Biochemical Applications of Gas Chromatography

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PREFACE

This book was written to fulfill a need for a text on gas chromatography that would supply both the theoretical background and details of the applications of this technique to biochemical problems. Several excellent books exist which review theory and instrumentation, but these do not contain sufficient experimental detail to be useful as laboratory manuals. On the other hand, the field has not matured sufficiently to make it possible to present a set of routine methods that can be used with confidence in every application. Therefore, experimental details of the most promising procedures are set forth together with enough theoretical background to enable the experimentalist to improvise adaptations without an extensive study of the literature.

Material is included that will be of interest to analytical biochemists working in a variety of fields. These fields include foods, essential oils, amino acids, carbohydrates, pesticides, clinical chemistry, and others. Although the book is organized along biochemical lines, information is included that will be of value to workers in other disciplines. For example, specific instructions are given for the analysis of atmospheric gases and for the resolution of isomeric aliphatic and aromatic hydrocarbons. This information should be useful in the solution of problems in air pollution and petroleum chemistry. In fact, it was necessary to draw upon the literature from these fields to round out the text in areas where biochemical applications were few or lacking altogether.

This book contains methods for the analysis of the principal groups of compounds of biochemical interest. However, the gas chromatographic method is so versatile that the material could not be organized coherently according to the functional groups occurring in the various compounds. For example, alcohols, aldehydes, and esters often coexist in a sample and can frequently be resolved in a single operation. Classification according to vapor pressure is also possible, but this does not allow for a logical presentation of biochemical data. Therefore, the methods employed for sample collection and pre-fractionation are used as the primary basis for chapter organization, since this permits an orderly arrangement of the material from the viewpoint of experimental techniques. Thus volatile components of tissues, essential oils, lipids, and nonvolatile components of tissues, are each treated as distinct groups, since the methods used for sample preparation within each group are similar. The chapters are then arranged in approximate order of decreasing vapor pressure of the compounds discussed in them, and subdivisions within chapters made according to elements and functional groups where this is

feasible. As a consequence of this arrangement, procedures for the analysis of compounds with the same functional groups may appear in several sections of the text. Thus methods for the chromatography of organic acids are found in the chapters on volatile compounds, resin acids, lipids, nonvolatile compounds, etc. Consequently anyone interested in compiling methods according to functional group should make liberal use of the subject indexes.

Although gas chromatography is the pivotal analytical technique described in this book it is not the only one. Other methods have been included, where required, to make the book as self-sufficient as possible within the scope of a single volume. Detailed methods for sample collection are provided for each group of compounds, and often pre-fractionation of samples by liquid-solid chromatography or ion exchange techniques is described. Finally, methods are provided for the preparation of stationary liquids and chromatographic columns, subtraction of unwanted compounds from the gas stream, and for the synthesis of derivatives of nonvolatile compounds which are amenable to gas chromatography.

The operating parameters used for gas chromatography are tabulated in standard form throughout the book. The conventions used are simple, and for the most part are self-explanatory. However, the meaning of the entries used to describe columns in series, in parallel, and multiply-packed columns may not be immediately clear. Therefore, the reader is strongly urged to consult pages 20 through 23 for a detailed account of the conventions employed.

In a number of cases, insufficient information was given in original research papers to allow for an adequate compilation of experimental methods. Therefore, it was decided that a more complete as well as a more accurate and up-to-date set of procedures could be obtained by submitting excerpts from the first draft of the manuscript to authors of the original papers for corrections and additions. About 320 methods were sent out and replies were received on 87% of them. Methods which have been verified by the original authors are marked with asterisks to acknowledge their contributions. Many valuable corrections and additions were obtained. In a number of cases, improved methods were submitted in advance of journal publication.

To insure further that the book would be reasonably up to date on publication, the manuscript was prepared during a relatively short time period—between August 1960 and February 1961. Additional material of major importance appearing through September 1961 was added in galley proofs, but coverage of the literature during this period was incomplete.

San Antonio, Texas
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January, 1962

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We greatly appreciate the suggestions of Dr. Richard J. Block who first perceived the need for such a book, and encouraged its completion.

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Chapter 1

General Techniques, Conventions, and Instrumentation

General Introduction

Chromatography has become an invaluable adjunct in so many fields of biochemical research that almost all workers are familiar with it. Consequently, there is no need to reintroduce the subject here. Gas chromatography differs from liquid–liquid chromatography or liquid–solid chromatography primarily in that a gas is used as the mobile phase, and the solute travels through the column as a plug of gas or vapor which is partly dissolved in, or adsorbed on, the stationary phase. The suggestion to use a gas as the mobile phase was made by Martin and Synge in 1941, but it was not implemented until the work of James and Martin (1952) on gas–liquid chromatography and the work of Cremer and Prior (1951) and Cremer and Müller (1951a,b) on gas–solid chromatography. Since then, the use of the technique has penetrated almost every area of analytical and biochemical research.

A. Theory and Nomenclature

I. Forms and Advantages of Gas Chromatography

Gas chromatography can be accomplished with either a liquid sorbed on an inert particulate support or an active solid as the stationary phase. Alternatively, the liquid can be coated on the internal surface of a long capillary tube with very small bore. Each of these procedures has special advantages that overlap only slightly. However, all forms of gas chromatography are more efficient in many respects than methods where a liquid is the mobile phase, because of unique features conferred by the low viscosity and high diffusivity of gases and vapors.

a. Gas–Liquid Chromatography (GLC)

The column in gas–liquid chromatography is a glass or metal tube, usually about 0.5 cm in internal diameter and 1 to 20 meters long. The tube is packed uniformly with a finely divided free-flowing powder prepared by impregnating an inert solid with a liquid of low volatility. The solid support should be absorbent in the sense that it is capable of imbibing and holding the stationary liquid without becoming greasy, but not adsorbent in the sense that it will bind components of the sample being analyzed by secondary

valence bonds. The main physical requirement for the liquid is that it must not be eluted from the column at the operating temperature employed.

A sample of the mixture to be separated is flash-evaporated at one end of the column and swept into it by a constantly flowing stream of carrier gas such as hydrogen, helium, or nitrogen (Fig. 1). The components of the

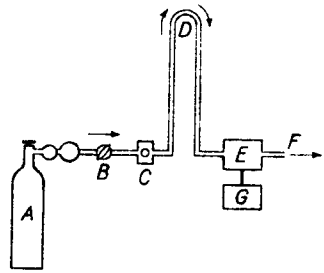

FIG. 1. Basic design of a gas chromatograph, arrows showing the direction of gas flow: (*A*) gas cylinder with reducing valve; (*B*) constant-pressure regulator; (*C*) port for injection of sample; (*D*) chromatographic column; (*E*) detector; (*F*) exit line; (*G*) strip chart recorder.

sample are carried through the column at different rates, which are governed by their partition coefficients between the gas phase and the stationary liquid phase. Ideally, they emerge from the other end of the column at different times. Their presence in the emerging carrier gas is detected by chemical or physical means, and the response of the detector is fed into a strip chart recorder. Generally, differential detectors are used, and the data are presented as a series of peaks spread out along a longitudinal time axis (Fig. 2). Each peak represents a discrete chemical compound, or a mixture

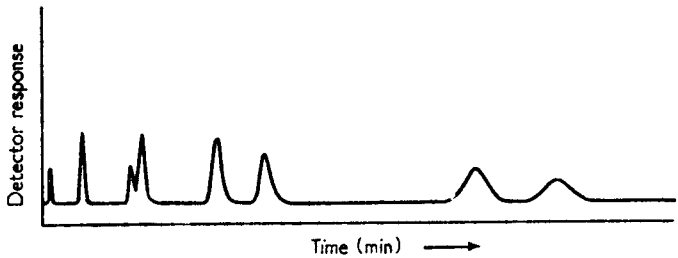

FIG. 2. Typical chromatogram showing separation of a hydrocarbon mixture. Note peak broadening at increased retention times.

of compounds with identical partition coefficients. The time required for each component to emerge from the column is characteristic of the compound and is known as its retention time. The area under the peak is proportional

to its concentration in the sample. This constitutes the principal primary information derived from the chromatogram.

It may be helpful to review briefly some elementary principles governing the rate of travel of chemical compounds through columns packed with a stationary liquid phase. Each compound has a characteristic partition coefficient, which is given by equation 1. Therefore, if the partition coefficient

$$K = \frac{\text{Weight of solute/ml stationary phase}}{\text{Weight of solute/ml mobile (gas) phase}} \quad (1)$$

of compound A is small, the amount of compound dissolved in the stationary liquid phase will be small compared to that in the gas phase. Consequently, the compound passes through the column rapidly, since it is not retarded by the stationary liquid. If the partition coefficient of compound B is large, the greater proportion of it resides in the solvent (stationary phase); therefore, passage through the column is slow, and this material passes through the detector and registers a peak at a later time than compound A. It must be remembered that the solute present in the gas phase is in dynamic equilibrium with the same solute in the liquid phase at all times. The vapor molecules cannot be swept through the column with the carrier gas, leaving the dissolved molecules behind; the only effect is to retard passage in proportion to the partition coefficient of the compound. This situation can be likened, however inaccurately, to a group of sailing ships of different draft being carried along by a high wind at sea. The vessels with greater displacement are slowed down by the drag of the water, whereas those with shallow draft skim along on the surface and pull ahead in the race.

The basic principles governing the separation of chemical compounds by gas chromatography are simple, but unfortunately, like in most other systems, behavior is not ideal. Therefore mathematical treatment of conditions as they supposedly occur in columns is moderately complex. This topic has been dealt with adequately by Keulemans (1959) and others and so will not be repeated here. Nevertheless, it may be worth while to point out a few consequences of departures from ideality, since they affect the symmetry and shape of chromatographic peaks and often must be dealt with experimentally. These are:

1. *Tailing.* Under ideal conditions, peaks on gas chromatograms should be symmetrical and resemble Gaussian distribution curves. This situation is often approximated in GLC, but sometimes the recorder pen does not return to the base line as rapidly on the descending side of the peak as it leaves it on the ascending side. This results in asymmetric peaks and is called tailing. Generally, tailing in GLC arises from adsorption of the solute on active sites of the solid support. It can be reduced and sometimes eliminated by deactivating the support (see this chapter, Section C.III). Peak asymmetry

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