CHROMATOGRAPHIC AND ELECTROPHORETIC TECHNIQUES

VOLUME I

PAPER AND THIN LAYER CHROMATOGRAPHY

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PREFACE TO THE FOURTH EDITION

THE subject and literature of paper and thin layer chromatography includes a spectrum of commercially available and home-made equipment. However, it is extremely difficult to find an adequate discussion of the most suitable techniques for a particular type of separation. Briefly, the purpose of this work is threefold: to bring together in as concise a form as possible tried and tested methods of paper and thin layer chromatography; to discuss all those methods which can be applied routinely; and to provide all the essential information to enable the reader to apply these techniques immediately and without recourse to the literature for further details of technique. Much of the work has been carried out on commercially available apparatus as this offers many advantages to the majority who prefer to buy rather than construct their own equipment. However, as much thin layer work is done on five and ten centimetre squares, some homemade equipment is also described.

Paper and thin layer chromatography continue to expand rapidly, whereas individual interests do not. We have therefore taken the decision to restrict the contents of this almost entirely rewritten and updated volume to the field of biochemistry and, in particular, to take most of the applications from clinical biochemistry and toxicology. Nevertheless, because of its extremely practical approach we feel that the work should appeal to all those concerned in the application of these techniques in the biological sciences.

January, 1976

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CHAPTER 1

(a) THE EARLY HISTORY OF CHROMATOGRAPHY

Trevor I. Williams

As with many great discoveries, the origins of chromatography are hard to discern. It is certain, however, that the popular attribution to M. S. Tswett, by L. Zechmeister[1] and others, does much less than justice to many much earlier workers. Tswett certainly provided convincing evidence of the power and versatility of the method, and was to some extent instrumental in introducing it into general chemical practice, but he was by no means its originator. We will return later to his contributions, which were considerable, but meanwhile must concern ourselves with those of his predecessors.

Various definitions of chromatography have been proposed[2,3,4] but none has proved wholly satisfactory in comprehending all those variations of the method that working chemists would now recognize. For present purposes we may consider chromatography as including separation processes based on differential distribution between two immiscible phases, one of which moves relative to the other. The element of motion may be provided not by movement of a phase but by movement within a phase, as in electromigration.

There is ample evidence that what may be called chromatographic effects were not only observed, but put to practical use, some two thousand years ago. Dyers were accustomed to judge the quality of their dyes, and in particular to detect the presence of adulterants by letting a drop of solution spread out on a piece of cloth or paper (papyrus). The fringe of colours formed at the boundary of the spot was usefully diagnostic. Such edge-effects were, of course, also of interest to the dyer because they might affect the quality of his wares. In some cases, the specificity of such tests was increased by impregnating the base material with a suitable reagent. Pliny,[5] for example, describes a spot-test for iron using papyrus impregnated with extract of gall.

Interesting though they are, it would be wrong to attach much practical significance to these very early tests, of whose extent and and importance we know very little. What is very significant, however, is that in the early years of the nineteenth century it was commonplace to test dyes and pigments by applying them to sheets or strips of absorbent material, sometimes impregnated with chemical reagents. Such tests were widely referred to in the chemical trade literature and would, therefore, have been well known to F. F. Runge, a well qualified industrial chemist who, from about 1830, devoted much of his time to the problems of dyes and dyeing. He wrote an important three-volume book entitled *Farbenchemie* (1834, 1842, 1850); the last volume of this clearly describes the separation of substances by capillary action: Runge thus has a strong claim to be the first identifiable practitioner of chromatography. A later book, now very rare, was his *Bildungstrieb*

der Stoffe (1855) which is illustrated with actual paper chromatograms individually prepared and pasted in by hand. While some of Runge's tests were carried out on paper impregnated with chromatic chemical reagents, making them more akin to spot-tests than true chromatograms, others depended simply on free radial diffusion on untreated paper. In his day, Runge was a chemist of some standing and his views would be widely known. His *Grundriss der Chemie* (2 vols., 1847/8), in which such tests were described, was in its day a standard work. Whatever chromatographic debt Runge owed to his predecessors, he handed on to his successors.

Among the most important of his immediate successors were C. F. Schonbein—best known for his work on ozone—and F. Goppelsroeder, both of whom favoured vertical ascent on strips of paper rather than Runge's radial flow. Schonbein is credited with having been the first to observe that solutes advanced less rapidly than the liquid front, but Runge's surviving chromatograms still show this effect so clearly that he cannot fail to have detected it. Schonbein and Goppelsroeder carried out some quite sophisticated experiments to measure the relative speeds of ascent of different substances under standard conditions. In effect, they measured what we now call R_F values. All these workers made some experiments also with three-dimensional methods—using, for example, blocks of wood or tubes filled with fine sand—but never pursued them very seriously.

If paper chromatography had its roots in the dyestuffs industry, column chromatography may be said to derive from the oil industry. There it was traditional to purify crude oils, generally vegetable but sometimes mineral, by allowing them to percolate through beds of carbon or bleaching earths. It was observed that some stratification of the removed impurities occurred in the filter-beds.[6,7,8,] In 1886, C. Engler and M. Boehm[9] showed that when crude petroleum was passed through tall, narrow filters containing carbon, the filtrate was rich in unsaturated hydrocarbons while saturated ones were retained at different levels in the columns. This technique was used as a basis for the manufacture of petroleum jelly.

This work was certainly known to the American petroleum chemist D. T. Day, as was that on the stratification of impurities in lamp-wicks, a phenomenon then being widely discussed in the technical journals. Day's interest was primarily in the variations in the compositions of different petroleums in nature. In 1897 he came to the conclusion that the main cause was percolation through natural mineral deposits, which retained some constituents but allowed others to pass through. Soil chemists were interested in similar phenomena, because of their effect on the distribution of mineral nutrients. Justus von Liebig, for example, demonstrated that potassium is generally retained in layers above those of sodium. In Britain, two soil chemists, H. S. Thomson and J. T. Way, described in the *Journal of the Royal Agricultural Society* experiments in which mineral salt solutions were percolated through columns of prepared soil. The interest was, however, in soil fertility, and possible analytical applications were not pursued. Day, however, was very conscious of the analytical aspect, and his work became

internationally known when he gave a detailed account of his experiments at the First International Petroleum Congress (Paris, 1900). In Europe, Engler, whose work we have already noted, developed his previous work. With E. Albrecht[10] he constructed a special column with sampling ports at intervals, to enable the process of separation to be followed, and they used a variety of physical methods to detect changes in the composition of the filtrate. This is interesting as an early example of the applications of the method to colourless substances. Engler and Albrecht well understood the analytical possibilities of the method outside the field of petroleum chemistry.

Only at this stage do we reach the work of M. S. Tswett, who, unlike most of his predecessors, was a botanist rather than a chemist. His interest was in plant pigments, many of which present peculiar analytical difficulties because of their lability and the complexity of the mixtures in which they occur. As a botanist, working in Warsaw, it is possible that Tswett was unaware of the then comparatively recent work of Day, in a field very different from his own, but he certainly knew something of the experiments of some of the earlier workers, such as Goppelsroeder, since he makes reference to them. Tswett's classic experiments are too well known to need description here. Briefly, he isolated the principal plant pigments—carotene, chlorophyll, and xanthophyll—by passing them, in solution in petroleum ether, through a column of powdered chalk. He enhanced the separation by 'developing' the chromatograms by washing the column with pure solvent. Unfortunately, Tswett's work at first attracted little attention, mainly because he originally published in an obscure journal, the *Journal of the Society of Naturalists of Warsaw*; we may perhaps make here a comparison with Gregor Mendel. After 1906, when he published in German, Tswett's work was more generally known, but not until long after his death, in 1919, was it widely acclaimed. In 1931, R. Kuhn, E. Lederer, and their co-workers showed its immense value in the field of carotenoid chemistry. It is probably because Tswett worked in the same field as the one in which chromatography achieved its first outstanding success that he is so widely identified with its discovery.

P. Karrer, at the Eleventh International Congress of Pure and Applied Chemistry (1947) succinctly reviewed the influence of chromatography on the development of organic chemistry:

> ". . . No other discovery has exerted as great an influence and widened the field of investigation of the organic chemist as much as Tswett's chromatographic adsorption analysis. Research in the field of vitamins, hormones, carotenoids, and numerous other natural compounds would never have progressed so rapidly and achieved such great results if it had not been for this new method, which has also disclosed the enormous variety of closely related compounds in nature."

At this point, we may pause to reflect why there was a lapse of some eighty years between the first clear demonstration of the possibilities of chromatography and its emergence as an outstandingly valuable analytical and preparative technique. The reason is implicit in Karrer's

comments. Not until the 1930s did chemists become keenly interested in the kind of problem that chromatography was uniquely able to solve; namely, the isolation of very minor constituents, often labile and of totally unknown chemical nature, from very complex mixtures. In the nineteenth century this sort of problem rarely arose. While chromatography might with advantage have been introduced much earlier, such classical techniques as crystallisation, distillation, sublimation, filtration, and so on could cope at least adequately with most problems then exercising chemists. Chromatography triumphed when other methods proved inadequate for new problems that had come to the forefront.

In our earlier brief definition of chromatography we referred to partition between two immiscible phases as being one of its essential features. Hitherto, we have considered only liquid/solid partition but if we consider also gas-phase partitions the number of basic possibilities increases greatly, embracing, for example, gas/liquid and liquid/liquid exchanges. Within each combination of phases we may have several possibilities, depending on the kind of chemical affinity utilized. As far as liquid/solid chromatography is concerned we have so far considered separations based on differences in adsorptive affinity, but differences in ion-exchange properties can also be utilized. Tswett himself realised that principles other than adsorption might be employed, and J. E. Meinhardt[11] listed seventeen different principles as a possible basis for chromatography.

Chromatography based on differential partition between two immiscible solvents was described by A. J. P. Martin and R. L. M. Synge[12] in 1941. In this, water was fixed within silica gel, prepared in powder form, and the mobile phase was chloroform or some other immiscible solvent. By facilitating the analysis of amino acids, the introduction of this method had a powerful effect on the development of protein chemistry but it was quickly extended to other classes of substance, especially—in the first instance—antibiotics. Later Martin and his collaborators[13] extended their method for qualitative purposes by using filter-paper saturated with water as the stationary phase. Two-dimensional dispersal on sheets of paper was affected by using two different solvents flowing successively at right-angles. By the standards of the day, this provided an extraordinarily delicate method of analysis for amino acids. From 10^{-4} g. of wool, it was possible to demonstrate the presence of all the amino acids that could be detected by other methods. Moreover, very large numbers of analyses could be made easily, swiftly, and cheaply.

The war-time Manhattan Project created many novel and urgent analytical problems. In particular, there arose a demand for rare earths of high purity in gram quantities, mainly because they are among the fission products of heavy elements. Because of their very great chemical similarity, their separation by classical methods is painfully laborious and slow. Separation processes based on percolation through large columns of synthetic ion-exchange resins, followed by development with citrate or other complexing agent, provided an extraordinarily effective method of quick and complete separation. The

effect on rare-earth chemistry, when details of the work were released by the American Atomic Energy Commission in 1947, can fairly be described as revolutionary.

Finally, among the major developments in chromatography, we must mention gas-phase chromatography. The solid phase may be either solid, or—more generally—liquid retained on some sort of solid support. The effective pioneers were A. T. James and A. J. P. Martin[14]—though earlier experiments were carried out by G. Hesse and by S. Claesson—who initially were interested in the separation of fatty acids. Very quickly, of course, the method was extended to cover a wide range of substances, and today gas chromatography—perhaps coupled with mass spectrometry—is standard technique in most chemical laboratories.

In addition to the major methods described above there are, of course, many minor, but nevertheless very important, variations. Such, for example, are thin-layer chromatography, first described in 1946 and the use of specially prepared impregnated papers in the chromatography of inorganic substances.

Effectively, chromatography is less than half a century old, for it was only in the 1930s that it made any substantial contribution to the solution of chemical problems. Since that time it has emerged—in its various manifestations—as perhaps the most powerful single analytical and preparative method available in the chemical laboratory. In some fields, such as protein and rare-earth chemistry, it has permitted tremendous advances where previously there was near-stagnation. In virtually every field of practical chemistry its influence has been very great. To the younger generation of research workers the method is commonplace, even slightly old-fashioned, but it is timely to remember how much is owed to the pioneers who developed it.

(b) INTRODUCTION TO PAPER AND THIN-LAYER CHROMATOGRAPHY

Ivor Smith and R. S. Ersser

SEPARATIONS on paper (PC) or thin layers (TLC) are the easiest of chromatographic techniques to perform and require simple apparatus. They readily provide qualitative information and, with careful attention to detail, it is often possible to obtain quantitative data. In the simplest form of the technique a drop of the solution containing the mixture of compounds to be separated is placed near one end of a strip of paper or thin-layer and allowed to dry. The strip is then placed so that the end with the application spot dips into a liquid (usually a mixture of solvents). It is essential that the dried spot is not immersed as it would then dissolve in the solvent and be lost. As the solvent travels towards the far end of the strip, the test mixture separates into various components. When the solvent reaches the far end of the strip or a convenient time has elapsed, the sheet is removed, rapidly dried

and the spots are detected using a suitable location reagent.

Separation is due to the small changes in physical and chemical properties, which result from the structural differences of the chemically related group of compounds which are under investigation. They therefore have relatively lesser or greater affinity for both the mobile or stationary phases of the chromatographic system. If paper acted merely as an inert support, separation of the components of the mixture would be caused exclusively by continuous partition between the solvent flowing along the paper and the water held in the paper—filter paper contains about 15 per cent of its weight of water—and this may be the case in some circumstances. More usually the paper does affect the separation process in a number of ways: it acts as an adsorbent in a similar manner to the alumina column; it has a strong affinity for polar molecules which are held by hydrogen bonding and van der Waal's forces; it functions as an ion exchange material due to its content of carboxyl groups. Which of these forces predominates in any particular situation depends on the type of compounds being separated and the solvents used. On heat-activated layers of silica gel and similar substances, when non-aqueous solvent systems are used, adsorption is the predominating process and this has been useful for separating hydrophobic substances such as lipids (see Chapter 14). When aqueous solvents are used on layers of crystalline material of large surface area and fine particle size, a situation between these two extremes exists and the magnitude of the influence of the individual components of this complex system are not easily assessed.

Where a mixture contains many components it will be obvious that complete separations of all of these on a strip of finite length may not occur, and many examples are known where two substances run to the same position on the chromatogram. In order to overcome this difficulty recourse has been made to the use of a number of different solvents with different properties, so that components running together in one solvent will probably separate in another solvent. Although many one-way chromatograms, each in a different solvent, could be compared, a great deal more information is obtained if two solvents are used in conjunction to prepare a two-way chromatogram than if the two are used to prepare two one-way separations (see Fig. 1.1.). A two-way chromatogram is prepared by placing a drop of the mixture near a corner of a square or rectangular sheet of paper or layer. Solvent is then allowed to travel up the whole sheet with the result that a one-way separation is obtained and then, after drying completely, the sheet is turned at right-angles and run in a second solvent which performs a further separation and causes the components to be distributed on the sheet in two dimensions instead of the previous one dimension.

The Chromatographic Method

Chromatographic procedures conform to a universal pattern which can be divided into the three major sections of sample preparation, separation and detection.

Sample collection, preservation and preparation. The problems of

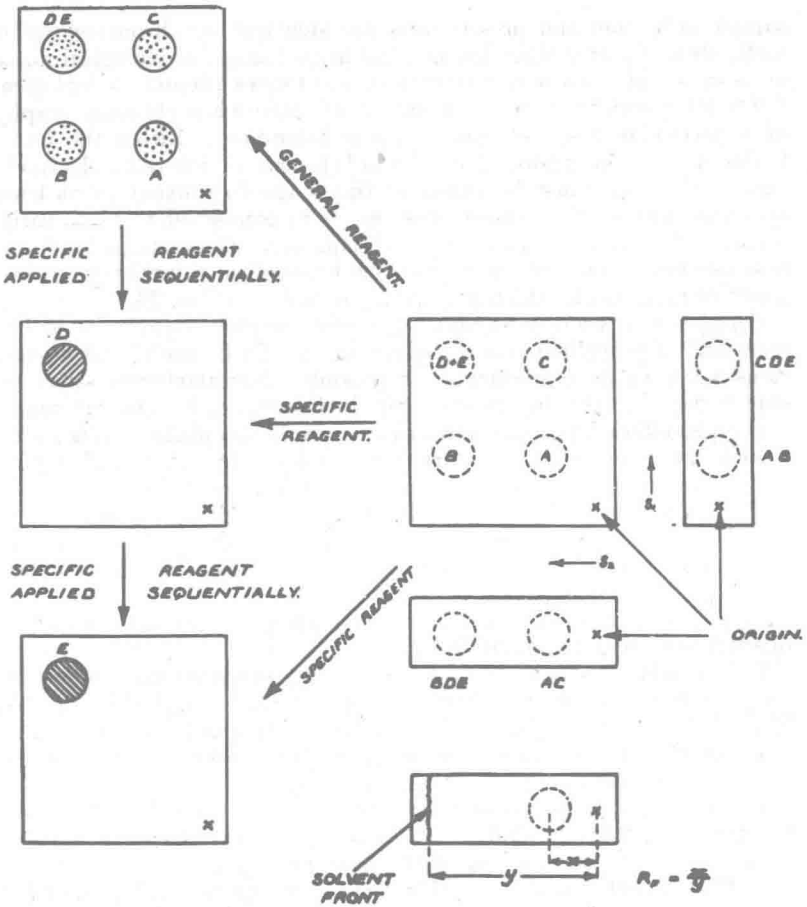

FIG. 1.1. Diagram illustrating one- and two-dimensional chromatography, the use of general and specific reagents and sequential application of reagents (multiple dipping). ABCDE are five components present in a mixture. In solvent Si the R_f values of A and B are equal; those of C, D and E are also equal. In solvent S_2 the R_f values of A and C are equal; those of B, D and E are also equal. A one-way chromatogram using either solvent therefore shows only two spots on location with a general reagent. A two-way chromatogram shows four spots on location with a general reagent, but by the application of specific reagents to duplicate chromatograms one of the spots is shown to be a mixture of two components D and E having the same R_f in both solvents. When the reagents can be applied sequentially only one chromatogram need be prepared, otherwise three separate chromatograms are necessary.

sample collection and preservation are identical for chromatographic methods as for any other biochemical investigation of biological fluids or tissues. The complex mixtures of substances present in biological fluids often results in their mutual interference when chromatography of a particular group of compounds is attempted. Either the interferences must be removed or the substances of interest selectively extracted. Care must be taken at this stage to prevent or at least minimize losses. Sometimes substances are converted to more satisfactory derivatives which are subsequently chromatographed for example labile keto-acids are converted to stable di-nitrophenyl hydrazones or aminoacids which may be separated by PC or TLC.

Separation (*Chromatography*). Chromatographic separation is the resultant of propelling and retarding forces. To be useful the separations must be as reproducible as possible. Manufacturers strive to supply papers or thin layers with reproducible separation characteristics and much effort is put into spreading similarly acceptable layers in the laboratory. Chromatograms are developed in air-tight tanks, which are saturated with solvent vapours at an even temperature.

The solvent used should ideally have the following properties:

1. The individual components of the solvent should be obtainable easily and at fairly low cost, but should be of sufficient purity for direct use.

2. The solvent should be stable in air and when mixed with small quantities of acid and alkaline vapours.

3. The solvent should be capable of being prepared as required by simple mixing, or of being prepared in bulk and stored till required. Thus, it is valuable to be able to prepare solvents composed of liquids as required, but where one of the components is solid it is more valuable to be able to prepare and store large amounts.

4. The components should be relatively non-volatile, or their volatilities should be similar in the closed apparatus so that they evaporate off the sheet at about the same rate.

5. The solvent should be capable of rapid, complete and easy removal from the sheet after the chromatogram has been run. Any traces of unremoved or unremovable solvent should be inert to the location reagents.

6. The solvent should remain homogeneous throughout the range of temperature experienced in the particular laboratory.

7. The solvent should not react with any of the substances to be separated.

8. The substances to be separated should be spread through the whole length of the sheet from just above the origin practically to the solvent front: i.e. the Rf values should vary from 5–95. If the front has run off the sheet, the fastest component should be near the front edge of the sheet.

Detection, identification and quantitation. Relatively few compounds are naturally coloured, fluoresce or absorb ultraviolet light and so after separation the majority of compounds are detected with a chemical location reagent.

The term "location reagent" is defined as the total material applied

to the dry chromatogram, after chromatography in order to locate or reveal the positions of the separated substances so that a colour is produced which is different to that produced when the reagent is applied to a blank sheet which has also been run in the solvent. Thus, the reagent includes: the chemicals (active constituents) which react to produce the colour, the solvent in which they are dissolved and any subsidiary substance which is added to strengthen or stabilize the colours so produced. The following characteristics are desirable in a location reagent:

1. It should be cheap, and pure.
2. It should be stable both alone and in the solution in which it is applied.
3. It should react rapidly with susceptible compounds, preferably in the cold.
4. It should be inert towards residual solvent on the paper.
5. If heating is required, no noxious or corrosive fumes should be evolved.
6. It should be capable of multiple dipping (see Chapter 2).
7. It should not be a health hazard.

Chemical reagents of this type suffer from the disadvantage, when compared to physical location methods (fluorescence, radio-activity, ultra-violet light, etc.) that they destroy the compounds being sought.

The identification of separated compounds is usually based on their reaction with suitable location reagents, and their position on the chromatogram. The fundamental measurement in chromatography is that of R_f, which is defined as follows:—

$$R_f = \frac{\text{distance substance travels from the origin}}{\text{distance solvent front travels from the origin}}$$

However, for convenience, many authors use $100 \times R_f$ so that an R_f is stated to be 54 and not 0·54; this system is used throughout the book.

This value, which is a physical constant of the substance concerned, should therefore be reproducible. However, it is commonplace that published R_f values vary, to a greater or lesser extent, from apparatus to apparatus. The reason for this is, of course, that it is seldom the case that the chromatographic system is fully described and so, in effect, every worker is using a different system. Such a description should include the following:

1. The dimensions of the apparatus.
2. The grade of paper, or nature and thickness of thin layer media.
3. Ascending or descending solvent flow and length of flow.
4. The volume used and composition of the solvent travelling along the chromatogram.
5. Any other liquid or vapours incorporated for special purposes.
6. Equilibration time (if any).
7. The temperature.
8. The nature of the mixture to be chromatographed and previous mode of treatment, e.g. type of desalting.

Any variant in any of the above conditions will affect the R_f value, although in many cases the effect will not be great enough to be