PRACTICAL CHROMATOGRAPHIC TECHNIQUES

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LONDON
GEORGE NEWNES LIMITED
TOWER HOUSE, SOUTHAMPTON STREET,
LONDON, W.C.2

First Published 1964

Printed in Great Britain by
Butler & Tanner Ltd., Frome and London

Newnes Practical Science Books

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PREFACE

Chromatography in one or another of its various forms has been successfully used for separating mixtures of so many different types of substances that in all probability it is now used more frequently for both qualitative and quantitative analysis and for preparative purposes than any other comparable method. While the use of all kinds of chromatogram has increased very greatly during the last 20 years, the growth of partition chromatography, which was introduced by A. J. P. Martin, and R. L. M. Synge in 1941, has been the most rapid. The present very wide employment of this method in many chemical and in the great majority of biochemical laboratories is sufficient indication of the importance of the advance which was made by Martin and Synge. One of the present authors (A. H. G.) was fortunate enough to be introduced to chromatography in 1941 by Dr. Synge. The first partition chromatograms were columns of silica gel coloured yellow by methyl orange. These were employed to separate mixtures of acetyl amino acids. The pink bands which moved rapidly downwards were a memorable sight.

While the early results obtained with partition chromatography suggested something of the potentialities of the method, the rapidity of its spread into many different fields certainly could not have been foreseen. Probably the main reasons why paper chromatography was quickly accepted as a useful laboratory method were its great simplicity and the very considerable amounts of information which can thus be obtained. Most other types of chromatogram require rather more attention but none of the manipulations is difficult to carry out. However, to obtain optimum results, care must be taken with certain crucial steps in the procedure, as well as in the design of apparatus. The primary function of this book, which is directed towards the interests of the beginner, is to describe established chromatographic techniques, with an emphasis on those practical points which are essential for good results. The early chapters introduce the nature of chromatography, its mechanisms, the essentials of its quantitative theory and a critical description of apparatus, without assuming any previous knowledge on the part of the reader.

Full exploitation of the chromatographic process, however, calls for more than a careful application of established methods. The separation of mixtures of unknown substances requires the use of an adequate adsorbent and solvent or solvent mixture, the selection of all of which may present difficulty. Success, in these circumstances, must depend both on knowledge of what has already been achieved with mixtures of similar substances and on those general principles which have been deduced from past experience. We have therefore attempted to lay emphasis, throughout the book, on a practical evaluation of the various materials suitable for the stationary phase of chromatograms, and on the selection of solvents in relation to both the properties of the substances being separated and to the probable mechanisms operating. The final chapter contains a description of thin-layer chromatography and specially developed chromatographic materials, including modified celluloses and dextrans. If this book succeeds in helping the reader to build up a working knowledge of chromatography, it will have served its purpose.

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ACKNOWLEDGEMENTS

The authors are grateful to a number of individuals and organizations for permission to reproduce in this book illustrations from their publications. In particular, thanks are due to Professor C. E. Dent, F.R.S., for Fig. 3.19, Dr. A. A. Leach for Fig. 6.8, Dr. S. M. Partridge for Figs. 6.5, 6.6 and 6.7, and Dr. R. L. M. Synge, F.R.S., for Fig. 2.12. We are indebted to the proprietors of the *Biochemical Journal* and the Cambridge University Press for permission to publish Figs. 2.12, 3.20 and 6.3–6.8, to the Elsevier Publishing Company for Fig. 7.1, to Brinkmann Instruments, Inc., for Fig. 7.2, to the Locarte Company for Fig. 3.11, to the Reinhold Publishing Corporation for Figs. 4.3 and 4.4, and to the American Association for the Advancement of Science for Fig. 3.19. We are especially grateful to Mr. E. B. Brain for the cover photograph.

The authors would like to thank the publishers and their staff for their help and patience during the preparation of this book. Dr. J. E. Eastoe wishes to thank Professor B. Cohen of the Royal College of Surgeons of England for his advice and encouragement and Mrs. Beryl Eastoe for her painstaking work in preparing the manuscript.

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CHAPTER 1

INTRODUCTION

The introduction of new experimental methods is often the main cause for a period of rapid scientific advance. The method of separation of dissolved substances known as chromatography, which forms the subject of this book, has already yielded a rich harvest of discovery in branches of chemistry as different as the separation of isotopes and the purification of new antibiotics. So much new data continues to be accumulated by means of chromatography that at the present time it is certainly the most widely practised, and most fruitful of all the methods which depend on differential migration of the substances under analysis.

Just as the rapid advance of organic chemistry in the nineteenth century was made possible by the techniques of crystallization and distillation, so the great development of biochemistry in the last twenty-five years has depended mainly on extensive application of chromatography and of zone electrophoresis. Like the leading techniques of nineteenth-century organic chemistry, the analytical applications of chromatography are just as important as its use for preparative purposes. Considerable attention will therefore be devoted to both these aspects of the subject in this book.

During the long working period when the average organic chemist was crystallizing and recrystallizing, distilling and redistilling and thus isolating and characterizing many thousands of new substances, a few workers in the applied disciplines allied to organic chemistry were feeling their way towards the new method, now known as chromatography, which was not to be fully exploited for nearly a century. Some reference to the work of these pioneers may be of interest because the discovery of chromatography has often been considered as an outstanding contribution by a single scientist. In fact many aspects of what is today called chromatography were well understood before the time of Mikhial Tswett (1872–1919), the Russian–Italian botanist, who first gave a name to the method. Some notes on the history of chromatography may also serve to emphasize that

a technique is not likely to become important until a sufficiency of clearly determined and potentially resolvable problems has made its appearance. Part of Tswett's brilliance was that he found a series of problems specially suited to the chromatographic method. As a botanist he was very interested in the green pigments of leaves. These pigments, which he was the first to separate on a column of calcium carbonate, are still excellent examples for demonstration of the powers of the chromatographic method.

Chromatography, as the name indicates, was evolved for work with coloured substances. An important first stage was described as early as 1850 by Runge who noticed the formation of clearly distinct zones of colour when solutions of certain dyestuffs were dropped on to blotting paper. The procedure must have developed from the method of spot testing in which a drop of the solution to be tested was placed on a specially impregnated piece of paper or cloth. With mixtures of dyes, continuous feeding of the solution into a piece of blotting paper leads to sharper zone formation than after the addition of only a single drop. Such methods were well known among dye technologists during the latter half of the nineteenth century and may well have come to the notice of Tswett. The complete separation of components which is characteristic of modern paper chromatography, was not achieved; for this to take place pure solvent must be allowed to enter the filter paper instead of further amounts of the solution containing the substances to be separated. In modern terms the chromatograms of Runge were examples of the process first examined in detail by Tiselius (1940) and used by him as a quantitative method under the name of *frontal analysis*. As will be described later, *frontal analysis* is applicable when a solution containing more than one solute is allowed to pass through a tube packed with an adsorbent. Since each solute is retarded to a different and characteristic degree, a number of fairly sharp increases in total concentration are observed as the *front* of each solute becomes superimposed on the solution already emerging.

Attempts were made by Reed as early as 1893 to broaden the scope of the method, later to be named chromatography, by using powders packed into glass columns. Unfortunately he was successful only with certain inorganic salts. In quite another field of technology, at only a slightly later date, powders packed into columns were used with much greater success. The American oil chemist, Day, obtained markedly different fractions from natural petroleum after its passage

through a column packed with fuller's earth. This work was reported by Day at the First International Petroleum Congress held at Paris in 1900 and was soon confirmed by oil technologists in various parts of Europe. Although these workers used quite large apparatus, including pumps and tubes arranged for sampling at various heights, only the enrichment and not the complete separation of individual components was achieved. Once again, as with Runge's experiments with dyes, no complete separations were possible because the mixed oil was introduced continuously into the column and developing solvent was not used.

The employment of pure solvent for the development of a chromatogram was first described in 1906 by Tswett. Undoubtedly this most important step forward transformed the technique from a potentially useful analytical method into one suitable for qualitative and quantitative analyses and separations on a preparative scale.

Tswett's investigations were not limited to the leaf pigments. In 1910 he described the chromatography of extracts of egg yolk on columns of inulin. Since some of the substances being separated were colourless a means for their detection not based on this property was required. For this purpose Tswett allowed drops of the eluate to evaporate from tissue paper. When fatty substances were present the spots thus formed remained transparent even after evaporation of the solvent. This work laid the basis for identification of colourless components after separation by chromatography. Unfortunately Tswett was not to live long enough to see any considerable spread of his new method. Indeed chromatography was so little known that it is usual to speak of its rediscovery in 1931 by Kuhn and Lederer. The gap between the work of Tswett and this rediscovery was probably due to several factors, mainly well beyond the control of the scientific world. The fact that Tswett's major work was published only in Russian must also have delayed the spread of his ideas. From another point of view, however, the delay can be taken as a measure of how far in advance he was of his scientific contemporaries. For instance, he must certainly have had a rather clear understanding of the process of adsorption, which was the mechanism chiefly concerned in his successful separation of the leaf pigments. Thus he was well aware that an adsorbent after saturation with one substance, can still take up others, and demonstrated this for the leaf pigments with pieces of filter paper from which he was then able to elute the various substances.

Indeed if Tswett's insight into the mechanisms of chromatography had been able to spread more rapidly through the scientific world, as might have happened but for the intervention of the 1914–18 war, the lag period in the history of chromatography might never have occurred. As it was, by 1922 Palmer was able to review considerable chromatographic work on the pigments of milk fat. When in 1931 chromatography began to grow more rapidly it was applied successfully to many of the problems on which Tswett had himself worked. By 1931 Kuhn and Lederer were able to isolate alpha- and beta-carotenes and thus fulfil an earlier prophecy made by Tswett. Many new groups of substances, mainly of natural origin, were then investigated. The size of chromatographic columns was increased so that recovery of the separated materials became practicable. Little improvement in theory or method occurred, however, until 1940–41 when two important steps were taken almost concurrently. In Britain, Martin and Synge (1941) introduced the partition chromatogram, and in Sweden, Tiselius (1940) and Claesson (1946) analysed the three main types of behaviour which take place, either separately or together, during adsorption chromatography.

Many factors now contributed to a very rapid development of the whole subject. Irrespective of their relative importance they may be noted as: (1) the new theoretical treatments due both to Tiselius and to Martin and Synge, (2) the existence of suitable problems, especially for the new technique of partition chromatography, and (3) the simplicity and easy availability of the apparatus needed for partition chromatography on paper.

Once it became possible to carry out chromatographic analyses of complex mixtures of amino acids with nothing more than a glass or wooden box, a trough for organic solvent, filter paper, a sprayer loaded with a solution of ninhydrin, and an oven, the spread of the method became extremely rapid. It may be worthy of mention that one of the observations which led to the development of the first paper chromatogram concerned the great sensitivity of the ninhydrin reaction for amino acids when carried out on filter paper. Since the essential requirements for the successful partition chromatography of water-soluble substances were already known it was then possible to take advantage of filter paper as a matrix for partition chromatography. To the delight of the inventors, the spots of amino acids after such separation, when revealed by heating with ninhydrin, were sharp and well separated and, in certain cases, even showed

slight colour differences which were useful for identification. Since as little as 5 μg of a single amino acid or peptide could thus be revealed, a method was at hand for the rapid identification of the very numerous hydrolytic products obtainable from even quite small amounts of proteins. Thus for the first time, a means of attack on the extremely difficult problem of the structure of proteins became available. Any description of its exploitation for the ultimately successful studies of the amino-acid sequences of such molecules as gramicidin S, ACTH, insulin and ribonuclease is beyond the scope of this book. Suffice to mention here that once the relatively easy and rewarding technique of paper chromatography became available, a rather different approach to such problems could sometimes be employed. Instead of starting with the mixture to be analysed and searching for the optimum chromatographic method for each problem, it was often worth while attempting to create problems to fit the method. In the study of amino-acid sequences in proteins, one of the conditions for rapid advance thus became the successful choice of methods of partial hydrolysis which would yield sets of peptides of the right degree of complexity for analysis by paper chromatography. Although many new mixtures of appropriate degrees of complexity were separated in this way, numerous other mixtures remained intractable and so challenged the ingenuity of the analyst. Preliminary separations by electrophoresis or other suitable methods often helped, but a powerful stimulus remained to increase the range and resolving power of the paper chromatogram itself. That this has now been largely achieved is shown by the considerable number of alternative solvent systems which have been described.

In this book, numerous detailed descriptions of well-tried chromatographic systems are given. These should facilitate the separation of many of the mixtures ordinarily encountered in the laboratory. On the other hand, the number of new mixtures still requiring analysis must far exceed all those for which appropriate systems have already been found. Since chromatography, and especially partition chromatography on paper, is a method of analysis which can be varied easily in many different ways it can be expected that numerous modifications and new applications will continue to be found. If in fact this turns out to be true, chromatography will maintain its lead over other methods such as countercurrent solvent extraction, electrophoresis and ultracentrifugation in the sense that more

separations of more individual substances will continue to be made in this way.

It is also true that other methods will certainly find many new applications, and powerful new methods will surely be discovered; thus how long chromatography will maintain its leading position can hardly be predicted, although its very convenience must tell in this direction. A safer prediction would perhaps be that the increasingly complex mixtures requiring analysis will need the successive use of several methods rather than the application of any single one.

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CHAPTER 2

PRINCIPLES

Definition and Scope

Chromatography has been defined as 'the technical procedure of analysis by percolation of fluid through a body of comminuted or porous rigid material irrespective of the nature of the physicochemical processes that may lead to the separation of substances in the apparatus' (Gordon, Martin and Synge, 1944). This definition adequately summarizes the main features of the chromatographic technique. It is essentially a practical method for separating the constituents of a mixture, achieved by passage through a system of two phases, which move relative to each other. Normally there is a *stationary phase*, consisting of a finely-divided solid or gel with a large surface area, in contact with a continuous, fluid *moving phase*, which completely fills the interstices. Separation occurs by virtue of differences in the distribution of constituents between the moving phase and either the bulk of the stationary phase or the interface between the two phases.

Initially, the substances to be separated are molecularly dispersed in a small volume of the moving phase. Thus the mixture of solids (or liquids) to be separated is dissolved in the liquid moving phase, or a mixture of their vapours (at a suitable constant temperature) is fed into a stream of carrier gas. The small volume of moving phase, containing the substances to be separated, then passes over the particles of the stationary phase, usually being immediately preceded and followed by the pure moving phase [Fig. 2.1(*a*)]. The dispersed substances would be swept past the stationary phase at the same rate as the moving phase were it not for the fact that there is usually some interaction between these substances and the stationary phase [Fig. 2.1(*b*)]. Their affinity for the stationary phase causes them to travel more slowly than the moving phase, and moreover, where the affinities of the various substances are not the same, they will be transported by the moving phase through the *chromatogram* at different rates [Fig. 2.1(*c*)–(*n*)]. Such differences in the rates of movement are the basis of chromatographic separation.

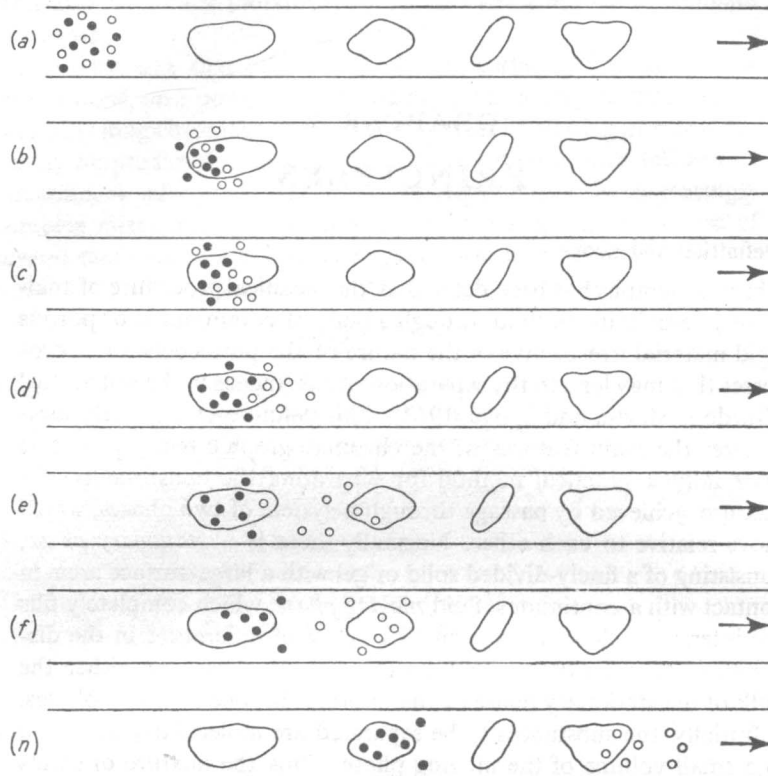

FIG. 2.1. The basis of chromatographic separation.
The affinity of the particles of stationary phase for the black molecules is greater than for the white ones. The white molecules are therefore able to follow the moving solvent more rapidly than the black ones. Separation of the two substances takes place gradually (*b*) to (*f*), being complete at (*n*) where there is pure solvent between the regions containing dissolved substances.

Chromatography provides a versatile method for separation since the choice of materials for both moving and stationary phases is extremely wide. After these have been broadly selected to suit the type of substances to be separated, either phase may be adjusted, as indicated by trial experiments, to give optimum results. Complete separation of substances differing only very slightly in structure may be effected by making the two-phase system, or in practice, the chromatographic *column* of sufficient length.

Differential Migration Methods

Chromatography is only one of a number of methods of separation depending on migration, which has been perfected during the last twenty years. Some features of the most widely used of these differential migration methods, including electrophoresis (electrochromatography or ionography) and multiple partition, which are most closely allied to chromatography, are summarized in Table 2.1.

TABLE 2.1. CHARACTERISTICS OF SOME DIFFERENTIAL MIGRATION METHODS

Method	*Force Causing Migration*	*Property of Substances to be Separated which Provides a Basis for Separation*
Chromatography	Differential movement of two phases due to gravity or pressure	Adsorption* Partition (solubility)* Ion-exchange*
Electrophoresis	Electrical potential gradient	Electrical mobility (charge in relation to molecular size, together with adsorption, partition, etc.)
Liquid–liquid extraction	Stepwise differential movement of two liquid phases	Partition between liquid phases
Mass spectrometry	Magnetic field	Ratio of ionic charge to mass
Differential sedimentation	Centrifugal force	Molecular weight and shape
Diffusion	Thermal agitation	Molecular weight

* Acting singly or in combination.

All these methods are characterized by the action of a driving force which promotes migration of molecules or ions in a permeable medium (solution, gel, gas, or vacuum). The migrating substances also meet with resistive forces which oppose migration. The rate of migration thus depends upon the resultant of the driving and resistive forces. Differences in the value of this resultant force for various substances enable their differential migration and hence separation to take place (Strain, 1960). Migration usually occurs either away from a narrow initial zone of the mixture (chromatography, electrophoresis