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Microcolumn Liquid Analysis of Lipids with Flame Ionization Detection

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Abstract

A system for the rapid analysis of compounds which may be either too large or too sensitive to be subjected intact to gas chromatographic or other methods is described. Capillary columns of 0.5 mm and 1 mm I.D. and various lengths are packed with very fine particle size, specially prepared, silica gel and other media. A simple pumping arrangement is employed for either single solvent or gradient elution, and the solute in the effluent is detected by means of a highly sensitive flame ionization detector. The application of this system is illustrated by the separation of microgram and submicrogram amounts of triglycerides, steroids, and other neutral lipids, as well as some phospholipids.

Introduction

THE DEVELOPMENT OF LIQUID column chromatographic techniques has lagged behind the rapid growth of thin-layer methods and gas chromatography, particularly in the field of lipid investigation. This may be due primarily to the fact that with these latter techniques very small samples may be employed, and this has in turn permitted high resolving power to be obtained. The chief obstacle to a similar utilization of liquid column systems is not a problem of theoretical resolution, but is a result of the lack, until recently, of a highly sensitive detection method which is not narrowly restricted to the detection of only one class of compound. Additionally, such a detector should not be affected by the eluting solvent, or changes in solvent composition. It is the purpose of this discussion to describe a system which overcomes these difficulties, to give some illustrations of its usefulness in combination with micro glass capillary columns, particularly with regard to problems of lipid analysis, and finally to explore some of the means by which it might eventually be automated.

Detection Systems

Two fundamentally different approaches may be taken towards developing a detector for liquid column effluents. Either the solute may be detected in the presence of solvent, or alternatively the solvent may be removed prior to the detection of the solute.

Several rather severe limitations are placed on any system which does not remove the solvent. The detector must then be of such a specific nature that it does not sense the solvent, or it must measure a change in some physical property of the solution. A further complication arises where solvent gradients must be employed. Detectors of this type include e.g., ultra-violet absorption, light absorption after color development with an external reagent, refractive index and ultrasonic devices. Each of these suffers from lack of general applicability, or has other problems related to the presence of the solvent.

It was thus considered by us as well as by other workers (1,3,5,7) that prior removal of solvent would have many advantages. The only obstacles to be overcome in this approach were technical, and not theoretical ones. Two aspects of the problem are apparent. The solvent must be removed selectively, and the residue must then be conveyed to the detector in a manner that reflects its original concentration. This requires that some sort of solvent evaporation chamber be placed between the columns and detector.

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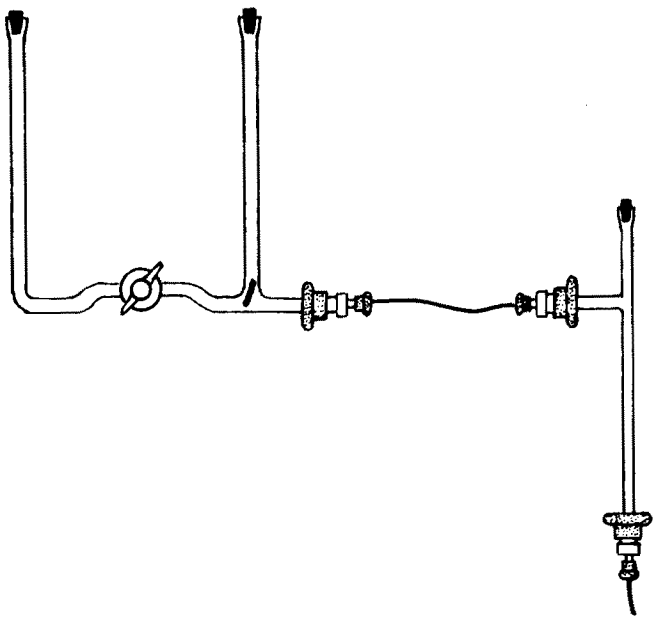

FIG. 1. Micro gradient mixer; the capacity of each arm is 3 ml. Both arms are connected across the top to equalize the pressure and obtain approximately linear gradients.

Both a moving wire (3) and a chain (1,2,7,8) have been used to transport the effluent from the column to the evaporation chamber. In our system we have preferred to use a chain because it has the advantage of being able to hold a much larger amount of solution for a given length than a wire, and also permits a slower speed through the detector. After solvent removal in a heated chamber, the sample is next introduced into the detector. The detector itself, has in every case been similar to one of those used in gas chromatography, based on either argon (3), or flame ionization (1,2,7,8).

Fundamentally different approaches have been taken, however, in the method of conveying the sample from the solvent evaporation chamber to the detector. The devices of James, Scott, and Ravenhill (3) and those of Karmen (4,5) depend on a prior pyrolysis or vaporization of the sample before it goes into the detector. While this may be suitable for

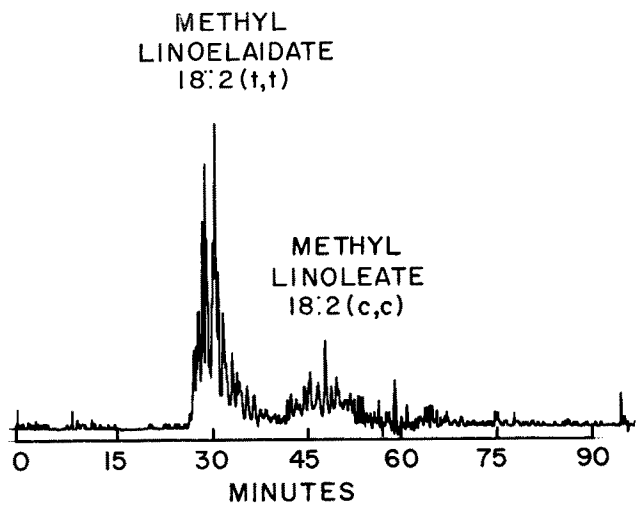

FIG. 2. Separations of cis-trans isomers of fatty acid methyl esters. Five micrograms of each component were injected on the column. Column: 1 mm × 600 mm Silica Gel H; 30% silver nitrate.

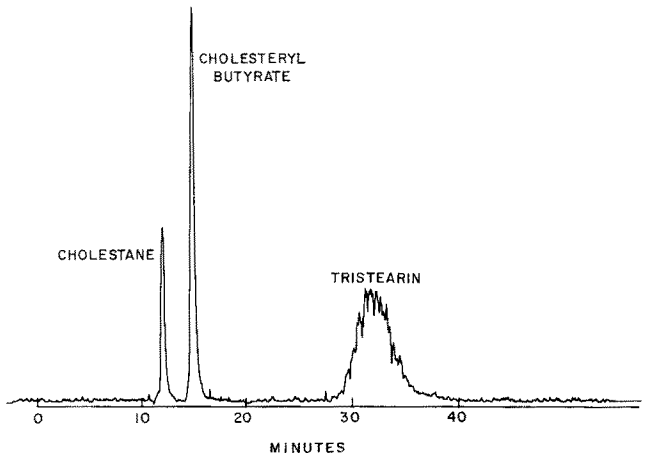

FIG. 3. Rapid analysis of a mixture containing a hydrocarbon, a sterol ester, and a triglyceride. Silica Gel H; solvent, benzene.

many classes of compound, it would seem that quantitative pyrolysis of some samples of substances such as lipoproteins, complex phospholipids, and other large molecules would be difficult in the short time permitted. Thus direct introduction of the intact sample into the detector would appear to be more desirable. This has been done in our case by passing the chain through the long axis of the burner tube into the hydrogen flame ionization cell, thus assuring complete combustion of the sample (7,8). A similar arrangement has also been described by Haahti (1,2). In the work discussed below we have employed a model 5400 Barber-Colman liquid chromatograph detector which is similar to the detector which we have developed.

Application of Microcolumn System to Lipid Separations

Considerable improvement in sharpness of separation can be achieved in reducing the size of the column and at the same time reducing the sample load. Such an effect is also seen in the differences between preparative and analytical scale separations in gas chromatography. By being able to detect very small samples in the column effluent by means of the detectors described above, it became possible to carry our column separations on miniature columns with a rapid flow rate, and achieve good resolution, with sample loads of only a few micrograms or even less (8). Applications of this technique to peptides, proteins and other macromolecules, including viruses, have been described by us previously (8,9). In most of these separations molecular sieving techniques or liquid-liquid partition have been employed. In the examples cited below for lipid separations we have utilized glass capillary columns packed with silica gel or silver nitrate impregnated silica gel. However, one is not restricted to using silica gel columns, and several interesting applications involving methylated Sephadex and reversed phase partition have been described by Haahti (2).

The glass capillary columns were connected to a solvent reservoir by means of fine bore Teflon tubing. The flow rate is adjusted by controlling the pressure applied from a cylinder of nitrogen to the solvent reservoir, and may be varied from about 200 μl per hour up to about 500 μl per minute. Simple linear solvent gradients may be established, if required, by

use of the miniature gradient mixing device shown in Fig. 1. While this may perhaps lack the versatility of a direct pumping arrangement, it does have the advantage of simplicity and yields reproducible results. The sample was loaded on the column by means of a microliter syringe through the septum in the screw cap on top of the column. The columns were packed with Silica Gel H, Merck, 5–25 μ particle size, which had been acid-washed by treatment with boiling concentrated hydrochloric acid followed by extensive washing with distilled water and methanol. The silica gel was activated prior to column packing by heating at 120C for one hour. The packing procedure requires only a few minutes and is essentially the same as for large columns.[1] The bottom of the column is fitted with a porous polyethylene or Teflon-coated glass fiber filter to support the silica gel. A similar procedure was used in preparing and packing the silver nitrate impregnated silica gel columns.

Fig. 2 shows an example of a separation of geometrical isomers in less than an hour using silver-nitrate-impregnated silica gel. This illustrates a type of separation which may have considerable utility, particularly with only a few micrograms of sample. A similarity with the kind of result obtainable by means of thin-layer chromatography (TLC) can be seen. The advantage of the microcolumn procedure is that detection of the compounds is made easier, and wtih further refinement of technique should lead to a simple method of quantitation, thus avoiding charring and densitometric scanning.

[1] It has recently been found that a dry packing procedure similar to that described by Nelson (6) leads to improved results. Column pressures of about 1–2 atmospheres are generally required for elution.

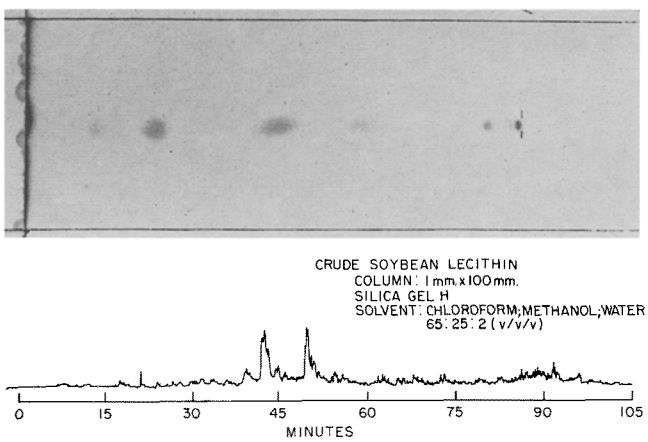

FIG. 4. Crude soybean lecithin fraction showing separation into two major peaks. Several minor components are also apparent. A comparison can be made with the same sample spotted on a thin layer plate and run in chloroform-methanol-water: 65:25:4 (v/v/v) on Silica Gel G as shown above.

Fig. 3 shows the separation of a mixture of 0.5 μg cholestane, 2.0 μg cholesteryl butyrate and 9 μg of tristearin on a 100 × 1 mm column of Silica Gel H. It is apparent that very much improvement has been obtained in reduction of signal noise level, and the result approaches that which one might expect from a gas chromatograph.

In Fig. 4 is seen the result of chromatographic analysis of a sample of crude soybean lecithin. A comparison with the result obtained by TLC is shown.

The illustrations cited above serve as representative samples of the type of separations which can be obtained with this system. It has the versatility of thin-layer, combined with a more general means of detection and possible quantitation. While this work has described an analytical system in which the entire column output is fed into the detector, it should be emphasized that the detector part of this system can also be employed to monitor large preparative columns. Two methods for doing this are available. The first involves stream splitting of the column effluent, and the second simply uses the detector to monitor samples collected in test tubes, by withdrawing 1 μl, or other suitable volume aliquots, and placing these on the chain with a microliter syringe. Such a procedure has yielded excellent, precise, quantitative data for protein hormone separations, with a degree of sensitivity far exceeding that obtained by other means (10).

Much further work remains to be done, particularly in improvements with regard to the solvent pumping system and conveyance of the sample to the detector in a smooth manner, before a truly automated system can be set up. However, the way is now open for these refinements to be made. Perhaps some of those features incorporated into the elegant system developed by Nelson (6) for handling larger samples could also be applied here.

ACKNOWLEDGMENT

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