8,9,11,12,14,15-$^3$H(N)]-Arachidonic acid [20:4(n-6)] was from New England Nuclear, Boston, MA. [1-$^{14}$C]Adrenic acid [22:4(n-6)], as well as isomers of 20:3 and 20:4 were generously provided by Dr. H. W. Sprecher, Columbus, OH. Isomers of 18:2 were provided by Dr. E. A. Emken, Peoria, IL. The columns, Zorbax ODS, 5–6 $\mu$m particle size, 25 cm × 0.46 cm (I.D.) and 15 cm × 0.46 cm (I.D.) or Zorbax C-8, 25 cm × 0.46 cm (I.D.), were from DuPont Company, Wilmington, DE. Column temperatures were controlled using a block heater (Jones Chromatography, Columbus, OH). Chromatography was done with a 322 M system and a Model 421 microprocessor from Beckman Instruments, Berkeley, CA. The detectors used were a Model LC-75, Perkin-Elmer, Norwalk, CT, and a Model 305, BioRad, Richmond, CA. Integration was performed with software and A/D interface purchased from Nelson Analytical, Cupertino, CA, on an HP-85 desk top computer (Hewlett-Packard, Palo Alto, CA). Fractions were collected in plastic minivials using a Model 328 fraction collector from Instrumentation Specialties, Lincoln, NE, and counted in an LS 7000 liquid scintillation counter (Beckman Instruments) after adding Aquasol-2 (New England Nuclear) or Ready-Solv-EP (Beckman) scintillation cocktails. A Model HP radioactive flow detector (Radiomatic Instruments & Chemical Co., Tampa, FL) was also used. Counting efficiencies were determined using [$^3$H] or [$^{14}$C]toluene standards (New England Nuclear).

### Preparation of free fatty acids and methyl esters

Phospholipid fractions were obtained from brain lipid extracts by silicic acid column chromatography (12). Free fatty acids were produced by alkaline or acid hydrolysis. Alkaline hydrolysis was done at room temperature by stirring the samples with 1 ml of 0.5 N NaOH in methanol–water 90:10 overnight under $N_2$. This was followed by acidification and two partitions into 3 ml of hexane. The hexane phase was washed twice with 3 ml of water and the solvent was evaporated under $N_2$. The free fatty acids were stored in hexane and dissolved in methanol just prior to injection. Acid hydrolysis was done by a new method with 0.5 N HCl in acetonitrile–water 9:1 at 100°C for 45 min.[3]

Methanolysis was catalyzed by acid (1 ml of 0.7 N HCl in methanol, 30 min, 100°C), alkali (1 ml of 0.5 N NaOH in methanol, 30 min, room temperature) or $BF_3$ (13). After adding 1 ml of water, methyl esters were extracted into hexane (3 × 3 ml) and purified by TLC using hexane–ethyl ether 95:5. The esters were visualized with 0.05% 2′,7′-dichlorofluorescein in methanol–water 50:50 (14) and eluted and washed using the solvents described by Arvidson (15).

Methyl esters prepared by alkaline methanolysis of 400 mg of a total lipid extract from bovine brain were separated according to unsaturation on a >1000 $\mu$m silica gel G plate containing 20% $AgNO_3$ by wt. Polyenoic methyl ester fractions (6 to 3 double bonds) were resolved with chloroform–methanol–water 80:20:2. The dienes to saturates, located in the solvent front, were eluted and then separated on a similarly prepared TLC plate by using chloroform–methanol 99:1. The fractions were located under ultraviolet light after spraying with dichlorofluorescein and eluted as described before. Esters were further purified by TLC on silica gel G with hexane–ethyl ether 95:5, located with the aid of standards, eluted as above, and partitioned with water. Methyl esters in the fractions were identified and quantitated by GLC using a 428 gas chromatograph (Packard, Downers Grove, IL) equipped with a glass column containing 10% CS-TO on Chrom WAW, 100–200 mesh (Alltech Associates, Arlington Heights, IL).

[3] Aveldano, M. I., and L. A. Horrocks. Unpublished results.

Column temperature was 195°C. In addition, unsaturated fatty acids were identified by hydrogenation. Platinum oxide was added to a solution of the methyl esters in 0.5 ml of methanol, and hydrogen was gently bubbled for 2 min. After addition of 1 ml of water, the esters were recovered by extraction with 3 ml of hexane.

### HPLC of fatty acids and methyl esters

Free fatty acids, dissolved in methanol, were separated using acetonitrile and aqueous phosphoric acid (pH 2, about 30 mM). A concentration of 8 mM $H_3PO_4$ (pH 2.5) was also sufficient to suppress ionization of fatty acids with maintenance of peak shape. Methyl esters, dissolved in acetonitrile, were resolved with acetonitrile–water mixtures. Injection volume was 20 μl, column temperature was 35°C, and flow rates were 0.5 to 2 ml/min. Radioactivity in $^3H$- and $^{14}C$-labeled samples was counted by collection of 1-ml fractions in counting vials with addition of 4 ml of liquid scintillation cocktail. Counting of $^{14}C$-labeled samples with a radioactive flow detector was done using a 1:3 eluent:cocktail ratio. Counting efficiencies were determined using [$^3H$] or [$^{14}C$]toluene standards (New England Nuclear). Calibrations for mass measurements were done as detailed in Tables 2 and 4. Elaidic acid, [*trans*-18:1(n-9)] either as the free acid or as the methyl ester, as well as methyl 19:1 (n-10), were used as internal standards (50 nmol/injection).

## RESULTS

### Separation of free fatty acids and methyl esters

The capacity factor (k′) of compounds to be resolved is a useful parameter for planning HPLC separations. This factor is equal to the difference between the retention time of the individual compounds and $t_o$, divided by $t_o$, which is the time required for the mobile phase (or any unretained compound) to move from one end of the column to the other. Members of any series of fatty acids having the same chain length and a different degree of unsaturation are always separable by reverse phase HPLC using aqueous $H_3PO_4$ and acetonitrile (**Fig. 1**). Capacity factors increase logarithmically with decreasing $CH_3CN$ concentration. However, the slopes of the lines differ slightly among members of each series, as is apparent for the 18 carbon fatty acids (**Table 1**). By decreasing acetonitrile concentrations, k′ increases more for 18:0 than it does for the unsaturated $C_{18}$ fatty acids.

Series of fatty acids of the same degree of unsaturation and different chain length are also readily separated by reverse phase columns. However, as seen in Table 1, the longer the carbon chain, the larger the

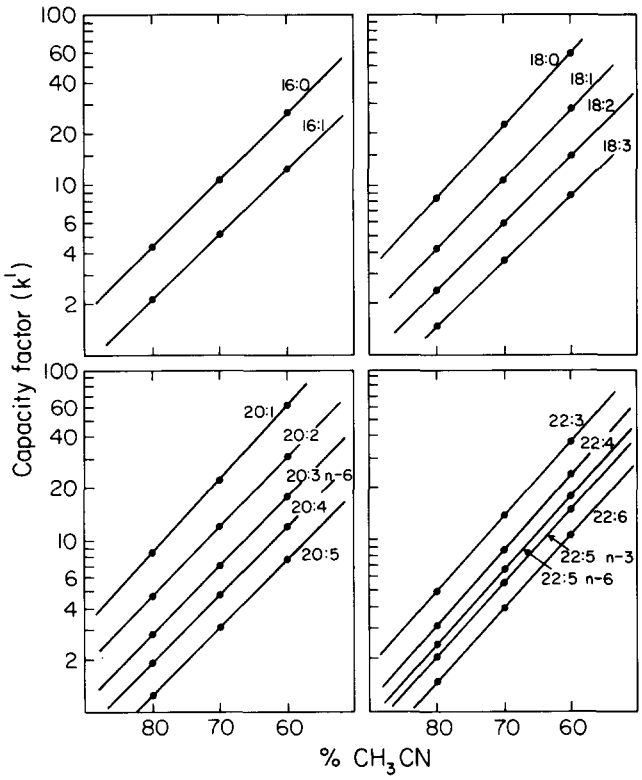


**Fig. 1.** Effect of acetonitrile concentration on capacity factors (k′) for fatty acids of different degrees of unsaturation. Fatty acid standards in 20 μl of methanol were injected onto two (25 + 15) cm × 0.46 cm I.D. Zorbax ODS columns connected in series, and eluted with aqueous $H_3PO_4$–acetonitrile at 2 ml/min. Retention times for fatty acids ($r_{fa}$) and solvent ($r_o$) were measured, and capacity factors were calculated as $k' = (r_{fa} - r_o)/r_o$. Each point is the mean of three determinations, with standard deviations lower than 3%.

increase in k′ with decreasing acetonitrile concentration. It can be concluded that the higher the hydrophobicity (the longer the chain length or the lower the unsaturation), the larger the increase in retention time with decreasing acetonitrile concentration. Conversely, as the solvent strength increases, retention times for the less hydrophobic compounds will decrease faster. Induced dipole interactions between the double bonds in the fatty acid and the triple bonds in the nitrile group of acetonitrile may play a role in this behavior.

The resolution by reverse phase HPLC of fatty acids in complex mixtures, such as those from natural sources, is therefore a difficult task, since changes in solvent strength will result in changes in the elution order of fatty acids. This is illustrated in the separations of free fatty acids from brain phospholipids shown in **Fig. 2.** A coelution of 16:1(n-9) and 20:4(n-6) is observed with 70% acetonitrile (Fig. 2A), whereas 16:1 elutes *before* 20:4 at concentrations lower than 60% (Fig. 2B), and *after* 20:4 at concentrations higher than 80%, as can be calculated from Table 1. This inversion in elution order with varying acetonitrile concentration is observed for

TABLE 1. Effect of acetonitrile concentration on capacity factors for free fatty acids

| Fatty Acid | Intercept[a] b | Slope[a] m | Ratios of k′ at 60% and 80% Acetonitrile[b] |
|---|---|---|---|
| 22:6 n-3 | 3.67 | −0.044 | 7.6 |
| 22:5 n-3 (n-6)[c] | 3.68 (3.65) | −0.043 (−0.043) | 7.3 (7.2) |
| 20:5 n-3 | 3.34 | −0.041 | 6.3 |
| 22:4 n-6 | 3.96 | −0.043 | 7.3 |
| 20:4 n-6 | 3.62 | −0.042 | 7.1 |
| 22:3 n-6 | 4.25 | −0.043 | 7.2 |
| 20:3 n-6 (n-3) | 3.83 (3.76) | −0.042 (−0.041) | 6.8 (6.7) |
| 18:3 n-3 | 3.30 | −0.039 | 6.1 |
| 20:2 n-6 | 4.10 | −0.042 | 6.9 |
| 18:2 n-6 | 3.58 | −0.040 | 6.3 |
| 20:1 n-9 | 4.46 | −0.044 | 7.6 |
| 18:1 n-9 | 3.96 | −0.042 | 6.9 |
| 16:1 n-9 | 3.37 | −0.038 | 5.9 |
| 18:0 | 4.41 | −0.043 | 7.3 |
| 16:0 | 3.79 | −0.039 | 6.1 |

[a] Values represent the intercepts (b) and slopes (m) of the regression lines for the equation log k′ = m%$CH_3CN$ + b.

[b] The ratios represent the increase in k′ from 80 to 60% $CH_3CN$, calculated from the equations.

[c] Values for isomers are given in parentheses.

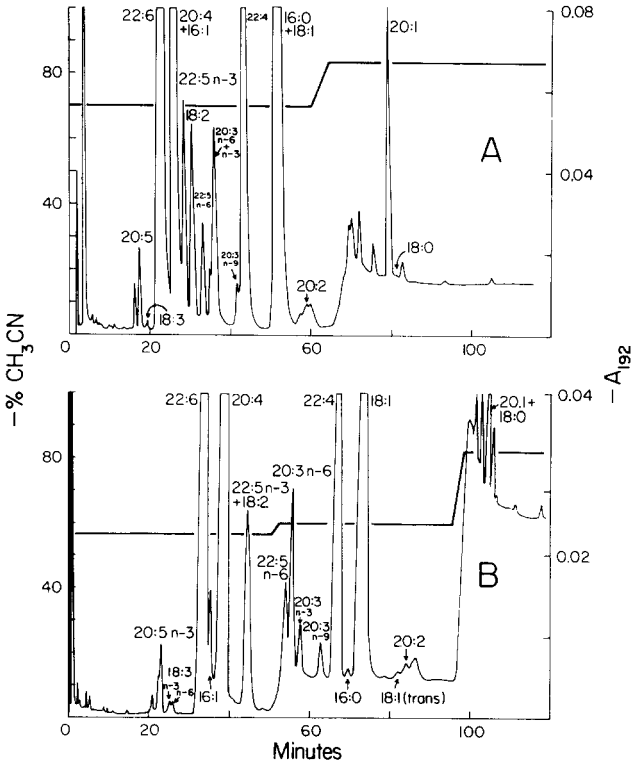


**Fig. 2.** HPLC separation of free fatty acids obtained from mouse brain glycerophospholipids. Phospholipids were subjected to alkaline hydrolysis and the released free fatty acids (140 μg/20 μl) were separated with the columns described in Fig. 1. A, Acetonitrile concentration was held at 70% for 60 min then increased to 83% over 4 min; flow rate: 1 ml/min. B, Acetonitrile concentration was started at 58%, raised at 50 min to 61% over a 2-min period, and then increased at 90 min to 83% over 2 min; flow rate: 2 ml/min.

several pairs, 22:6(n-3)–18:3(n-3), 22:5(n-3)–18:2(n-6), 22:4(n-6)–20:3(n-6), 16:0–18:1(n-9), 18:0–20:1(n-9), etc. This behavior, however, allows one to fine-tune separations of fatty acids of interest by simply modifying the solvent strength, the result being comparable to changing column polarity in GLC. Aliquots of the same sample can be chromatographed at two different solvent strengths to achieve resolution of major fatty acids as shown in Fig. 2. Alternatively, unresolved pairs can be collected to be reinjected and eluted at a second acetonitrile concentration.

When working with labeled compounds, the change in relative positions of fatty acids with varying solvent strength enables one to ascertain if a peak of radioactivity is actually associated with a particular fatty acid. **Fig. 3A** shows the resolution of labeled free fatty acids obtained from brain phospholipids after intraventricular injections of [$^3$H]20:4 and [$^{14}$C]22:4. Using 70% acetonitrile, the tritiated peak eluting after 20:4 appeared to be associated with 18:2. Using 58% acetonitrile, the unidentified labeled peak elutes before 18:2.

Methyl esters behave like free fatty acids as far as changes in elution order with varying solvent strength are concerned. Since they are less polar than the free acids, the esters are more strongly retained by the reverse phase columns. Resolution of major unsaturated FAME from brain phospholipids takes longer than that of the free acids, even when a shorter column and a higher solvent strength is used (Fig. 3, A and B). Although resolution of polyunsaturated FAME was at-

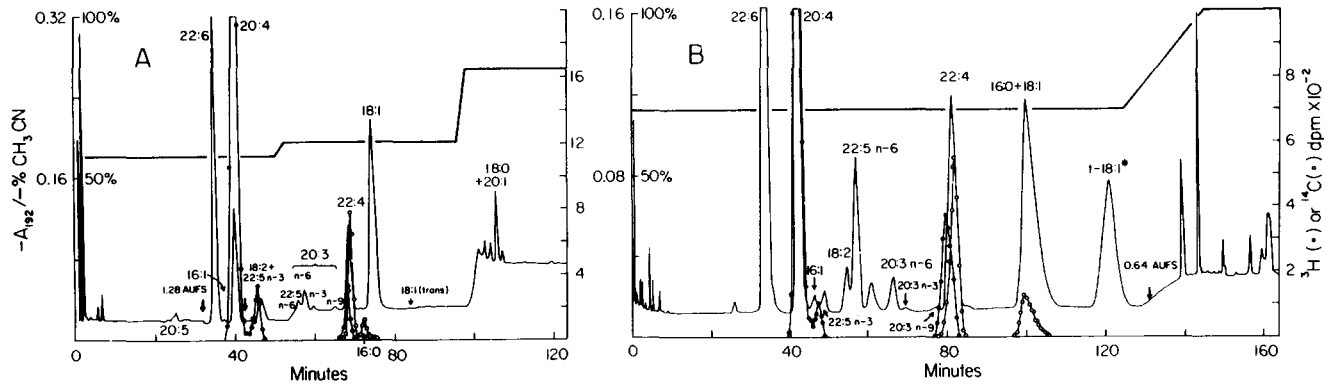


**Fig. 3.** HPLC separation of labeled free fatty acids (A) and methyl esters (B) from mouse brain phospholipids after simultaneous intraventricular injection of $^{3}$H-labeled 20:4 and $^{14}$C-labeled 22:4. Six $\mu$Ci of each fatty acid was complexed with bovine serum albumin and injected into the ventricles of 12- (A) or 2- (B) month-old mice through chronically implanted cannulae. Free fatty acids were prepared and resolved as detailed in Fig. 2B. Methyl esters, prepared by acid methanolysis of phospholipids, were resolved on a 25 × 0.46 cm Zorbax ODS column using 70% acetonitrile–water at 2 ml/min. (*), Fifty nmol of methyl *trans*-18:1, added as internal standard. After elution of *trans*-18:1, $CH_3CN$ concentration was increased to 100% over 20 min. About 150 $\mu$g of FFA or FAME were injected.

tained using 70% acetonitrile, 16:0 was more difficult to separate from 18:1 as FAME than as FFA. Thus, for resolution of complex mixtures, free fatty acid separations are advantageous over those of methyl esters.

A major application of the separation of FFA or FAME by HPLC is in studies of metabolic conversions of fatty acids. Tritiated fatty acids can be used to study various aspects of fatty acid metabolism. Double-label experiments using $^{3}$H- and $^{14}$C-labeled fatty acids of similar radioactivities can be conducted, since low levels of tritiated metabolites can be measured with good efficiency by collection and liquid scintillation counting. Fig. 3 shows that 1 hr after intraventricular injection of $^{3}$H-labeled 20:4, about 10% was elongated to $^{3}$H-labeled 22:4. A high degree of $\beta$-oxidation of 1-$^{14}$C-labeled 22:4 is also apparent from the radioactivity present in 16:0. Both precursors, as well as the products of their metabolic conversions, were incorporated into phospholipids.

The percentage distribution of radioactivity among fatty acids can be readily established by HPLC. **Fig. 4** shows the separation of labeled fatty acids incorporated into brain phospholipids after intraventricular injections of [$^{14}$C]acetate. Labeled FAME are separated by using pure acetonitrile at 0.5 ml/min. Measurable areas of saturated fatty acids can be attained at these low flow rates, as well as improved counting efficiencies with radioactive flow detectors, with the additional advantage of economy in the use of HPLC solvents and scintillation cocktail. This very simple procedure is particularly useful for studying rates of desaturation and elongation of fatty acids, since labeled methyl esters differing by a single double bond or in chain length are very easily separated.

Methyl esters prefractionated according to unsaturation by argentation TLC can be rapidly and completely separated by reverse phase HPLC using 100% acetonitrile. Complete resolution of major methyl esters obtained from a total lipid extract after argentation TLC can be accomplished with a total HPLC analysis time of about 2 hr (**Fig. 5**). The injection volumes of the different fractions can be adjusted as desired to enable quantitation of low ultraviolet-absorbing com-

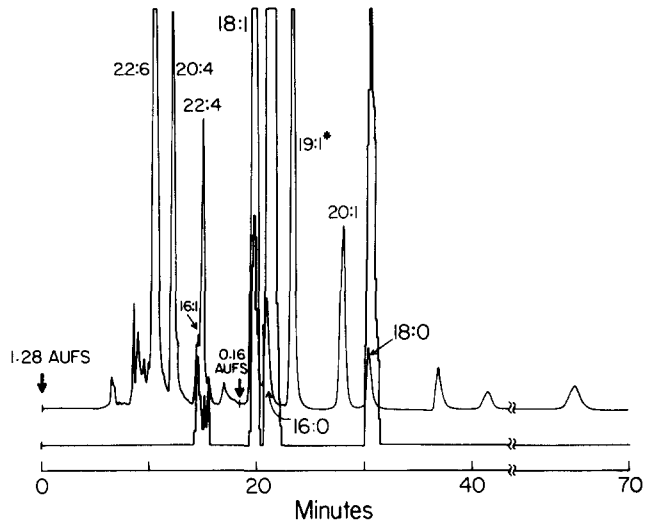


**Fig. 4.** Distribution of radioactivity among fatty acids from brain phospholipids after intraventricular injections of [1-$^{14}$C]acetate. Injections (40 $\mu$Ci each) were done every 30 min for 2 hr. One hour after the last injection, phospholipids were isolated and methanolyzed. One-hundred-ninety-one $\mu$g of methyl esters were injected onto a 25 × 0.46 cm Zorbax ODS column which was joined to a 5 × 0.46 cm guard column packed with Permaphase ODS (DuPont). Methyl esters were eluted with 100% $CH_3CN$ at 0.5 ml/min. Radioactivity was measured with a radioactive flow detector (counting efficiency 75%) set at $10^2$ full scale units per 6 seconds. Total radioactivity injected was 6400 dpm. Distribution of radioactivity was as follows: 16:1, 0.9 ± 0.3%; 18:1, 6.2 ± 0.6%; 16:0, 77.6 ± 1.3%; 18:0, 14.3 ± 1.8% (mean ±S.D. from five determinations). Absorbance at 205 nm and the radioactivity tracing are depicted. (*), Fifty nmol of methyl 19:1 added as internal standard.

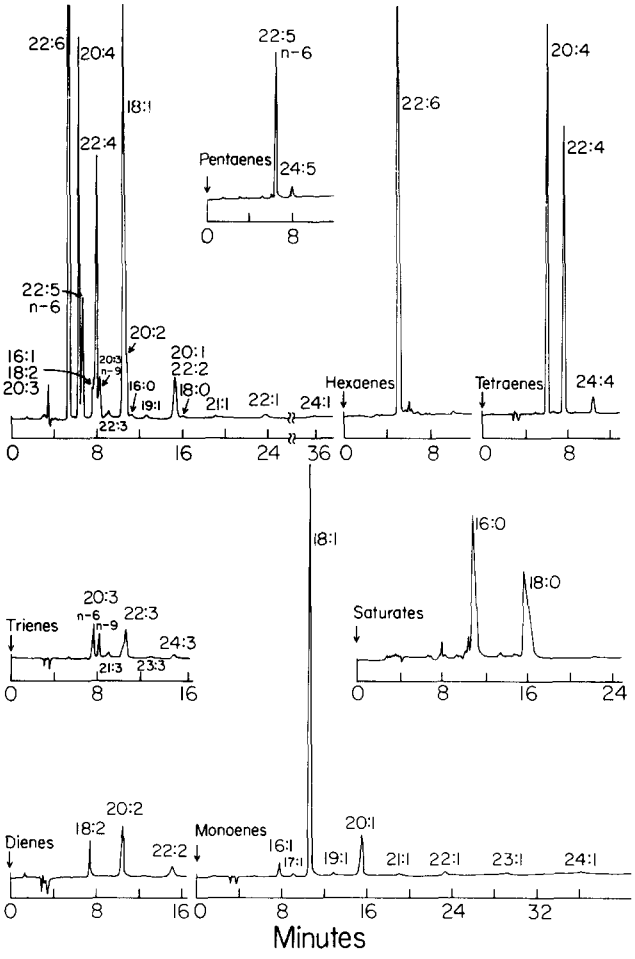


**Fig. 5.** Rapid separation of fatty acid methyl esters by HPLC after argentation TLC. Methyl esters were prepared by alkaline methanolysis of lipid extracts from bovine brain, purified by TLC, and fractionated according to unsaturation by silver ion TLC. The esters were dissolved in acetonitrile. All fractions were taken to the same volume, except the saturates (100 times more concentrated), and 20 μl of each was injected. The detector was set at 0.64 AUFS for all fractions, except dienes and saturates (0.32 AUFS). A 25-cm Zorbax ODS column and 100% acetonitrile at 1 ml/min were used. Amounts injected were: saturates, 330 μg; total FAME, 25 μg; monoenes, 8 μg; and tetraenes, 1.8 μg. The rest of the fractions contained less than 1 μg FAME/20 μl.

pounds, since fatty acids in fractions are spaced enough to avoid overloading with neighbor peaks. Thus, measurable areas of 16:0 and 18:0 can be attained if the saturated fraction is injected separately (e.g., Figs. 5 and 7). The selection of an appropriate internal standard is much easier for fractions than for the complex mixtures described above.

Argentation TLC–HPLC can be used to resolve minor fatty acids present in biological samples. For example, a small but detectable amount of elaidic acid could be separated from oleic acid in the monoenoic fraction from bovine brain lipids (**Fig. 6A'**). Positional isomers of 20:4 and 20:3 were also resolved (Fig. 6, C and D). The double peak of 22:3 is probably due to partially resolved positional isomers of this fatty acid. Both fatty acids were converted to 22:0 after catalytic hydrogenation. Minor odd-chain trienoic fatty acids were also detected in this fraction. Geometrical isomers of methyl 18:2 were resolved, although they were not found as naturally occurring fatty acids in the dienoic fraction from brain (Fig. 6B). Minor dienoic fatty acids including 20:2, 22:2, and 24:2 (not shown) were observed in this fraction. Since resolution increases logarithmically with decreasing $CH_3CN$ concentration, the column loading can be increased and $CH_3CN$ concentration decreased for preparative isolation of methyl esters from natural sources.

Being less retentive than the octadecylsilyl columns, an octylsilyl stationary phase permits faster separations of very long-chain fatty acids. The accuracy of the quantitation can be improved with slower flow rates without unacceptable increases in retention time. In addition to 16:0 and 18:0, other long-chain saturated fatty acids in the saturated fraction from brain could be detected at 205 nm (**Fig. 7A**). The use of these columns permits the detection and very rapid separation (60 min) of very long-chain monoenoic fatty acids including those with odd and even numbers of carbon atoms in glycerophospholipids (Fig. 7B). Monoenes up to 28 carbon atoms have been described in brain cerebrosides (16, 17) but, due to the low level and broad width of these peaks, no conclusive evidence for their presence was obtained using capillary GLC analysis of total brain lipids including sphingolipids (18). The latter are excluded as a source of the fatty acids shown in Fig. 7B since the methyl esters were obtained by a mild alkaline methanolysis procedure. The injection of large amounts of the isolated monoenoic fraction makes possible the isolation of significant amounts of these minor components for further studies. The major fatty acid methyl esters from brain sphingomyelin were eluted with 90% acetonitrile in order to separate monoenoic from saturated fatty acids (Fig. 7C). The position on the chromatogram of the latter could be ascertained by monitoring at 205 nm.

### Quantitation of individual free fatty acids and methyl esters

Since the absorption of fatty acids at 192 nm depends on the presence of double bonds, different detector responses are obtained according to the degree of unsaturation. Quantitation of all unsaturated fatty acids in a complex mixture would require, in addition to complete resolution, as many calibration curves as classes of fatty acids are present in the sample. Absorption of saturated fatty acids, major components of most mam-

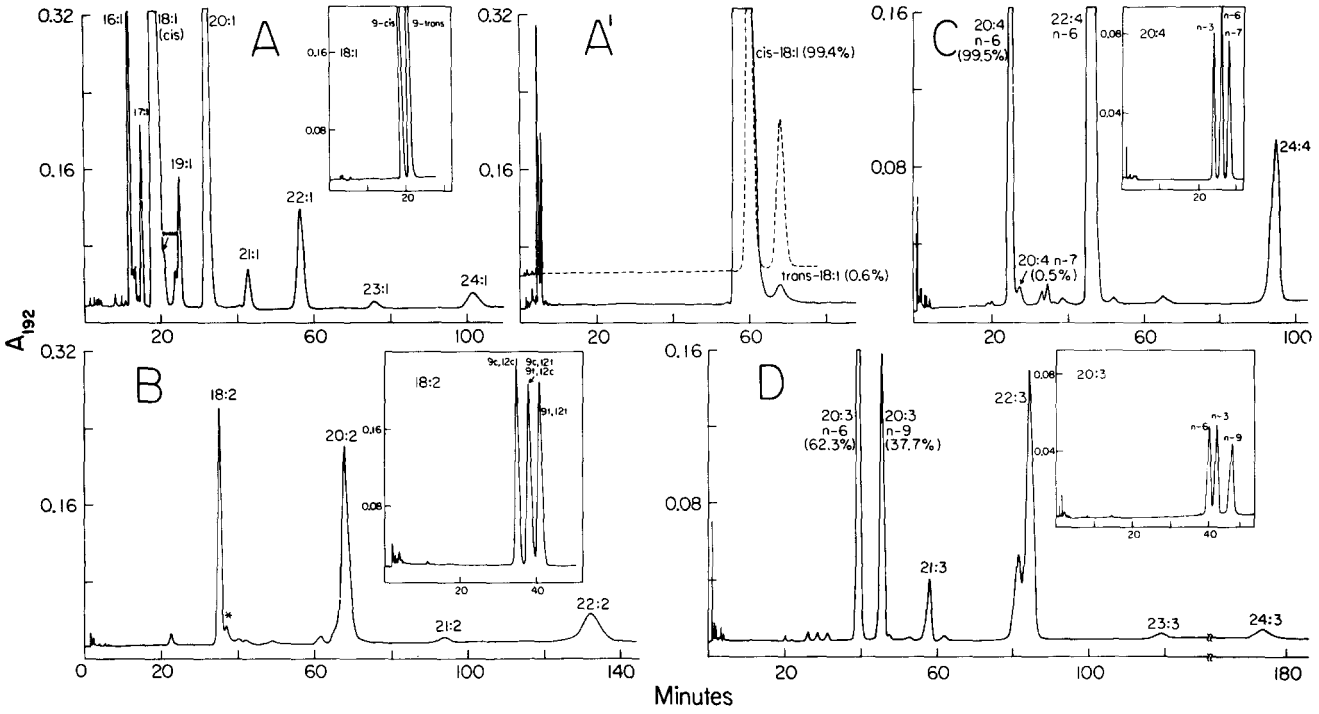


**Fig. 6.** Resolution of geometrical and positional isomers of fatty acid methyl esters derived from glycerophospholipids of bovine brain. Separations of standards are shown in the inserts. Flow rates of 1 ml/min were used in A and B and of 2 ml/min in C and D. Numbers in parentheses indicate isomer proportions on an area basis. The same fractions shown in Fig. 5, without dilution, were injected. A, The monoenoic fraction (800 μg) was run at 90% acetonitrile. Although the standards were completely separated, the major peak of methyl oleate overlapped methyl elaidate from brain. The entire 18:1 peak was collected, taken to dryness in vacuo, and chromatographed using 80% acetonitrile (A'). The dotted line in A' shows a separation of standards. B, Dienes (30 μg) were eluted with 80% acetonitrile. The retention time of the peak marked * did not correspond to any of the standards. It is probably a positional isomer of 18:2. C and D, Tetraenes (180 μg) and trienes (50 μg) were eluted with 75% acetonitrile.

malian tissue lipids, is too low to allow quantitation in the conditions required to resolve unsaturates (e.g., see 16:0 in Figs. 2 and 3A). Conversely, a condition that permits good detection of saturated fatty acids (e.g., Fig. 4) does not allow resolution of unsaturates. Therefore, the procedure is not recommended for compositional studies. However, individual fatty acids or methyl esters can be quantitated, provided they are resolved and that sufficient amounts to give measurable areas can be injected. Specific radioactivities of labeled fatty acids can therefore be determined.

Fatty acids or methyl esters can be measured using external or internal standard procedures. Areas are little affected by changes in acetonitrile concentration but obviously depend on flow rates. Calibrations of six fatty acids gave straight lines over the range of 5 to 100 nmol using the conditions shown in Fig. 3 for FFA and FAME. The absorption was approximately proportional to the number of double bonds (**Table 2**). The internal standard was *trans*-18:1 since it appears in a relatively vacant section of the chromatograms (Fig. 3) and is present in insignificant amounts. Area ratios for FFA (or FAME) and a fixed amount (50 nmol) of *trans*-18:1 gave straight lines in the range 5–100 nmol for all unsaturated fatty acids.

Quantitation of saturated fatty acids is possible under certain conditions. The sample size can be considerably increased for methyl esters since they are more soluble in acetonitrile than the respective free acids. Lower flow rates also increase areas. Saturates are detected better (1.8 times more area/μmol) at 205 nm than they are at 192 nm (**Table 3**). Methyl 19:1 was chosen as internal standard because, for a separation like that shown in Fig. 4, methyl *trans*-18:1 would coelute with 16:0. Since the absorbance of 19:1 is 8.1-fold lower at 205 nm than at 192 nm, the area ratios of saturates to 19:1 are 14-fold higher at 205 than they are at 192 nm.

**Table 4** gives examples of the determination of specific radioactivities of fatty acids using the HPLC separation shown in Fig. 3A. **Table 5** shows the specific radioactivities of fatty acids separated from brain phospholipids after injections of [1-$^{14}$C]acetate. A reasonable agreement was obtained for fatty acid mass with the area ratio procedure and GLC using a different internal standard. Mass and radioactivity can be measured on the same aliquot of the sample by HPLC.

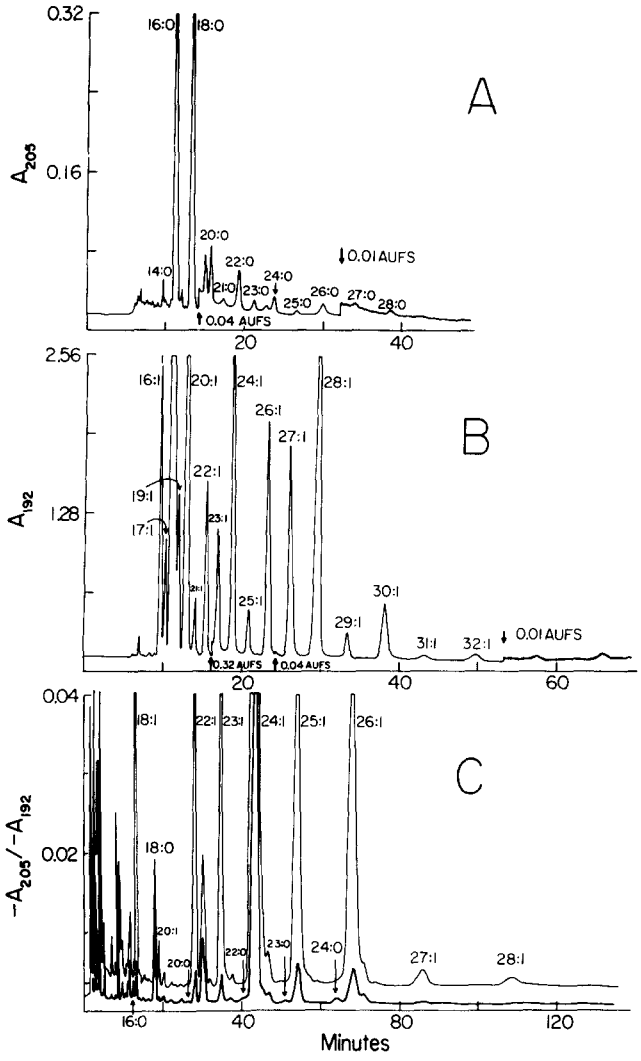


**Fig. 7.** Use of a Zorbax C-8 column for the rapid separation and improved detection of long chain monoenoic and saturated fatty acids. A, The saturated fraction (0.7 mg) was eluted with 100% acetonitrile at 0.5 ml/min. B, Monoenes (0.8 mg) chromatographed with the same conditions as A. C, Methyl esters (0.1 mg) obtained from bovine brain sphingomyelin which had been isolated by two-dimensional TLC (21). Methyl esters were prepared with 14% $BF_3$–methanol (13) and purified by TLC using hexane–ether 95:5. The separation was done with 90% acetonitrile at 1 ml/min.

## DISCUSSION

Detection at 192 nm is highly selective for unsaturated fatty acids, the sensitivity increasing with the number of double bonds. Detection of saturates can be somewhat improved at 205 nm but then the sensitivity for unsaturates, particularly monoenes, decreases markedly. Dual wavelength detectors can be useful for the simultaneous detection of unsaturated and saturated fatty acids, and help in the identification of the latter, since they are the only components in a fatty acid mixture for which the 205/192 nm ratio is higher than unity. Detection at 192 nm restricts the number of commercially available solvents, apart from acetonitrile, that meet the requirements of transparency and water solubility. Purity of the water is also of utmost importance, particularly when including gradient elution or when using the procedure as a preparative technique. Organic impurities in the water are concentrated by the reverse phase columns and appear as spurious peaks on the chromatograms when the acetonitrile concentration is raised. Using the procedure described for water purification, only very small peaks were detected at 192 nm after passing 600 ml of water through an octadecylsilyl

TABLE 2. Quantitative behavior of unsaturated fatty acids in HPLC separations at 192 nm

| Fatty Acid | nmol/$10^5$ Area Units[a] | | Area Ratio to *trans*-18:1[b] | |
|---|---|---|---|---|
| | FFA | FAME | FFA | FAME |
| 22:6 n-3 | 6.24 | 5.94 | 5.47 | 6.24 |
| 22:4 n-6 | 8.66 | 8.88 | 3.79 | 4.12 |
| 20:4 n-6 | 9.15 | 9.02 | 3.66 | 3.88 |
| 20:3 n-3 | 11.70 | 11.72 | 2.86 | 3.09 |
| 18:2 n-6 | 16.58 | 16.22 | 2.04 | 2.18 |
| 18:1 n-9 | 37.80 | 34.50 | 0.95 | 0.96 |

[a] Values are the reciprocal of the slopes of the regression lines for area vs. nmol.

[b] The ratios represent the slopes of the regression lines for the areas of the fatty acids divided by the area of the internal standard, as a function of the amount of fatty acid.

Twenty μl of mixtures of fatty acids or methyl esters containing 5 to 100 nmol of each fatty acid and 50 nmol of *trans*-18:1 (FFA or FAME) as internal standard were chromatographed as shown in Fig. 3. Correlation coefficients for regression lines were greater than 0.999.

TABLE 3. Responses of saturated methyl esters at 192 and 205 nm

| Methyl Ester | μmol/$10^5$ Area Units[a] | | Area Ratio to Methyl 19:1[b] | |
|---|---|---|---|---|
| | 192 nm | 205 nm | 192 nm | 205 nm |
| 12:0 | 2.59 | 1.40 | 0.0036 | 0.050 |
| 14:0 | 2.70 | 1.43 | 0.0035 | 0.052 |
| 16:0 | 2.57 | 1.40 | 0.0037 | 0.052 |
| 18:0 | 2.56 | 1.37 | 0.0037 | 0.051 |

[a] Values are the reciprocal of the slopes of regression lines for area vs μmol.

[b] The ratios represent the slopes of regression lines for the areas of the fatty acids divided by the area of the internal standard, as a function of the amount of fatty acid.

Twenty μl of mixtures containing 0.05 to 1.5 μmol of methyl esters and 50 nmol of methyl 19:1 were injected, and chromatographed using 100% acetonitrile at 0.5 ml/min. The absorbance of 50 nmol of methyl 19:1 was 567 ± 38 and 70 ± 5 × $10^3$ area units at 192 and 205 nm, respectively. Correlation coefficients were greater than 0.996.

TABLE 4. Specific radioactivities of fatty acids of brain phospholipids after intraventricular injection of [$^3$H]arachidonic and [$^{14}$C]adrenic acids

| Time after injection | Fatty Acid | Mass | Collected dpm | | Specific radioactivities: dpm/μmol | | Specific radioactivities: μmol/mol | |
|---|---|---|---|---|---|---|---|---|
| | | | $^3$H | $^{14}$C | $^3$H | $^{14}$C | $^3$H | $^{14}$C |
| *min* | | *nmol* | | | | | | |
| | 20:4 | 72.2 | 9517 | | 132 | | 1.0 | |
| 4 | 22:4 | 22.8 | 110 | 423 | 5 | 19 | 0.04 | 411 |
| | 16:0 | 129.6 | | 49 | | 0.4 | | 9 |
| | 20:4 | 69.3 | 15350 | | 222 | | 1.6 | |
| 15 | 22:4 | 21.4 | 211 | 785 | 10 | 37 | 0.07 | 801 |
| | 16:0 | 119.5 | | 114 | | 1.0 | | 24 |
| | 20:4 | 39.4 | 17072 | | 433 | | 3.2 | |
| 60 | 22:4 | 11.8 | 711 | 1111 | 60 | 94 | 0.43 | 2035 |
| | 16:0 | 66.7 | | 379 | | 5.7 | | 134 |

Six μCi of $^3H_8$-labeled 20:4 and $^{14}$C-labeled 22:4 ($62.2 \times 10^3$ and 21 Ci/mol, respectively) were injected into the brain of 2-month-old mice through chronically implanted cannulae. The brains were removed at the specified intervals and lipids were extracted. Free fatty acids were prepared from phospholipids by acid hydrolysis and resolved by HPLC as shown in Fig. 3A. Counting efficiencies for $^3$H and $^{14}$C were 21 and 82%, respectively. The mass of 20:4 and 22:4 was calculated from the area ratios with 50 nmol of *trans*-18:1 which was added as an internal standard. The mass of 16:0 was estimated by GLC.

column when the concentration of acetonitrile was increased to 100%.

The limitations concerning the acetonitrile separations are: *1*) the difficulties in resolution of all fatty acids in a single run, and *2*) the relatively long analysis time per sample. Complete resolution of methyl esters by GLC is also difficult on most packed columns by GLC. Using a single stationary phase, fatty acids of similar ECL (equivalent chain length) are not resolved. After argentation TLC of the fractions obtained from bovine brain, it was observed by isothermal GLC analysis that 18:3–20:1, 20:2–21:1, 21:2–22:1, 22:0–20:3 (n-6), 23:0–22:2, 23:1–22:3, 24:1–22:4, 23:3–22:5 (n-6), 24:2–25:0, 22:5 (n-3)–24:3–25:1, 22:6–26:0, and 24:4–26:1 coeluted using a polar packing.

Similar changes in elution order with variation of solvent strength were observed using octadecylsilyl columns from different sources (Altex, Waters, DuPont). However, the selectivities at a given acetonitrile concentration differ among columns. The order of elution may be different in two different columns at a given acetonitrile concentration, or resolutions that require 70% acetonitrile in one column may be attained at 60% in another. A semilogarithmic graph of capacity factors for fatty acid standards as a function of acetonitrile concentration in the particular column available, such as that shown in Fig. 1, will allow one to predict the separation of fatty acids of interest at any given acetonitrile concentration. Differences in column selectivities may be useful to achieve resolution by "column switching" techniques. As an example, 100% acetonitrile did not resolve monoenoic from saturated methyl esters of similar hydrophobicity on an octylsilyl column (e.g., 18:1–16:0, 20:1–18:0, Fig. 7A and B) but these are resolved with the same solvent on an octadecylsilyl column (Figs. 4 and 5). Thus, by switching the unresolved pair to a second column as it elutes from the first one, separation may be achieved on a single run.

The advantages of free fatty acid over methyl ester analysis include: *1*) faster separations; *2*) no derivatization required; and *3*) the Schmidt decarboxylation procedure (19) can be directly applied to the eluted compounds to study intramolecular distribution of radio-

TABLE 5. Specific radioactivities of brain phospholipids after intraventricular injections of [1-$^{14}$C]acetate

| Fatty Acid | Mass: HPLC | Mass: GLC | Radioactivity | Specific Radioactivity |
|---|---|---|---|---|
| | *nmol* | | *dpm* | *dpm/nmol* |
| 16:0 | 160 | 163 | 4966 | 31.0, 30.1 |
| 18:0 | 154 | 153 | 915 | 5.9, 6.0 |
| 18:1 | 132 | 135 | 396 | 3.0, 2.9 |

The mass values by HPLC were obtained using area ratios (Table 3) with an assumed area ratio of 1.00 for 18:1 with methyl 19:1 as the internal standard. The chromatogram is shown in Fig. 4. The mass values by GLC were obtained with methyl 21:0 as the internal standard.

activity. The advantages of methyl ester over free fatty acid analysis are: *1*) increased sample capacities, due to stronger retention by the columns; *2*) higher solubility of methyl esters, especially saturates, in acetonitrile; *3*) greater sample stability since free fatty acids can form methyl esters when stored in methanol; *4*) simplicity and economy, since free fatty acids require an acid medium for ion suppression that may shorten column lifespan; and *5*) methyl esters can be prefractionated using argentation TLC or HPLC (20), which allows: *a*) rapid resolution of major methyl esters without overlapping, *b*) improved detection of minor or low ultraviolet-absorbing components in the samples, and *c*) accurate measurements of specific radioactivities of minor components.

Reverse phase HPLC separations of free fatty acid and methyl esters allow for: *1*) studies of distribution of radioactivity among fatty acids (only resolution of the labeled compounds is required (see Fig. 4); *2*) determination of specific radioactivities of fatty acids from various lipid classes (This obviously requires that the fatty acid under study can be adequately quantitated and that it is separated from other compounds. By varying solvent strength, separations of most naturally occurring fatty acids can be achieved.); *3*) accurate chromatographic identification of unknowns by measuring their retention times as free fatty acid and as methyl ester on the same column; *4*) preparative isolation, either from natural sources or after organic synthesis; and *5*) using the ability of the columns to resolve geometrical isomers as a prefractionation procedure to separate *cis* from *trans* groups. Positional isomers in each group can further be analyzed by capillary GLC. This combination of methods has recently been applied to characterize positional and geometrical isomers of monoenoic fatty acids (20).

Although the sensitivity for mass measurements can be enormously increased by using aromatic derivatives (4–9), advantages of FFA and FAME over these are as follows. *1*) Simpler and less expensive methods are available to prepare FFA and FAME in quantitative yields. *2*) FFA (or FAME) prepared by alkaline hydrolysis (or methanolysis) of lipids and extracted into hexane, are practically devoid of other ultraviolet-absorbing compounds and can be directly analyzed by HPLC. On the other hand aromatic derivatives must be purified by TLC before HPLC to eliminate residues of strongly ultraviolet-absorbing reagents used in their preparation. *3*) Since reverse phase separations are based on subtle differences in hydrophobicity, the addition of a bulky hydrophobic tag to the compounds may decrease these differences and make resolutions more difficult. These derivatives have not been employed to separate fatty acids from mammalian tissues.

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