CHROMATOGRAPHIC METHODS

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PREFACE

In recent years the techniques of chromatography have progressed rapidly. However, the aims and objectives of the First Edition, as quoted below, are just as relevant today as they undoubtedly were in 1963.

'The various methods of separating mixtures which are grouped under the general name *chromatography* are now well known and widely used. Since the inception of chromatography as a column technique in 1903, the principal landmarks in its progress have been its virtual rediscovery in the 1930s, the invention of synthetic resins in 1935, the introduction of paper chromatography in the early 1940s and finally, the development of gas solid and gas liquid chromatography in the late 1940s and early 1950s.

Subsequent expansion in the use of chromatographic methods has been rapid and continuous, with the result that in the last 15 years a substantial volume of literature on the subject has appeared, dealing not only with particular separations but also in much specific detail with improvements in technique.

Many specialist books have been published. Some are concerned only with particular aspects of the subject. Others are essentially literature surveys which are usually very comprehensive (though somewhat uncritical) and hence rather formidable to someone seeking an introduction to chromatography. The present book aims to present a short account of the techniques in current use.'

The new edition of *Chromatographic Methods* reflects the many changes that have occurred right across the field of chromatography. Development of new materials, for instance adsorbents and polymers, and advances in electronic instrumentation and computing techniques have radically changed the practice and implementation of the various chromatographic techniques.

The principles of chromatography remain the same however, and therefore several aspects of the earlier editions have been retained, but with a new emphasis. Thus, there is an updated but more condensed coverage of plane

chromatography; an expanded chapter on gas chromatography to reflect the developments of the past ten years, particularly those advances in column and detector design; a new substantial chapter on high performance liquid chromatography, currently the most rapidly expanding analytical technique. Developments in spectroscopic instrumentation have enabled combination techniques such as GC–MS, GC–IR, HPLC–MS, HPLC–IR to increase in importance and these are discussed in Chapter 7.

The march of technology has included rapid developments in both analog and digital electronics, which is reflected in the changes in instrument design and capabilities. This includes control of instrument parameters and collection and processing of data. Chapter 8 presents an overview of the subject to assist the analyst's understanding and evaluation of modern instruments and data processing techniques. The final chapter comprises a considerably extended and modified series of experiments which reflect the current practice of chromatography.

The individual chapters have been written, in general, to be self-contained so that readers may dip into the text and pursue the study of particular topics without necessary reference outwith the particular chapter.

Although the chromatographic instrument capable of totally automated method development is now available in, for instance HPLC, there is no substitute for a sound understanding of the principles and practice of chromatography. The role of the chromatographer may have changed in recent years but his expertise is still a valuable essential in the analytical laboratory.

The authors gratefully acknowledge the help, advice and criticism from colleagues and particularly the preceding authors, R. Stock and C. B. Rice. Thanks are also due to the various manufacturers who have generously provided information and permission to use diagrams, etc, as noted in the text.

Finally we would like to acknowledge the patience and support shown by our wives during writing of the text, and particularly to May for preparation of the typescript.

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1 INTRODUCTION

1.1 INTRODUCTION TO CHROMATOGRAPHY

The first person to use the term chromatography was Tswett (1872–1919) the Russian chemist. He used chromatography, from the Greek for colour – chroma and write – graphein to describe his work on the separation of coloured plant pigments into bands on a column of chalk [1,2]. It was not until the 1930s that chromatography in the form of thin-layer and ion-exchange chromatography became a regularly used technique. The 1940s saw the development of partition chromatography and paper chromatography with gas chromatography following in 1950. The 1960s saw a rapid rise in the routine use of chromatography as a universal technique, particularly in chemistry, biology and medicine. It is now used as a production process and yet is sensitive enough for trace analytical techniques.

Two Nobel prizes have been awarded to chromatographers, Tiselius (Sweden) in 1948 for his research on 'Electrophoresis and Adsorption Analysis', and Martin and Synge (UK) for the 'Invention of Partition Chromatography'. Recent developments and new technologies, such as microelectronics and microcomputers have enabled manufacturers to produce instruments that are reliable, with parameters that can be precisely set and measured, to give reproducible chromatograms.

Chromatography is a flexible yet powerful analytical procedure but remains a separation technique. However, the ideal instruments for complete analysis of complex mixtures are becoming more feasible with the coupling of rapid scanning spectroscopic instruments to chromatographs providing spectro-analytical information for each separated component.

1.1.1 Definition of chromatography

Tswett (1906) stated that: 'Chromatography is a method in which the compo-

nents of a mixture are separated on an adsorbent column in a flowing system'.

Recently the International Union of Pure and Applied Chemistry has defined chromatography as:

'A method used primarily for the separation of the components of a sample, in which the components are distributed between two phases, one of which is stationary while the other moves. The stationary phase may be a solid, or a liquid supported on a solid, or a gel. The stationary phase may be packed in a column, spread as a layer, or distributed as a film, etc.; in these definitions 'chromatographic bed' is used as a general term to denote any of the different forms in which the stationary phase may be used. The mobile phase may be gaseous or liquid.' [3].

1.2 HISTORICAL ASPECTS OF CHROMATOGRAPHY

Although chromatographic-like separation processes occur in nature – for example, migration of solutions through soils, clays and porous rocks – the value of such processes was not recognized until the 19th century. Runge (1850) developed methods for testing dyes and bleaches produced from coal tars used in dye chemistry [4]. He demonstrated the composition of dye colours by spotting the mixtures on to special paper producing colour separations. Examples of Runge's work are illustrated and discussed in a recent paper by Cramer [5]. Groppelsroder (1861) developed 'capillary analysis', a form of paper chromatography, using paper strips, with the ends dipping into an aqueous solution, to separate coloured materials in solution, but he was unable to explain the process [6]. It was to be 75 years before this work was followed up and paper chromatography as we know it developed [7, 8, 11].

Tswett's early papers are regarded as the first to describe the separation processes involved during the extensive studies on plant extracts and ligroin solutions using a range of over 100 adsorption media. More comprehensive descriptions are given in Tswett's own book, which was unfortunately only published in Russian.

The next major developments occurred in the 1930s when Lederer and co-workers (1931) separated lutein and zeaxanthine in carbon disulphide on a column of calcium carbonate powder and xanthophylls from egg yolk on a 7 cm diameter column [8, 9]. Further developments soon followed from Khun, Karrer and Ruzicka, who applied chromatography to their own fields of interest, the work being recognized by the award of the Nobel prize (1937, 1938, 1939 respectively). 'Flow Through Chromatography' rapidly gained acceptance and by the 1940s liquid adsorption column chromatography was an established laboratory separation technique on both preparative and analytical scales.

Tiselius (1940) and Claesson (1946) developed the classical procedures by observing the properties of solutions in the chromatographic process and

classifying these into three groups, differing in the principle of the separation, *viz.* frontal analysis, displacement chromatography, elution chromatography [10]. Gradient elution was introduced at a later date (1952). Tiselius was awarded the Nobel Prize in 1948 for his contribution to chromatography. At about the same time Martin and Synge were developing a separation procedure for the isolation of acetylated amino acids from protein hydrolysates by extraction from an aqueous phase into a chloroform organic phase. A series of 40 extraction funnels were used in which the acetylated amino acids could be separated according to their distribution ratio and partition coefficients between a counter current of water and chloroform [11].

They soon replaced this with a chromatographic column filled with silica gel particles with water retained on the silica gel, and the chloroform flowing through the column. This system successfully separated the acetylated amino acids according to their partition coefficients and marked the beginning of partition chromatography. The silica gel was soon replaced by cellulose removing the need to derivatize the amino acids [12]. Martin and Synge were awarded the Nobel Prize in 1952 for this work.

Evidence of the importance of the technique was the method development work sponsored by the American Petroleum Institute for the analysis of constituents in petroleum products; for example, ASTM D-1319 (1954) details the procedure for the determination of saturates, alkenes and aromatics using a fluorescent indicator on silica gel adsorbent with isopropanol as eluent [13].

During the 1930s and 1940s chromatography progressed rapidly with several parallel developments of the earlier work which have resulted in the various chromatographic techniques we use today. A brief note on the historical developments of the main techniques is presented below.

Paper chromatography (PC)

This technique arose from the early work mentioned above with the developments due to Martin and co-workers [14]. Their work on partition column chromatography (*vide supra*) required an adsorbent that would hold water more efficiently than silica gel. This led to the use of cellulose, and hence filter paper as the 'column'. They were able to separate successfully over 20 amino acids by a two-dimensional technique using ninhydrin to locate the spots. The simplicity of paper chromatography ensured its rapid acceptance and reference texts were soon produced detailing organic and inorganic applications thus illustrating the importance of the new analytical technique [15, 16].

Thin layer chromatography (TLC)

TLC originated from the work of Izmailov and Shraiber (1938), who analysed pharmaceutical tinctures by spotting samples on to a thin layer of alumina adsorbent on a glass plate and applying spots of solvent to give circular chromatograms [17]. Later Meinhard and Hall (1949) used a starch binder with a mixture of celite and alumina on microscope slides, still obtaining circular

chromatograms [18]. Kirchner *et al.* (1951) used an ascending development method analogous to paper chromatography [19]. It was, however the work of Stahl, and the development of standardized commercially available adsorbents that provided the impetus for the widespread use of TLC [20], as illustrated in his book, a reference text on TLC [21].

Ion exchange chromatography (IEC)

This technique came into prominence during the Second World War as a separation procedure for the rare earth and transuranium elements. The technique was first used by Taylor and Urey (1938) to separate lithium and potassium isotopes using zeolite resins [22], and Samuelson (1939) demonstrated the potential of synthetic resins [23]. A summary of developments that resulted from work on separation of the transuranium elements is described in Seaborg's book, based on the work he carried out with Thompson. Such texts assisted the general acceptance of the technique [24, 25].

Gel permeation chromatography (GPC)

GPC, sometimes called gel filtration, uses material with a controlled pore size as the stationary phase. The discovery by Flodin and Porath (1958) of a suitable cross-linked gel formed by reaction of dextran with epichlorohydrin provided the breakthrough [26]. Subsequently the commercial development of dextran and similar hydrophilic gels (e.g. agar), ensured rapid acceptance and application of GPC. Development of polystyrene and similar hydrophobic gels with their semi-rigid structure and wide range of pore sizes permitted organic solvents to be used [27]. Analysis by GPC of polymeric materials has revolutionized molecular weight analysis and preparative separation of high molecular weight synthetic polymers.

Affinity chromatography (AC)

This is a relatively recent development attributed to Porath *et al.* (1967) [28]. The stationary phase is a peptide or protein covalently bonded to a ligand such as a nucleic acid or an enzyme on an inert open matrix such as cellulose or agar and is used for the separation of protein molecules.

Gas chromatography (GC)

One of the most important techniques, GC evolved from eariler work on the adsorption of gases on various materials which had been observed for many years, and the pioneering work of Martin on partition chromatography. Martin, with co-worker James, developed and refined this earlier work to develop gas–liquid chromatography (GLC), a technique that has revolutionized analytical chemistry. They used a gas (nitrogen) instead of a liquid mobile phase and stearic acid stationary phase on a celite support to separate C_2–C_4 fatty acids [29]. The

apparatus for gas chromatography rapidly developed and by 1956 GLC was widely used as a routine analytical technique.

The theoretical aspects of chromatography were first studied by Wilson (1949), who discussed the quantitative aspects in terms of diffusion, rate of adsorption and isotherm non-linearity [30]. The first comprehensive mathematical treatment describing column performance (using the height equivalent to a theoretical plate HETP) in terms of stationary phase particle size and diffusion was presented by Glueckauf (1949) [31]. However, it was van Deemter and co-workers (1956) who developed the rate theory to describe the separation processes following on earlier work of Lapidus and Admunson (1952) [32]. Column efficiency was described as a function of mobile phase flow rate, diffusion properties and stationary phase particle size. It was many years before Giddings (1963) pointed out that if the efficiencies of gas chromatography were to be achieved in liquid chromatography, then particle sizes of 2–20 μm were required [33]. This would require high mobile phase inlet pressures. When such systems were demonstrated high column efficiencies were obtained and high performance (or pressure) liquid chromatography (HPLC) had arrived and was to have just as significant an effect on analytical chemistry as GC had a decade earlier.

There has been continuous development in chromatography, particularly techniques and practice, materials and refinement of instrumentation which has resulted in the efficient, reliable and sensitive chromatographic methods in use today and which form the backbone of modern analytical procedures and routine laboratory analysis.

1.3 CLASSIFICATION OF CHROMATOGRAPHIC METHODS

1.3.1 According to separation procedure

Chromatography encompasses a number of variations on the basic principle of the separation of components in a mixture achieved by a successive series of equilibrium stages. These equilibria depend on the partition or differential adsorption of the individual components between two phases; a mobile phase (MP) which moves over a stationary phase (SP) composed of small particles and therefore presenting a large surface area to the mobile phase. The sample mixture is introduced into the mobile phase and undergoes a series of partition or adsorption interactions between the mobile and stationary phases as it moves through the chromatographic system. The differences in physical and chemical properties of the individual components determine their relative affinity for the stationary phase and therefore the components will migrate through the system at differing rates. The least retarded component, having an equilibrium ratio which least favours the stationary phase, will be eluted first, i.e. moves fastest through the system. The most retarded component moves the slowest and is

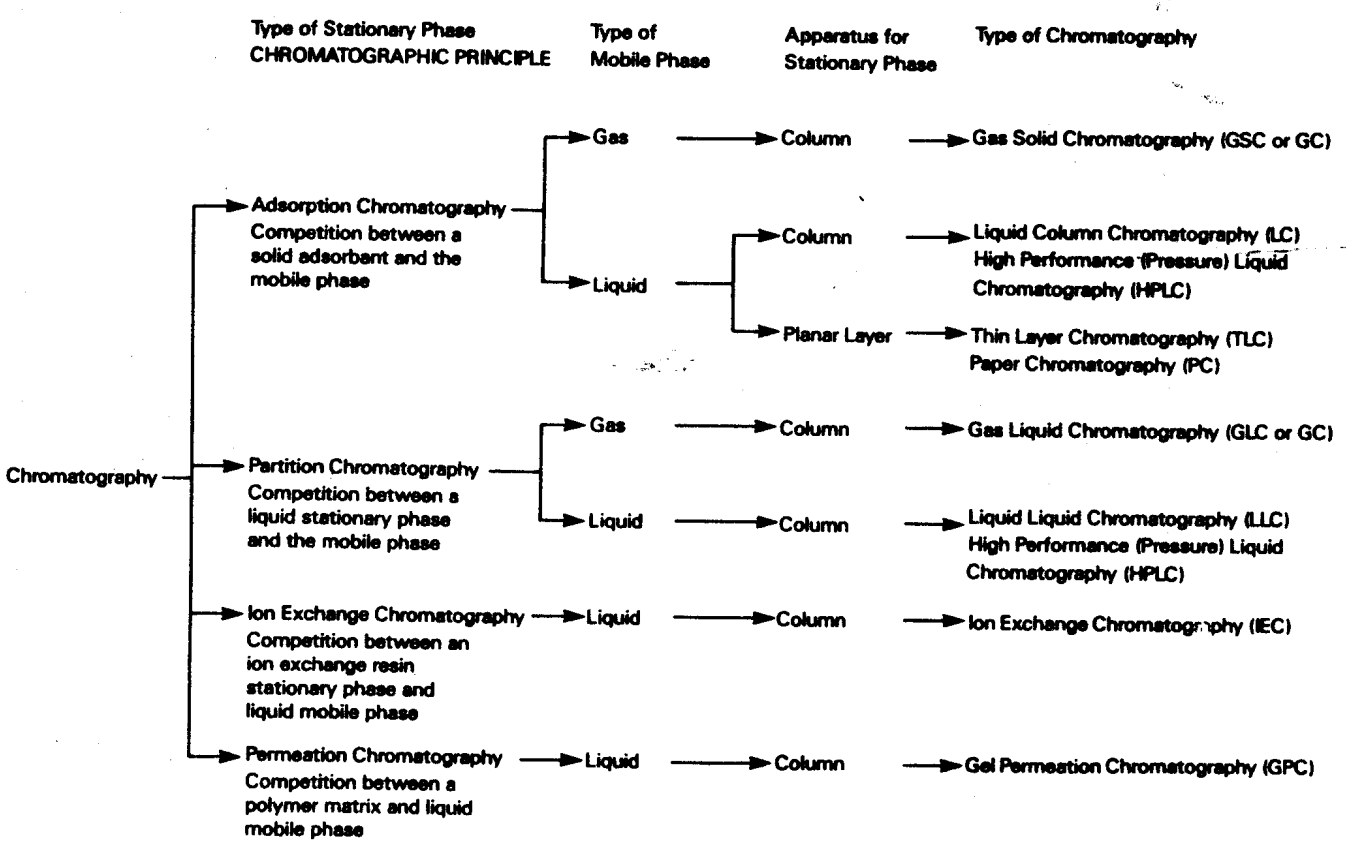

Fig. 1.1 Classification of chromatographic methods.

eluted last. A wide range of stationary and mobile phases can be used making it possible to separate components with only small differences in their properties.

The mobile phase can be a liquid or a gas and the stationary phase a liquid or a solid. Separation involving two immiscible liquid phases is referred to as partition or liquid–liquid (partition) chromatography and when physical surface forces govern the retention properties of the component on a solid stationary phase liquid–solid (adsorption) chromatography is involved; when the mobile phase is a gas we have gas–liquid chromatography (GLC) and gas–solid chromatography (GSC) respectively. The classification of chromatographic methods is shown in Fig. 1.1 and a simplified diagram of the apparatus of chromatography is shown in Fig. 1.2.

1.3.2 According to development procedure

Tiselius (1941) classified chromatography according to the separation principle, *viz.* elution development, displacement development and frontal analysis [10]. In

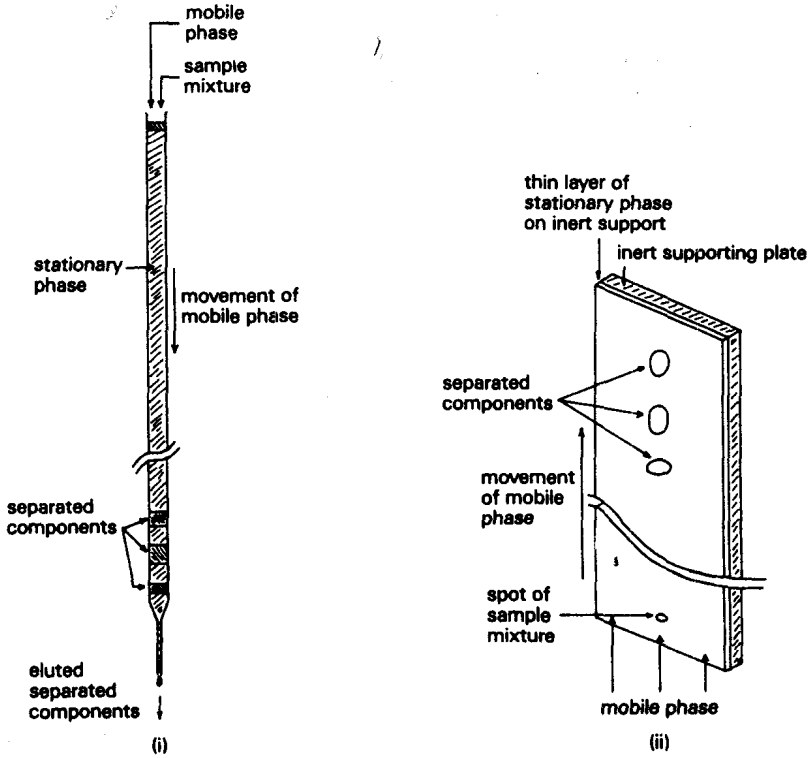

Fig. 1.2 Simplified apparatus for chromatography: (i) column chromatography; (ii) planar chromatography (tlc).

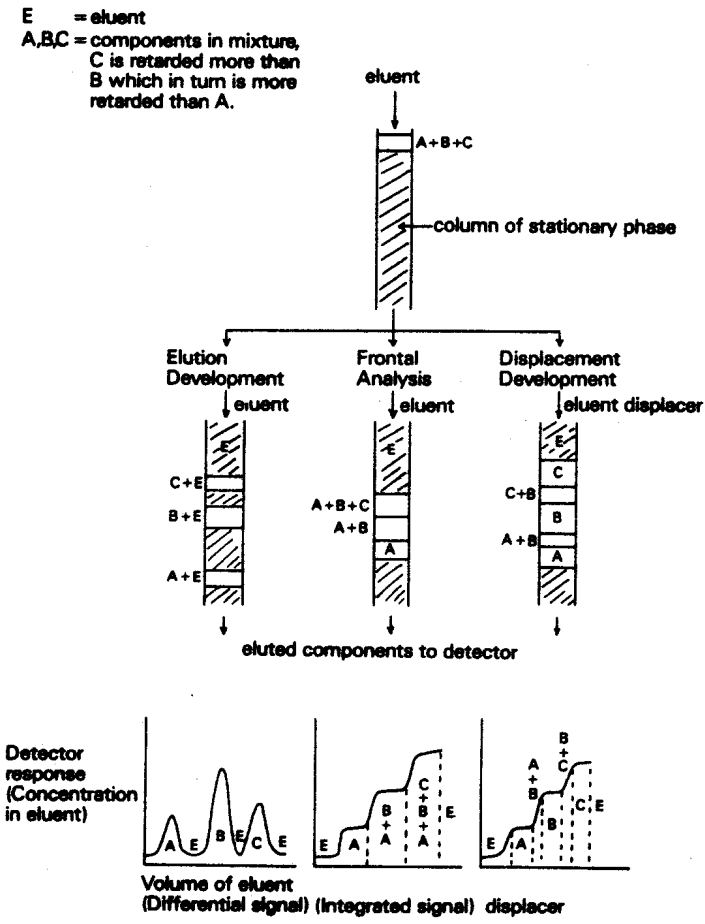

Fig. 1.3 Classification of chromatographic methods according to development procedure.

practice only elution and to a lesser extent, displacement development are commonly used (Fig. 1.3.).

Elution development

Elution development is the technique most widely used in the various methods of chromatography (GC, GLC, LLC and LSC). A small sample mixture is introduced on to the column and is eluted with a moblic phase which has a lesser affinity for the stationary phase than the sample components. The components therefore move along at a rate determined by their relative affinity for the stationary phase but at a slower rate than the eluent. The components are eluted

in order of their affinities but their migration is determined by the ternary interaction between components, stationary phase and mobile phase. Since the components can be completely separated with a zone of mobile phase between them, elution chromatography is used for analytical separations, with some variation possible. In simple elution chromatography the column is eluted with the same solvent all the time. This is most suitable when the components have similar affinities for the stationary phase and are therefore eluted rapidly, one after another.

Stepwise elution is carried out by changing the eluent after a predetermined period of time. The eluents are chosen to have increasing eluting power, that is, increasing affinity of the mobile phase for the components remaining on the column, and therefore 'releasing' them from the stationary phase, enabling them to move through the system.

Gradient elution uses a gradual change in composition of the eluting solvent to achieve separation of components of widely varying affinities for the stationary phase. The ratio of two or more solvents is gradually changed to increase slowly the eluting power of the mobile phase. Thus the tailing part of a component zone or peak emerging from a column is eluted by a solvent of slightly higher eluting power than the leading part. This eluent gradient narrows the zones and reduces tailing. The solvent composition gradient may be linear, steadily increasing or decreasing, or logarithmic, and may be a concentration, pH, polarity or ionic strength gradient.

Displacement development

This consists of elution or development of the separation procedure by a solvent which has a greater affinity for the stationary phase than the sample components. The sample mixture is first introduced on to the column, and adheres to the stationary phase. Elution occurs when a displacing solvent is passed through the column, displacing the components on the stationary phase which also separate due to their varying partition or adsorption properties.

Generally displacement development does not produce completely separated components in bands separated by eluent. Between the zones of pure component there are adjacent zones containing mixtures and therefore the central parts are collected if preparative work is being carried out.

Frontal analysis

This consists of the continuous addition of a sample mixture on to the column. Initially the component with the least affinity for the stationary phase will pass along the column while a strongly adsorbed or attracted component builds up on the stationary phase at the beginning of the column. However, there is a limit to the capacity of the stationary phase and when this is exceeded this component also migrates along the column. Therefore the first component is eluted from the column, firstly in a pure form, then as a mixture with the next components to be