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Factice chromatography: An automatically monitored, liquid-gel system for the separation of nonpolar lipids*

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SUMMARY

A technique of column chromatography using factice, a hydrophobic polymer, as stationary phase, and aqueous acetone as moving phase is described. With this liquid-gel system, the major nonpolar lipid classes (cholesterol esters, triglycerides, cholesterol, diglycerides, and monoglycerides) are easily separated from each other. Furthermore, separations of components with different fatty acid composition within the same lipid class can also be obtained. Thus, cholesterol esters with fatty acid chain lengths from 2 to 18 carbons are eluted as individual peaks. Similar separations are possible with glycerides or with mixtures of methyl esters of fatty acids. However, with the exception of cholesterol esters, lipids within a given class containing fatty acids of equivalent polarity (2-carbon shortening equivalent to one double bond) are inseparable. Elution is performed with a single solvent mixture and detection of lipid in the effluent stream can be achieved by the use of automatic differential refractometry. In this way, the entire procedure can be automatically performed. Free fatty acids and phospholipids are poorly separated and must be removed from the lipid mixture prior to chromatography.

The separation of components in complex mixtures of lipids remains an essential aspect of many studies in lipid chemistry and metabolism. This report describes a system of liquid-gel chromatography that has proved useful for many types of nonpolar lipid separation. With this method, lipids are eluted by a single solvent mixture from a column of hydrophobic polymer swollen to the gel state by organic solvent. The unchanging composition of the eluant provides a constant solvent background in the effluent, permitting automatic detection of lipid by the technique of differential refractometry (1). This automatic feature as well as the unusual degree of resolution of some components, heretofore not possible, suggest that this method may be a valuable adjunct to chromatographic methods currently in use.

In early work with this method, powdered rubber was used to form the stationary or gel phase (1). Columns of powdered rubber had been used successfully by others for the separation of terpenes[1] and fatty acids (2). After examination of many types of polymers, however, polymerized vegetable oil or "factice" was found to be least subject to deterioration and most valuable for the lipid separations to be described (1).

With factice as stationary phase and aqueous acetone as moving phase, it has been possible to effect rapid and complete separation of the major nonpolar lipid classes: cholesterol esters, cholesterol, triglycerides, and partial glycerides. Furthermore, certain *intra*-class separations, depending on fatty acid differences, can also be accomplished. Phospholipids and free fatty acids are poorly separated by this method and can interfere with other separations; hence, they must be removed from the lipid mixture prior to chromatography. The least polar lipids such as waxes and long-chain hydrocarbons, which are insoluble in aqueous acetone, also cannot be successfully chromatographed.

METHODS

Materials and Ancillary Methods. Large quantities of acetone are used; hence redistillation may not be practical. Baker and Adamson Acetone (reagent grade) was used since it was found to contain less than 1 μg/ml of solute residue. However, each new lot

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[1] Trowbridge, J., and A. Herrick, personal communication.

TABLE 1. SOURCES OF CHROMATOGRAPHIC TEST SUBSTANCES

Compounds	Sources
Cholesteryl acetate, trihexanoin, trioctanoin, trilaurin, methyl butyrate, methyl hexanoate, methyl octanoate, methyl decanoate, methyl laurate, methyl myristate	Eastman Organic Chemicals, Distillation Products Industries, Rochester, New York
Triolein, tripalmitin, methyl palmitate, methyl oleate, methyl linoleate methyl linolenate	The Hormel Foundation, Austin, Minnesota
1-Monostearin, 1-monopalmitin, 1-monolaurin, 1,3-distearin, 1,3-dipalmitin, 1,3-dilaurin, dicaprin	The Proctor and Gamble Company, Cincinnati, Ohio courtesy of Dr. F. H. Mattson
Cholesteryl myristate, cholesteryl stearate	Dougherty Chemicals, Richmond Hill, New York
Cholesteryl palmitate, cholesteryl oleate	K and K Laboratories, Jamaica, New York
Linseed oil	Archer-Daniels-Midland Co., Minneapolis, Minn.
Tributyrin, cholesterol	Amend Drug and Chemical Co., New York
Trimyristin	Paragon Division, The Matheson Co., East Rutherford, N. J.

was tested to insure low solute contamination. Other solvents were reagent grade and were distilled in an all-glass apparatus before use.

Various compounds used singly and in test mixtures are listed with their sources in Table 1. The cholesterol esters of butyric, hexanoic, octanoic, decanoic, linoleic, and linolenic acids were prepared by esterification of the appropriate acid chloride with cholesterol. Thionyl chloride was used for the preparation of saturated acid chlorides and oxalyl chloride for unsaturated acids. The procedures followed were essentially those reported by Swell and Treadwell (3) and Wood et al. (4).

Serum lipid extracts were prepared by the method of Folch et al. (5). Gas-liquid chromatography (GLC) of fatty acids was carried out as described by Farquhar et al. (6). Quantitative reliability was assured by testing for detector linearity with fatty acid standards A-F of the U.S. Public Health Service Metabolism Study Section of the National Institutes of Health. Thin-layer silicic acid chromatography (7) was used as a rapid means of identifying effluent peaks and assessing their purity. Plates were developed in 28% or 35% ethyl ether in petroleum ether (v/v). The former solvent mixture was useful in separating cholesterol esters from triglycerides; the latter provided better identification of partial glycerides and nonesterified cholesterol.

Preparation of Stationary Phase. Factice produced by polymerization of soybean oil with sulfur monochloride was obtained from the Carter-Bell Manufacturing Co., Springfield, New Jersey. The variety "31-B, Coarsely Ground" was found most satisfactory.

Factice is a light tan, crumbly elastomer, which is stable at room temperature. To obtain factice of suitable particle size and in quantity sufficient for 2–3 columns, approximately 300 g of the starting material was slurried in acetone and poured onto a #60 mesh sieve (U.S. Standard Sieve Series). The slurry was gently rubbed by hand through the sieve and small amounts of acetone were intermittently poured onto the sieve. The particles that passed the #60 sieve were caught below in a #140 sieve. Since factice crumbles easily and produces exceedingly fine particles that pass both sieves, the acetone used for sieving becomes turbid and should be periodically clarified by filtration through Whatman #1 paper in a large Buchner funnel. In less than 1 hr, most of the initial 300 g can be forced through the #60 sieve.

Particles retained in the #140 sieve were placed in a 2-liter beaker filled with glacial acetic acid. The mixture was stirred gently for at least 5 min and the particles separated by filtration. The glacial acetic acid wash was then repeated. These washes remove magnesium salts used as stabilizers during factice polymerization and small amounts of a brown viscous liquid (most likely incompletely polymerized vegetable oil). The acetic acid-washed particles were then washed in acetone. Very small particles formed during these washes must be removed by resieving in small batches. Ten- to fifteen-gram amounts of factice were placed successively on the #140 sieve and rinsed copiously with acetone. The final product retained by the #140 sieve was used for constructing chromatographic columns. These particles may be stored indefinitely in acetone. However, immediately prior to use, the factice should be sieved once again to remove fine particles formed during storage.

Preparation of Columns. A thin slurry of factice particles was poured into a jacketed glass column, fitted below with a sintered glass plate (Fig. 1). It is essential to build the column with the same solvent that will be used as eluant, since changes in solvent may cause the column to shrink and fracture. As the factice settles, the finest particles settling most slowly were sucked off before adding more slurry. When the level of settled particles neared the top of the column, pressure was applied to pack the factice and produce a column that flows in normal usage (gravity flow *without* applied pressure) at 1 ml/min. Ten to 20 lbs of pressure from a nitrogen cylinder applied for $^1/_2$ to 2 min usually suffices; however, the flow rate (gravity without applied pressure) should be checked frequently during pressure packing. After a filter paper disk was gently placed at the upper end of the column, a continuous flow of solvent was maintained

by siphoning from a large reservoir of solvent placed above the column.

Equilibration of stationary phase with solvent was achieved by elution of several column volumes of solvent. At a flow rate of 1 ml/min, this required no more than 4 hr. If the separation is to be monitored refractometrically, a stable baseline indicates equilibration.

With refractometric recording, solvent flows first to the reference cell of a differential refractometer and then to the column. The components shown in Fig. 1 have been found useful; however, the method should be easily adaptable to other types of glassware, columns with different dimensions, and other techniques of detection. The glassware (Fig. 1) can be obtained from the Scientific Glass Co., Bloomfield, N. J. (Parts I, II, and III, catalog number: JC 1125); the Teflon tubing from Pennsylvania Fluorocarbon Co., Philadelphia 4, Pa. (AWG #18TW Natural).

Eluants. Many solvent combinations, including acetone, chloroform, methanol, methyl ethyl ketone, isopropanol, ethyl acetate, petroleum ether, and water, have been evaluated. The effects of pH and ionic strength of the eluant have also been studied. Acetone and water mixtures have provided the best separations to date.

Factice swells in nonpolar solvents and shrinks with the addition of water or other polar solvents, so that minor changes in composition of the eluant can cause disruption of the column. Therefore, each run must be carried out with a single solvent system; however, any column can be used indefinitely with the same eluant.

The amount of water in the solvent mixture is critical in determining the outcome of the separation. With decreasing water concentration, peaks are more rapidly eluted, but closely related substances may be less well separated. For the separation of the major nonpolar lipid classes (cholesterol esters, triglycerides, partial glycerides, nonesterified cholesterol), 2% H_2O–acetone (v/v) is useful. The same solvent system also separates the cholesterol esters from each other. For the resolution of triglyceride mixtures, 5% H_2O–acetone is used; for methyl esters of fatty acids, 12–15% H_2O–acetone. Usually, the most aqueous water-acetone mixture that will dissolve the lipid sample in 1–2 ml of solvent will also provide the most discriminating separation.

Temperature. Most separations are performed at room temperature. However, cholesterol esters separate poorly from each other at temperatures above 20°; for this separation, the column jackets should be connected to a circulating water bath maintained at 18–

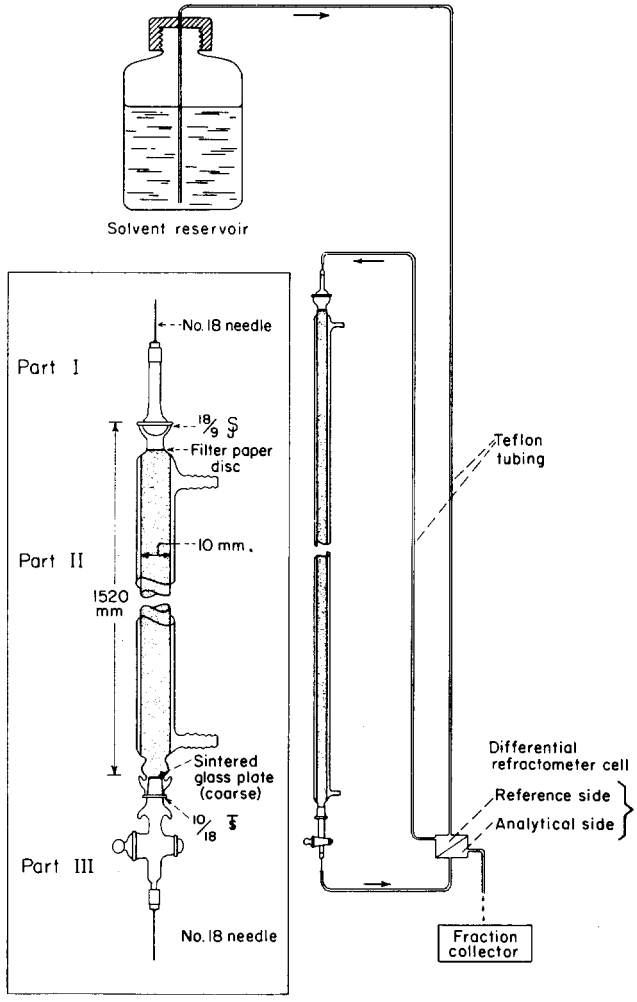

FIG. 1. Glassware and auxiliary equipment. The solvent reservoir is a 20-liter polyethylene bottle. A small port in the cap for air entry allows gravity flow.

20°. In general, an increase in column temperature has the same effect as a reduction in water content of the eluant. With the exception of cholesterol esters, no advantages or better separation could be obtained by varying the column temperature over the range from 2–30°.

Charging the Column. Phospholipids and free fatty acids are eluted from factice columns in long "smears" and must be removed from the lipid mixture before it is charged on the column. Phospholipids were removed by rapid passage over columns of Florisil according to the technique of Carroll (8) or by silicic acid chromatography (9). Silicic acid in a batch process also removes phospholipids effectively and easily. The lipid extract in petroleum ether was shaken with adsorbent (at least 60 mg silicic acid/mg phospholipid). The petroleum ether was pulled from the mixture through a small glass adaptor packed with glass wool.

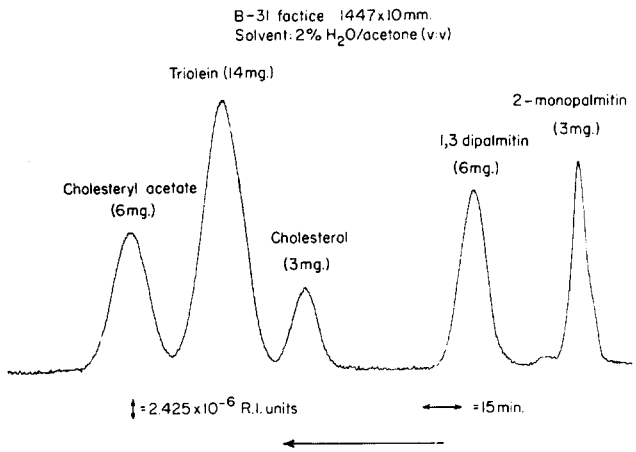

FIG. 2. Refractometric recording of a lipid class separation by factice chromatography, proceeding from right to left. The small vertical scalar representing approximately 2.5 units in the 6th decimal place of refractive index change indicates the sensitivity of this system of differential refractometry. This record was obtained with the Phoenix Refractometer (see text).

The silicic acid was washed several times with ethyl ether, and these washes were added to the petroleum ether extract. The pooled phospholipid-free extract was then dried, dissolved in the appropriate eluant, and charged on the factice column. Phospholipids can be recovered quantitatively by washing the silicic acid with methanol.

In the silicic acid method for removing phospholipids, free fatty acids remain with the nonpolar lipid fraction. With Florisil, nearly all of the free fatty acids are retained on the column with phospholipids, but a small amount of inorganic material from Florisil might be present with the eluted nonpolar lipids.

The free fatty acids and inorganic material can be removed from nonpolar solvents with 0.05 N aqueous sodium hydroxide. To avoid troublesome emulsions, the lipid extract was carefully layered over the aqueous alkaline phase, and the interface was slowly broadened several times by tipping the vessel. This alkaline wash was repeated several times, using small amounts of ethanol to break emulsions. The extract was then prepared for charging on factice columns.

Columns were charged by pipetting the lipid, dissolved in the smallest possible volume of eluant, directly on the column. If the volume of charge exceeds 2 ml, separations become blurred. As much as 100 mg of any solute in the lipid mixture can be eluted without significantly overloading the column. At times, such large amounts might be difficult to dissolve in small volumes of solvent; in such cases, the lipid may be placed on the column as an emulsion or suspension. However, if the charge is not sufficiently soluble in aqueous acetone, long, erratic elution peaks will result; this requires that the water content of the eluant be reduced.

When the elution is monitored with a refractometer, it is important to remove all traces of solvents other than the eluant from the charge since other solvents produce large refractive index changes at the solvent front.

Detection and Collection of Lipid. It is time-saving but not essential to monitor lipids in the effluent stream with an automatic differential refractometer. Fractions can be collected and lipid detected by other techniques; i.e., carboxyl-ester determination (10) or total weight of solute (11). Two automatic recording refractometers have been used in this laboratory: Model 5610 of the Phoenix Precision Instrument Co., Philadelphia, Pa.; and Model 21 of Waters Associates, Framingham, Mass. The latter instrument is more sensitive but requires more careful temperature control of the optical system. Both instruments detect as little as 5 μg of lipid/ml of eluant; peaks containing as little as 1 mg of any nonpolar lipid are reliably shown. A detailed account of the principles and techniques of refractometric monitoring has been published elsewhere (1). The column effluent is collected in a standard fraction collector giving 4–6 fractions/hr (10–15 ml/fraction).

These highly sensitive differential refractometers record changes in refractive index in the seventh decimal place; hence small changes in solvent composition might cause large distortions in refractive index. A small change in refractive index occurs briefly even when factice columns are charged with *solute-free* eluant. This is most likely the result of minor changes in solvent composition due to evaporation when the column stream is interrupted for charging. It produces a small peak or "solvent front disturbance" after one column volume of elution. Since the solvent front disturbance signalizes passage of a single column volume, the first recorded peak might represent both the solvent disturbance and the solute eluted in one column volume.

A small amount of column "bleed" is found in each fraction. Columns of factice, prepared as described, bleed less than 10 μg/ml of eluant. Exhaustive Soxhlet extraction of factice with organic solvents does not eliminate this bleed, nor will continuous elution of the column with aqueous acetone for many weeks. The "bleed" is composed in large part of polymerized soybean oil containing numerous components of different polarity. Much of this contamination can be eliminated from the eluant fractions by partitioning the lipid into petroleum ether (30–60°), and washing the petroleum ether upper phase three times with 50% H_2O–acetone (v/v). Only traces of "bleed" will remain with

the lipids in the upper phase. Different batches of factice obtained from the Carter-Bell Manufacturing Co. and treated by the above methods produce columns with similar amounts of "bleed."

RESULTS

A. Separation of Lipid Classes. Factice chromatography provides excellent separation of lipid classes in mixtures from which phospholipids and free fatty acids have been removed. Continuous elution with 2% water–acetone (v/v) yields the classes in the order expected for reversed-phase partition chromatography; i.e., elution of polar components before nonpolar. Figure 2 is an example of such a separation, refractometrically monitored. The solvent front disturbance is noted only as a slight distortion of the ascending limb of the monoglyceride peak. The small peak immediately following the descending limb may be due to a contaminant in the charge. At a flow rate of 1 ml/min, this first peak appeared approximately 1 hr after charging the column, and the entire separation shown in Fig. 2 was complete in less than 5 hr. Cholesteryl acetate slightly overlapped triolein, but sterol esters of long-chain fatty acids are eluted much later and do not contaminate the triglyceride peak. Likewise, triolein was almost completely separated from cholesterol, but triglycerides more polar than triolein can be eluted with cholesterol or even with the partial glycerides.

Figure 3 illustrates the overlaps found with many representatives of the commonly encountered lipid classes. The peaks are represented as blocks and their appearance time during elution is given in column volumes. One column volume is approximately equal to 1 hr of elution time. Inspection along the vertical axis indicates the overlaps, such as dilaurin and trioctanoin, distearin and trilaurin, etc. Furthermore, in this system of chromatography, shortening of a fatty acid by 2 carbons is equivalent to the insertion of one double bond; hence, tripalmitin-triolein, trimyristin-trilinolein, and trilaurin-trilinolenin are inseparable pairs. Thus, it can be predicted from Fig. 3 that trilinolenin (inseparable from trilaurin) will overlap with distearin.

The equivalence of one double bond to 2-carbon shortening leads to certain predictions for triglyceride separation outlined in Table 2. Assuming a triglyceride mixture composed of the 10 fatty acids shown in the upper portion of the table, 10 groups of triglycerides would be separated from each other by factice chromatography. As the lower portion of Table 2 indicates, most of these 10 peaks would contain many different

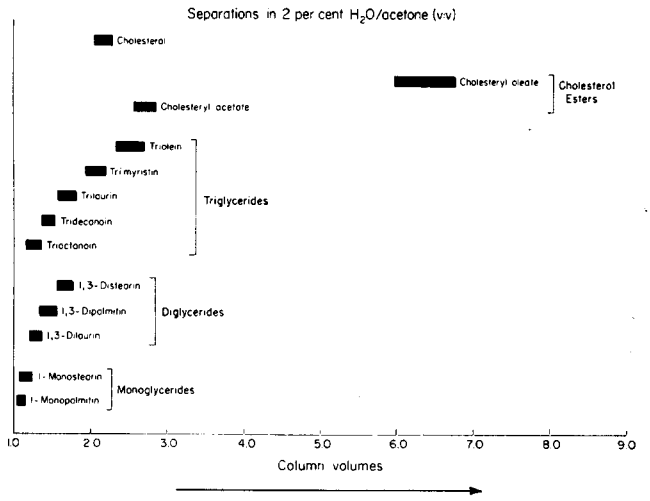

FIG. 3. Results of a series of separations indicating the time of elution of each compound in column volumes. One column volume equals approximately 60 ml or 1 hr of elution time. The length of each bar, also expressed in column-volumes, was determined by measuring the length of baseline intercepted by straight lines tangential to each limb of the chromatographic peak at its inflection point. This baseline length was then divided by the length of baseline required for the passage of one column volume. Thus each bar length represents the fraction of a column volume needed for the elution of roughly 95% of that component.

triglycerides. Fortunately, many triglyceride mixtures of biologic origin are composed predominantly of only three or four fatty acids. Hence, relatively pure triglyceride species can be isolated from naturally occurring mixtures, as will be illustrated subsequently.

The lipid classes in most naturally occurring mixtures are almost completely separable by factice chromatography, and recoveries are close to 100% (Table 3).

Compounds recovered from factice columns do not appear to have been altered or decomposed in any way; examination of highly unsaturated components by infrared and ultraviolet spectroscopy before and after chromatography shows no evidence of *cis-trans* isomerization, conjugation, or peroxidation.

B. Intra-class Separation. Cholesterol Esters. Cholesterol esters, the least polar of the major classes in naturally occurring lipid mixtures, can be easily separated from more polar lipids by factice chromatography. Furthermore, elution in 2% water–acetone (v/v) permits complete resolution of the *n*-saturated series of cholesterol esters from cholesteryl acetate to stearate. Each of these sterol esters was chromatographed alone and in combinations to permit precise determination of elution order. Figure 4 illustrates complete separation of a synthetic mixture of these sterol esters (5–15 mg of each component) in a 14-hr run (cholesterol, triolein, dipalmitin, and monopalmitin were also included). The identity and purity of each

TABLE 2. CHROMATOGRAPHIC EQUIVALENCE OF FATTY ACIDS AND THEORETICAL RESOLUTION OF TRIGLYCERIDE GROUPS

Equivalent Fatty Acids

	Group A	Group B	Group C	Group D
Double Bond Equivalence	(:0)	(:1)	(:2)	(:3)
Components	18:0	18:1 16:0	18:2 16:1 14:0	18:3 16:2 14:1 12:0

Theoretical Triglyceride Separations

	Fatty Acid Composition of Triglyceride in Each Peak									
Double Bond Equivalence of Peak	(:0)	(:1)	(:2)	(:3)	(:4)	(:5)	(:6)	(:7)	(:8)	(:9)
	AAA	AAB	ABB AAC	BBB ABC AAD	ABD ACC BBC	ACD BCC BBD	ADD BCD CCC	BDD CCD	CDD	DDD

TABLE 3. RECOVERY OF SOLUTES AFTER FACTICE CHROMATOGRAPHY

Substance	Amount of Charge	Recovery	Recovery
			%
Cholesterol	18.60 mg	18.25 mg	98.1
Dipalmitin	22.20 mg	22.75 mg	102.5
Monopalmitin	12.8 mg	11.9 mg	93.0
Trilinolein	48.60 mg	47.50 mg	97.7
	64.95 mg	60.75 mg	93.5
Chol. acetate	27.3 mg	26.00 mg	95.2
Rat adipose glycerides (H^3-labeled)	21,150 cpm	20,721 cpm	98.0

sterol ester was ascertained by GLC analysis of the fatty acid moieties. The C_{14}, C_{16}, and C_{18} esters showed less than 5% contamination with compounds from adjacent peaks.

The elution time of cholesterol esters is a logarithmic function of chain length (Fig. 5), a fact that is useful in identifying unknown components. The deviations of cholesterol (zero on the horizontal axis) and cholesteryl acetate from an otherwise orderly progression of elution is entirely reproducible.

Unsaturated sterol esters are also separable, but, unlike the triglycerides, the 2-carbon and one double bond equivalence does not pertain strictly in this lipid class. Thus, Fig. 6 demonstrates that cholesteryl oleate is seen as a leading shoulder on the peak of cholesteryl palmitate. Similarly, cholesteryl linoleate precedes the myristate, and linolenate precedes the laurate peak.

Glycerides and Methyl Esters. The *n*-saturated series of triglycerides (tributyrin to tristearin) and of methyl esters (C_4–C_{26}) are eluted in a predictable procession: the logarithms of emergence times after the solvent front are directly proportional to chain lengths. For triglycerides, elution with 5% H_2O–acetone is most effective; for long-chain methyl esters, 12% H_2O; and for short-chain or highly unsaturated methyl esters, 15% H_2O–acetone. In each case, there is precise equivalence between 2-carbon shortening and one double bond; thus methyl palmitate and oleate are inseparable at all column temperatures from 2–30°.

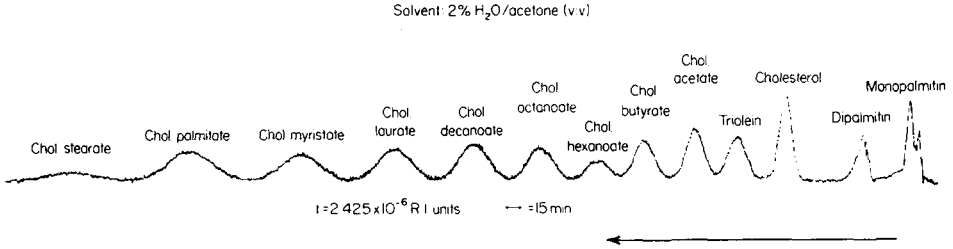

FIG. 4. Separation of the saturated series of cholesterol esters from triolein, cholesterol, and partial glycerides. At the beginning of the run on the far right, a sharp peak of solvent front disturbance is seen. The asymmetry of the dipalmitin peak is most likely due to the presence of the 1,2- and 1,3-isomers.

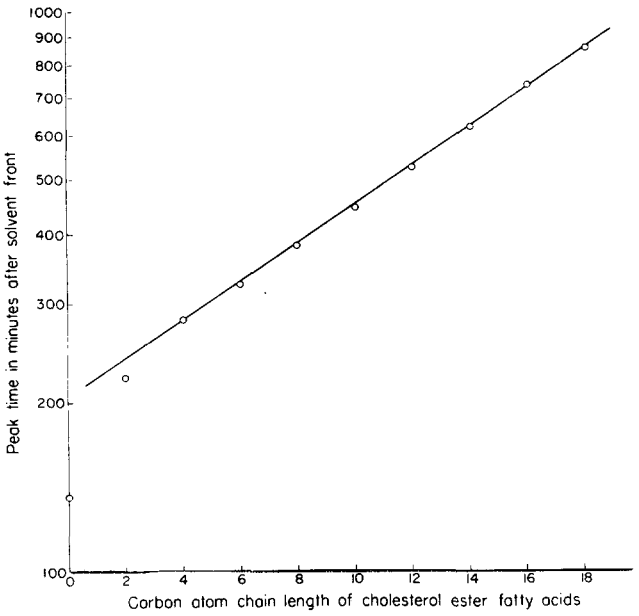

FIG. 5. The emergence time of cholesterol esters after the solvent front as a logarithmic function of fatty acid chain length. The point shown at 0 on the abscissa is the emergence time of nonesterified cholesterol.

A separation of short-chain methyl esters is illustrated in Fig. 7. Methyl butyrate appears immediately after the solvent front disturbance. Thereafter, each even-numbered ester emerges completely separated from its nearest homolog. In other experiments, methyl linoleate and linolenate eluted with methyl myristate and laurate, respectively, and the C_{18} homologs (18:0, 18:1, 18:2, and 18:3) are eluted in four separate peaks.

Further triglyceride separations are shown in Fig. 8. Partial separation of such closely related glycerides as trilinolein, dilinoleo-linolenin, dilinoleno-linolein, and trilinolenin has been accomplished. In spite of the overlaps shown and those predicted theoretically (Table 2), the analysis of glycerides by factice chromatography provides a basis for the precise structural analysis of these compounds.

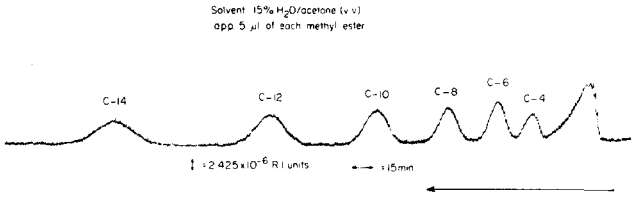

FIG. 7. Separation of methyl esters of fatty acids. The large initial peak at the right is the solvent front disturbance of refractive index. Unsaturated esters separate with the equivalent saturated esters; e.g., 18:2 with 14:0 and 18:3 with 12:0.

With appropriate acetone–water mixtures, certain mono- and diglyceride combinations can be resolved (Fig. 9). As with silicic acid thin-layer chromatography, the 1,2- and 1,3-diglycerides are separable, but 1- and 2-monoglycerides are not (12). Diglyceride isomers are more poorly separated in less aqueous eluants.

C. Separations with Naturally Occurring Mixtures. Analyses of a vegetable oil (linseed), human serum lipids, and adipose tissue by factice chromatography illustrate the utility of this method in studies of biologic mixtures. Factice chromatography of linseed oil yielded eight separate triglyceride peaks. Analysis of the fatty acids in each peak by GLC demonstrated that the first peak contained only linolenic acid or nine double bonds per molecule of triglyceride (:9 in Table 2). The second peak contained 1 mole of linoleic acid per 2 moles of linolenic acid, or eight double bonds per molecule (:8 in Table 2). Subsequent peaks contained more complex mixtures of fatty acids, since linseed oil contains C_{16} and C_{14} acids as well as C_{18}. However, the rules defined in Table 2 predicted the fatty acid composition of each peak: each successive peak had one less double-bond equivalent. A more detailed quantitative analysis of these peaks has been presented elsewhere (1).

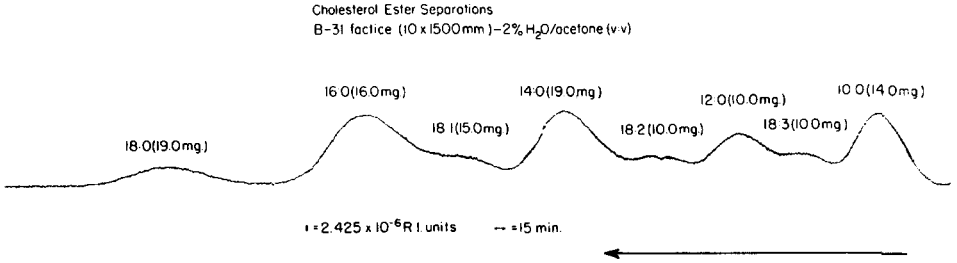

FIG. 6. Separation of cholesterol esters. Cholesterol esters with fatty acids of equivalent polarity (e.g., 16:0 and 18:1) do not completely overlap. The identity of each peak was confirmed by fraction collection and GLC analysis of the fatty acid moieties.

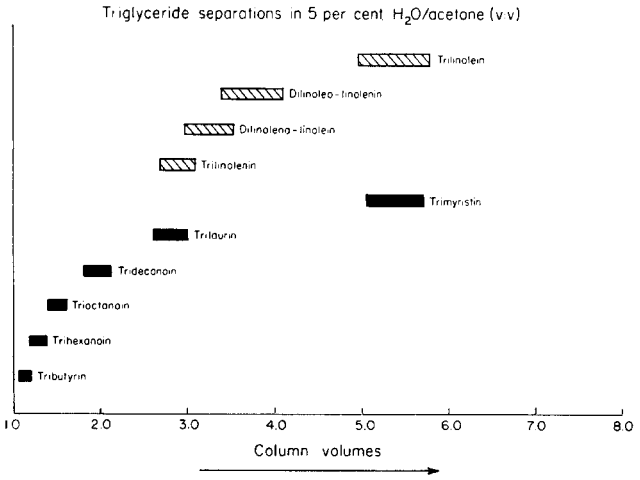

FIG. 8. Elution of four closely related unsaturated triglycerides (cross-hatched bars) with 5% H_2O–acetone (v/v). The elution in column volumes is compared with a series of saturated triglycerides.

An analysis of human serum lipids is shown in Fig. 10. The first peak at the extreme right is the solvent front disturbance. Monoglycerides and diglycerides, if present, would have appeared as separate peaks prior to the first triglyceride peak TG (I). The partial glycerides in this serum sample must have comprised less than 1% of the total lipids. The triglyceride peak labeled TG (I) contained a mixture of the following acids: 14:0, 16:0, 16:1, 18:1, and 18:2. However, 18:2 and equivalent acids (16:1 and 14:0) were present in amounts double those of 18:1 and 16:0. Hence this peak contained triglycerides with five double-bond equivalents (BCC of Table 2).

Peak TG (II) contained triglycerides with four double-bond equivalents as well as unesterified cholesterol. As shown in Fig. 3, unesterified cholesterol overlaps with triglycerides of four, five, and six double-bond equivalence (i.e., eluted with trimyristin and with other triglycerides up to triolein), but the majority of the cholesterol is eluted with the triglycerides of four double-bond equivalence. Better separation of the cholesterol from these triglycerides can be obtained with 5% water in acetone (v/v). Peak TG (III) appeared as two incompletely separated small peaks containing the three and two double-bond equivalent glycerides.

The cholesterol esters emerged in three distinct peaks. It was determined by GLC that CE-I was primarily cholesteryl arachidonate, but small amounts of other compounds were noted. CE-II was exclusively cholesteryl linoleate, and CE-III a mixture of cholesteryl oleate (ascending limb of the peak) and cholesteryl palmitate (descending limb). In other serum lipid samples, small amounts of cholesteryl stearate have been found in a small peak following and completely separated from CE-III.

Partial glycerides in human plasma can be increased in concentration by the incubation *in vitro* of plasma containing lipoprotein lipase (13). This preparation was obtained by the intravenous administration of heparin to a normal post-prandial subject. A separation of hydrolytic products is shown in Fig. 11. The pre-heparin plasma showed a series of triglyceride peaks (TG) containing cholesterol (C), with very small amounts of mono- and diglycerides (MG and DG). During *in vitro* lipolysis, MG increased as TG decreased. During this hydrolysis, however, there was little increase in diglyceride, as previously reported by Carlson and Wadstrom (13).

Triglycerides obtained by the extraction of rat epididymal fat pads are resolved into six peaks by factice chromatography. These correspond to triglycerides with 7, 6, 5, 4, 3, and 2 double-bond equivalents. The 4 and 5 double-bond peaks comprise about two-thirds of the total. The fatty acid compositions of all peaks conform to the double-bond equivalence rule for triglycerides set forth in Table 2.

DISCUSSION

Factice is a polymerized or vulcanized vegetable oil that has considerable industrial use as a rubber extender or substitute; hence the origin of the word "factice" from "caoutchouc factice," the French expression for artificial rubber. Factice can be prepared either by the vulcanization of unsaturated oils with sulfur at high temperature or by the reaction at

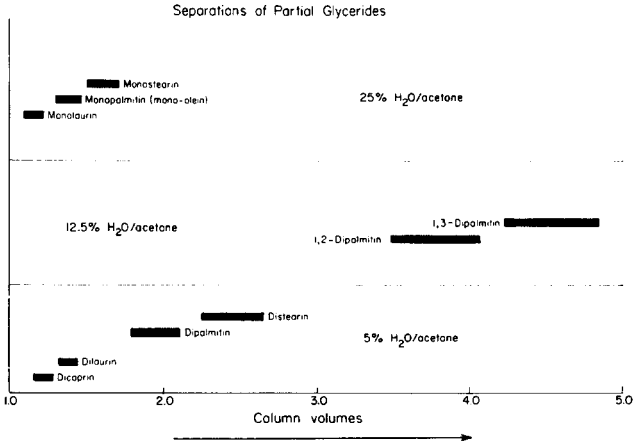

FIG. 9. Separations of partial glycerides. Partial glycerides are separated best by more aqueous H_2O–acetone mixtures than are useful for lipid class separations. Several mono- and diglyceride intra-class separations are illustrated.

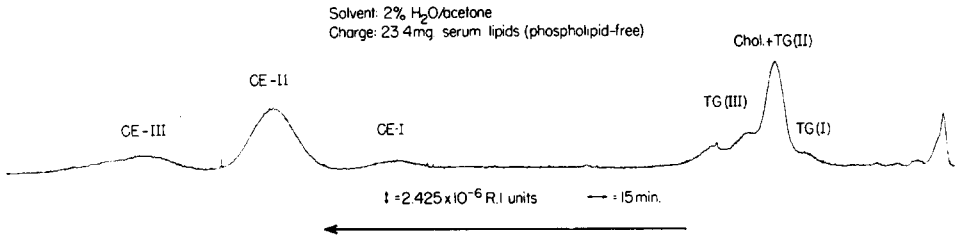

FIG. 10. Serum lipid extracts, freed of phospholipids, separated by factice chromatography. TG and CE peaks are triglyceride and cholesterol ester, respectively. The fatty acid composition of these peaks is discussed in the text.

room temperature between sulfur monochloride and vegetable oil. The latter reaction produces "white" factice, which was used in these studies. In either case, a triglyceride polymer is formed with a molecular weight most likely under 10,000 (14). The reaction mechanism is not completely understood, but most likely involves the formation of disulfide linkages between fatty acid double-bonds, thus linking fatty acids within triglyceride molecules as well as linking one triglyceride molecule to another (14).

The final product is an elastomer that readily forms gels with a variety of organic solvents. Although this is most striking with petroleum ether or chloroform, a particle of factice exposed to acetone vapor will swell and show approximately a 50% increase in weight. This suggests that the stationary phase in factice chromatography is an acetone-factice gel that can be made to shrink or expand, depending on the water content of the surrounding acetone–water mixture. With a moving phase of aqueous acetone, the system is most conveniently thought of as a form of liquid-gel partition chromatography.

Other similar hydrophobic polymers such as natural rubber or styrene-divinyl benzene co-polymers also can be used as stationary phases for lipid separation with some success. After examining more than 20 different hydrophobic polymers, however, it was found that factice was the most stable polymer for discriminating and reproducible liquid-gel chromatography of nonpolar lipids (1). Since small changes in the water content of the eluant cause the gel to shrink or expand markedly, and simultaneously there are sharp changes in the chromatographic separations achieved, it was initially thought that the gel might separate lipids in part by a molecular sieving action. However, an examination of the partition ratios of different solutes in factice–aqueous acetone mixtures reveals that a simple liquid-gel partition scheme adequately explains the results described.

The applications of factice liquid-gel chromatography are numerous. The technique lends itself to preparative work as well as to analytical studies. It performs separations within the glyceride and sterol ester classes that heretofore have been either impossible or extremely tedious. Factice chromatography can well replace countercurrent distribution for isolation of specific fatty acids in amounts as large as 100 mg of each component; fractional distillation of methyl esters must be used with either procedure to make a prior separation on the basis of chain length. Di- and monoglycerides, which often are small but biologically important constituents of tissue lipids, can easily be isolated from triglycerides and sterol esters without the "trailing" inherent in silicic acid chromatography (9). Finally, the use of a single solvent system for elution permits automatic monitoring of elution by refractometry.

The use of a sensitive recording differential refractometer is not essential but is highly valuable as a time-saving device. On the basis of the recorded peaks,

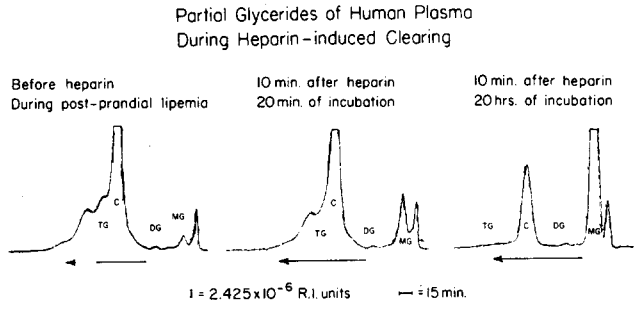

FIG. 11. Partial glycerides of human plasma during heparin-induced clearing. During *in vitro* lipolysis of serum triglycerides induced by lipoprotein lipase, there is a marked increase in the content of monoglycerides as shown by the separations in the center and on the right of this figure. Small amounts of partial glyceride were also seen in this post-prandial serum, prior to the heparin-induced release of lipoprotein lipase. The subject, a 33-year old normal male, was given 0.1 mg/kg of heparin intravenously.

fractions can be isolated for further identification and analysis. Although refractive index increments produced by all solutes are linear with solute concentration, precise quantification cannot be based on area measurements alone since refractive index increments produced by the various lipids are sometimes strikingly different. For instance, compounds rich in double bonds have almost twice the refractive index of the saturated homologs. The mixture shown in Fig. 2 contained dipalmitin, cholesterol, triolein, and cholesteryl acetate in a ratio of 6:3:14:6 by weight. By triangulation of peaks, these areas are found to be in a ratio of 6:2.6:13.3:6.2. (The monopalmitin peak was omitted from consideration because of errors induced by the solvent front disturbance.) If the esters in Fig. 2 had been rich in highly unsaturated acids, estimates of composition based on uncorrected areas would have been in error by as much as 100%. With mixtures of known components, however, triangulation or planimetry of the recorded peaks, combined with refractive index corrections for each peak, can result in exceedingly accurate quantitative analysis.

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