PAPER CHROMATOGRAPHY AND CHEMICAL STRUCTURE

I. TANKLESS OR FLAT-BED CHROMATOGRAPHY A METHOD FOR THE ACCURATE DETERMINATION OF R_M VALUES

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INTRODUCTION

The theoretical basis for the relation between R_F values in partition chromatography and chemical structure was first proposed by CONSDEN, GORDON AND MARTIN[1] and later by MARTIN[2], who deduced that, for ideal solutions, the partition coefficient (α) of a substance A between two phases is related to the free energy required to transport one mole of A from one phase to the other, by the expression

$$\ln \alpha = \frac{\Delta\mu_A}{RT}$$

MARTIN showed that addition of a group X to the substance A should change the partition coefficient by a factor depending only on the nature of X and the two phases, but not on A itself. Hence when A is substituted by n groups X, m groups Y etc.

$$RT \ln \alpha = \Delta\mu_A + n\Delta\mu_X + m\Delta\mu_Y + \ldots \text{ etc.}$$

and since

$$\alpha = \frac{A_L}{A_S}\left(\frac{1}{R_F} - 1\right)$$

where A_L = cross-sectional area of the mobile phase, A_S = cross-sectional area of the stationary phase,

$$RT \ln \frac{A_L}{A_S}\left(\frac{1}{R_F} - 1\right) = \Delta\mu_A + n\Delta\mu_X + m\Delta\mu_Y + \ldots \text{ etc.}$$

BATE-SMITH AND WESTALL[3] introduced the term

$$R_M = \log\left(\frac{1}{R_F} - 1\right)$$

and showed experimentally that the relationship predicted by MARTIN was followed for a number of flavones, anthocyanins and some related compounds. However, because of the nature of the substituent groups they studied (for example, hydroxy groups), their data was necessarily restricted to a small range of compounds. Since that

time, several workers have experienced difficulties in demonstrating the experimental validity of MARTIN's theoretical postulates. BATE-SMITH AND WESTALL themselves, from their own findings, were led to question one of the main conclusions that must arise if MARTIN's equation is in fact completely valid; *i.e.*, that the R_M increment (ΔR_M) for any given substituent should be a constant, irrespective of the remainder of the molecule—always providing that other molecular interactions are absent. Thus they found that in several series of anthocyanidin glucosides, the value of ΔR_M(OH) tended to decrease as R_M itself increased and they suggested that the change in chemical potential caused by a substituent might decrease as the polar substitution increased. LEDERER[4] studied several homologous series and concluded in 1957 that sufficient data had already accumulated to show that for these different series, $\Delta R_M(CH_2)$ was in fact a constant. Nevertheless, other workers have questioned this, and HOWE[5], more recently, after a study of several series of compounds, drew two conclusions about $\Delta R_M(CH_2)$: (i) that the majority of series investigated (which included carboxylic acids, dicarboxylic acids, aminocarboxylic acids, etc.) did indeed show a close approximation to linearity, when R_M was plotted against the number of CH_2 groups, and (ii) that $\Delta R_M(CH_2)$ was not a constant in all the series (non-parallelism of slopes). HOWE was therefore led to regard MARTIN's postulates as being an approximation only and was thus prevented from recognizing the importance of certain constitutive effects. Some of these and their theoretical implications will be dealt with in the succeeding paper: this communication is mainly concerned with the technical requirements for the determination of R_F and ΔR_M.

LIMITATIONS OF CONVENTIONAL CHROMATOGRAPHY AND SOME SUGGESTED SOLUTIONS

The accurate determination of R_F values is at the very centre of all attempts to correlate chromatographic behaviour with chemical structure; and, in fact, the practical difficulty of doing this for many series of compounds is still a serious stumbling block to the rigorous examination of MARTIN's postulates. This almost certainly accounts for many of the anomalies in the literature and for at least some of the differing conclusions as to the correctness of MARTIN's postulates. It is important, first, to bear in mind that many of the R_F values reported in the literature have been obtained by workers interested chiefly in the practical aspects of separation rather than theoretical considerations and make, in fact, no pretension to be accurate reflections of chromatographic behaviour. The recent attempt, therefore, by FRANC AND JOKL[6], to use such data to demonstrate the failure of the MARTIN equation and hence of the BATE-SMITH AND WESTALL relationship is open to criticism on these grounds. BATE-SMITH AND WESTALL carefully drew up five conditions, which they considered essential if accurate and reproducible R_F values are to be obtained. These may be briefly summarized as follows: (1) the temperature must be constant, (2) the solvent mixture must be at equilibrium, (3) the paper, after spotting, must be equilibrated for 24 h in the tank, (4) a control substance must be run and, if its R_F value differs by more than 0.02 from standard, the run must be discarded and (5) development must be for at least 30–35 cm. Detailed analysis of the 37 chromatographic runs considered by FRANC AND JOKL reveals that these conditions were in fact only rarely obeyed and that much of the data can be dismissed for various technical reasons, such as the following: (i) the use of

ascending chromatography[7–12], which cannot give accurate R_F values; (ii) inclusion of R_F values outside the acceptable range[13–14]; (iii) solvent interactions—for example, chromatography of acids with solvents containing ammonia[16–18], or bases with solvents containing acid[7,19]; and (iv) pronounced lack of equilibration[20,21], commented on by the authors. Apart from these technical reasons, failure to obtain constant ΔR_M values is, we believe, in a residue of cases due to certain structural characteristics of the compounds used; particularly in the lowest members of homologous series: the significance of these will appear from the succeeding papers in this series.

If it is assumed therefore that, in the absence of recognizable steric or electronic interactions in molecules, departure from the MARTIN equation during the running of homologous series is likely to be due to failure, even under the best chromatographic runs, to obtain ideal conditions, it is essential to examine further the reasons that might account for this. Even if all five conditions adumbrated by BATE-SMITH AND WESTALL are rigorously observed, ideal equilibration can probably only rarely be obtained in a tank. Apart from the practical difficulties, there are theoretical reasons why this might be so. The vapour pressure over a curved surface is not the same as that over a flat surface and this difference is considerable for a meniscus of small radius[22]. Since filter paper consists of a network of capillaries, which may be only partially filled during the chromatographic run, the solvent in the tank cannot be in dynamic equilibrium with that in the capillaries, so there will be a passage of solvent molecules between the paper and the vapour phase in a direction that will depend on the curvature of the capillary menisci. The gross effect will be that the amount of solvent appearing to have passed over the paper will be different from the true amount and the magnitude of the discrepancy will vary according to the length of the run, temperature, types of solvent, etc. Analysis of the literature[6–21] indicates that MARTIN's formula is, even under non-ideal conditions, obeyed better when polar, especially hydroxylic, solvents are used. Such solvents, especially water and alcohols, cause swelling of the paper and close the small capillaries; they also form hydrogen bonds with cellulose, penetrate deep into the structure and consequently both condensation and evaporation are slowed down. Another source of difficulty in conventional paper chromatography is the present impossibility of adequately defining the nature of the stationary phase.

It seems clear that there are some inherent limitations in the accuracy with which R_F values can be determined by conventional paper chromatography and these are, in the main, due to failure to obtain real equilibrium between the paper and vapour phase. If this is so, the following conclusions may be drawn.

(1) It is logical to attempt to remove the vapour phase altogether. This implies removal of the tank *qua* tank—that is, an empty receptable in which papers are suspended.

(2) If the nature of the stationary phase is uncertain and leads to unknown and probably variable effects in partition coefficients, the system should be inverted and reverse-phase chromatography used. If an inert, non-volatile substance is used as stationary phase, its nature is then defined.

(3) The mobile phase can now always be chosen so that no problems concerning changes in its composition need arise. Being neutral, no interactions with acidic or basic functions need occur.

(4) Horizontal chromatography should—ideally—replace vertical chromatography.

TANKLESS CHROMATOGRAPHY

Several attempts were made to put these ideas into practice. After a number of trials, the following system, which we have called "tankless" or "flat-bed" chromatography, was adopted.

The method

Sheets of Whatman No. 1 filter paper, measuring 30 × 60 cm and cut in the same direction from larger sheets, are impregnated with the stationary phase as described below. Each sheet is spotted with the test compounds in the usual manner (about 15 cm from one edge) and then placed between two sheets of thin aluminium foil (as sold for domestic use, about 25 μ thick), carefully smoothed first to remove folds and creases. The aluminium sheets should be about 5 cm wider than the papers and rather longer. The sandwiched paper is then placed horizontally on a pad of filter papers, about 1 cm thick, resting on a flat table-top. The process is repeated with further sheets of paper, each paper being separated from the ones below and above by an aluminium sheet (Fig. 1). As many papers as required can be prepared in this way: we have successfully used up to fifty sheets in one run. When the pile is complete, each paper is sealed at both sides and at the far edge by folding the overlapping sheets of foil. The pile is then compressed evenly by placing a flexible polythene sack of sand, weighing about 50 kg, on top. It is important to distribute the weight evenly, as spot migration is affected by pressure differences. The ends of the papers that protrude from the pile are sealed at their lateral edges by folding the overlapping aluminium foil, and then dipped into a tray of solvent. The system is then rendered vapour-tight with aluminium foil as shown in Fig. 1.

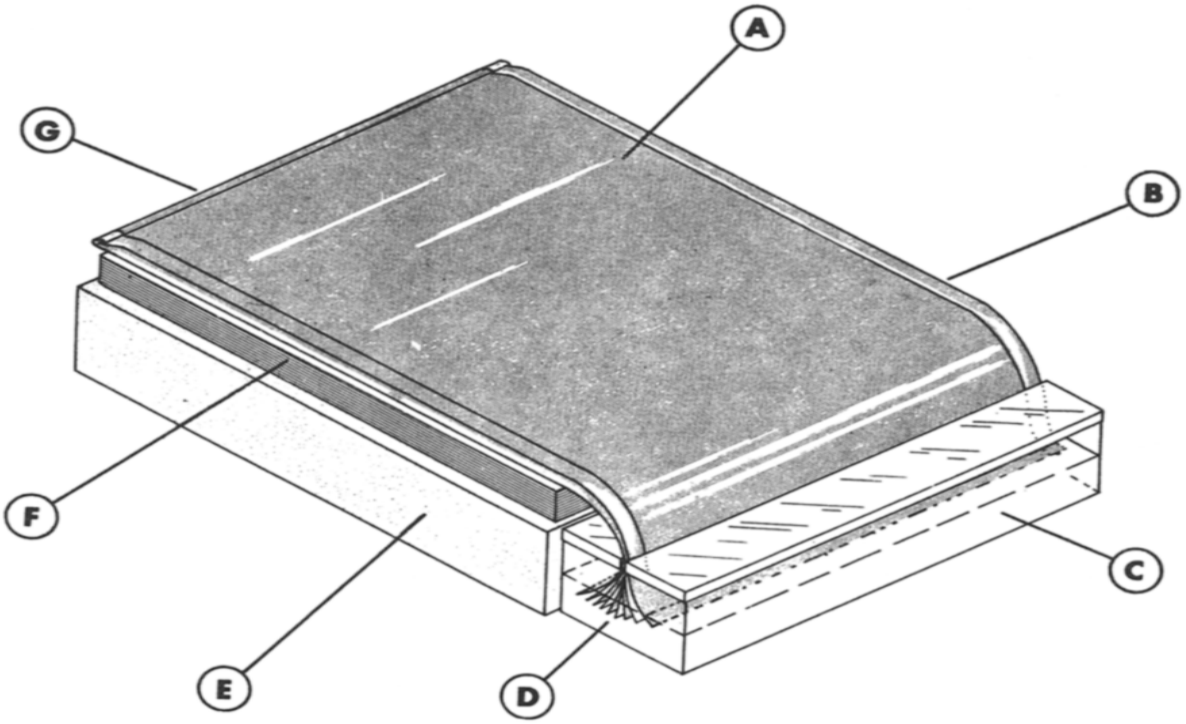

Fig. 1. Flat-bed or tankless chromatography. A. Sheets of paper alternating with aluminium foil. B. Edge of aluminium folded over. C. Solvent trough. D. Papers separated in solvent. E. Rigid support. F. Pile of filter papers. G. Ends of aluminium sheets folded over.

With the system described above and with aqueous ethanol as solvent, chromatography runs of about 40 cm take about 16 h, but the time can be varied by altering the weight used to compress the papers. It is important that sufficient compression be used to eliminate all micro-channels between the aluminium and paper sheets. If folds are present in the foil or if for some other reason contact is faulty, channelling occurs. The aluminium sheets can be used over and over again, but each time they must be carefully smoothed to remove creases and folds. Glass plates should not be used in the pile to give even contact: they are flexible enough to produce local pressure differences in the pile.

Preparation of papers

When papers are normally prepared for chromatography by impregnating them with a stationary phase, this is usually carried out by dipping them in a solution of the latter and allowing the solvent to evaporate. This method is not suitable for the determination of R_F values with the accuracy required for testing MARTIN's equation. It can easily be shown by comparing chromatographic runs on such papers in different directions across the paper that a gradient of stationary phase is produced by gravity in the direction that the paper is hung for drying. This phenomenon is normally undetectable when papers are run in the same direction, but its effect is quite sufficient to account for a severe departure of observed R_F values from true R_F values. The following technique was found suitable for the preparation of impregnated papers and should be used for the accurate determination of R_F values.

A pad of 50 sheets of chromatographic paper is immersed in a bath containing the solution of stationary phase (such as liquid paraffin) in a very light solvent such as diethyl ether. After a few minutes, the pad is removed, drained rapidly and then placed horizontally on another pad of paper. The impregnated papers are then covered with a third pad of dry papers and the pile compressed with the bag of sand. Under these conditions, the papers in the middle of the pile dry out from the edges and are quite dry from the light solvent in about an hour. They are then removed and about 2 cm of paper is cut from each edge, where there is usually a slight build-up of stationary phase. Papers prepared in this fashion have been checked several times and do not show a concentration gradient in any direction.

Advantages and disadvantages of the method

1. Tankless chromatography ensures that as many compounds as required can be run at one time under virtually identical conditions. Duplicate and triplicate papers can be used *ad lib.* throughout the pile to check running conditions and provide statistical data on R_F deviations. It is usually never possible to do this in a tank, for here R_F values vary according to the dimensions of the tank, degree of saturation, and particularly, the actual position of the papers relative to each other and the walls of the tank. In experiments to be described in succeeding papers we have sometimes obtained data on as many as 200 spots on the same day.

2. The run can be adjusted so that in the required time (usually overnight) the solvent front traverses the paper completely. Because there is no vapour and the system is sealed, there occurs at this point virtually a dead stop to chromatography; in tank chromatography this does not happen and, as the front nears the end of the paper, considerable distortion of the R_F values is produced. For accurate calculation

of R_F values, of course, the front must not be allowed to "run out", even in tankless chromatography.

3. Reproducible R_F values varying by less than 0.01 can be produced over the range 0.12–0.90.

4. It is not suggested that tankless chromatography is a substitute for conventional descending chromatography, which for practical purposes is more convenient. It is, however, the only means we have found of studying R_M values over a large range of compounds accurately enough to test MARTIN's equation exhaustively. For much work of this nature, providing accurate R_F values of key compounds have been found under tankless conditions, comparison with unknowns *of similar R_F range* can be carried out in tanks with sufficient accuracy. When this is done, two precautions are necessary. (1) R_F values must be chosen to lie between 0.20 and 0.80 under the normal descending conditions, (2) the time of development must not exceed about 5 h. It is important to bear in mind that R_F values by tank chromatography are usually somewhat different from those obtained under tankless conditions and they *cannot be directly compared with each other*: but the ΔR_M values are nearly identical under both sets of conditions, when the tank chromatography is carried out as nearly ideally as possible.

5. The use of reversed phase chromatography covers a wide range of organic compounds. There are obvious limitations, however. It is not easy to obtain data directly from compounds such as amino acids or carbohydrates, which have little organic-phase solubility. However, as will be shown in a succeeding paper, this is only a practical and not a theoretical limitation, since the required data on compounds can usually be obtained from suitable derivatives.

EXPERIMENTAL

Much detailed experimental work with the aid of the new method is described in the succeeding paper and it is sufficient here to give a single example. We have chosen for this purpose the chromatography of straight-chain alkyl 3,5-dinitrobenzoates. This is a readily available series of compounds that has been studied by several other workers. It is of particular interest since it appears as one of the worst examples from the

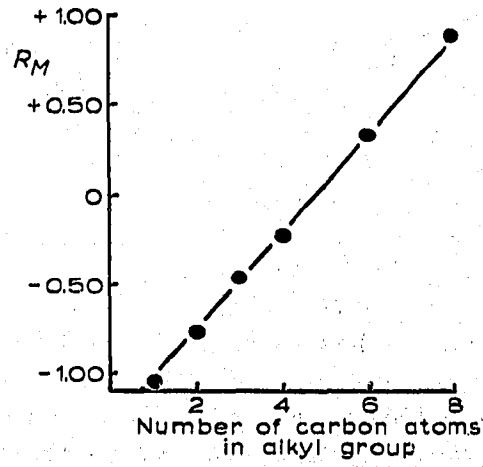

Fig. 2. Relationship between R_M and number of carbon atoms in *n*-alkyl dinitrobenzoates.

point of view of the MARTIN equation in the survey by FRANC AND JOKL[6]: the three plots of ΔR_M against number of carbon atoms given by these authors are all non-linear. Fig. 2 illustrates the chromatography of methanol, ethanol, *n*-propanol, *n*-butanol, *n*-hexanol, *n*-octanol, all as dinitrobenzoates, under tankless conditions. The stationary phase was liquid paraffin (papers impregnated with a 5 % solution of liquid paraffin, B.P., in ether); the mobile phase was 50 % aqueous ethanol. The plot of R_M against alcohol carbon number is seen to be completely linear.

SUMMARY

Attempts to correlate the chromatographic behaviour of substances with their chemical structure must be based on accurate knowledge of R_F values and a survey of published work indicates that these may often be in error because of the practical difficulties of carrying out chromatography under ideal conditions. The theoretical limitations of chromatography in tanks are also discussed. In order to overcome certain of these difficulties, a new method for the accurate determination of R_F values, tankless or flat-bed chromatography, has been studied and its advantages examined. Chief among these are its extreme reproducibility, the possibility of running very large numbers of compounds together under near-ideal conditions and the precision of the experimental R_F values. A series of alkyl dinitrobenzoates was shown, by this method, to obey MARTIN's equation with respect to $\Delta R_M(CH_2)$.

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