CHROM. 4478

SEMI-AUTOMATIC CHROMATOGRAPHIC DETERMINATION OF NEUTRAL LIPIDS

N. T. WERTHESSEN, J. R. BEALL AND A. T. JAMES

Institute of Health Sciences, Brown University; Department of Gynecology and Obstetrics, University of Oklahoma School of Medicine; and the Unilever Research Laboratory, Sharnbrook, Bedford (Great Britain)

(Received October 20th, 1969)

SUMMARY

A new type of detector for liquid chromatographic columns is described based on the continuous evaporation of the eluate and redissolution of residues in a fixed solvent that is fed to a recording diffractometer. Some of the limitations of the technique and its application to the quantitative determination of neutral lipids are de-

INTRODUCTION

Although liquid chromatographic techniques are essential in much biochemical research, particularly in the lipid field, there is still a dearth of automatic recording detectors suitable for such separations. The recording diffractometers so useful for separations using a single solvent are virtually useless when applied to systems involving a solvent change or gradient elution. We have therefore combined the principles of solvent evaporation described earlier as a basis for solute detectors[1] and that of continuously dissolving the residue in a single solvent that is passed to a recording diffractometer.

In this paper we describe an apparatus developed along these lines and its application to a particular lipid separation required in metabolic studies[2]. The instrument has the following advantages:

(1) It can detect and quantitatively determine as low as 10 to 20 μg of various lipids at one-half its full sensitivity under routine working conditions (see Table I).

(2) So long as silica gel chromatography and evaporation of solvent under an inert gas (N_2 or CO_2) do not alter the lipids under study, then the present form of the apparatus is suitable.

(3) After measurement of the concentration of the lipid, the total quantity is available for further study if this is desirable, *e.g.* for determination of carbon-14 content of a fraction.

GENERAL CONSIDERATIONS

In the system described here, the total eluate from a column is evaporated on a moving inert plastic tape (Mylar or Teflon tape, 1.375 in. wide). The residue is then dissolved in a suitable solvent which passes continuously through a recording refractometer (Type R4, manufactured by Waters Associates). The change in refractive index of the solvent is shown on a potentiometric recorder, the magnitude of change being a function of the concentration of the lipid.

Successful operation of the device is dependent upon: (1) making certain that the tape is clean, (2) having rapid and complete evaporation of eluates from the column, (3) preventing continuous redissolution of eluted lipid by succesive volumes of eluate, (4) ensuring that the solvent which redissolves the lipid is maintained at a constant level of purity (which specifically includes constant concentration of dissolved gas), (5) having a constant rate of flow of solvent on the tape followed by its complete removal, (6) having complete flow control of column eluate, and (7) having controlled tape speed.

OPERATION OF THE APPARATUS

The plastic tape moves through a continuously changing solvent bath in chamber A (Fig. 1) to be washed and then it proceeds over rollers through the gas lock (1) to chamber B. In this chamber, the tape is guided below the horizontal through a drying oven (7). The tape, now carrying eluate residues, emerges from the oven and is returned to the horizontal plane by a roller before leaving chamber B.

Immediately after entry into chamber B, the tape receives the eluate from the column (2). By crimping the tape into a trough by guides on the outside edges of the drying oven, the eluate is contained in the center of the tape. Nitrogen or other inert gas is forced (via 3) over a 50-W heater in the oven and through fine holes lining the sides of the base of the oven, so that the heated gas strikes the tape and is deflected towards the central pool of eluate. Two heating elements and gas streams at different temperatures are necessary for complete control of the solvent evaporation.

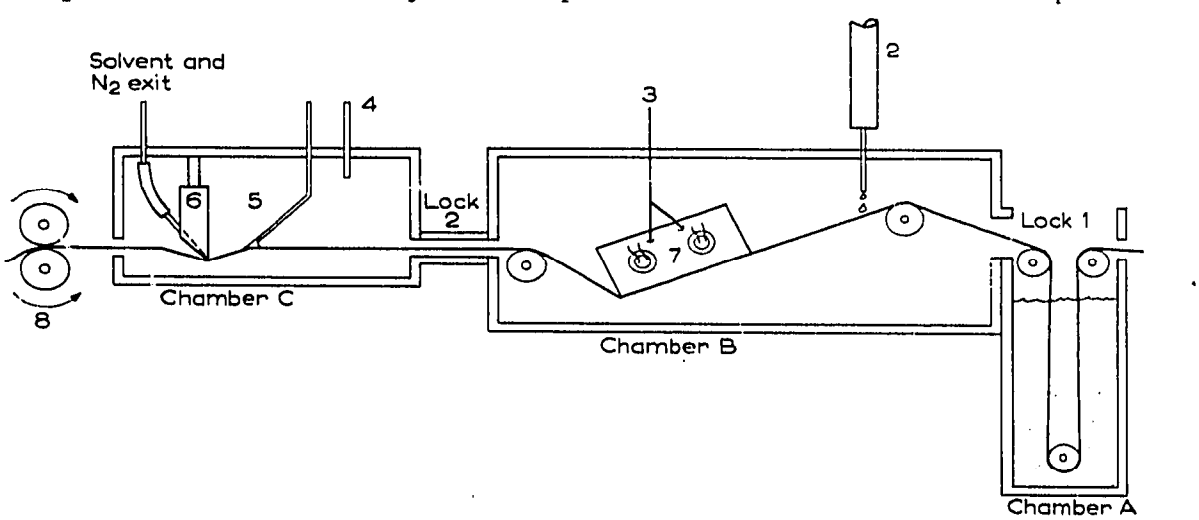

Fig. 1. Diagrammatic sketch of eluate collection, evaporation and resolution system. For key, see text.

Column eluate flows on to the tape in pulses. The pulsation coupled with the slope at which the tape is held prevents the following solvent from redissolving the residue from earlier portions of eluate. The solvent pool "buds off" under these conditions, producing discrete separate large drops which remain separate as they evaporate. Eluates which form crystalline residues show the residue as small circles after evaporation is complete.

An exhaust vacuum applied to the drying chamber removes the evaporated solvents.

For both collection of the material and observation of changes in refractive index, the tape moves into the redissolving chamber C through gas lock 2. Nitrogen saturated with the solvent used to redissolve the residue is continuously passed through C (via 4) to completely exclude air and to prevent loss of solvent in the redissolving phase and removal from the chamber.

The redissolving solvent flows to the tape via a Teflon applicator (5), 1 in. wide. This is at such a height above the tape that at the flow rate employed, the solvent forms a bridge between the applicator and the tape.

After passing under the applicator, the tape is inclined downwards to the solvent pick-up unit (6). This inclination is produced by having the pick-up unit press the tape down into a gap between two guide plates whose upper surfaces match the convex base of the pick-up unit. The pick-up unit pressing on the tape is a heavy metal section whose convex base is also cut and polished to a fine edge. At the lowest point of the base is an orifice (0.040 in.) that connects to the negative pressure produced by the pseudo-aspirator at the base of the condenser described later.

The negative pressure sucks the solvent, plus nitrogen, from the chamber up to a liquid–gas separator mounted above the refractometer. The separator unit is a glass Y-shaped tube which allows the solvent to flow from the bottom to the refractometer while the nitrogen passes to the aspirator. If the refractometer is not used, an appropriate vertical length of tubing is needed to permit the pick-up and Y tube combination to function and deliver samples to collection vials. Unless some negative liquid pressure is available at the base of such a liquid–gas separation unit, it cannot function. Flow rate of liquid from the Y tube is easily adjusted by maintaining the exit port at an appropriate level below the separator.

When the refractometer is employed, the ambient temperature in the Y tube must be greater than the internal temperature of the refractometer. Careful adjustment of the negative pressure determined by the level of the exit port is also necessary. Because the solvent contains nitrogen these adjustments must be properly made or small gas bubbles will form in the refractometer and render the instrument useless. Fortunately, gas bubbles are recognizable by pulses in the recording and so are easily differentiated.

TAPE DRIVE

The tape is drawn through the apparatus by a capstan (8, Fig. 1) having one metal and one rubber roller, driven by a synchronous motor via gearing to provide a range of speeds (minimum 1 in./min). The pressure exerted by the rollers of the capstan is sufficient to ensure that changes in tape resistance to movement will not cause changes in tape speed.

Cog tape drives, normally used for ensuring constant tape speed, were avoided because of the higher tape cost and increased difficulty in tape manipulation within the instrument. By using the cheaper unperforated tape, it is practical to use the tape only once.

In addition, using a tape only once is necessary when isotopic labelling is used, since failure to completely remove material containing label from the tape does not then cause any error in subsequent usage.

SOLVENT FLOW SYSTEM

The system consists of a still located at floor level. Vapor from the still rises through a partially insulated column to ceiling height and enters a condenser. The condensed liquid flows into a reservoir with two exits (1, Fig. 2)

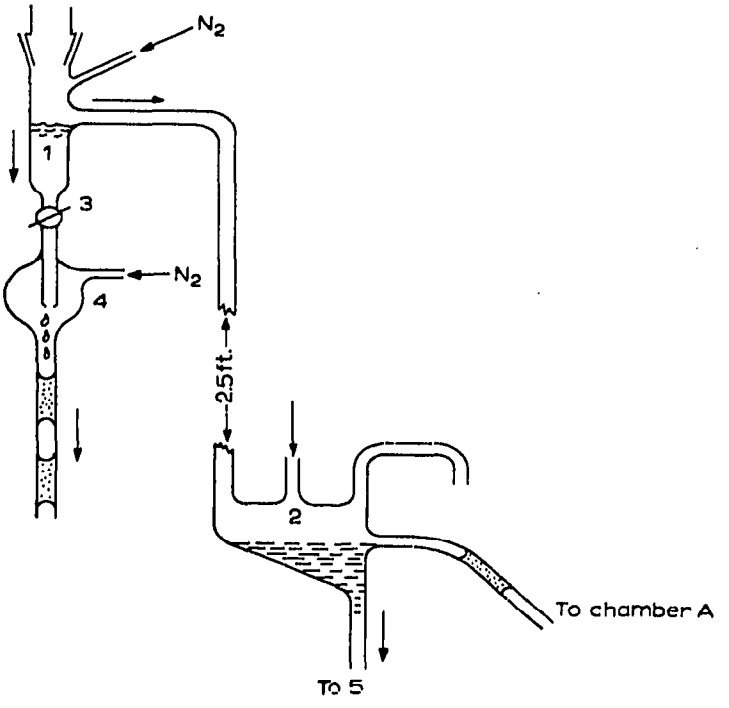

Fig. 2. Diagram of solvent system. For key, see text.

From the upper exit (an overflow), the solvent flows to a constant level reservoir (2) which feeds the applicator (5, Fig. 1). Adjustment of the height of this reservoir provides the desired flow rate to the tape. Overflow from reservoir 2 runs into the tape washing unit (Chamber A, Fig. 1). Overflow from here is returned to the still.

The second exit from reservoir 1 is fitted with a fine teflon stopcock to control flow (3, Fig. 2), the condensate then passes into the aspirator (4). The flow from the stopcock is set at a level such that discrete slugs of liquid form in the exit tube and draw nitrogen in through the side tube. The negative pressure so obtained is used to "lift" the redissolving solvent from the tape.

This simple system for producing constant flow and constant negative pressure was found satisfactory for providing the absolute constancy of flow needed. It has

additional advantages which are due to the use of the still as the source of driving power. First, constant distillation of the redissolving solvent through a fractionation column assures a minimum of change in the redissolving solvent. Secondly, the adjustments that control flow rates are working against gravity. Therefore, once set and locked they can be expected to remain constant; only changes in flow resistance can then alter flow rates. By adjusting the distillation rate to exceed flow through the aspirator and the redissolving unit, *only* the amount of fluid entering the washing chamber is subject to change as a function of room temperature and/or main voltage changes which may affect distillation rates.

It should be noted, however, that temperature of the redissolving solvent affects its viscosity. Thus its flow rate through a fixed resistance at a constant pressure head will change. Ambient temperature changes around these lines must therefore either be controlled or flow rate measured as part of the calibration procedure.

The flow rate of the redissolving solvent is in the range 0.6 to 0.8 ml/min. Since the refractometer is measuring concentration, fluctuations in rate of more than 1% or 0.0006 to 0.0008 ml are undesirable during the analysis. A change in flow rate could be reflected as a significant change in calibration value. Equally important—changing flow rates cause much "noise" and poor baselines in the recordings. No inexpensive mechanical pumps have been found capable of this degree of constancy at such low flow rates.

The importance of flow rate constancy becomes apparent when the manner of operation of the refractometer is considered. The instrument is best described as a differential refractometer in the sense that it reads the difference in refractive index (at a sensitivity of 10^{-6}) between the refractive index of fluid in the reference cell and the sample cell. If the solvents in the two chambers are maintained constant, a zero reading (arbitrarily set on the recorder scale) results.

The refractive index of a liquid is altered, at *constant* temperature, by any impurity, *e.g.* dissolved gases, liquids, or solids. The change of refractive index is a function of (a) the concentration of impurity, and (b) the refractive index of the dissolved impurity.

Thus a change in flow rate of the redissolving solvent during the period in which lipid is being redissolved will change the calibration.

OPERATION OF THE COLUMN

The column used consists of a glass tube 46 cm long, 6 mm I.D., containing 4 g of silica gel. The base of the column is fitted with an ultrafine Teflon stopcock, the volume between the sintered glass filter at the column base and the stopcock being 0.02 ml. The column was kept at constant temperature by passing water at 19° through a concentric jacket. The top of the column terminated in a standard taper (10/30) male joint, below which was fitted a Teflon collar. The solvent line was connected via a Teflon cylinder into whose base was cut a female standard taper. This cylinder was secured to the column top by adjustable connection to the teflon collar. The cylinder was bored out to a diameter of 0.040 in. which connected with two ports. The stainless-steel solvent line from the column pump was fitted to one of these ports and a small needle valve to the other. In this way, any gas at the top of the column could be extruded through the valve by the solvent.

Into the solvent line either a manifold fed by several pumps, or a mixing chamber, were connected so that both gradient elution or discrete changes of solvent could be used.

The glass tubing leading from the base of the Teflon stop-cock at the bottom of the column was drawn to a fine point. Eluate dropped into a narrow short funnel (9 mm I.D. at top, capillary at base). The funnel fitted into a 2 cm length of teflon tubing which in turn was connected to the stainless-steel (I.D. 0.040 in., O.D. 0.0655 in.) tubing which directed the eluate to the tape. The base of the tubing was held about 5 mm from the tape.

Such a multi-diameter line, in which a portion is capillary tubing, will retain fluid flowing through it until the fluid seeping into the larger diameter tubing below the capillary exerts sufficient negative pressure to pull air through the capillary base of the funnel. This is true provided, of course, that the flow rate into the funnel is not too great to prevent a break. By this simple means, a pulsating flow rate can be developed from the constant flow rate of eluate issuing from the base of the column.

Solvent pumps of various types have been employed. To date the most satisfactory in our hands have been the constant infusion pumps manufactured by the Harvard Apparatus Company. These are no more than glass syringes driven by constant-speed drives and serve well for solvents other than ethyl ether when high-resistance columns are used; for low-resistance columns they are satisfactory even with ether. When the higher pressures necessary for high-resistance columns are used, then syringes with Teflon plungers must be utilised, otherwise the low viscosity of lipid solvents will permit considerable leakage between the piston and cylinder walls.

Only Teflon, glass and stainless steel, can be used in these pumping systems. Plastics other than Teflon will continuously leach plasticizers into the solvents even after months of use.

The standard squeeze bottle, unless made of Teflon, cannot be used to store solvents employed in making lipid extracts to be analysed with this system. Metals other than stainless steel appear to release oil into the solvents; the oil apparently penetrates the metal during the machining operations and leaches out slowly.

PROBLEMS POSED BY ELUTION SOLVENTS

Because the detection system employed is sensitive to any compound soluble in the redissolving solvent, the minimum of non-volatile residue in the eluting solvents is critical; even hexane requires passage through large silicic acid columns followed by distillation.

As a purity test, 10 ml of hexane were evaporated on to a small area of the tape; when no signal was obtained, the hexane was considered sufficiently pure. Ether and methanol of analytical reagent grade usually passed this test at the first stage of the development of the system without further purification. Later a more dependable methanol ("Nanograde" provided by Mallinckrodt) has proven satisfactory with limitations (see below).

There are several escapes from the dilemma of working in a range where the solvents employed can, by the limits of purity they possess, interfere with the observations the analyst is attempting. These are: (1) blank runs, (2) solvent purification and (3) working in a restricted range.

The blank run approach is relatively simple but requires precision of operation and careful preplanning so as to minimise non-productive runs. In Fig. 4 is presented a recording from a blank run. It can be seen that there are no significant peaks recorded until methanol was used in the eluant sequence.

In this run, had the tape been stopped as each eluant came from the column, a small peak would have been seen. But since this peak, with these particular solvents, would not have exceeded 0.02 sq. in., it became invisible within the baseline variations when stretched out over the 15 min of elution and evaporation. The methanol in the eluate, however, appears to extract from the column that portion of impurity in the prior eluates which is absorbed by the column.

Successful application of the blank run principle requires, therefore, (a) precision operation (b) operation of a suitable column in a fashion which (1) minimises the solvent needed, and (2) moves the desired fraction off the column in as sharply defined fashion as possible.

It is not usually necessary to use a column capable of separating the components of 5 mg of assorted lipids when observations at the 10 to 50 μg level are to be made. This was the case in the work on which this description is based. A reduced column size reduces the solvent volume proportionately. At appropriate levels of choice of column and solvent volume, the level of impurity in the solvents employed in our studies would not interfere in the accuracy of determining 10 to 50 μg of lipid.

COLUMN COMPOSITION AND USE

With some grades of silica gel, regeneration of the column is possible, a suitable grade is "ultra pure" silica gel (0.05–0.2 mm) (Brinkman Instrument Company). Regeneration is performed by passing three column volumes of methanol, followed by a similar volume of ethyl ether through the column. Refilling the column with hexane or 5 % ethyl ether in hexane completes the procedure.

With continued use of the overall system, it has been found necessary to develop criteria for (a) properly constructed columns and (b) the continued ability of a column to function. It is not yet certain that the following criteria will prove permanently satisfactory. As of this date, however, they have resolved several situations and seemingly prevented potential difficulty. They are:

(1) When a column is constructed, several blank runs are made. Unless the column conforms (see Fig. 4) to expected blank run standards, it is discarded. If it conforms, frequent later runs are made to assure that the blank run values are maintained.

(2) An artificial mixture of known compounds (in our case sterol ester and free sterol) is placed on the column. If the results of this test conform to the expected values, the column is ready for use on unknown mixtures.

(3) A column use history is maintained. Failure to conform to expectations on test runs terminates use of the column. Several columns have functioned well through 50 determinations.

CHOICE OF "REDISSOLVING SOLVENT"

The sensitivity of the refractometer assay system is at a maximum when the solvent used has a refractive index far removed from that of the solutes. The most

suitable such solvent from the refractive index aspect for most blood and tissue lipids is methanol. Unfortunately, however, triglycerides are poorly soluble in methanol. The best compromise of both refractive index, solubility etc. is ethanol. A composite solvent such as chloroform–methanol cannot be employed if a still is used for solvent distribution and re-use. "Constant boiling" mixtures prove difficult because changes in barometric pressure alter the output of the still.

Test system

Because of our biological interests, development of the overall system was limited to accurate separation and measurement of sterol esters, triglycerides, free sterols and free fatty acids as discrete *classes*.

The elution sequence employed in the first evaluation of the analytical system was as follows:

(1) 5 % ethyl ether in hexane was used to fill the column after regeneration and to dissolve the lipid samples applied to the column.

(2) After application of the lipids to the column, 5 % ether in hexane was passed through for 10 min at the rate of 1 ml/min.

(3) Continuous gradient elution was then begun by adding 100 % ether to the 90-ml volume of 5 % ether in hexane in the mixing chamber at a constant rate.

(4) When the peak corresponding to the free sterols was seen, gradient elution was stopped and 100 % methanol was put through the column at a lower rate. The resulting peak would be expected to contain free fatty acids, residual phospholipids and possibly other very polar lipids. This fraction in the first stage of development was used only to prove recovery of lipids by tracer analysis.

The material present in each peak was determined by adding known quantities of specific substances to the standard extract described below. Defined quantities of each material had previously been deposited directly on the tape so that detector sensitivity could be predicted.

By finding a specific peak increased by the expected area, it was demonstrated (a) that the peak contained the substance and (b) that the added material was totally eluted from the column. Then by maintaining constant all volumes of solvent and the rates of change of solvent in the gradient elution, it became possible to predict within 2 to 3 min when a peak could be expected to appear. Sterol peaks were also identified by the Liebermann–Burchard reaction.

As a reference standard of biological mixture of lipids instead of a synthetic mixture, 50 ml of human plasma which had been deep frozen were extracted with chloroform–methanol (2:1). The extract was evaporated to an oil which was then re-extracted with chloroform. The chloroform was reduced in volume and dry acetone added. The mixture was evaporated to small volume and acetone again added. This procedure was repeated three times. The final volume reduction gave an oil.

The oil was then extracted with dry acetone. To the acetone extract, $MgCl_2$ was added to precipitate phospholipids. The concentration employed was 0.5 ml of a 95 % ethanol solution (50 g $MgCl_2$ per 100 ml) per 10 ml of acetone. The dissolved phospholipids were permitted to precipitate overnight at 4°. Removal of the phospholipids was completed by filtration through a sintered glass filter covered by a 2-cm-thick layer of ground filter paper. The final product was dissolved in 5 % ether in hexane. Although this procedure can be quantitative, no effort was made in

this preparation to recover all of the desired lipids available in the sample. The prime concern was exclusion of the phospholipids because of their interference with neutral lipid separation.

Aliquots of the extract ranging from an equivalent of 0.015 ml of plasma to 0.125 were assayed with the elution sequence described above. The calibration curves resulting for the sterol esters, triglycerides and free sterol fractions are presented in Fig. 3. The free fatty acid levels were not observed because (a) they were low and (b) at this stage of development of the procedure the blank methanol fraction showed erratic peaks due to solvent contaminants.

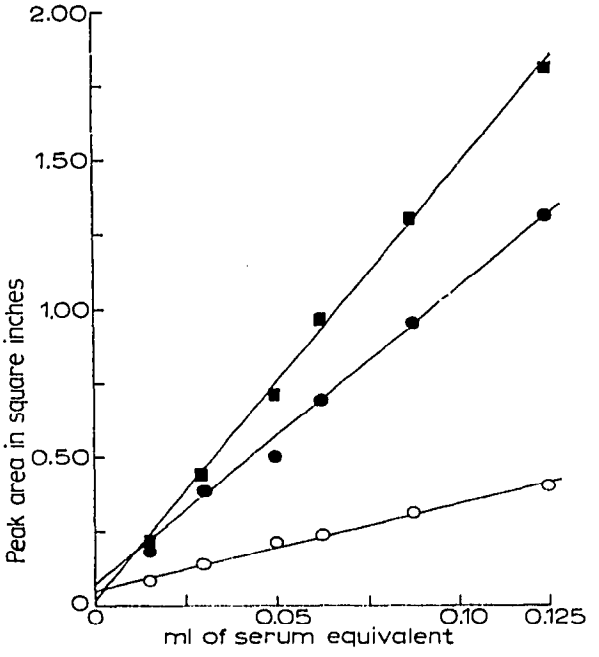

Fig. 3. The relationship between the area of the peak in square inches and the quantity of serum equivalent of the extract described in the text. Solid squares represent triglycerides; solid circles represent sterol esters; open circles represent free sterol.

As Fig. 3 shows, the device showed a linear response for each of the three lipid classes. However, only the triglyceride line passed through the origin. Computation of linearity by the method of least squares gave intercepts of 0.038, 0.050, and 0.010 sq. in. for the sterol ester, free sterol and triglyceride lines, respectively.

Theoretically, each of these lines should pass through O. A blank run through a column to which no extract has been added showed no peaks at the expected times on the recording.

Explanations of the observed discrepancy *at the time* were (a) that the solvents were *not* completely free of residue and (b) that when a lipid class moved down the column it carried with it an accumulation of residue that was not moved in similar fashion by the solvents alone.

Another possibility could have been the incomplete removal of solvent from the lipids. Retention of solvent, if it is constant and directly related to the amount of lipid deposited on the tape, would fit the observations. It was, however, difficult to accept this possibility. Neither rate of evaporation of samples of lipids nor the nature of solvent employed to deposit a particular sample on the tape was found to influence

the resulting signal. Had retained solvent contributed to the size of the signal, then deposition of a sterol ester sample on the tape dissolved in methanol should have produced a markedly different peak from the same amount of ester when it was deposited in a chloroform solution. Checks of this sort were applied early in development of the system and found to give the desired result: to wit, constant area per unit of lipid irrespective of solvent employed.

It was not until the refractometer was improved and a different system of elution was employed that it became clear that solvent impurity was the primary source of the difficulty. This is discussed below.

As one part of the original calibration of the system, sterol esters, triglycerides (as olive oil) and cholesterol were added to serum aliquots. The area obtained for each peak was corrected by subtraction of the area expected for the serum aliquot alone.

It was found in the initial study that with an ethanol flow of 0.78 ml/min, with the original refractometer at one-half its full sensitivity, that 0.02 sq. in. peaks were easily seen. In Table I are presented the final calibration values for that stage of development.

TABLE I

MICROGRAMS PER SQUARE INCH OF PEAK OF THREE LIPID CLASSES

Sterol ester	268 μg
Triglyceride	390 μg
Free cholesterol	206 μg

TABLE II

AMOUNT OF LIPID IN THE SERUM EXTRACT

	According to Dr. Kritchevsky (*mg/100 ml*)	*New system* (*mg/100 ml*)
Sterol esters	156	161
Triglycerides	595	579
Free cholesterol	51	58

Through the kindness of Dr. DAVID KRITCHEVSKY, an independent analysis of the three major classes of lipids in the serum extract was obtained. The sterol ester and free sterol areas obtained by use of the new system were corrected, for this comparison, by the value of the area at the intercept; the triglyceride value was not corrected. Comparative results are shown in Table II.

The values for the sterol esters and triglycerides lie within the probable error of both techniques. The free cholesterol values are significantly different and illustrate the inherent problem of any assay system of the type being described. The higher free cholesterol value obtained by using the refractive index method is undoubtedly due to the well known fact that other substances besides cholesterol are eluted by the solvent mixture which elutes cholesterol from a column of this type.

The completion of this series of studies satisfied us as to the usefulness of the

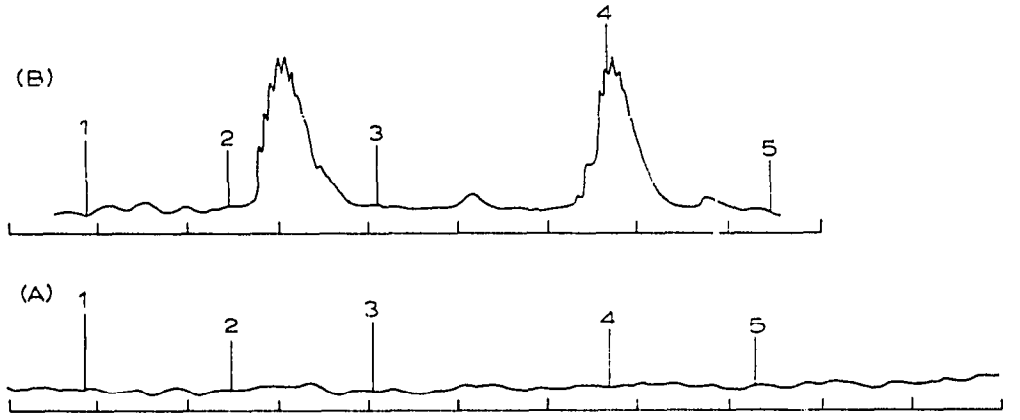

Fig. 4. Presented in this figure are tracing of column calibrating experiments done at maximal sensitivity. (A) represents a blank run and illustrates the peak seen when methanol strips the column of residue deposited by the earlier eluants. The vertical lines at the base of each tracing represent 10-minute intervals. The vertical, numbered lines represent the onset of pumping the following eluants through the column: (1) 12.0 ml of 5% ethyl ether in hexane; (2) 12.0 ml of 20% ethyl ether in hexane; (3) 20.0 ml of 50% ethyl ether in hexane; (4) 12.0 ml of 50% ethyl ether in methanol; (5) 12.0 ml of methanol. All eluants, except methanol, were pumped at 0.84 ml/min. Methanol was pumped at 0.4 ml/min. (B) 97.2 μg of cholesterol and 114 μg of cholesterol linoleate were placed on the column. The expected peaks were 1.13 and 0.98 sq. in., respectively. The areas found were 1.02 and 0.104. The column was regenerated by passing through 30 ml of methanol, 30 ml of ethyl ether and 15 ml of 5% ether in hexane. The column volume was 9 ml.

TABLE III

REPRESENTATIVE CALIBRATION VALUES DETERMINED ON NEW INSTRUMENT EMPLOYING PLANIMETER ACCURATE TO 0.01 sq. in.

The varying concentrations of purified cholesterol (via dibromination) and cholesterol linoleate (synthetic) were placed on the tape through the same funnel system used for column eluates. A wash of 0.3 ml of 5% ether in hexane was used to ensure complete deposit of standard on the tape. Redissolving solvent flow rate was 0.714 ml/min. Instrument sensitivity was maximal.

Micrograms	*Square inches*	*Micrograms per square inch*
Cholesterol determinations		
97.2	1.163	83.6
121.5	1.463	83.0
145.8	1.673	87.1
48.6	0.550	88.4
24.3	0.293	82.9
97.2	1.093	88.9
Calibration value = 85 ± 2.8 μg/sq. in.		
Cholesterol linoleate determination		
28.5	0.250	114.0
57.0	0.500	114.0
85.5	0.763	112.1
114.0	0.890	128.1
142.5	1.190	119.7
171.0	1.520	112.5
Calibration value = 116.7 ± 6.2 μg/sq. in.		

overall system and now a more sensitive refractometer has been obtained which provides a more stable baseline. A typical run is shown in Fig. 4. Calibration values are listed in Table III.

ACKNOWLEDGEMENT

Supported in part by United States Public Health Service Grant No. HD02599.

REFERENCES

1 A. T. JAMES, J. R. RAVENHILL AND R. P. W. SCOTT, *Chem. Ind. (London)*, (1964) 746.
2 J. A. MERRILL AND N. T. WERTHESSEN, *Am. J. Obstet. Gynecol.*, 96 (1966) 619.