The Separation of the Long Chain Fatty Acids by Gas-Liquid Chromatography

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All chromatographic systems consist of two phases: one phase is held stationary, the other moves through it. The stationary phase may be a solid or a liquid; in the latter case it is distributed over the surface of a finely divided microporous inert (i.e., nonabsorptive) solid, usually Celite or paper. The support and stationary phase are usually packed into a column. The moving phase can be either liquid or gas; thus we speak of a liquid-solid, a liquid-liquid, a gas-solid, or a gas-liquid chromatogram. Any substance added to a two phase system, when equilibrium is reached, will be found to have distributed itself between the two phases. The ratio of concentrations in the two phases is known as the distribution coefficient (α), and is a constant for the substance distributed, the two phases and the temperature. Any mixture of substances can be separated on a chromatogram provided all the components have different distribution coefficients.

Let us consider two substances A and B. Substance A at equilibrium has 90 per cent in the stationary phase and 10 per cent in the moving phase. Substance B however, has only 10 per cent in the stationary phase and 90 per cent in the moving phase. If a mixture of A and B is applied to the chromatogram and washed through with solvent, then B will move as a zone at nine tenths of the rate of the moving solvent (i.e., $R_F = 0.9$) and A will move as a zone at one tenth of the rate of the moving solvent (i.e., $R_F = 0.1$). Clearly substance B will move rapidly away from A and can be eluted from the chromatogram quite separate from substance A.

In the gas-liquid chromatogram (the latest type) the substances to be separated are moved through the column by a stream of permanent gas instead of by a solvent. This use of a gas as moving phase confers a number of advantages, viz:

(1) The compressibility of the gas allows the use of long thin easily packed columns that give high rates of flow by the application of quite small pressures.

(2) High diffusion rates in the gas allow high column efficiencies to be attained even when separations are carried out rapidly.

(3) It is easier to detect the zones, which emerge as vapors in the gas stream, than substances in solution and the detectors are easily made self recording, so that after loading the column the whole process is automatic.

The experimental arrangement of the gas-liquid chromatogram is very simple. The column itself consists of a length of glass or metal tubing packed with a mixture of a microporous support (Celite or crushed firebrick) of known particle size (usually 80–100 or 100–120 mesh) and the liquid phase a substance liquid at the column temperature but having a very low vapor pressure (10^{-2} to 10^{-3} mm). The column is maintained at a constant temperature by a heating jacket and is connected to the vapor detector. The latter records automatically against time the concentration of vapor in the zone blown out of the column by the stream of gas at a constant flow rate. Despite this basic simplicity it is surprising how complicated an apparatus many workers have constructed.

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Gas-liquid chromatography has proved to be the most refined and rapid method for the quantitative and qualitative analysis of the long chain fatty acids.[1,2] In a great many problems it is necessary to deal with the smallest possible sample, e.g., the determination of fatty acid composition of repeated blood samples. The size of sample necessary is determined entirely by the sensitivity of the detector employed and unfortunately many detectors fall in sensitivity when the temperature of the apparatus is raised to the levels necessary for analysis of the long chain acids (170° → 250° C).

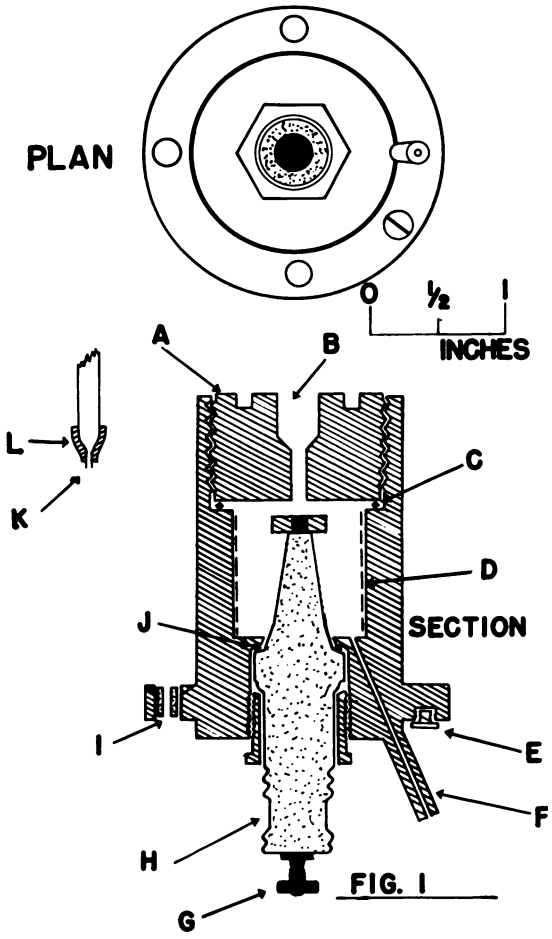

Fig. 1. Plan and section of argon β-ray ionization monitor. A. removable cap for insertion of Sr^{90} foil; B. column seating; C. copper gasket for top cap; D. Sr^{90} foil (Silver); E. screw contact for H.T. input; F. gas outlet; G. input contact; H. center section of demountable spark plug; I. ceramic insulator for mounting screws; J. copper gasket for center electrode; K. column base; L. silicone rubber sleeve to provide gas tight seal in column seat.

Recently a new detector has been developed by Lovelock[3] that has the merits of very high sensitivity even at elevated temperatures and also great simplicity. Its construction has recently been still further simplified.[4] The detector consists of a small chamber containing a cylindrical radioactive source (Sr^{90}, 10 millicuries) emitting β-particles. In the center of the chamber is placed an insulated electrode consisting of the center section of a demountable automobile spark plug fitted with a small disc electrode (Fig. 1). A high d.c. potential (300 to 1,200 volts) is maintained across the cell giving an ionization current of 10^{-8} amperes when pure argon (a commercial product) is used as mobile phase of the chromatogram. As soon as a zone passes into the chamber a large signal is generated that is amplified by a simple electronic circuit and fed to a recorder. The response is linear and independent of molecular weight for fatty acid methyl esters of molecular weight greater than 100. The detector is insensitive to changes in gas flow rate and relatively insensitive to temperature variations so that highly constant thermostatic control is unnecessary.

The column heater consists of a cylindrical bar of aluminium $2^1/_2$-inch diameter and 4 ft 3 in. long. A slot $^1/_2$ inch wide is machined along almost the whole length of the bar (Fig. 2) and partially closed by insertion of a length of $^1/_2$ inch section aluminium, leaving a square section hole to contain the column (4 ft packed length, 4 mm internal diameter). A cylindrical space is turned in the bottom of the bar to accommodate the detector cell and a hole is bored through to break into the slot. The detector is insulated electrically from the bar by a gasket of silica loaded silicone rubber and held in place by screws passing through ceramic insulators. The bar is heated by a double winding of glass insulated Nichrome wire (a total dissipation of 150 watts will give temperatures up to 250° C), a resistance thermometer being used to control the switching of one of the windings, the input to both windings is controlled by a variable transformer. The apparatus has proved cheap to construct, robust and

TABLE I

Comparison of Sensitivity of Various Detectors

Type of detector	Approximate useful sensitivity achieved in terms of concentration of vapor detectable at 200° C	Approximate loads of fatty acid esters necessary for analyses of fatty acids in the range C_6–C_{22}
Katharometer	1 in 10^5	3 to 20 mg
Gas density meter[5]	1 in 5×10^5	1 to 3 mg
Hydrogen flare detector[6]	1 in 10^9	100 μg to 1 mg
Argon β-ray detector[3]	1 in 10^{12}	10 to 100 μg

highly sensitive. In Table I is given a comparison of the sensitivity of various detectors. The quoted load for the argon detector is larger than necessary at maximal sensitivity but it is difficult to load a column accurately with quantities of less than 50 μg. A typical record is shown in Figure 6.

FACTORS INFLUENCING THE SEPARATION OF THE LONG CHAIN SATURATED AND UNSATURATED FATTY ACIDS

The relative position of the zones on a gas-liquid chromatogram is controlled by the differences in partial vapor pressure of the substances when dissolved in the column stationary phase. The nature and magnitude of the cohesive forces between solute and solvent molecules defines the partial vapor pressure of the solute.

When the stationary phase of the chromatogram is a saturated paraffin hydrocarbon, the cohesive forces between the solvent and solute molecules are solely of the nonpolar Van der Waals type. These forces decrease with decrease in molecular weight, hence an unsaturated compound will be held less strongly than the corresponding saturated compound. In Figure 3 is shown a plot of log time of emergence of the center of the zone (relative to a master substance, *n*-pentane) against number of carbon atoms in the molecule for a variety of unsaturated hydrocarbons. In this case the stationary phase is a saturated paraffin hydrocarbon. The dotted line represents the same data for the corresponding straight chain saturated compounds. It can be seen that the unsaturated straight chain hydrocarbons move more rapidly through the column than the corresponding saturated compounds. The same is true for the long chain fatty acid methyl esters. In Figure 4 is shown a separation of these acids using as stationary phase Apiezon L grease. Both linoleic and oleic acids move ahead of stearic acid though linoleic and linolenic acids are not resolved. However, this type of column possesses the advantage that zone position is affected not only by number of double bonds but also by their position and configuration (i.e., whether *cis* or *trans*). The peak in Figure 4 following closely on oleic acid is due to positional isomers of oleic acid.

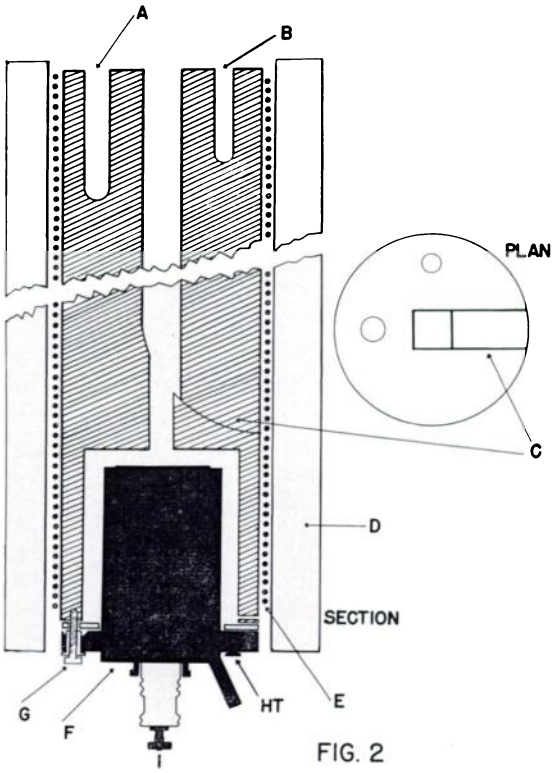

Fig. 2. Plan and section of heating jacket. A. pocket for resistance thermometer; B. pocket for thermometer; C. inserted section to form space for column; D. fiberglass pipe insulation; E. heater winding; F. detector cell; G. holding screws passing through ceramic insulator.

When a more polar liquid is used as the stationary phase new types of solute-solvent interaction come into play. In addition to Van der Waals forces, polar groups such as double bonds show a selective interaction with the

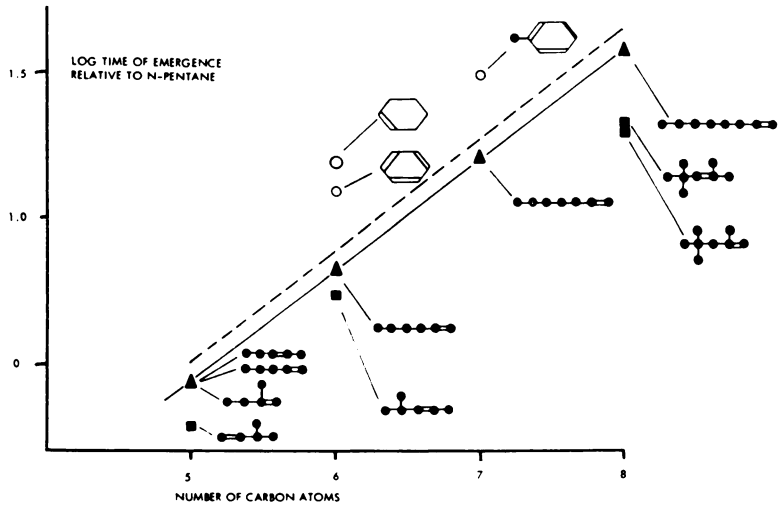

Fig. 3. Relationship between log-time of emergence (relative to *n*-pentane) and number of carbon atoms in the molecule of some unsaturated hydrocarbons. The dotted line refers to the straight chain saturated hydrocarbons. Each dot represents a carbon atom. Stationary phase—liquid paraffin at 78°.

stationary phase. This is demonstrated in Figure 5 for the same unsaturated compounds as Figure 4 but in this case the liquid in the column is an aromatic hydrocarbon. Now the unsaturated hydrocarbons are held back relatively to the corresponding saturated hydrocarbons. Similar effects will occur in polar liquids with long chain fatty acids. The first demonstration of these effects was given by Callen and Orr[7] using a polymer of propylene

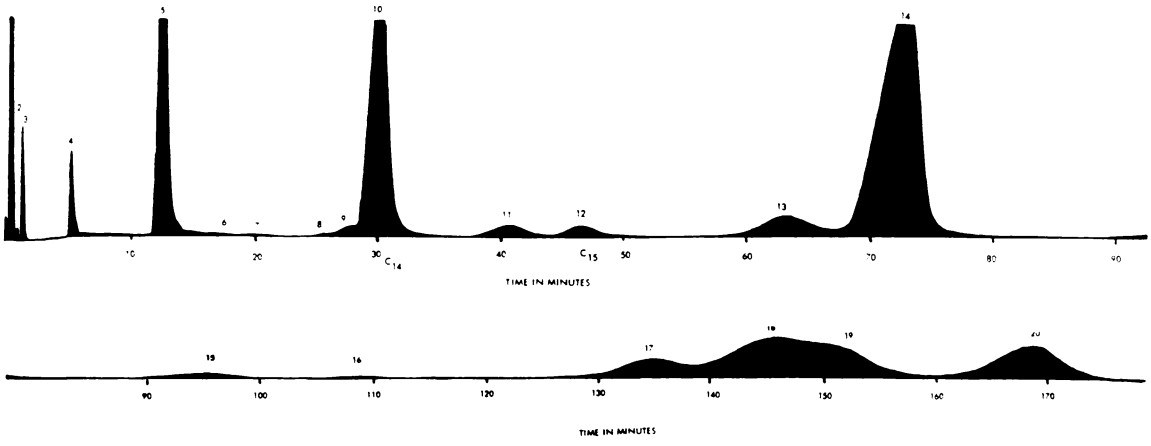

Fig. 4. Separation of 3.6 mg of fatty acid esters from human fecal fat. Stationary phase—Apiezon L. vacuum stopcock grease. Temperature 197° C. Column length 4 ft, nitrogen pressure-inlet 65 cm above atmospheric, outlet at atmospheric pressure. Detector—gas density meter.

Peaks in order of appearance: (1) Arr.; (2) *n*-hexanoic; (3) *n*-octanoic; (4) *n*-decanoic; (5) *n*-dodecanoic; (6) branched decanoic; (7) *n*-tridecanoic; (8) highly branched tetradecanoic; (9) branched saturated + mono unsaturated tetradecanoic; (10) *n*-tetradecanoic; (11) *iso* + ante *iso* branched pentadecanoic; (12) *n*-pentadecanoic; (13) branched saturated + mono unsaturated hexadecanoic; (14) *n*-hexadecanoic; (15) *iso* + ante *iso* branched heptadecanoic; (16) *n*-heptadecanoic; (17) linoleic; (18) oleic; (19) positional isomers of oleic largely vaccenic acid; (20) *n*-octadecanoic.

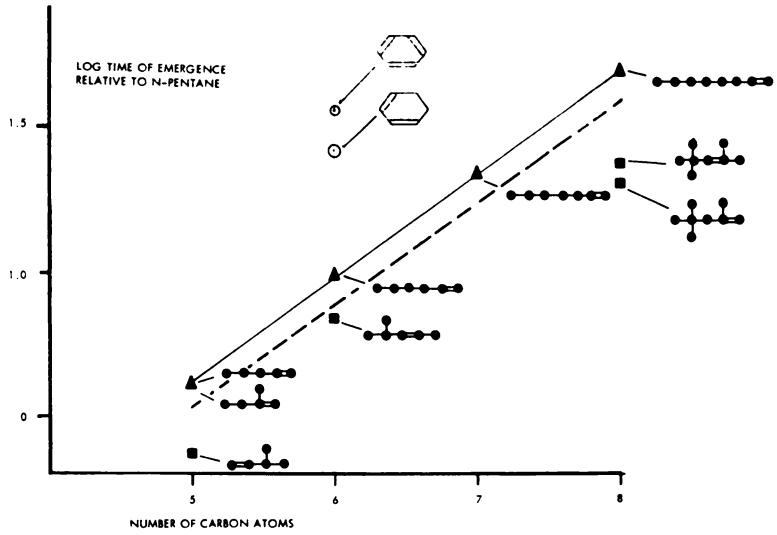

Fig. 5. Relationship between log-time of emergence relative to *n*-pentane and number of carbon atoms in the molecule for some unsaturated hydrocarbons. Stationary phase—benzyldiphenyl at 78°.

glycol and adipic or sebacic acids (Reoplex 400, Geigy Chemical Co. Ltd.). In my hands, however, such polymers have proved thermally unstable and have been stripped rapidly from the column at 180–200° C. I have preferred to use the easily synthesized polymers of ethylene glycol and adipic acid since these are thermally stable and give excellent separations of all the common saturated and unsaturated acids. Double bond position does not, however, alter chromatographic behavior. Lipsky and Landowne[8] have reported the use of polymers of diethylene glycol and adipic acid and have shown what refined separations are now possible. In Figure 6 is given an example of the analysis of only 100 μg of mixed fatty acids on a 4 ft column with a stationary phase of polyethyleneglycol-adipate. The column efficiency is 3,500 theoretical plates. All the polar stationary phases mentioned possess the advantage that the retention volumes of the acids (i.e., time of emergence × flow rate of carrier gas) are lower than with the saturated hydrocarbon stationary phases, so that analyses can be carried out more rapidly even at lower temperatures.

The only other micro method for the analysis of the polyunsaturated acids, the alkali isomerization technic, is rather time consuming. A comparison has been made of the results obtained on similar samples by this technic and with the gas-liquid chromatogram. The comparison of analysis for a wide range of linoleic and linolenic acids is given in Table II (a), and for arachidonic acid in Table II (b). Good agreement is obtained suggesting that

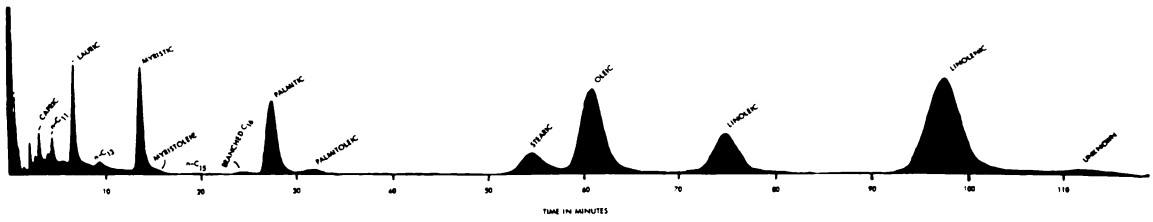

Fig. 6. Separation of 100 μg of fatty acid methyl esters. Stationary phase polyethylene glycol adipate. Temperature 175° C. Column length 4 ft. Argon pressure at inlet 65 cm above atmospheric; at outlet atmospheric pressure. Detector—Argon β-ray monitor.

TABLE II

Comparison of Alkali Isomerization and the Gas-Liquid Chromatogram in the Analyses of Polyunsaturated Acids

(a) Linoleic + linolenic acids

By alkali isomerization			By gas chromatogram
Linoleic %	Linolenic %	Total %	%
48.1	4.8	52.9	59.5
44.0	7.0	51.0	52.5
30.4	5.2	35.6	33.6
14.0	7.8	21.8	21.9
2.6	1.25	3.85	4.6

(b) Arachidonic acid

% Arachidonic	
By alkali isomerization	By gas chromatogram
0	0
0.3	0.5
4.9	4.5
1.5	1.33
22.9	22.8

the gas chromatogram is capable of providing accurate analyses for these acids as well as for the saturated acids. It should, however, be mentioned that the lack of even impure samples of the long chain penta- and hexaenoic acids has prevented any study of their behavior on the chromatogram; this remains to be done.

To summarize the present position, it can be said that a relatively cheap and simple apparatus can now be constructed that will allow automatic analyses of microgramme quantities of long chain fatty acids to be carried out rapidly and accurately so that studies of lipid metabolism will be greatly facilitated.

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