A QUANTITATIVE APPROACH TO UNIDIMENSIONAL MULTIPLE CHROMATOGRAPHY*

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One very successful but infrequently-used technique for the resolution of closely related compounds is unidimensional multiple chromatography (UMC)[1–3], (any procedure involving the repeated irrigation of chromatographic supports in one direction). The excessive time consumed by repeated irrigations using this technique, has largely been circumvented by the speed of thin-layer chromatography (TLC)[4,5] and centrifugally accelerated chromatography[6]. UMC has some desirable features which make it an extremely useful analytical tool. A number of advantages are readily evident when UMC is used in conjunction with TLC or "open column chromatography". For example, time consuming labors are eliminated such as packing columns, extruding and streaking for band detection or monitoring large volumes of eluate or the contents of a large number of fractions, etc. Furthermore, no special or expensive apparatus is required for UMC, the traditional and simple equipment employed for ascending chromatography being adequate. While continuous chromatography (solvents allowed to drip from support) can theoretically afford greater resolution than UMC[3], the latter method remains a practical technique because of its ease of operation. Thus, the advantages of UMC described above will generally more than compensate for the greater resolving efficiency of continuous chromatography.

In a recent communication[3], the theory of UMC was developed and its practical and theoretical potentialities and the limitations discussed. In that article, a theoretical method for the selection of solvent systems which might separate closely related compounds was suggested, but the excessive labor involved in accumulating the relevant data made the approach founded in theory impractical. On that account, a simple empirical procedure based upon a few guiding rules was proposed for the selection of an appropriate chromatographic solvent. To aid in placing these guides on a more quantitative basis, tables have been compiled which relate the R_F values of two compounds to the number of solvent passes required for resolution by a preselected increment. Thus if the R_F values of two compounds are known or can be estimated, it will be possible, by consultation of Tables I–IV, to ascertain if the solutes can be resolved with the test solvent and when separation is possible, how many solvent excursions will be required.

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When the detection of an elongated spot on a chromatogram leads to the suspicion of the presence of two or more solutes, the normal procedure is to search for another solvent mixture which will resolve the spot into two bands. UMC is an alternate approach to the problem which is more attractive because it is more certain of success and may be less time consuming than extensive experimentation with other solvents. The rationale behind the use of UMC is that very similar compounds which are partially separated on one solvent pass, in most instances, can be completely resolved by further solvent irrigations. On the other hand, there is never any guarantee that replacing one solvent by another will ever lead to complete or even increased resolution of closely related solutes[3]. As an example, a survey of the literature and our own experience with several solvents failed to reveal a single solvent system (of 16 tested) which was capable of resolving fructose and arabinose on a single pass. However, the solutes are readily separated by UMC. The use of Tables I–IV for selecting a suitable solvent for the resolution of the two closely migrating saccharides is illustrated below.

EXPERIMENTAL

Computations

The following equation[1–3] relates the true or single pass R_F values of two compounds to their relative separation Δp after p passes:

$$\Delta p = (1 - R_{Fa})^p - (1 - R_{Fb})^p \qquad (1)$$

where R_{Fa} and R_{Fb} are the R_F values of compounds A and B, respectively. Equation (1) was found to be more useful for computational purposes when rearranged into the form of eqn. (2):

$$\Delta p - [(1 - R_{Fa})^p - (1 - R_{Fb})^p] \geq 0. \qquad (2)$$

The values of p in Tables I–IV were determined on the Indiana University IBM 650 computer programmed to select the smallest integral values of p, p_{min}, which produced solution of eqn. (2) (provided a solution of the equation was possible). The value of p_{min} was found by incrementing p by unity until the condition demanded by eqn. (2) for a given set of experimental conditions was satisfied. Various experimental conditions were simulated by altering the values assigned Δp, R_{Fa} and R_{Fb}. After solution of eqn. (2) for the initial conditions, the values of Δp, R_{Fa} and R_{Fb} were then appropriately incremented until all useful combinations were considered.

Multiple chromatography

To demonstrate the use of Tables I–IV and illustrate the principles involved in UMC, sample chromatograms were run by the ascending technique[7]. Two sugars, arabinose and fructose, having similar R_F values were spotted at the origin of Eaton and Dikeman No. 613 papers, 23 cm in length, exposed 8 h to solvent vapors and irrigated at room temperature (26° ± 1°), with various water–ethanol–nitromethane solvents. After the solvents had reached the tops of the papers, the strips were withdrawn, air dried, and the sugar locations detected by developing the strips with the silver nitrate–sodium hydroxide reagents[8] by the dip technique. Another paper strip spotted with the two sugars was given two irrigations at room temperature with the solvent, water–ethanol–nitromethane (10:25:65 % by vol.), and developed as described above.

R_F, starting position and solvent proportions

To investigate the effect of starting position and solvent proportions on the R_F values for ascending chromatography, glucose and methionine were placed at various positions on Eaton and Dikeman No. 629 and 048 papers and irrigated with water–*tert.*-butanol and water–acetone solvents at 25° ± 0.1°, respectively. The same experiment was then repeated with varying solvent proportions which altered the single pass R_F values. Glucose was detected as described above and methionine was detected by spraying with ninhydrin[9].

RESULTS

Figs. 1 and 2 show reproductions of the developed chromatograms of arabinose and fructose after one and two ascents, respectively. The predicted difference in the centers of the spots after two ascents was calculated by eqn. (1) to be 1.8 cm and this is in moderate agreement with the observed separation of 2.0 cm.

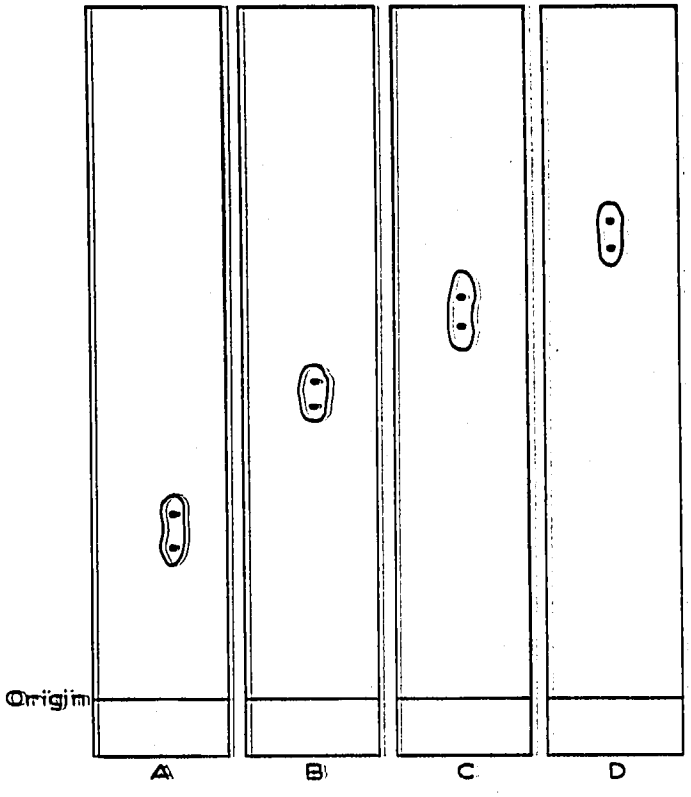

Fig. 1. Chromatography of arabinose and fructose with various water–ethanol–nitromethane solvents on Eaton and Dikeman No. 613 paper at 26° ± 1°. Arabinose is the more rapidly migrating component. The chromatograms were developed by the solvents listed (% by vol.).

Chromatogram	*H_2O*	*EtOH*	*$MeNO_2$*
A	10	25	65
B	12.1	39.4	48.5
C	17.6	42.8	39.6
D	24.1	47.0	28.9

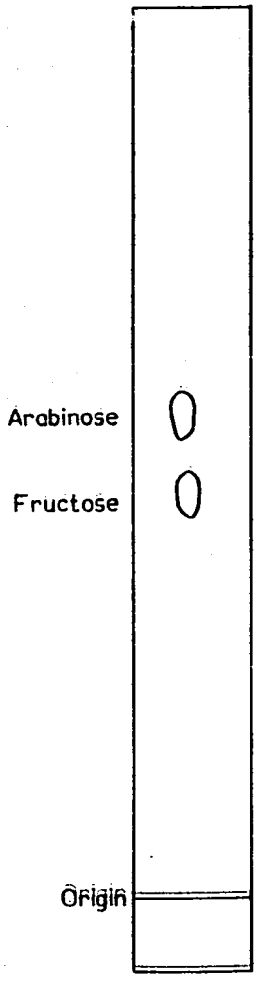

Fig. 2. Separation of arabinose and fructose with water–ethanol–nitromethane (10:25:65, % by vol.) by UMC with 2 solvent passes under conditions in Fig. 1.

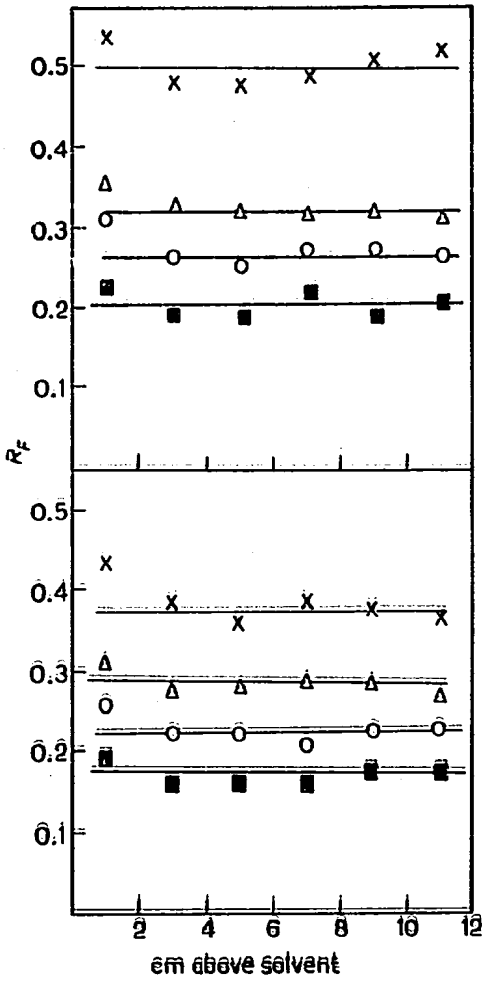

Fig. 3. Dependence of R_F on starting position on Eaton and Dikeman No. 629 paper. Upper plot, methionine; lower plot, glucose. Solvents are composed of water–*tert.*-butanol. Temperature was 25° ± 0.1°. Paper length was 23 cm (origin to top). % water by vol.: ■ 11.3; ○ 13.6; △ 16.1; × 23.1.

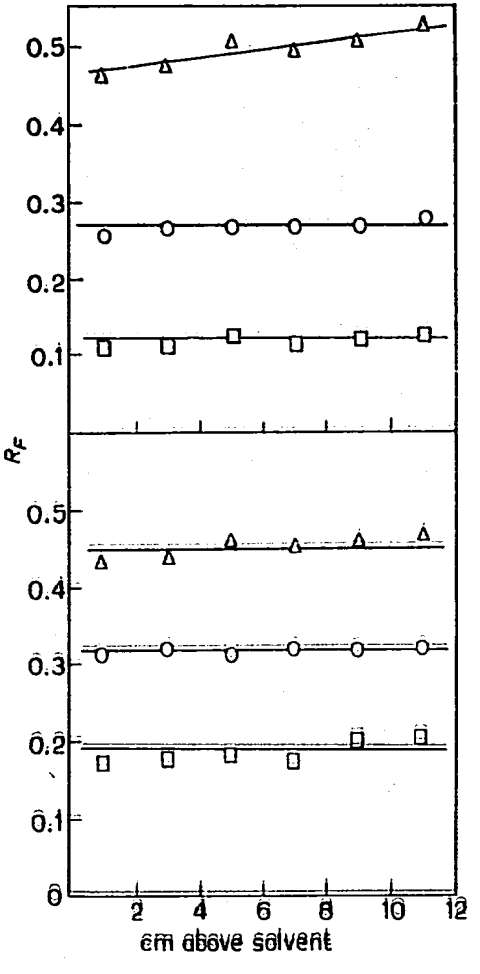

Fig. 4. Dependence of R_F on starting position on Eaton and Dikeman No. 648 paper. Conditions as in Fig. 3 except solvent was water–acetone. % water by vol.: □ 9.4; ○ 13.9; △ 16.5.

Fig. 3 depicts the dependence of the R_F value of glucose and methionine on their starting position on Eaton and Dikeman No. 629 paper with water–*tert.*-butanol as the irrigating solvent at 25° ± 0.1°. The ascending technique of chromatography was employed without prior exposure of the supports to solvent vapors.

Fig. 4 is a plot of the migration rates of glucose and methionine as a function of their starting position on Eaton and Dikeman No. 048 papers with water–acetone solvents at 25° ± 0.1°. The ascending technique of chromatography was used without prior exposure of the supports to solvent vapors.

The results of the calculations on the Indiana University IBM 650 computer relating the R_F values to the number of solvent passes required for a given degree of resolution ranging from 0.04 to 0.10 are listed in Tables I–IV. The degree of resolution, DR, is defined by eqn. (3):

$$DR = \frac{\Delta p}{L} \tag{3}$$

where L is the length of the support in cm and Δp is the separation in cm of the two compounds after p passes.

DISCUSSION

Assumptions

In programming the IBM 650 computer to determine the minimum number of solvent irrigations required to separate two compounds by some preselected distance, it was assumed that R_F values were independent of paper position. A number of inconsistent reports about the effect of starting position on the R_F of various compounds have appeared in the literature[1–3,10,11]. Because of the wide variety of experimental conditions employed, inconsistent reports are not at all surprising. By and large, it has been our experience that when a solute is spotted on the lower portions of rectangular paper supports and irrigated with water miscible mixtures which do not produce R_F values in excess of 0.3–0.4 the R_F of the compound is relatively independent of starting position. With solvents producing R_F values below 0.2, practically no deviation in R_F was found with starting position. When papers of unusual geometries are used, particularly those which are fed with wicks or when the solutes are placed a long distance from the solvent, fluctuating R_F values are found[10,11]. R_F variation also seems to occur when irrigation with water immiscible systems is attempted[10].

In view of the observations that solvent gradients occur along the paper[10,12,13], particularly when using ascending chromatography, and because of the possible variations of the composition of solvents along the support[14], it was expected that the R_F value would appear to be relatively sensitive to starting position. It has been shown that the R_F function is influenced by at least three variables at constant temperature: the chemical potential of transport, the ratio of the cross-sectional areas of the stationary and mobile phases[15,16] and the ratio of the average velocity of the local solvent (velocity at solute band) to the velocity of the solvent front[10]. It seems entirely possible that as the height on the support increases, the ratio of the cross-sectional area decreases while the local solvent velocity increases because of solvent concentration gradients along the paper. The former effect would tend to decrease the migration rate of the band while the latter effect would tend to increase it. If these two variables of chromatography compensate each other quite closely, the net result

TABLE I

NUMBER OF SOLVENT PASSES REQUIRED TO SEPARATE TWO SOLUTES 0.1 TIMES THE LENGTH OF THE SUPPORT

R_F of faster moving solute × 100	R_F of slower moving solute × 100 — Number of solvent passes for required separation								
	2	3	4	5	6	7	8	9–14	Impossible
30	23–21								24–29
29	22–20								23–28
28	21–19	22							23–27
27	20–18	21							22–26
26	19–17	20							21–25
25	18–16	19							20–24
24	17–15	18							19–23
23	16–14	17	18						19–22
22	15–13	16	17						18–21
21	14–12	15	16						17–20
20	13–11	15–14							16–19
19	13–10	14							15–18
18	12–9	13			14				15–17
17	11–8	12		13					14–16
16	10–7	11	12						13–15
15	9–6	10	11						12–14
14	8–5	9	10						11–13
13	7–4	8	9			10			11–12
12	6–3	7	8		9				10–11
11	5–2	6	7	8					9–10
10	4–1	6–5		7					8–9
9	3–1	5–4		6					7–8
8	2–1	4–3		5				6	7
7	1	3–2	4				5		6
6		2–1	3			4			5
5		1	2			3			4
4			1		2			3	
3					1			2	
2								1	

To determine the number of passes for separation, locate R_F of slower moving compound in the row occupied by R_F of faster moving compound. The required number of passes is given by heading of column in which smaller R_F appears.

would lead to the observation of constant R_F values along the paper provided that the ratio of the transport potentials also remains constant.

Thus Tables I–IV will only give an exact value for p_{min} (minimum number of passes for preselected separation) when the R_F is constant; it will yield an approximate value when R_F is variable. It should be noted from eqn. (2) that no provision was made for "rounding off" the calculated difference between the two compounds after p irrigations. Consequently, unless the calculated separation is *exactly* equal to or greater than the preselected separation Δp, the computer will indicate separation is impossible regardless of how closely Δp and the calculated separation may agree. For example, if the extent of separation between two compounds was 0.079 the computer would indicate that a separation of 0.06 but not 0.08 could be achieved after an appropriate number of irrigations (computations made in increments of 0.02 for DR).

To demonstrate the relative constancy for R_F *versus* paper position for the type of systems described above (rectangular papers and water miscible solvents), glucose

TABLE II

NUMBER OF SOLVENT PASSES REQUIRED TO SEPARATE TWO SOLUTES 0.08 TIMES THE LENGTH OF THE SUPPORT

R_F of faster moving solute × 100	R_F of slower moving solute × 100								
	Number of solvent passes for required separation								
	2	3	4	5	6	7	8	9–14	Impossible
30	24–23								25–29
29	23–22	24							25–28
28	22–21	23							24–27
27	21–20	22							23–26
26	20–19	21							22–25
25	19–18	20							21–24
24	18–17	19							20–23
23	17–16	18							19–22
22	17–15		18						19–21
21	16–14		17						18–20
20	15–13	16							17–19
19	14–12	15							16–18
18	13–11	14							15–17
17	12–10	13							14–16
16	11–9	12		13					14–15
15	10–8	11		12					13–14
14	9–7	10	11						12–13
13	8–6	9	10						11–12
12	7–5	8	9						10–11
11	6–4	7	8						9–10
10	5–3	6	7				8		9
9	4–2	5	6			7			8
8	3–1	4	5		6				7
7	2–1	4–3			5				6
6	1	3–2		4					5
5		2–1		3				4	
4		1		2				3	
3				1				2	
2								1	

To determine the number of passes for separation, locate R_F of slower moving compound in the row occupied by R_F of faster moving compound. The required number of passes is given by heading of column in which smaller R_F appears.

and methionine were chromatographed as a function of starting position and solvent proportions. The data collected were graphed in Figs. 3 and 4. Methionine was selected as one of the solutes because in an earlier communication[3], it was noted that the behavior of this material on paper seemed to be anomalous when contrasted to that of other amino acids. Figs. 3 and 4 suggests that these peculiarities are not reflected as R_F variations with starting position.

On Eaton and Dikeman No. 629 paper there does appear to be a slight increase in the R_F values of the compounds when they are started very close to the solvent front (1 cm). Since the R_F which is determined on the first pass will be used to estimate the number of solvent passes required to separate the compounds, it is necessary that the measured R_F on a single pass be representative of that when compounds are started higher on the support. To fulfill this condition, the compound ought to be spotted 2–3 cm above the solvent.

Because the R_F value is somewhat influenced by loading, it is wise to use a

TABLE III

NUMBER OF SOLVENT PASSES REQUIRED TO SEPARATE TWO SOLUTES 0.06 TIMES THE LENGTH OF THE SUPPORT

R_F of faster moving solute × 100	R_F of slower moving solute × 100 — Number of solvent passes for required separation								
	2	3	4	5	6	7	8	9–14	Impossible
30	25	26							27–29
29	24	25							26–28
28	23	24							25–27
27	23–22								24–26
26	22–21								23–25
25	21–20								22–24
24	20–19								21–23
23	19–18								20–22
22	18–17		19						20–21
21	17–16		18						19–20
20	16–15		17						18–19
19	15–14	16							17–18
18	14–13	15							16–17
17	13–12	14							15–16
16	12–11	13							14–15
15	11–10	12							14–13
14	10–9	11				12			13
13	9–8	10			11				12
12	8–7	9		10					11
11	7–6	8		9					10
10	6–5	7	8						9
9	5–4	6	7						8
8	4–3	5	6						7
7	3–2	4	5						6
6	2–1	3	4					5	
5	1	2	3					4	
4		1	2				3		
3			1			2			
2						1			

To determine the number of passes for separation, locate R_F of slower moving compound in the row occupied by R_F of faster moving compound. The required number of passes is given by heading of column in which smaller R_F appears.

solute concentration just above the limits of detection by the chromogenic reagents used for color development. The effects of loading on R_F are pictorially represented in a paper by STAHL AND KALTENBACH[17]. Reducing solute concentration will also help to minimize streaking.

Selection of p_{min}

Values of p_{min} were compiled for all combinations of R_F values ranging from 0.30 to 0.01. Larger values of R_F were not considered because the maximum extent of separation achieved by UMC occurs when the average apparent R_F of the two compounds is $1 - e^{-1}$ or 0.632 times the length of the support[3]. Thus, compounds having average R_F values in the range 0.3–0.4 will achieve maximum separation with two solvent passes and compounds having average R_F values greater than 0.4 will achieve maximum separation after one pass. Tables I–IV then will be most useful for the study of compounds possessing small R_F values; a condition necessary according to theoretical

TABLE IV

NUMBER OF SOLVENT PASSES REQUIRED TO SEPARATE TWO SOLUTES 0.04 TIMES THE LENGTH OF THE SUPPORT

R_F of faster moving solute × 100	R_F of slower moving solute × 100 — Number of solvent passes for required separation								
	2	3	4	5	6	7	8	9-14	Impossible
30	27								28–29
29	26								27–28
28	25								26–27
27	24								25–26
26	23								24–25
25	22								23–24
24	21								22–23
23	20								21–22
22	19								20–21
21	18		19						20
20	17		18						19
19	16	17							18
18	15	16							17
17	14	15							16
16	13	14							15
15	12	13							14
14	11	12							13
13	10	11							12
12	9	10							11
11	8	9							10
10	7	8						9	
9	6	7				8			
8	5	6			7				
7	4	5			6				
6	3	4			5				
5	2	3		4					
4	1	2		3					
3		1		2					
2				1					

To determine the number of passes for separation, locate R_F of slower moving compound in the row occupied by R_F of faster moving compound. The required number of passes is given by heading of column in which smaller R_F appears.

considerations for the production of good resolution[3]. This phenomenon was predicted because a solvent which decreases the average R_F value of two compounds increases the ratio of the R_F values and is capable of discriminating more efficiently between them.

This experimentally supported prediction[3] made it possible to suggest a number of simple guiding rules for the selection of appropriate solvents to resolve closely related compounds. It was proposed for UMC that a solvent composition be chosen for reasons of economy, flow rate, etc. and the molar proportions altered to vary the R_F of the two components. The solvent should contain two miscible components; one in which the solutes are very soluble and the other in which the solutes are relatively insoluble. Appropriate proportions might then be selected which would achieve resolution of the compounds provided an appropriate number of irrigations were given. Tables I–IV help to quantitate these simple rules.

The techniques of UMC ought to prove valuable when it is known from chemical

experience that there exists in a mixture two or more closely related compounds which cannot be resolved with a single solvent irrigation or when a mixture is suspected from the appearance of an oblong spot on a chromatogram. To separate the mixture in the first instance, if pure materials are available, their R_F values can be determined and the number of solvent passes required to separate them found directly from Tables I–IV. If pure samples of the compounds are unavailable for chromatographic tests, then their resolution might be effected by following the guide lines above. The resolution of fructose and arabinose described below illustrates the utility of this suggested approach to UMC.

For the disengagement of these two compounds, a solvent system containing water–ethanol–nitromethane was selected because of its relatively rapid flow rate on paper. The molar proportions of water, ethanol and nitromethane were then altered so that the R_F values ranged from approximately 0.2 to 0.7. On these chromatograms, reproduced in Fig. 1, it will be noted that all the spots are oblong, indicative of a mixture, but complete separation is not achieved by any solvent mixture. It was assumed that the spots are composed of two bands whose centers are indicated by the dots. The R_F's of these dots were then measured and Tables I–IV were consulted for the appropriate number of solvent developments. It was learned that if a preselected degree of separation of 0.06 were required, the solvent, water–ethanol–nitromethane ((10::25::65 %, by vol.)), giving chromatogram A ought to resolve the compounds after two solvent excursions. Fig. 2 shows that the compounds are well resolved as predicted and the extent of separation of the two spot centers is 2.0 cm which agrees reasonably well with that calculated from eqn. ((1)), 1.8 cm.

The selection of the extent of separation, Δp, which will be required to discern two visible spots is somewhat arbitrary, but a safe rule of thumb is that a 1.5–2 cm disengagement of spot centers will suffice. However, the practitioner will probably not experience much difficulty in the selection of Δp because previous experience with chromatography of related systems will probably furnish the necessary information. For the arabinose–fructose system described above, the extent of separation was arbitrarily set at 1.4 cm ((experience indicated that 1.3–1.5 cm is generally sufficient for such systems)) and since the paper is 23 cm long (origin to top), the relative separation ((separation in cm//length of paper)) is 1.4 cm/23 cm = 0.061. This value is approximately 0.06 and according to Table III for compounds having R_F values of 0.22 and 0.27, two solvent passes will be required. Other examples of the usefulness of UMC for the resolution of mixtures have been illustrated in a prior manuscript[3].

If the extent of separation required lies within the values tabulated, the required number of passes can be evaluated by interpolating. For example, if two compounds have R_F values of 0.07 and 0.05, for a separation of 0.09, it can be seen from Tables I and II that 7 passes will be required. Since the increments in Tables I–IV are small, interpolation ought to be accurate to within one pass. The number of solvent developments can be reduced if the bands are confined to small areas.

The degree of resolution, DR, required for separation will vary with experimental conditions and depend upon the length of the paper, extent of loading, sharpness of the zones, etc.[18–30]. The actual preselection of the required degree of resolution will be left to the judgment and previous experience of the investigator. A theoretical approach to the selection of DR is beyond the scope of this paper. For practical in-

stances, a theoretical evaluation of band dimensions seems impossible because the effects of adsorption, ion-exchange, etc. on band distortion cannot be predicted or anticipated. However, it should be pointed out, that on the second and subsequent passes during UMC, the solvent flows over the trailing edge of the band before reaching the leading edge. This effect aids in sharpening the bands. Therefore, when a solute moves an equal distance during continuous chromatography and UMC, the vertical spot dimensions on the latter chromatogram will be equal to or smaller than the vertical spot dimensions on the former chromatogram. This sharpening effect, of course, aids resolving the compounds.

Elongated spots which are often observed may be the result of adsorption, ion-exchange, non-linear isotherms, etc., which may cause a single pure material to appear as a distorted band. If the band is a single component, it will be expected that this distortion will become more extensive as the number of solvent passes is increased. On the other hand, if the solute is a mixture of more than one component, it ought to be possible to separate them with a sufficient number of solvent passes. Consequently, the probability of success in ascertaining the purity of the spot using UMC (provided the R_F values were small) would seem to be very high.

NOTE ADDED IN PROOF

After submission of this manuscript a related paper by LENK[21] came to my attention.

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SUMMARY

Tables relating the R_F value of two solutes to the number of solvent passes required for their separation by a preselected amount have been compiled for unidimensional multiple chromatography, UMC. These tables will help quantitate some simple guiding rules which were proposed for the selection of solvents for chromatographic separations. The use of the tables is illustrated and the rationale for the high probability of success of UMC is discussed.

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