METHODS OF BIOCHEMICAL ANALYSIS

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VOLUME VIII

INTERSCIENCE PUBLISHERS, INC., NEW YORK
INTERSCIENCE PUBLISHERS LTD., LONDON

Library of Congress Catalog Card Number 54-7232

Interscience Publishers, Inc., 250 Fifth Avenue, New York 1, N. Y.

For Great Britain and Northern Ireland:

Interscience Publishers Ltd., 88/90 Chancery Lane, London, W. C. 2

PRINTED IN THE UNITED STATES OF AMERICA BY MACK PRINTING CO., EASTON, PA.

PREFACE TO THE SERIES

Annual review volumes dealing with many different fields of science have proved their value repeatedly and are now widely used and well established. These reviews have been concerned primarily with the results of the developing fields, rather than with the techniques and methods employed, and they have served to keep the ever-expanding scene within the view of the investigator, the applier, the teacher, and the student.

It is particularly important that review services of this nature should now be extended to cover methods and techniques, because it is becoming increasingly difficult to keep abreast of the manifold experimental innovations and improvements which constitute the limiting factor in many cases for the growth of the experimental sciences. Concepts and vision of creative scientists far outrun that which can actually be attained in present practice. Therefore an emphasis on methodology and instrumentation is a fundamental need for material achievement to keep in sight of the advance of useful ideas.

The current volume is the first of a series which is designed to try to meet this need in the field of biochemical analysis. The topics to be included are chemical, physical, microbiological and, if necessary, animal assays, as well as basic techniques and instrumentation for the determination of enzymes, vitamins, hormones, lipids, carbohydrates, proteins and their products, minerals, antimetabolites, etc.

Certain chapters will deal with well-established methods or techniques which have undergone sufficient improvement to merit recapitulation, reappraisal, and new recommendations. Other chapters will be concerned with essentially new approaches which bear promise of great usefulness. Relatively few subjects can be included in any single volume, but as they accumulate these volumes should comprise a self-modernizing encyclopedia of methods of biochemical analysis. By judicious selection of topics it is planned that most subjects of current importance will receive treatment in these volumes.

The general plan followed in the organization of the individual chapters is a discussion of the background and previous work, a critical evaluation of the various approaches, and a presentation of the procedural details of the method or methods recommended by the author. The presentation of the experimental details is to be given in a manner that will furnish the laboratory worker with the complete information required to carry out the analyses.

Within this comprehensive scheme the reader may note that the treatments vary widely with respect to taste, style, and point of view. It is the editor's policy to encourage individual expression in these presentations because it is stifling to originality and justifiably annoying to many authors to submerge themselves in a standard mold. Scientific writing need not be as dull and uniform as it too often is. In certain technical details a consistent pattern is followed for the sake of convenience, as in the form used for reference citations and indexing.

The success of the treatment of any topic will depend primarily on the experience, critical ability, and capacity to communicate of the author. Those invited to prepare the respective chapters are scientists who either have originated the methods they discuss or have had intimate personal experience with them.

It is the wish of the Advisory Board and the editor to make this series of volumes as useful as possible and to this end suggestions will always be welcome.

DAVID GLICK

Minneapolis, Minnesota
January, 1954

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CONTENTS

Qualitative and Quantitative Determination of the Fatty Acids by Gas-Liquid Chromatography

A. T. JAMES, *National Institute for Medical Research, London*

I. INTRODUCTION

Apart from the petroleum hydrocarbons the fatty acids represent perhaps the most complex group of naturally occurring substances. The problem of separating and determining all the components of such mixtures has occupied biochemists for many years, and until recently no simple technique was available. Where high resolving power is required for mixtures of such closely related substances, chromatography is the technique of choice. Unfortunately, the liquid–liquid chromatograms used in the past for separation of the fatty acids possess a number of disadvantages:

1. The columns must be run very slowly to obtain maximum efficiency.
2. Continuous analysis of the column effluent is difficult.
3. With the solvent systems used, introduction of a double bond into a fatty acid molecule alters the R_F by an amount similar to that caused by removal of two carbon atoms from the chain, so that complete resolution of saturated and unsaturated acids is never attained.

However, the use of a gas as the moving phase of the chromatogram gives rise to a number of distinct advantages:

1. Since, unlike a liquid, the moving phase is compressible, it is possible to use long, thin, easily packed columns which have high efficiencies even at rapid rates of flow.
2. Automatic continuous analysis of the column effluent is easily achieved by a variety of physical techniques.
3. Since molecular interactions are of importance only in the stationary phase, liquids can be chosen that show selective interactions with particular structures in the compounds to be separated.

II. NOMENCLATURE

In this section use is made of some of the recommendations on nomenclature and presentation of data drawn up by a group under the auspices of the Analytical Section of the International Union of Pure and Applied Chemistry. The group consisted of the following: Dr. D. Ambrose, Chairman (Great Britain), Dr. A. T. James (Great Britain), Professor A. I. M. Keulemans (The Netherlands), Dr. E. Kovats (Switzerland), Dr. R. Rock (Germany), C. Rouit, Ingr. Dr. (France), and Dr. F. H. Stross (United States).

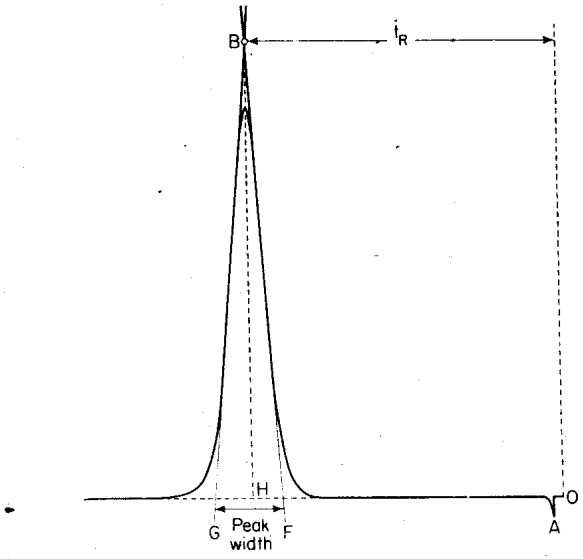

Fig. 1. Schematic diagram of the parameters used in measuring retention volumes, column efficiency, etc.

1. R_F = rate of movement of center of zone relative to the rate of movement of the mobile phase of the chromatogram.

2. Partition coefficient K = (weight of solute/ml. of stationary phase) ÷ (weight of solute/ml. of mobile phase at equilibrium).

3. Retention volume (uncorrected), V_R, is the volume of gas required to elute the compound under study and is given by

$$V_R = t_R F_c$$

where t_R is the retention time of the peak center measured from the time of sample loading (O, Fig. 1), and F_c is the volumetric flow rate of the carrier gas measured at the outlet pressure and the temperature of the column. This is conveniently measured with a soap film flowmeter (Fig. 2).

4. Corrected retention volume, $V_R°$, is the retention volume (V_R) corrected for the pressure drop in the column caused by the compressibility of the moving phase:

$$V_R° = fV_R$$

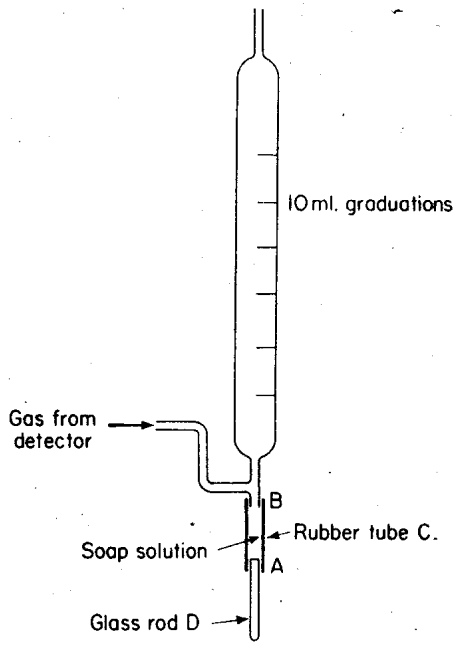

Fig. 2. Soap film flowmeter. An aqueous solution of soap or detergent is placed in the vertical section AB to a level such that when the rubber tube C is compressed the liquid level rises to occlude completely the glass tube above B. A bubble is formed when a silicone rubber tube from the flowmeter inlet is attached to the detector outlet, and the movement of this bubble is timed between the volumetric graduations.

where f is the pressure correction factor given by

$$f = {}^3/_2[(p_i/p_o)^2 - 1]/[(p_i/p_o)^3 - 1]$$

p_i is the gas pressure at inlet of column; p_o is the gas pressure at outlet of column.

5. Reduced retention volume, V_R', is given by

$$V_R' = V_R - V_M$$

where V_M is the retention time of a nonabsorbed sample (nitrogen, air, etc.). This is the time OA in Figure 1, where A represents the inflection point of a peak due to air introduced in loading the sample. Thus

$$V_R' = \text{time } AH \text{ (Fig. 1)} \times F_c$$

6. Net retention volume, V_n, is given by

$$V_n = fV_R'$$

where f is the pressure correction factor.

7. Specific retention volume, V_g, is the corrected retention volume at 0°C. per gram of liquid phase.

$$V_g = 273\ V_n/TW_L = 273\ K/T\rho_L$$

where T is the column temperature, W_L is the weight of liquid phase in the column, and ρ_L is the density of the liquid phase at the column temperature.

8. Relative retention, r, is given by

$$r_{1,2} = K_1/K_2 = (V_R')_1/(V_R')_2$$

where the subscripts refer to components 1 and 2, component 2 being the standard.

9. CH_2 separation factor, ΔCH_2, is given by ΔCH_2 = Reduced retention volume of substance $R(CH_2)_nX$ ÷ reduced retention volume of substance $R(CH_2)_{n-1}X$

III. THE GAS–LIQUID CHROMATOGRAM

1. Principles

In essence, all chromatograms operate in the same fashion: one phase of an immiscible pair is held stationary in a column, and the second phase is moved continuously through it. Any mixture applied to the top of the column and washed through by the moving phase will separate into individual components provided these components possess sufficiently different partition coefficients in the two phases employed. The amounts of substances to be separated are kept small so that the partition coefficient is independent of concentration and symmetrical zones result.

The essential features of the technique are as follows: (*a*) a source of permanent gas at constant pressure; (*b*) a column containing a mixture of the stationary phase (a substance liquid at the column temperature but having a low vapor pressure, 10^{-3} mm. or less) and an inert microporous support for the stationary phase; (*c*) a heating

jacket for both column and detector; (*d*) the detector, whose function it is to measure the concentration of vapor in each zone leaving the column; and (*e*) a recorder to present the information supplied by the detector.

2. Apparatus

Since a wide variety of commercial instruments are now available, there is little need for detailed description of apparatus. Table I lists

TABLE I
Gas Chromatographic Apparatus

Type of detector	Manufacturer	Approximate weight of sample of long chain acids necessary for an analysis in the range C_6–C_{20} at 200°C. using packed columns
Catharometer	All manufacturers of gas chromatographic apparatus	3–10 mg.
Gas density meter	Griffin & George Ltd., Ealing Road, Alperton, Middlesex	1–3 mg.
Hydrogen flame	Shandon Scientific Co. Ltd., 6 Cromwell Pl., London S.W. 7	1–3 mg.
Hydrogen flame ionization (suitable for capillary columns)	Shandon Scientific Co. Ltd. (see above) Perkin Elmer Instrument Division, Norwalk, Conn.	30–80 μg.
Argon ionization monitor (suitable for capillary columns)	[a] Pye Scientific Instruments Ltd., Cambridge, England (U.S. agent: Jarrell-Ash Co., Newtonville, Mass.) [a] Perkin Elmer Instrument Division (see above) [a] Wheelco Division of Barber-Colman, Rockford, Ill.	30–80 μg.[b]

[a] These companies also manufacture capillary columns.

[b] With nitrogen or hydrogen as carrier gas there is no upper limit of load when used with preparative columns.

the types of detector obtainable, together with a rough indication of the loads of long chain fatty acids required for a reasonably accurate analysis (the load is determined by the sensitivity of the detector).

Accurate temperature control of the column and often of the detector is necessary when highly reproducible results are required. Of the two methods, vapor heating and electric heating, the former has the convenience of good stability but conditions cannot be changed rapidly. A list of suitable liquids for vapor jackets is given in Table II.

TABLE II
Stable Liquids Suitable for Vapor Jackets

Substance	Boiling point, °C.
Ethyl alcohol	78.6
Water	100
Ethylene glycol monoethyl ether	137
o-Dichlorobenzene	180
Cyclohexyl acetate	177
Ethylene glycol	197

It is convenient to attach to most instruments a time switch operating the recorder alone, so that an analysis can be started at the end of the working day and carried out during the operator's absence. The switch should be set to close in the morning about an hour before the apparatus is required, so that it is in operating condition throughout the whole of the working day. Heating jackets, amplifiers, etc., should be left on all the time because this increases their life and avoids waste of time waiting for equilibrium to be established. It is also useful to have a magnetic valve in the gas line set to cut the flow to a low level overnight and so save gas, yet keep the column in usable condition.

3. Preparation of Supports for the Stationary Phase

Kieselguhr is the most widely used support and is prepared in the following manner. Celite 545 (Johns Mansville Ltd.) (200 g.) is stirred with tap water in a 5 liter beaker until suspended and then allowed to settle for 3 minutes. The material still suspended is poured off, the residue is stirred with more water and allowed to settle for 2 minutes, and the supernatant is rejected. More water is added, the Celite is resuspended by stirring, and the precipitate obtained after 2 minutes is added to the initial precipitate. Residual fines are removed by resuspending and rejecting the supernatant obtained after

TABLE III

Composition of Stationary Phases Used in the Separation of the Fatty Acids

Stationary phase	Chemical type	Temperature stability	Useful temperature range, °C.	Range of fatty acids suitable	Remarks	Source
10% stearic acid in DC-550 silicone	Silicone polymer having methyl and phenyl side groups, containing a long chain acid	Good	100–140	C_1–C_6	Suitable for free acids only	Silicone from Dow Chemical Co., Midland, Mich., or Midland Silicones Ltd., 19 Upper Brook St., London W. 1
15% sebacic acid in dioctyl sebacate	Ester containing a dicarboxylic acid	Not known	Up to 150	C_1–C_6	Suitable for free acids	Laboratory suppliers
Dioctyl or dinonyl phthalate	Ester	Good	80–150	C_1–C_8	Suitable for methyl esters	Laboratory suppliers
Liquid paraffin or low-melting paraffin wax	Saturated hydrocarbon	Fair	60–150	C_1–C_8	Suitable for methyl esters	Laboratory suppliers
Apiezon M grease	Saturated hydrocarbon	Good	Up to 200	C_5–C_{22}	Suitable for methyl esters	Laboratory suppliers

Apiezon L grease	Saturated hydrocarbon	Good	Up to 300	C_5–C_{30}	Suitable for methyl esters	Laboratory suppliers
Silicone greases	Silicone polymer containing some aromatic side groups	Fair	Up to 250	Up to C_{30}	Poor separations of satd. and unsatd. acids	Laboratory suppliers
Epoxy resins (e.g., No. 1001)		Good	Up to 250	Probably up to C_{30}	Good separations of most acids except stearic and oleic	Shell Chemicals Ltd.
Polyethylene glycol adipate	Polyester	Good	Up to 200	Up to C_{26}	Good separations of all acids except isomers of the monoenes	By synthesis
Reoplex 400	Polyester	Fair	Up to 200	Up to C_{26}	Good separations of all acids except isomers of the monoenes	Geigy Chemicals Ltd., Manchester, England
LAC-4-R777	Polyester	Good	Up to 200	Up to C_{30}	Good separations of all acids except isomers of the monoenes	Cambridge Industries Co., Inc., Cambridge, Mass.

3 minutes. The residue is treated with concentrated HCl to remove iron, washed free of acid with distilled water, and oven-dried. The dry product is then sieved in standard mesh sieves to give four fractions—60–80, 80–100, 100–120, and 120–140 mesh. Any of these fractions can be used, but the 100–120 mesh material gives the best results.

When nonpolar stationary phases such as Apiezon L or M greases are used, it is necessary to pretreat the Celite to prevent adsorption. The Celite is washed until almost neutral with 2% methanolic KOH followed by dry methanol, filtered, and oven-dried. It is then resieved to remove large particles. This alkali-treated material must be stored in a desiccator over solid KOH, since contact with laboratory air for any length of time produces a new layer of adsorbent material (probably silicic acid) on the Celite surface. Ground furnace brick has been used as a support, but with polar substances such as the fatty acid or esters adsorption and peak skewing occur.

4. Preparation of Stationary Phases

A summary of the properties of a number of stationary phases is given in Table III. The most useful materials for the long chain fatty acids are the Apiezon greases and the polyesters. The polymer of ethylene glycol and adipic acid is prepared as follows.

Preparation of polyethylene glycol adipate: 1.05 moles of ethylene glycol (65.1 g.) is added to 1 mole of adipic acid (146 g.) in a 500 ml. round-bottomed flask fitted with a side arm. A slow stream of nitrogen is passed into the flask which is heated to 180°C. in a silicone oil bath. When the mixture has melted, 10–25 mg. of *p*-toluenesulfonic acid is added to act as an acid catalyst. The water evolved during the reaction is swept out by the stream of nitrogen. After 2 hours the nitrogen stream is turned off and the flask is evacuated with a water pump for a further hour to remove any residual water and excess glycol. The resulting viscous liquid is cooled and poured into a glass-stoppered bottle. The polymer solidifies at room temperature and melts at 40–60°C. A similar procedure can be used for other polymers of similar chemical type.

The stationary phases are weighed out and mixed with the Celite by dissolving in a volatile solvent (40–60° petroleum ether for the nonpolar and chloroform for the polar substances). A weighed amount of

Celite is added, and the solvent is evaporated first on the water bath and then in an oven at 150°C.

Proportions of stationary phase to support that give good columns are: (*a*) 2.0 g. Apiezon L or M to 8 g. Celite and (*b*) 2.5 g. polyethylene glycol adipate to 8 g. Celite. Provided column loads are limited (e.g., with the high sensitivity detectors), smaller amounts of stationary phase can be used, (viz., 1 g. of stationary phase to 7 g. Celite) (see Section IV.1.C).

The silicone–stearic acid columns used for short chain acids (not esters) are prepared by mixing crystalline stearic acid (10 or 15% by wt.) with DC-550 or MS-550 silicone and heating until dissolved. The required amount of Celite (100–120 mesh) is added (8 g. to 3 g. of stationary phase) and carefully mixed with a glass rod for 15–20 minutes. Care should be taken to mix the Celite and not to grind it. Packing is carried out in the manner described in Section III.6.

5. Column Construction

The precise shape of column depends on the apparatus used. Many commercial gas chromatograms use spiral metal columns; these

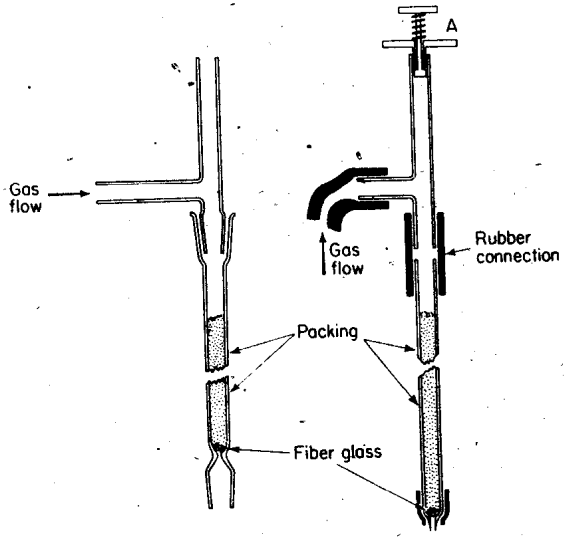

Fig. 3. Two types of straight column. *Left:* Sealed by glass cone in silicone rubber seal of detector. *Right:* Sealed by silicone rubber joint. *A*, Compression gas seal.