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Norman K. Freeman, Frank T. Lindgren,
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August 23, 1956

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ABSTRACT

A tentative method has been developed for the analysis of serum lipides, using chromatography and infrared spectrophotometry. The extracted lipides are separated into three fractions by successive elutions from a silicic acid-celite column with chloroform-hexane (1:19), chloroform, and methanol. By suitable infrared absorption measurements of these fractions (redissolved in carbon disulfide), the amounts of cholesteryl esters, glycerides, total phosphatides, cholesterol, and free fatty acids can be estimated. The accuracy for a given component is somewhat dependent on the composition of the sample. In general the probable error is about ± 10% for the major components, slightly greater for unesterified cholesterol and fatty acids.

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INTRODUCTION

In an earlier paper[1] we discussed the infrared absorption spectra of some lipoproteins and related lipides, and described a crude analytical procedure which included infrared spectrophotometric measurements of the main lipoprotein constituents as they were separable by simple solvent extraction. For the advancement of this method to a firmer and more generally useful status, one of the steps proposed was to achieve a better separation of the lipide components by the use of chromatography. We have attempted to carry out this development, using silicic acid–celite adsorption columns in essentially the manner described by Borgstrom.[2] Analysis of the eluted fractions by infrared absorption measurements provides a neat complement to the chromatographic separation, and the combination of these two techniques may be regarded as an integrated system of lipide analysis. Our purposes here are to describe this method in its present state of development, and to discuss its merits and limitations of usefulness so far as we are able to judge them from our experience to date. Brief outlines have been given elsewhere,[3,4] and the results obtained in the study of lipoprotein composition have also been presented.[5]

Perhaps the outstanding virtue of this method is its capability of yielding a large amount of information from a single relatively small lipide sample, by the use of a set of uniform operations. For appropriate purposes, and with due consideration of their accuracy requirements, it can replace a battery of separate chemical determinations--i.e., total fatty acids, free fatty acids, total cholesterol, free cholesterol, and lipide phosphorus. Insofar as degradation can be avoided during handling, the method is nondestructive of the lipide components, which are still available for other investigations or tests. We have applied the procedure with some success in studying the compositions of samples as small as 5 mg of total lipide extracted from serum or from serum fractions. It is now being undertaken, by use of different infrared equipment, to reduce the required sample to less than 1 mg of total lipide.

The accuracy obtainable with this method is subject to some variation among the various components, depending on the amount present, and to some extent on the composition of the mixture. Specific factors bearing on the accuracy are pointed out in later discussions, but ± 10% is a rough working estimate for the probable error for any component that is present to the extent of at least 0.5 mg and comprises more than about 10% of the total lipide. (Unesterified cholesterol is not only a small fraction, but it is also subject to an additional error inherent in the technique.) It seems

probable that further refinements of the method can lead to greater accuracy.

At the present stage the measurements of most individual components--e.g., cholesterol--by this method do not offer any advantage over established chemical methods. Glycerides may be an important exception, however, since they are measured as esters in a fraction from which other classes of esters have been separated. This is essentially a direct measurement, in contrast to the customary determination by multiple difference.

The estimation of total phosphatides in serum has been reasonably consistent with lipide phosphorous determinations. Work is in progress on a more detailed analysis of this class of lipides, and information should be obtained relative to the validity of this measurement for more variable types of phosphatide distributions. Until such information becomes available, the application of this method to other lipide systems, such as tissue extracts, should be made with caution.

EXPERIMENTAL

The procedure will be described for the treatment of a 1-ml serum sample, ordinarily yielding 5 to 10 mg of total lipides. For larger samples the operations should be scaled up proportionately with respect to size of column, quantity of adsorbent, and volumes of extracting and eluting solvents. (A twofold variation in amount of lipide for a given set of column conditions is apparently tolerable with respect to the quoted over-all accuracy of the method.)

Materials Required

- Methanol, cp, reagent grade
- Ethyl ether, peroxide-free
- Hexane or petroleum ether (commercial grade hexane must be redistilled)
- Chloroform, reagent grade (contains 0.5 to 1% ethanol)
- Carbon disulfide, cp, reagent grade
- Silicic acid, cp, precipitated
- Celite (analytical filter-aid, Johns-Manville)

Purity of solvents can be checked by recording the infrared spectra of their nonvolatile residues. Complete solvent blanks may also be run and the absorbances determined at the analytical wave lengths for each eluted fraction. Corrections may be applied if necessary, or further purification may be indicated.

The adsorbent is prepared by sifting and mixing together thoroughly two parts (by weight) of silicic acid and one part of celite, washing the mixture with methanol, drying it, and finally heating it to 120° C for 24 hours. It may be stored in a closed vessel, or preferably in a desiccator.

Extraction of Lipides

Any method that extracts the lipides completely would presumably be satisfactory, except that if complete extraction is attained, considerable amounts of nonlipide substances are also extracted. Excessive amounts of

such impurities are undesirable, and some provision should be made for removing them. The two-phase method described below appears to be satisfactory in this regard, and it also allows acidification, to ensure that free fatty acids are extracted in the carboxyl form. (If the latter consideration is of no interest, acidification may be omitted.)

The procedure is as follows: 4 ml of methanol is measured into a clean 40-ml screw-cap vial (the cap is lined with a thin disc of polyethylene or Teflon); 1 ml of serum is pipetted slowly into the methanol, which is gently shaken and swirled during this addition. The vial is placed in a hot water bath or heating block at 50°-60° C for about 15 min. After cooling, 5 ml of water and 20 ml of ethyl ether are added. The vial is then tightly capped and shaken in a mechanical shaker for 4 min. The phases are separated by centrifugation and the ether layer is siphoned off into a 125-ml Erlenmeyer flask. The aqueous layer is acidified with 6 drops of 6 N HCl and the extraction is twice repeated with 15-ml portions of ethyl ether. The combined ether extracts are evaporated to dryness under a hood at room temperature. The maintenance of a nitrogen atmosphere during this and subsequent evaporations does not appear to be necessary for the present level of accuracy. It is a desirable precautionary measure, however, especially if tests other than infrared measurements are contemplated (see also discussion section).

Chromatography

For a 5- to 10-mg lipide sample, the type of adsorption column we have used consists of a vertical glass tube about 15 cm long and 5 mm i.d., with the lower end drawn to a fine tip. This constricted end is plugged with glass wool. The adsorbent (0.25 to 0.30 g) is added over the glass wool, most conveniently as a thin slurry in hexane. The amount can be controlled with sufficient accuracy by determining the required column height (~35 mm) and adjusting it to that level. Packing is accomplished by the application of pressure from a rubber bulb or from a low-pressure nitrogen or air line. During this step, and throughout the remainder of the chromatographic operation, the solvent level should not be allowed to fall below the top of the adsorbent bed. A 15-ml centrifuge tube is placed under the column for the collection of Fraction I. The sample is transferred quantitatively to the column with three or four small portions of hexane. The final rinsing of the flask may be made with the first elution solvent, 5% chloroform in hexane. Eight ml of 5% chloroform in hexane is then passed through the column to elute Fraction I. (For this size column we have found it convenient to couple the top of the tube to a 10-ml syringe containing the required volume of solvent. The coupling consists of a rubber serum-bottle closure which fits the top of the tubing as a cap. This is pierced with a short hypodermic needle and mounted in a plastic frame in the proper orientation so that the column fits on the underside with the needle projecting a short distance down into it. The syringe is fitted to the needle from above and is held in vertical alignment by a ring or clip. A small brass weight, recessed on the bottom to fit over the plunger of the syringe, serves to apply pressure to the column. Since the system is closed, the solvent in the column stops flowing after the plunger reaches the bottom of the cylinder. Accidental drying out of the adsorbent is thereby effectively prevented, and close attention to the solvent level is not required.)

After the elution of Fraction I, a clean 15-ml centrifuge tube is placed under the column and Fraction II is eluted with 8 ml of $CHCl_3$. Fraction III is eluted into a third tube with 8 ml of methanol. All fractions are evaporated to dryness under a hood at a temperature not exceeding 50° C. A current of nitrogen may be used to hasten the evaporation. Fraction III is usually contaminated with a small amount of column material, which may be evident before or during evaporation. This is removed by centrifugation after about half of the methanol has evaporated. After evaporation of all fractions it is necessary to rinse down the walls of the tubes carefully with small amounts of chloroform and again evaporate to dryness. This concentrates the material in the bottom of the tube and facilitates subsequent handling in small volumes. If it is not possible to proceed with the infrared measurements within a few hours, the samples should be stored in a vacuum desiccator.

Infrared Measurements

All our measurements have been made with a Baird Associates double-beam recording spectrophotometer, equipped with a sodium chloride prism. The absorption cell used has an optical path of 0.9 mm and a volume of 0.15 ml.

For infrared measurement, the sample (eluted fraction) is brought into solution in a known volume of carbon disulfide,* and the absorption cell is then filled with this solution. The measurement of solution volume is made in one of two ways, depending on the sample size. The larger samples, amounting to about 2 mg or more, are quantitatively transferred to calibrated volumetric tubes of 0.5, 1.0, or 2.0 ml capacity, by use of capillary pipettes and small volumes of carbon disulfide. With the smaller samples--of the order of 1 mg or less--a graduated 0.2-ml pipette can be employed as the measuring device. Approximately 0.2 ml of carbon disulfide is added to the sample in the centrifuge tube, and the tube is swirled and agitated to dissolve the sample. The solution is drawn up completely into the pipette, the volume is noted, and the cell is filled directly from the pipette. It is helpful to have the tip of the pipette drawn or ground to a size commensurate with the cell opening. The pipette is operated by a 0.25-ml syringe to which it is connected by plastic tubing.

The spectrum of each eluted fraction, in a measured volume of CS_2, is recorded from 5 to 11 microns. Over the bands whose absorbances are to be measured the scanning rate should be slow enough to allow full response of the recorder. In our instrument speeds of 2 to 3 minutes per micron have been used. The use of a cell containing pure solvent in the reference beam is optional, since for the desired precision it is necessary in either case to run background curves of solvent contained in the sample cell. Absorbances at the specified absorption maxima are calculated in the customary way as $\log_{10} (T_0/T_s)$, where T_0 = transmittance of the solvent and T_s = transmittance of the sample solution.

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* It scarcely needs emphasizing that all procedures with CS_2 should be carried out under a hood.

Typical spectra obtained for the chromatographic eluates of a serum lipide sample are shown in Fig. 1. (The region from about 6 to 7.5 microns is obscured by a strong absorption band of CS_2 and has been deleted.) The dotted curve in Fig. 1A is the solvent transmission curve.

Fraction I (Fig. 1A) consists of cholesteryl esters. Their concentration is determined by measuring the peak absorbance of either the 5.8-μ band or the 8.55-μ band and reading from the appropriate calibration curve (absorbance vs concentration; see below).

Fraction II (Fig. 1B) contains glycerides, unesterified fatty acids, and unesterified cholesterol. For three components it is necessary--in general--to use three absorption bands, each calibrated for all three components. In this instance the problem is simplified in that the absorption of cholesterol at 5.75 μ and 5.85 μ is extremely small and can be neglected. These two absorption bands can therefore be used to calculate fat (glycerides) and fatty acids as a two-component system. This is most conveniently done by the mathematics of linear simultaneous equations (see Mellon, Ref. 6), provided the Beer's law relationship holds. Briefly the calculation involves first writing the equations for the absorbances A_1 and A_2 (at wave lengths 1 and 2) as functions of the concentrations and the slopes ($a = A/c$) of the calibration curves:

$$A_1 = a_{11}c_1 + a_{12}c_2,$$

$$A_2 = a_{21}c_1 + a_{22}c_2.$$

The cell thickness is a constant term implicit in all the a's.

Solution of these equations gives explicit expressions for the concentration in terms of the measured A's and a set of constants derived from the a's:

$$c_1 = k_1A_1 + k_2A_2,$$

$$c_2 = k_3A_1 + k_4A_2.$$

If we substitute a typical set of calibration values, the equations become

$$A_{5.75\ \mu} = 0.100\ C_{fat} + 0.016\ C_{fat\ acid},$$

$$A_{5.85\ \mu} = 0.009\ C_{fat} + 0.166\ C_{fat\ acid},$$

and the solutions in terms of numerical constants and measured absorbances are

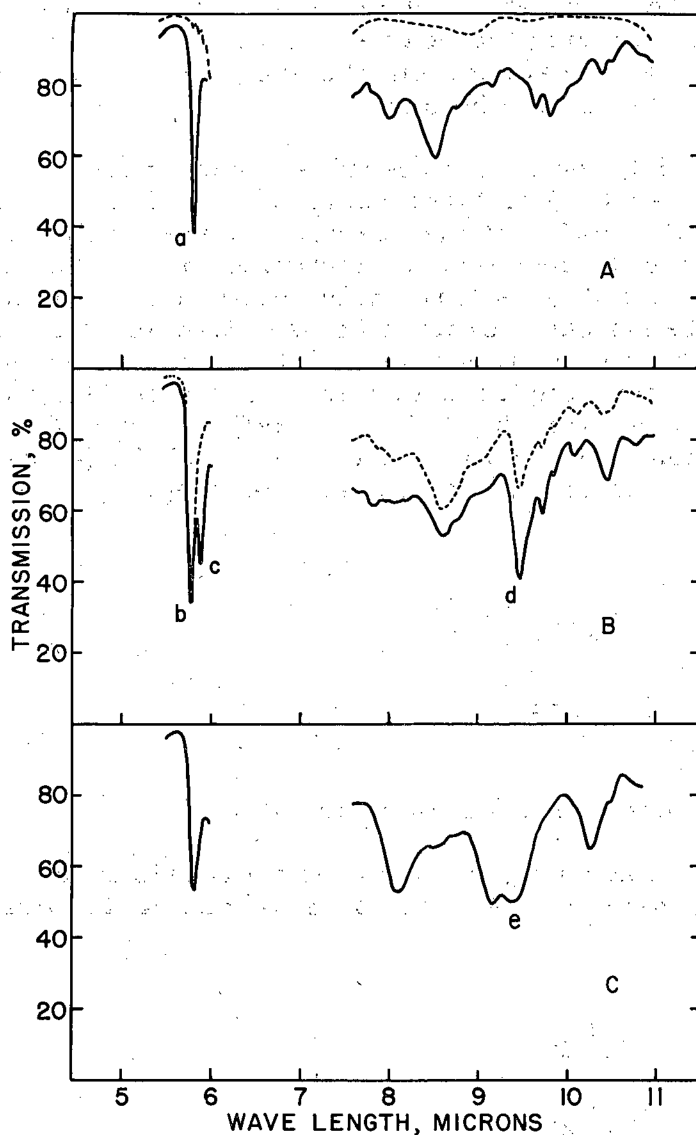

Fig. 1. Infrared spectra of serum lipide fractions eluted from silicic acid-celite. Cell thickness = 0.9 mm.

A. Solid line, Fraction I: cholesteryl esters, 5.2 mg in 1.0 ml CS_2. Broken line, CS_2 transmission curve.

B. Solid line, Fraction II: glycerides, 0.87 mg; free fatty acids, 0.36 mg; cholesterol, 1.53 mg; volume of CS_2, 0.2 ml. Broken line is a similar fraction in which free fatty acids are not present in sufficient amounts to give a discrete band at 5.85 μ.

C. Fraction III: phosphatides, 2.28 mg in 0.5 ml CS_2.

$$C_{fat} = 10.07\ A_{5.75} - 0.97\ A_{5.85},$$

$$C_{fat\ acid} = -0.55\ A_{5.75} + 6.09\ A_{5.85}.$$

Such a set of equations need be obtained only once for a given set of calibration data, and may be used as long as the slopes of the A-vs-c curves do not change.

After the concentrations of fat (C_f) and fatty acids (C_{fa}) are determined, the contributions of these two components to the absorbance at 9.5 μ are calculated from their calibration curves at that wave length. These quantities are applied as corrections to the measured absorbance of the cholesterol peak. In the particular cell for which the above calibration data were obtained, the A/c values at 9.5 μ were 0.010 and 0.006 for fat and fatty acids respectively.
Thus, for example,

$$A_{9.5}\ (\text{corrected}) = A_{9.5}\ (\text{measured}) - 0.01\ C_F - 0.006\ C_{FA}.$$

By use of this corrected value the concentration of cholesterol can be found from its calibration curve. The low order of accuracy in the determination of this component results partly from the fact that the measured absorbance at 9.5 μ is usually small (for the conditions described), and partly from the necessity for applying a double correction.

If the amount of free fatty acids is so small that no absorption peak at 5.85 μ is resolved from the glyceride band, the measurement of absorbance at that point becomes inaccurate. However, it is possible to make an estimate of the upper limit of free fatty acids, and ignore their contribution to the glyceride band at 5.75 μ. The absorbance of that band can be used directly for the estimation of glycerides from the appropriate calibration curve. Only one correction need then be applied to the 9.5-μ cholesterol band.

Fraction III contains the phosphatides, and their total concentration is determined from the absorbance of the 9.35-μ band.

Once the concentration of any component in the cell has been found from the infrared measurements, that value is multiplied by the volume of CS_2 used. This gives the actual weight of that component, i.e.,

$$\text{wt of component} = \text{conc. in cell} \times \text{vol of } CS_2.$$

Concentration of the component in the original sample, e.g., in mg per 100 ml, is given by

$$\text{mg}\ \% = \text{mg} \times 100/\text{vol of sample}.$$

Calibration

The spectra of the substances used as reference standards are shown in Figs. 2 and 3A. The principal calibration bands are indicated by small letters, which serve to identify the bands with the corresponding ones in the lipide fraction spectra, Fig. 1, and with the calibration curves in Fig. 4.

In order to prepare calibration curves (absorbance vs conc.) it is necessary to choose some representative lipide of each class as a standard, and to demonstrate that reasonable or anticipated variations in structure or fatty acid composition do not strain the limits of accuracy imposed by other methodological factors. Of the components being measured, only cholesterol can be dealt with as a specific chemical compound.

Taking fatty acids first as an example, one uses the 5.85-μ absorption band, which is a measure of carboxyl groups. Hence it is necessary--just as in titration--either to express the results on a molar basis or to make assumptions about the nature of the mixed fatty acids and their average molecular weight. We have chosen the latter alternative in using oleic acid as a calibration substance and considering that its molecular weight is reasonably close to an average for the fatty acids that are found in extracted serum lipides. We have verified that calibration curves for lauric, palmitic, stearic, and elaidic acids are all in substantial agreement with the oleic acid curve when their slopes are adjusted by using molecular weight ratios as multiplying factors.

Similarly, the 5.75-μ and 5.8-μ carbonyl absorption bands are measures of ester groups. We have used commercial olive oil as a representative triglyceride, and find satisfactory conformity with its calibration curve by tripalmitin, tristearin, corn oil, coconut oil, and tung oil--again making the necessary adjustments for molecular weight. Since our standard for cholesteryl esters has been synthetic cholesteryl laurate, the slope of the curve obtained for this substance has been adjusted to correspond to cholesteryl oleate. Substantially the same curve is obtained in a similar way from either cholesteryl palmitate or cholesteryl stearate.

Calibration for total phosphatides is contingent on the existence of an infrared absorption band that occurs with approximately the same intensity in the spectra of the principal component phosphatides. From a qualitative examination of the spectra in Fig. 3 it is evident that regions of absorption at 8.1 μ and 9.1 to 9.4 μ are potentially suitable for this purpose. Both of these regions are believed to be associated with phosphoric ester groups.[7] (It should be noted that the 5.8-μ ester carbonyl band is not found in the sphingomyelin spectrum, and the 10.3-μ band is not present in the cephalin curve.) Since lecithin is the major component, we have used either of its two absorption maxima at 9.15 μ and 9.35 μ as the analytical wave length. (The 9.15-μ band was used in certain cases where it was desirable to use $CHCl_3$ as a solvent.) Until recently egg lecithin prepared from egg yolks and purified by repeated precipitation with acetone has been used as a calibration standard. A typical preparation of this material contained 4.17% P and about 80% of phosphatidyl choline recoverable by the alumina-column treatment of Hanahan et al.[8] Recently it has been possible to calibrate with synthetic dioleyl lecithin,[2] and to compare its spectrum with that of egg phosphatides. Their absorptivities differed by 10% at 9.15 μ, and by only

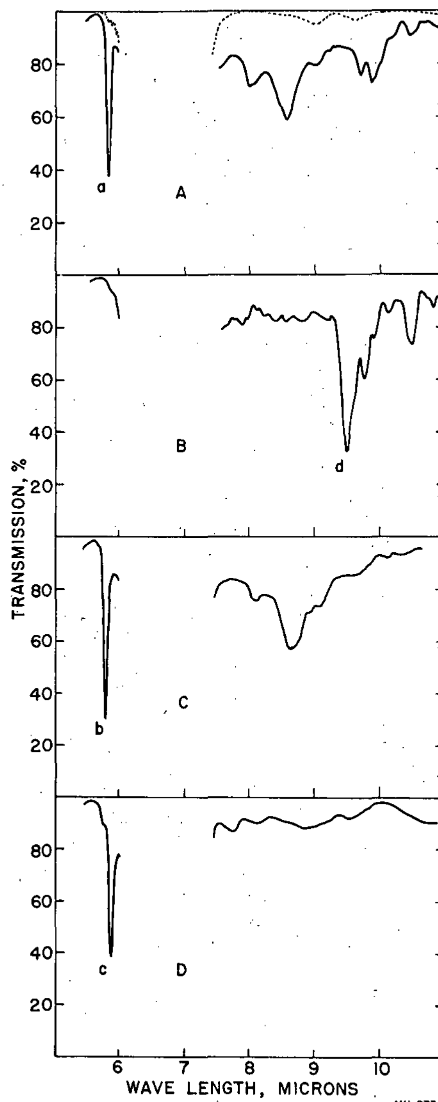

Fig. 2. Infrared spectra of nonphosphatide reference materials from 5 μ to 11 μ. CS_2 solutions, cell thickness = 0.9 mm. Small letters indicate principal bands used for measurement.

A. Cholesteryl laurate, 7.16 mg/ml, mp 77−78° C (prepared by esterification of cholesterol with lauric acid).

B. Cholesterol, mp 149° C (repurified via dibromide), 11.57 mg/ml.

C. Olive oil (commercial), 4.96 mg/ml.

D. Oleic acid (Hormel Institute), 2.50 mg/ml.

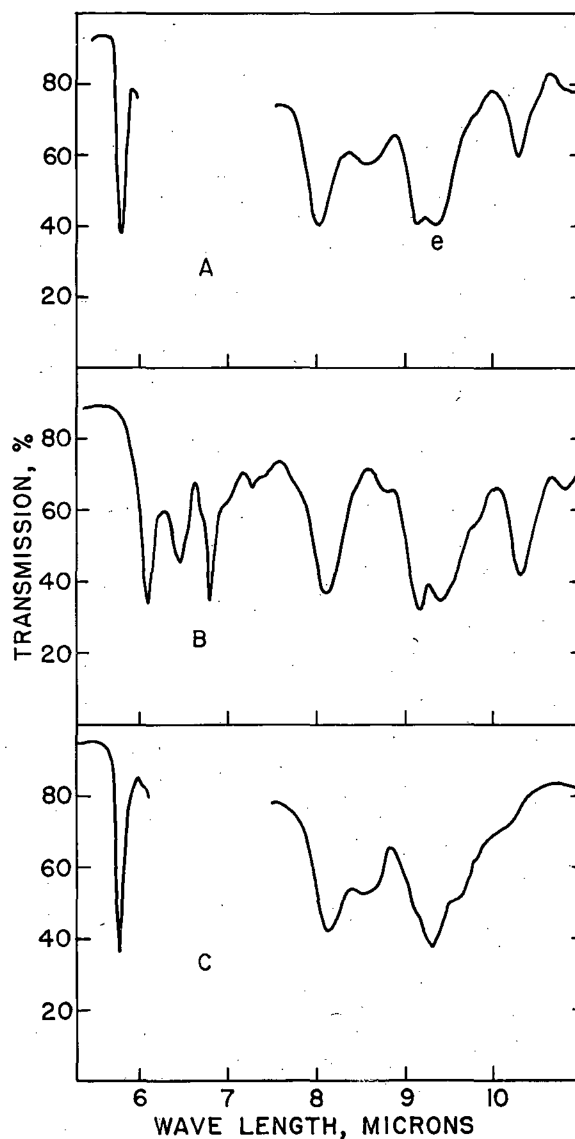

Fig. 3. Infrared spectra of phosphatide constituents from 5 μ to 11 μ. Cell thickness = 0.9 mm (for A and C).

A. Egg phosphatides (reprecipitated several times with acetone; 4.17 % P), 6.26 mg/ml in CS_2. Band designated e corresponds to calibration curve in Fig. 4.

B. Sphingomyelin (H. E. Carter, University of Illinois), solid film between salt plates.

C. Cephalin (prepared from egg phosphatides by the chromatographic procedure of Lea, Rhodes, and Stoll; II 4.0% P, 5.06 mg/ml in CS_2.

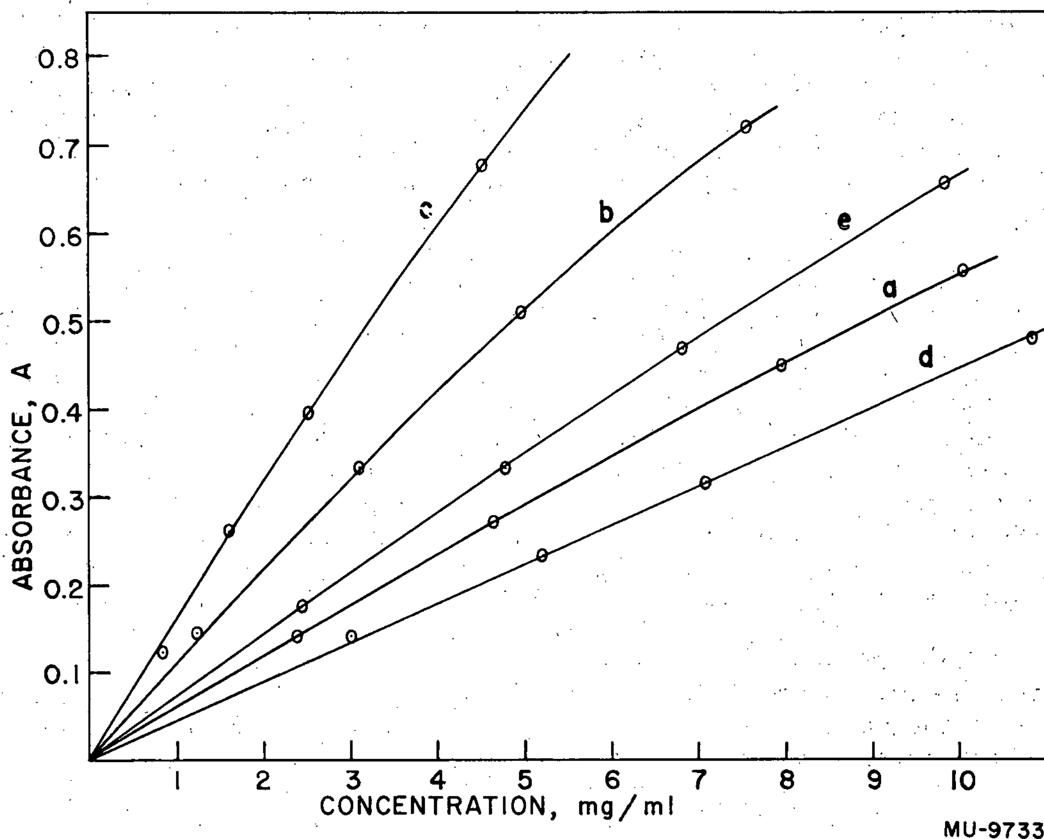

Fig. 4. Absorbance vs concentration curves at the principal wave lengths for the reference materials in CS_2 solution.

a. Cholesteryl laurate, 5.8 μ (corrected to cholesteryl oleate, see text).
b. Olive oil, 5.75 μ.
c. Oleic acid, 5.85 μ.
d. Cholesterol, 9.5 μ.
e. Egg phosphatides, 9.35 μ.

3% at 9.35 μ. These differences are consistent with the presence of cephalin in the egg material, since--as may be seen in Fig. 3c--the cephalin spectrum in this region exhibits a single peak at 9.3 u rather than the doublet pattern of the lecithin curve. The measured absorptivity of this isolated cephalin fraction at 9.35 μ (slightly off the peak) is the same as that of lecithin. Because purified sphingomyelin* has a limited solubility in CS_2, it can be compared only in chloroform solution at 9.15 μ. From limited data, its absorptivity at that wave length is about 20% less than that of lecithin. Qualitative comparison of the curves of solid films in this region suggests that the intensity correspondence should be about the same at both wave lengths. Use of the lecithin absorptivity to measure total phosphatides leads to a 4% error if the sphingomyelin content is assumed to be approximately 20%. With present calibration data and over-all measurement accuracy it is not considered worthwhile to use a weighted absorptivity for the mixed phosphatides. From the empirical standpoint, the validity of the infrared measurement is upheld by comparison with lipide phosphorous determinations in serum (Table III).

A typical set of calibration curves is illustrated in Fig. 4. Varying degrees of nonlinearity are apparent in them, which are of no consequence when the graphs are read directly. For absorbance values up to about 0.5 the deviations from linearity are small, and straight-line approximations up to that value have been used to obtain the slopes required for the two-component calculation described above. Very high absorbances, as well as very low ones, are to be avoided as less accurate on general spectrophotometric principles. This may sometimes necessitate repeating the infrared measurement at a difference concentration in cases where the estimate of required CS_2 volume fails to give a concentration for which the absorbance falls in the range of about 0.1 to 0.7.

The calibration values should be checked periodically. We have found them to remain stable within a few percent over periods of several months, although they are subject to variation from changes in cell characteristics or instrumental conditions if these are not well controlled.

RESULTS AND DISCUSSION

The effectiveness of the chromatographic separation was verified in two ways. First the procedure was carried out with a known mixture (simulating serum lipides) and with a lipide extract from human serum lipoprotein (S_f 6), both on 1-g columns. The samples were about 25 mg each. Figure 5 shows an elution curve obtained by evaporating successive 2-ml fractions to dryness, redissolving each in 0.2 ml of hexane, and measuring their refractive indices. The curve is a plot of Δn (= $n_{solution} - n_{solvent}$) versus the cumulative volume of each elution solvent; i.e., ordinate points are plotted at 2-ml intervals of abscissa x, and correspond to the measurement for the 2-ml volume fraction from x^{-2} to x.

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* We are extremely grateful to Prof. Erich Baer of the University of Toronto for the synthetic dioleyl lecithin, and to Prof. H. E. Carter of the University of Illinois for the sphingomyelin.

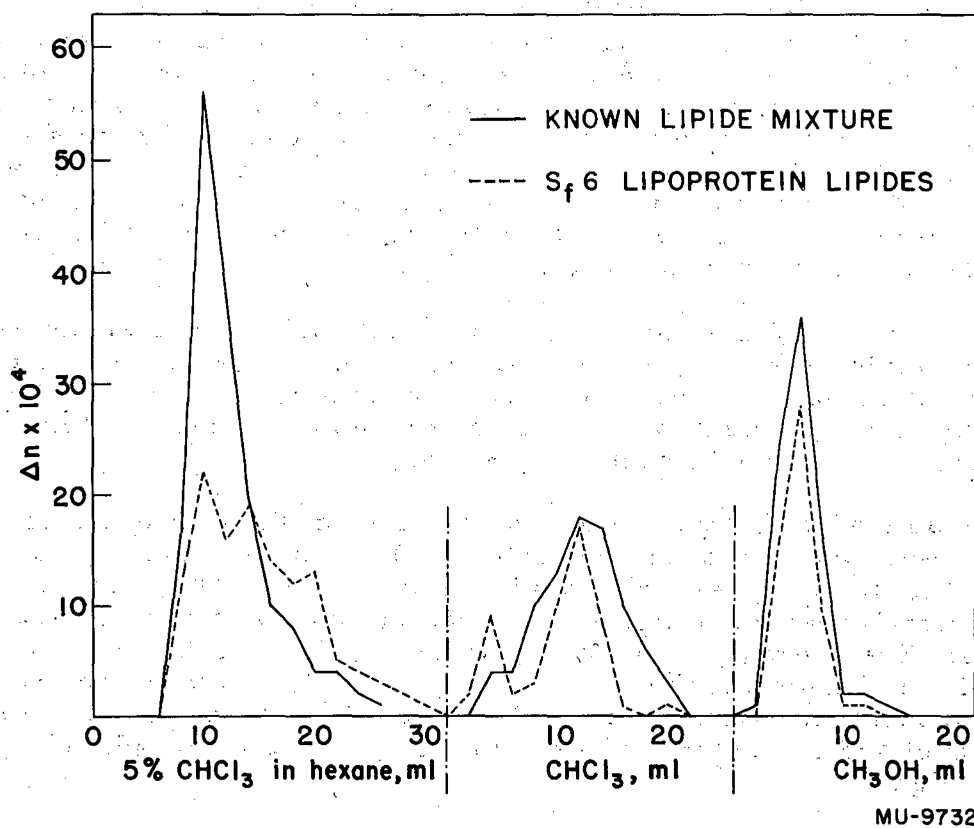

Fig. 5. Lipide elution curves obtained by refractive index measurements, 25-mg samples, 1-g columns; 2-ml fractions collected, evaporated to dryness, redissolved in 0.2 ml hexane for refractive index measurement. Δn = n solution - n solvent.

A second verification of the separability under these conditions is given in Table I. These results were obtained from three analyses of triplicate 12.3-mg aliquots of a total lipide extract from serum. In this instance 1-g columns were used, and some of the conditions for elution were altered to show the effects of these variations on the over-all results. Column A contained the standard adsorbent mixture, activated as described. Column B contained the same adsorbent mixture except that it was not heat-activated. Column C contained activated adsorbent, but the elution solvent for Fraction I was changed from 5% chloroform to 10% chloroform in hexane. For all columns the elution of Fraction I was broken into several subfractions in order to obtain a rough distribution curve. Furthermore, this elution was carried well beyond the standard elution volume (32 ml for a 1-g column). Fraction II was broken into only two subfractions, with a 25% extension of the standard volume. No breakdown was made of Fraction III. Weight recovery and total phosphatide values check well in all three analyses. From the reproducibility of the individual components it seems evident that the net results are but little affected by either of the variations employed. The chromatographic operating conditions chosen as standard are therefore considered to be adequate and to have some latitude--at least for the level of accuracy attained at present.

In certain applications a procedure essentially like that just described has been employed as a more precise control of the separation of Fractions I and II. A series of small intermediate fractions is eluted with 5% $CHCl_3$, and if any ester is detectable it is classified as cholesteryl ester or fat by its absorption peak position at 5.8 μ or 5.75 μ.

As an evaluation of the method exclusive of the extraction step, some analyses were made of a known mixture. The composition of this mixture and the results of five separate analyses are given in Table II. The weight of each sample was 4.29 mg, and chromatography was carried out on 0.25-g columns. The average error for all values in the table is about 7%. For unesterified cholesterol the average error is 14%; but with the exception of this component only three individual values are in error by more than 10%. Cholesterol and cholesteryl esters tend to be too low; phosphatides are slightly high.

Table III gives the results obtained in the complete analyses of some randomly selected serum samples, run in a routine manner as three different sets at different times. Total cholesterol and total phosphatide values obtained by this method are compared with chemical determinations of the same components. The samples had been analyzed for total cholesterol as a routine measurement in connection with other studies in progress in this laboratory. The modified Schoenheimer-Sperry method used has been described by Colman and McPhee.[9] From chromatographic-infrared measurements, total cholesterol is calculated as the sum of unesterified cholesterol plus 0.60 x cholesteryl esters. Phosphorus was determined in lipide extracts from 0.1-ml serum aliquots by an adaptation of the method of Griswold, Humoller, and McIntyre.[10] The factor 25 was used to convert phosphorus to equivalent phosphatide. In two sets of duplicates (Samples 10 and 11, Set 3) the agreement for all components is well within 10%.

Table I

Analyses of lipide extract from serum on 1-g columns,* showing discreteness of fractions and the effects of certain chromatographic variables. (Triplicate samples, 12.3 mg each. Amount of each fraction is given in mg as measured by infrared.)

Fraction		I a	b	c	d	e	II a	b	III	Total Component (mg)
vol (ml)		16	8	8	16	16	24	16	24	
Cholesteryl Esters	A	5.1	1.65	0.45	0.25	< .1				7.45
	B	6.95	0	0	0	--				6.95
	C	6.0	0.93	0.16	0.1	0				7.20
Cholesterol	A					<0.08	1.53	0.11		1.64
	B				.34	0.77	0.81	<0.05		1.92
	C					0	1.53	0.1		1.63
Glycerides	A					<0.05	0.83	<0.05		0.83
	B					<0.05	0.79	<0.05		0.79
	C					0	0.87	<0.05		0.87
Fatty Acid	A					<0.02	0.37	<0.02		0.37
	B					<0.02	0.40	<0.02		0.40
	C					0	0.36	<0.02		0.36
Phosphatides	A							<0.05	2.15	2.15
	B							<0.05	2.28	2.28
	C							<0.05	2.25	2.25

Phosphatides from chemical determination of lipide phosphorous: 2.08, 2.24 mg.

Sums of measured lipides: A 12.44 mg

B 12.34 mg

C 12.31 mg

* Column A: adsorbent and eluting solvents as described in text;

* Column B: adsorbent not heat-activated;

* Column C: adsorbent activated; eluting solvent for Fraction I changed to 10% $CHCl_3$ in hexane.

Table II

Quintuplicate analyses of a known mixture of lipides
(Values are in mg)

Component	Amount Present	Amount Found 1	2	3	4	5	Av Error 1%
Cholesteryl esters	2.09	1.83	1.95	1.92	2.05	1.99	6.8
Unesterified cholesterol	0.32	0.26	0.32	0.28	0.23	0.28	14.1
Glycerides	0.53	0.55	0.60	0.52	0.53	0.53	3.8
Unesterified fatty acids	0.21	0.20	0.21	0.21	0.21	0.20	2
Phosphatides (egg)	1.14	1.23	1.33	1.22	1.18		8.2
		Average error for all components					7.0%

Table III

Results of chromatographic-infrared serum lipide analyses. Comparison of phosphatides and total cholesterol with chemically determined values. (All concentrations are mg per 100 ml of serum.)

Sample	CE	UC	GL	UFA	PH	Chem PH	ΔPH(%)	Total Cholesterol Calc IR	Total Cholesterol Chem	Total Cholesterol Δ(%)
1	220	32	79	35	154	180	-14	163	141	+16
2	321	54	194	33	243	238	+ 2	245	239	+ 3
3	402	70	155	27	258	261	- 1	309	333	- 7
4	236	26	145	23	181	192	- 6	167	173	- 4
5	246	40	48	25	174	195	-11	188	209	-10
6	202	41	84	29	174	174	0	162	179	-10
7	272	63	147	9	254	256	- 1	226	243	- 7
8	510	91	124	23	327	350	- 7	397	428	- 7
9	101	24	93	21	140	139	+ 1	85	111	-23
10a	317	52	82	21	209	206	+ 2	241	241	0
10b	300	50	78	21	209	184	+13	229	241	- 5
11a	397	67	104	50	269	249	+ 8	303	321	- 6
11b	392	63	100	47	273	252	+ 8	296	321	- 8
12	200	29	37	46	163	152	+ 7	148	162	- 9
13	267	42	100	31	196	195	+ 1	201	231	-13
14	257	36	45	38	181	196	- 8	189	191	- 1
15	259	40	110	33	209	196	+ 7	194	216	-10

CE = Cholesteryl esters
UC = Unesterified cholesterol
GL = Glycerides (presumably triglycerides)
UFA = Unesterified fatty acids
PH = Phosphatides

In the set comprised of Samples 5 to 9, the sums of lipides measured by infrared were compared with the weighed total lipide extracts. The calculated values ranged from 94% to 97% of the weighed amounts.

Source of Error

Consideration of the most likely sources of error may not only suggest possible improvements, but may also help to avoid pitfalls. The principal errors can be segregated as they arise in extraction, chromatography, infrared photometric measurement, or calibration.

The extraction procedure described has been demonstrated to yield 96% or more of the total lipides of serum; i.e., on saponification of the extracted residue, an amount of fatty acid can be recovered that is 4% or less of the weighed extract. From most lipoproteins (from which the greatest part of serum proteins has been separated) the unextracted lipide is usually less than 1%. Other extraction methods may prove to be equally good or superior.

As indicated by the data in Table I and Fig. 5, the chromatographic separation is generally satisfactory. Small amounts of cross-contamination between Fractions I and II are difficult to detect in the spectra, since the positions of the principal bands of the ester group of cholesteryl esters are very close to the corresponding ones of glycerides. The differences are sufficient, nevertheless, that gross overlapping of these components causes measurable shifts from the correct band positions. Premature elution of Fraction II constituents in Fraction I has not been observed in the total serum lipide samples so far encountered. It has occurred, but infrequently, for other lipide mixtures containing high relative proportions of glycerides, apparently by exceeding the capacity of the column. There is for occasional samples evidence of cholesteryl esters' being carried over into Fraction II. The amount involved is ordinarily a small percentage of the total cholesteryl esters, and is only detectable if the amount of glycerides is small. Therefore it may in such cases represent a larger relative error in glycerides if that component is calculated from the total ester absorption. A carry-over of cholesteryl esters could conceivably occur as a result of oxidation, since inert atmospheres have not customarily been used in our work. On rechromatographing cholesteryl ester fractions that have been exposed to air in the dry state for long periods of time (weeks), it is found that they are distributed over all fractions. Whether rigorous exclusion of oxygen from the samples during evaporation and handling would improve the apparent overall accuracy of the method has not been investigated.

No measurable phosphorus has been found in Fraction II, but on the other hand Fraction III may contain as much as 10% of non-phosphatide material. This material has not been well characterized as yet, but it contains a hemelike pigment and has a considerably lower absorptivity than the major phosphatides at 9.35 μ. The estimated error in total phosphatides due to its presence is about 3% or less.

The concentration error resulting from infrared measurement error has been estimated as about 4%. The precision of the photometric measurements would presumably be improved if they were made at stationary wavelength settings instead of from recorded curves. However, during the developmental stage it has been helpful to have an extended spectral region in which to verify qualitatively the identities of the separated components, and to recognize accidental contaminations. (Fraction II has occasionally been found to contain phthalate esters, probably originating from impure solvents or accidentally extracted from poorly protected vial cap liners.)

In the determination of calibration curves, the error can be made small by averaging several measurements at each concentration. A greater inaccuracy undoubtedly arises from the arbitrary choice of standards, as discussed under calibration. This is a necessary compromise involving a degree of nonvalidity that is difficult to assess without more detailed information concerning the compositions of the individual lipide fractions.

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