

SOME FUNDAMENTAL ASPECTS OF CENTRIFUGALLY ACCELERATED PAPER CHROMATOGRAPHY*

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In recent publications from this laboratory, the technique of centrifugally accelerated paper chromatography has been described^{1, 2, 3}. The apparatus employed consists essentially of a carefully balanced, motor driven, disk-shaped head, rotating in a horizontal plane, and inclosing a circular sheet of filter paper, held in position at the center. While rotating, the paper sheet assumes a taut, horizontal position. The solution to be fractionated is applied, by means of a micropipet, to the paper surface, in the form of dots or partial rings. While the disk is rotated at from 300 to 1000 r.p.m., the developing solution is added to the paper disk, at a point away from its

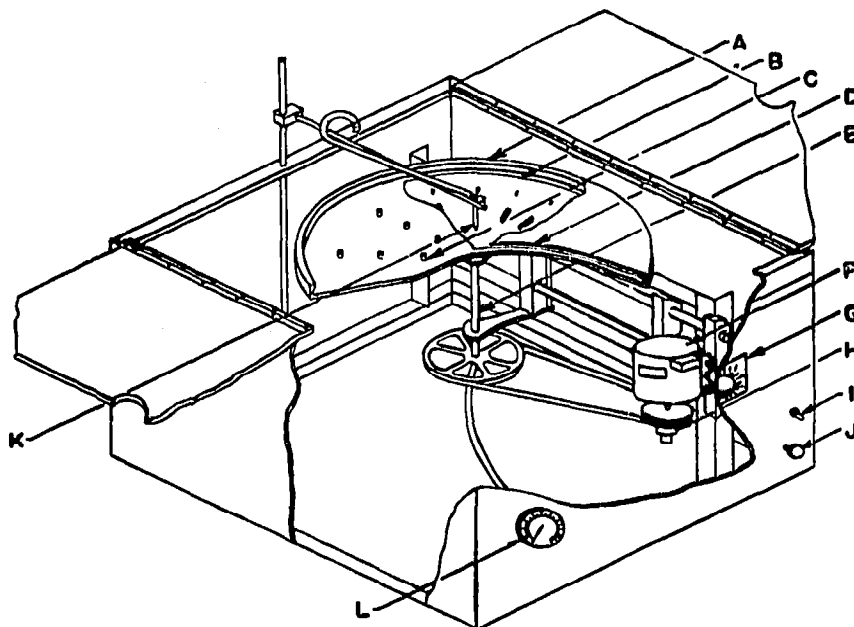


Fig. 1. Cut-away drawing of apparatus for centrifugally accelerated paper chromatography. A, rotor, outside diameter, 50 cm; B, circular paper sheet, 45 cm in diameter; C, pegs, to hold paper sheet away from lower inside surface of rotor; D, removable cover; E, rotor shaft; F, motor 1/6 horse power; G, combined variable speed control, 250-1000 r.p.m., and automatic timer; H, variable-pitch pulley to adjust speed of rotor; I, on-off switch; J, brake, for rapid stopping of rotor; K, micropipet for adding developing solution to paper sheet; L, tachometer, for reading r.p.m. of rotor.

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center, as a fine, continuous stream. In appearance, the developed chromatograms are not unlike those resulting from the classical circular paper method but the development time for an acceptable pattern is reduced markedly. The apparatus employed in these investigations is shown in Fig. 1.

The objective of this investigation was to explore the factors which are important in obtaining the best possible separations and the most reproducible chromatograms, and to study the variables which influence R_F values.

Materials

Whatman No. 1 filter paper cut to circular shape, 45 cm in diameter, from sheets $18\frac{1}{4} \times 22\frac{1}{2}$ inches; veronal buffer solution, pH 8.7, ionic strength 0.05; dye mixture of 2.0% bromphenol blue (BPB), 2.5% methyl orange (MeO) and 3.0% methyl red (MeR). All the experiments reported on in this paper were carried out at a temperature of approximately 26° C.

Rotational speed versus R_F values

In determining the effect of changes in the rotational speed of the rotor on R_F , 1 λ aliquots of the above dye mixture were placed on the circular sheet approximately 7.0 cm from its center. The veronal buffer was applied as a fine stream at a point

TABLE I

r.p.m.	R_F		
	BPB	MeR	MeO
364	0.86	0.64	0.22
480	0.86	0.63	0.22
600	0.86	0.64	0.23
742	0.86	0.64	0.21
866	0.85	0.61	0.21
925	0.85	0.61	0.20

approximately 5.0 cm from the center of the paper sheet, and at a rate of 1.15 ml/min, unless otherwise specified. To secure a flow rate of the buffer solution of 1.15 ml/min, a 5 λ pipet was used in conjunction with a helium pressure of 11.0 lb./in.².

The R_F of the dye mixture was determined at the following velocities; 364, 480, 600, 742, 866 and 925 r.p.m. At each velocity, the runs were terminated after a period of 7 min. As shown in Table I, there were no significant differences in the R_F values of the dyes at the various velocities. For a given component of the dye mixture, the mean deviation, expressed as per cent of the average of the R_F values for that component, at all measured rotational speeds, were computed to be 0.3, 2 and 4% for bromphenol blue, methyl red and methyl orange, respectively.

In the above operations, the solvent was continually added throughout the process. At a given velocity, however, if after the addition of a definite volume of buffer, the flow of liquid is stopped and the spinning continued, the R_F values of the dyes decrease progressively. This may be illustrated in the following way. At 540

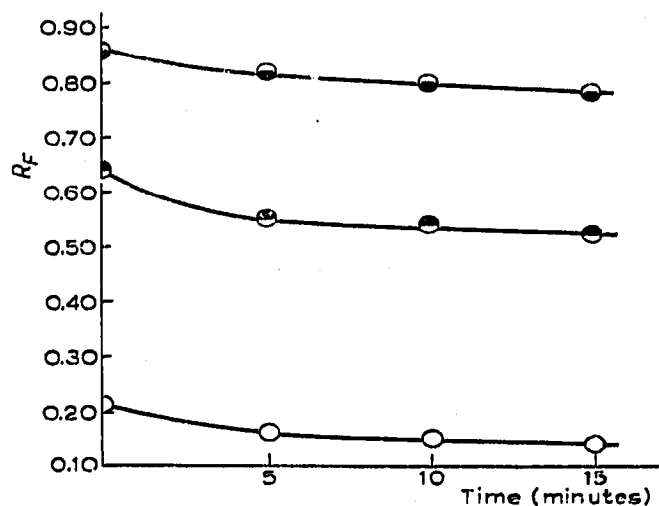


Fig. 2. R_F values of methyl orange (open circles), methyl red (circles, bottom half open) and bromphenol blue (circles, upper half open) plotted as a function of the time, in min, elapsing between the completion of the addition of 6.5 ml of buffer solution to the paper disk during a period of 5 min, and the stopping of the rotor.

r.p.m. the run was continued for periods of 5, 10 and 15 min after the addition of 6.5 ml of buffer (5 min at a flow rate 1.3 ml/min). From the data shown in Table II and Fig. 2, it is evident that there is a progressive decrease in R_F values in these time intervals.

Solvent flow rate versus R_F

The R_F values depend, in part, on the solvent flow rate. A flow rate of 1.3 ml/min was found to be too great for velocities below 300 r.p.m., adequate for velocities between 300–500 r.p.m. and satisfactory for velocities above 500. A flow rate of 1.15 ml/min is low enough to be used at all rotational speed above 250 r.p.m.

Using a flow rate of 1.3 ml/min, at moderate and low velocities (300–500) (100–300) in which the run and the solvent flow are terminated at the same time, it was found

TABLE II

Time, min	R_F values		
	BPB	MeR	MeO
5	0.82	0.55	0.16
10	0.80	0.54	0.15
15	0.78	0.52	0.14

that the flow rate was such that the wetted area of the paper sheet continued to increase in size while drying. In such cases, the solvent front should be marked after drying rather than before or discrepancies in R_F values may result. If at these velocities the run is continued for approximately one minute, after cessation of solvent flow, the wetted area of the paper no longer continues to increase on stopping the rotor, but there may be a small difference in the R_F value, obtained under these conditions.

It is obvious therefore, that the ideal flow rate ought to be such that on stopping

both the flow of solvent and the rotor, the perimeter of the wetted area does not increase appreciably on standing. At this flow rate the paper is said to be "minimally wet". At still higher flow rates, a flooding effect is obtained, that is, some of the water is sloshed across the surface of the paper, carrying the dyes along with it. On such chromatograms, this flooding effect is evident in the diffuse colored zones exhibited by the dyes. To obtain chromatograms with reproducible R_F values, the flow rate should be sufficiently low that the possibility of flooding is eliminated.

Grain of the paper versus chromatographic profile

The chromatograms that have been obtained are not circular in shape, as might have been expected, but elliptical. This elliptical outline was found to be related to the arrangement of the fibers in the paper. The R_F values of the separated dye components were found, within the limits of experimental error, to be the same whether the separation was carried out in a direction parallel to the long axis of the ellipse or at right angles to it.

Before cutting the rectangular sheets of Whatman No. 1 filter paper, lines were marked in the circle to be cut, parallel to the long sides of the paper sheets, that is, in the machine direction. In this way, it was possible to determine whether the elliptical pattern could be traced to some property of the paper or whether it was due, perhaps, to an imperfectly balanced rotor. It was found that, regardless of the velocity of rotation of the head, the major axis of the ellipse was always parallel to the long side of the original rectangular paper sheet. The ellipse was not altered to any great extent by pre-wetting the paper disk with solvent before adding the spot of dye mixture and the stream of developing liquid.

To confirm the observation that the flow rates are different in directions parallel to and at right angles to the machine direction of the filter paper, and that a non-circular pattern should therefore develop, strips were cut from the shorter and the longer sides of the original Whatman No. 1 filter paper sheets and were placed in identical, glass-covered cylinders containing the same amount of veronal buffer, and equilibrated in the same manner. At intervals of 3 and 6 min for an overall period of 1 hour, the height of buffer in the strips was measured. The height of the buffer was consistently higher in the strip cut from the longer side of the original paper sheet. The difference in height increased to a maximum of 1.1 cm after a period of approximately 40 min.

Factors affecting development

It has been observed that at higher velocities a more complete separation of the dyes was obtained, especially between methyl red and bromphenol blue, which at lower velocities and shorter runs, exhibit a zone of incomplete separation. For good separations, velocities of 500-700 r.p.m. are suggested, and at flow rates of 1.15 ml/min, the operation should last approximately 8 min. At these velocities and higher, the material to be separated may be placed closer to the center. This procedure results in a longer run which is another factor favoring better development. The point where

the material to be separated is placed on the paper sheet is therefore of some importance in developing a good chromatogram.

Factors affecting wetness of paper

As mentioned earlier, the flow rate of solvent is of foremost importance in the development of satisfactory chromatograms. If the flow rate is too high for a particular velocity, a flooding effect is observed and when the run is stopped, the center of the

TABLE III

Velocity—540 r.p.m.	
Time, min	Wetted area, cm ²
5	564
10	700
15	717
20	740

TABLE IV

Velocity, r.p.m.	Wetted area, cm ²
370	668
482	687
600	725
742	737
866	744
980	740

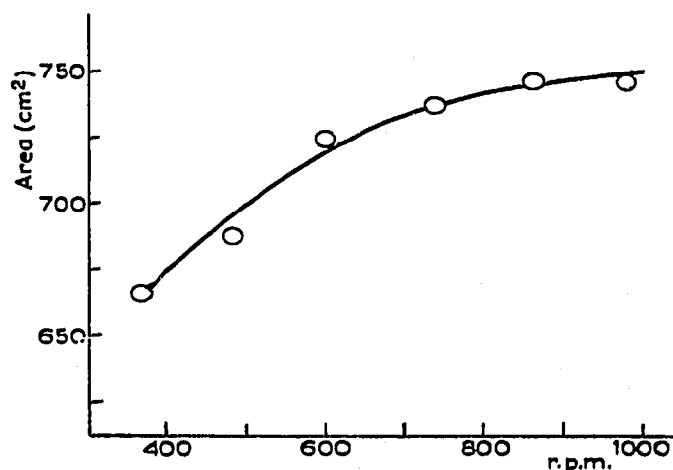


Fig. 3. The area of paper wetted by 8.05 ml of solvent applied during a run of 7 min, plotted as a function of the r.p.m. of the rotor.

paper may contain a shallow pool of water. From general considerations of this method of chromatography, one would expect that the amount of water per cm² would decrease with an increase in the distance of the particular area under observation from the center of the paper sheet. This conjecture was checked at a flow rate of 1.3 ml/min and 540 r.p.m.

By cutting out several pie-shaped sections of paper and dividing the sections into three parts along a radius of the original sheet, the conjecture was confirmed. Using this same method, it was found that if the solvent flow was stopped and the run continued, the water continues to move out in the form of a circular band of increasing diameter.

At 5 and 10 min, respectively, the concentration of water was greatest in the middle, then in the outer third, and eventually a relatively even wetness was obtained.

It was also observed that the weight of water per cm^2 was greater along the long axis of the ellipse.

From the above observations, it may be concluded that for a given velocity and a given volume of liquid, the area wetted by the liquid increases rapidly with time up to a certain point and then tapers off. This is evident from the data in Table III. However, for the same volume of liquid, 8.05 ml, and the same length of run, 7 min, the area of the paper wetted by the liquid increases with velocity as is evident from the data in Table IV and from Fig. 3. The areas were measured by means of a Keuffel and Esser compensating polar planimeter, reading areas directly in square centimeters to 0.1 cm^2 .

Effect of various filter papers on characteristics of the chromatograms

The question was raised as to the relative merits of different types of filter paper on the chromatographic separations. To answer this question, chromatograms were developed with the following filter papers: Eaton-Dikeman, No. 613, Schleicher and Schuell, No. 413, and with Cremer-Tiselius-Munktells chromatographic paper. A flow rate of 1.3 ml/min and a velocity of 600 r.p.m. were used in all experiments.

With Cremer-Tiselius-Munktells filter paper, sharply defined zones were obtained, but because of the thickness of the paper, a longer time was needed for adequate fractionation. Approximately 12 min were required for a separation which, with Whatman No. 1 paper, required only 7 min. The same type of elliptical outline was obtained as with Whatman No. 1.

With Eaton-Dikeman No. 613 filter paper, the elliptical outline was accentuated. The separations were not as clear and sharp as with Cremer-Tiselius-Munktells paper. This result may possibly be due to the fact that the flow rate was too high for this thickness of paper.

The Schleicher and Schuell filter paper gave a separation of the methyl orange component from the other components of the dye mixture, but bromphenol blue and methyl red were streaked as if following parallel grooves in the paper. The general outline of the solvent front was also elliptical, the major axis of the ellipse being parallel to the long axis of the original paper sheet and very irregular.

The R_F values of the dye mixture with these papers, except Schleicher and

TABLE V

	<i>BPB</i>	<i>MeR</i>	<i>MeO</i>
Whatman No. 1	0.86	0.64	0.22
Eaton-Dikeman No. 613	0.87	0.65	0.21
Cremer-Tiselius-Munktells	0.87	0.66	0.23
Schleicher and Schuell No. 413	—	—	0.26

Schuell, were not markedly different from those determined with Whatman No. 1. With Schleicher and Schuell, the R_F of methyl orange was found to be 0.26, a figure which is significantly different from the rest. No R_F values of bromphenol blue and

methyl red could be determined on this paper, because of the diffuseness of the zones on the chromatogram.

Separation of amino acids

To investigate the separation of a mixture of amino acids, organic solvents were employed. The solvent system used was butanol-glacial acetic acid-water, in the volume ratio 40:10:10, respectively. The amino acids leucine, methionine, and glycine were made up to 0.01 *M* in an aqueous solution and 5 λ aliquots were applied as spots to the paper. The zones on the chromatograms representing the various amino acids were developed by spraying the paper surface with a 0.2% by weight solution of ninhydrin in butanol (95% butanol and 5% 2 *N* acetic acid).

When early experiments showed that Whatman No. 1 filter paper did not yield adequately defined zones, Whatman-3MM paper was then employed. When the disk was rotated at a speed of 975 r.p.m., the Whatman-3MM paper gave zones which are sharply defined. It was found that solvent flow rates of 1.2 to 2.1 ml/min were satisfactory. The time of development was 15 min for a flow rate of 1.2 ml/min, and 7 min for a flow rate of 2.1 ml/min.

The R_F values obtained in the above experiments were found to be 0.85 for leucine, 0.56 for methionine, and 0.20 for glycine. In all experiments, the rotating head and the solvent flow were stopped at the same time. It was observed that the R_F values increased slightly with an increase in solvent flow rate, and that leucine, the leading component, exhibited the greatest change in R_F .

SUMMARY

A study has been made of some of the fundamental factors involved in obtaining good separations and reproducible chromatograms, with the technique of centrifugally accelerated paper chromatography. A study was also made of variables which might influence R_F values. Mixtures of bromphenol blue, methyl orange and methyl red were fractionated as well as solutions of leucine, methionine and glycine.

For the separation of the dyes, Whatman No. 1 filter paper was used with a veronal buffer solution of ionic strength 0.05 and having a pH of 8.7. For this system, it was found that the R_F values of the dyes were essentially unaffected by changes in rotational speed of the rotor from 300 to 925 r.p.m. It was found that the rate of addition of solvent was important in developing satisfactory chromatograms. A flow rate of 1.15 ml/min was found to be satisfactory at all rotational speeds above 250 r.p.m. The shape of the developed chromatogram was elliptical rather than circular, for all four types of paper which were studied. For Whatman No. 1, the major axis of the ellipse was parallel to the machine direction of the paper. The R_F values for the dye components were essentially the same for the following papers: Whatman No. 1, Eaton-Dikeman No. 613, and Cremer-Tiselius-Munktells.

The amino-acid mixture was fractionated using Whatman-3MM paper and a solvent system of butanol-glacial acetic acid and water, in the volume ratio 40:10:10, respectively. The disk was rotated at 975 r.p.m. With a solvent flow rate of 1.2 to 2.1 ml/min, sharply defined zones were obtained. The time of development was 15 min for a flow rate of 1.2 ml/min and 7 min for a flow rate of 2.1 ml/min. The R_F values obtained were 0.85 for leucine, 0.56 for methionine and 0.20 for glycine.

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