CHREV. 88

PROGRAMMED MULTIPLE DEVELOPMENT

BRIEF REVIEW AND STUDY OF EXTENDED PROGRAMS

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CONTENTS

1. SPOT SHAPE AND LOCATION *VERSUS* MULTIPLE DEVELOPMENT TECHNIQUE

Jeanes *et al.*[1] introduced unidimensional multiple chromatography (UMC). UMC improves the resolution obtainable from a given chromatographic system.

In UMC, the solvent front is allowed to advance a given distance, usually the length of the bed of stationary phase. The solvent is then evaporated from the bed, whereupon the same solvent is again allowed to advance the same distance. The process is repeated as often as desired.

With the advent of thin-layer chromatography (TLC)[2], Thoma[3] suggested that the "superb resolving power of UMC" be combined with the convenience of TLC: "A combination of TLC and UMC should prove to be an ideal way to resolve mixtures."

In UMC, the distance of solvent advance is held constant. Alternatively, the solvent can be allowed to advance successively greater distances. Saini[4], for example, in one instance allowed three successive solvent advances through one-third, two-thirds, and finally the whole of the bed length. This clearly improved the separation of the near-front components over that afforded by UMC.

Whether the solvent advance distance is held constant or increased, each solvent advance causes a clearly describable interaction of the solvent front with solute molecules already deposited on the bed. As a solvent front advances through already deposited solute molecules, the solute molecules behind the front advance in the direction of solvent flow at a fraction R_F of the solvent velocity toward those motionless solute molecules still beyond the front. We shall refer to this interaction as spot reconcentration by the solvent advance mechanism.

If the centers of two statistical aggregates of given-R_F molecules initially a

distance X_i apart (as projected along the direction of solvent flow) are traversed by n advancing solvent fronts, these centers become finally only a projected distance X_f apart. The relationship has been described by Jupille and Perry[5,6]:

$$X_f = (1 - R_F)^n X_i \tag{1}$$

Thoma[3] showed that the conventional, single-development R_F is related to the apparent $R_{F,n}$ that exists after the traversals of n UMC advancing solvent fronts, as follows:

$$(1 - R_F)^n = 1 - R_{F,n} \tag{2}$$

It follows that in chromatograms multiply developed by solvent advances alone[7]

$$X_f/X_i = 1 - R_{F,n} \tag{3}$$

X_f/X_i expresses the degree of mutual closing in the direction of solvent flow of the aggregate centers, because $(X_f/X_i)(X_i) = X_f$.

Eqns. 1 and 3 describe spot reconcentration by the solvent advance mechanism. The effects of the operation of this mechanism depend on R_F and, for a given spot, exponentially on the number of solvent advances. The effects, however, do not depend at all on the duration, linear extent, or constancy of the successive solvent advances, as long as all the molecules to be affected are traversed.

During a given solvent advance, the relative positions of identical R_F molecules do not change further once all such molecules have been traversed by the advancing front. Thus eqn. 3 describes spot shape distribution not only in any UMC chromatogram but also in any multiple-development chromatogram involving only solvent advances. No matter how many such multiple developments are carried out, the spot shape distribution is the same. Near the solvent front, where the apparent $R_{F,n}$ is near unity, the spots become almost lines drawn at right angles to the direction of solvent flow. Near the spot origin, however, spots remain almost unchanged.

In contrast, uniform spots result from programmed multiple development (PMD)[5-16]. To illustrate this contrast, two chromatograms differing only in the method of multiple development were prepared: one was developed by UMC, the other by PMD[8]. To show more clearly the effect of these methods of development on spot shape and spot shape distribution, the spots were deposited as rings.

Such comparable UMC and PMD chromatograms are shown in Figs. 1a and b, respectively. The UMC chromatogram shows the dependence of spot shape on apparent $R_{F,n}$ that is predicted by eqn. 3. The PMD chromatogram, on the other hand, shows essentially uniform spots that do not reflect either the spot R_F or the origin shape (or, thus, the origin location).

PMD differs from UMC because, in the former, the spot reconcentrating solvent advance mechanism is supplemented by a second spot reconcentrating mechanism, that of solvent removal. This mechanism is unique to PMD.

In PMD, the chromatographic bed —here, the adsorbent layer on the thin-layer plate— remains at all times in contact with the solvent reservoir. By capillary
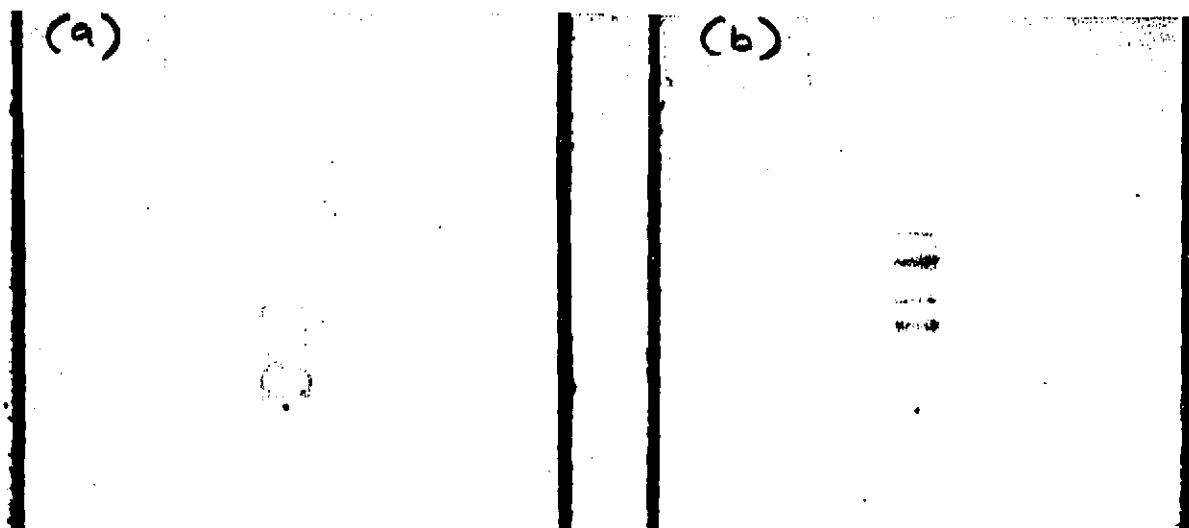
Fig. 1. The spot shapes obtained from solvent advances alone range from "lines" near the solvent front to "circles" near the origin, as shown by the UMC chromatogram (a). Spots reconcentrated by both solvent advances and removals tend to be all "lines", as shown by the PMD chromatogram (b). See also ref. 8. UMC: Three 200-sec advances of benzene through dye mixture. PMD: Three cycles, Mode 1, 100 sec unit time for advance, 100 sec fixed time for removal at power 10. Benzene, same dye mixture.

action the solvent flows at all times toward the solvent front. However, in PMD the rate of evaporation from the bed is controlled. The controlled rates of solvent evaporation during PMD allow the front to move forward into the bed during solvent advance and cause the front to move back toward the solvent reservoir during solvent removals.

In spot reconcentration by the solvent removal mechanism, the solvent flows into, and evaporates from, a receding solvent front. Because of this, solute molecules move into, and are deposited in, the receding front. Solute molecules behind the receding front continue to move toward those motionless solute molecules already deposited from it.

The higher the ratio of the spot velocity to the front recession velocity, the greater the efficiency of spot reconcentration by the solvent removal mechanism[6,8,9].

The rate of solvent removal in PMD can be controlled by any of several means, most commonly radiant heating, a counter-current stream of gas, or both. Usually, when gas is used, the flow-rate is held to a minimum and the gas flow is supplemented by gentle IR radiant heating (see Figs. 2a and b). However, gas alone can be used if the solute is particularly thermally labile.

The suggested actions of the solvent advance and removal mechanisms in reconcentrating spots are shown in Fig. 3.

The mechanisms that reconcentrate spots also align them, as illustrated in Figs. 4a and b.

Thus, the uniformly dense and precisely located spots of the PMD chromatogram do not reflect such normally unfavorable origin characteristics as being spread out or displaced.

Fig. 2. In PMD, the solvent can be removed by heat alone (a), mainly by gas flush (b), or solely by gas flush. IR radiation (at 5.1 μm wavelength) carries only about 0.05 as much energy per photon as UV at 254-nm wavelength, and so causes little photo-decomposition, but can cause thermal decomposition, in which case solvent is removed mainly or solely by gas. The thermally caused ghost spots shown in (a) are absent in (b)[17]. See also ref. 19. Samples: estrone, estradiol, and estriol, 0.5 mg/ml each, in methanol. Spotted volumes: 1, 5 and 10 μl. Plates: Quantum Q5DF.

To date, most applications of PMD have corresponded to the current applications of TLC; namely, the separation of relatively few components from each other and, at times, from a more complex matrix. PMD has usually been applied to improve a recalcitrant separation or to increase the molecular density within a spot and, thus, the detectability of the spot.

We cite a few examples. An example in which special sensitivity is desired is the moderately difficult separation of deoxycorticosterone (DOC), testosterone, 17-hydroxyprogesterone (17-OH Pr), and progesterone. The difficulty arises because high laboratory humidity plus the use of (possibly) wet nitrogen for solvent removal can deactivate the adsorbent enough to prevent the separation of DOC from 17-OH Pr. In the separation shown in Fig. 5, the solvent was removed with a combination of dried nitrogen and gentle heating.

This separation also serves to illustrate the high reproducibility that is characteristic of PMD spot positions. In PMD, spot positions are measured not from the
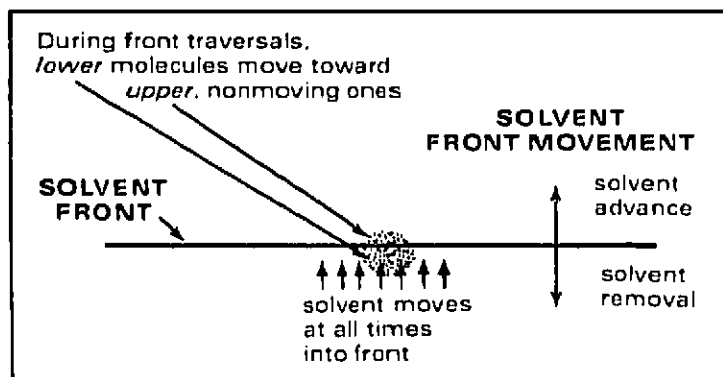

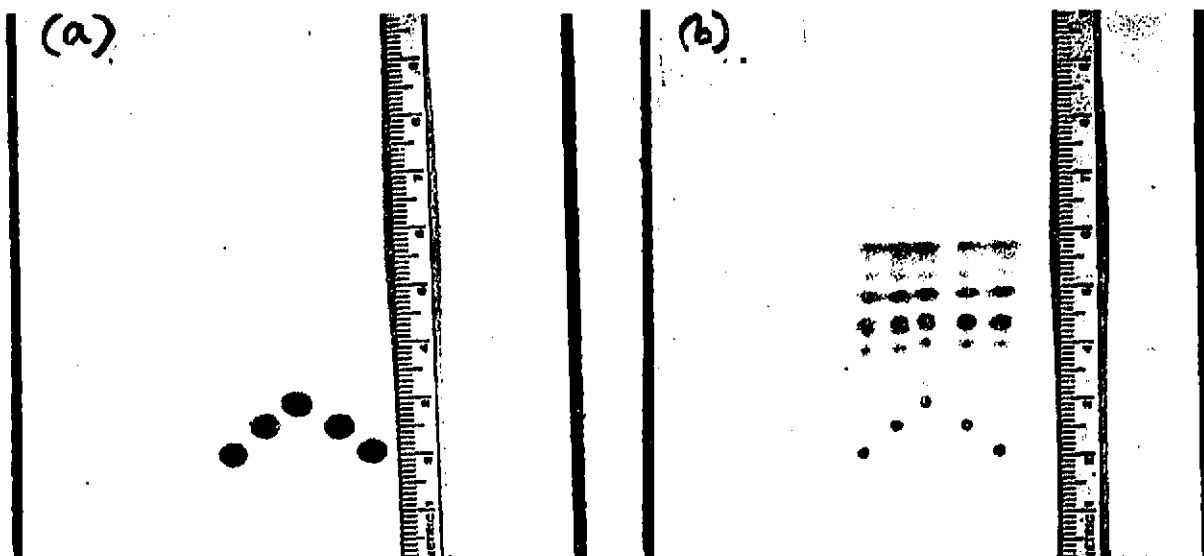Fig. 3. Spot reconcentration mechanisms in PMD.

Fig. 4. The PMD mechanisms that reconcentrate spots also align them. (a) original spots. (b) Spots developed by PMD. (For other examples, see refs. 11 and 14.)

origin nor with reference to the solvent front, but simply from the edge of the plate. The measurements cited were taken from four plates, two samples per plate (Table 1).

Another test[13] was made using twelve plates with one sample per plate. The samples were deposited as single 5-μl volumes of a solution containing, per μl, 0.2 μg each of amphetamine and methamphetamine. Spot positions were measured, as usual, from the edge of the plate (Table 2).

The solvent in this separation was acetone, whereas in the first example cited it was a mixed solvent, *viz.* ethylene dichloride-ethyl acetate (8:13). The positional
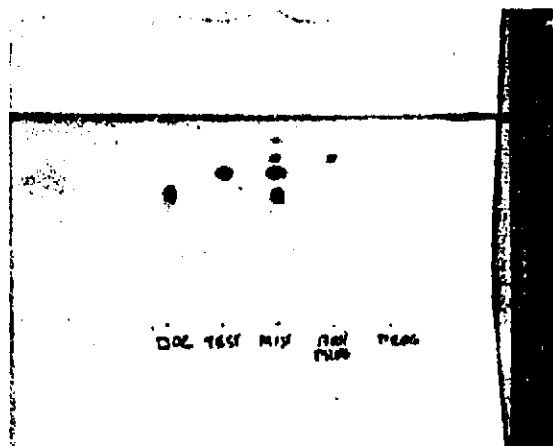

Fig. 5. PMD separation of sterones that requires dried nitrogen for solvent removal. Five cycles in Mode 2 were used, requiring about 41 min overall[18].

TABLE 1

REPRODUCIBILITY OF PMD SPOT POSITIONS USING A MIXED SOLVENT, ETHYLENE DICHLORIDE–ETHYL ACETATE

Compound	Distance (mm)		Relative S.D. (%)
	Mean	S.D.	
DOC	57.0	0.84	1.5
Testosterone	62.0	1.22	2.0
17-OH Pr.	65.1	1.61	2.5
Progesterone	68.8	1.47	2.1

reproducibility of PMD spots does not depend on whether the solvent is simple or mixed.

The positions of PMD spots can be precisely measured not only because the spot locations are highly reproducible but also because the spots themselves are not diffuse. The minimum top-to-bottom "width" of such spots on commercial plates made with silica gel particles of 20–40 μm in diameter is around 1 mm, i.e. about 25–50 particle diameters. The usual "working" top-to-bottom spot "width" is less than 2.5 mm[14].

PMD is often applied to improve trace detectability. Fig. 6 shows four chromatograms on one plate, each chromatogram containing 100 ng of estrone and estradiol deposited either from dilute or concentrated solutions. Unlike the ruined conventional chromatogram shown, the PMD chromatogram made from a single deposition of 100 μl of less concentrated solution shows about the same resolution and molecular density per spot as that made from 1 μl of the 100-times more concentrated solution[19].

Fig. 7 shows PMD chromatograms involving origins both contaminated and extended. Made from quinine-spiked raw urine, the origins extended some 20 mm, top to bottom[12]. The developed quinine spots, however, measure about 3 mm top to bottom and allow a good detectability of about 5 μg/μl.

Molecular density, and thus trace detectability, can be still further increased by either narrowed channels or centered PMD. The channels are made about 2 mm wide, rather than narrower, primarily because channels much narrower are hard to make and hard to judge with a pipet. (For very demanding separations and determinations, such narrower channels should be considered.) The channels can be made either from a conventional plate, that is, with the adsorbent bed lying on a supporting plane[11], or with a grooved plate, that is, with the narrow adsorbent beds lying in the grooves[20]. Of the two, the grooves yield the more dense and uniform spots. The

TABLE 2

REPRODUCIBILITY OF PMD SPOT POSITIONS USING A PURE SOLVENT, ACETONE

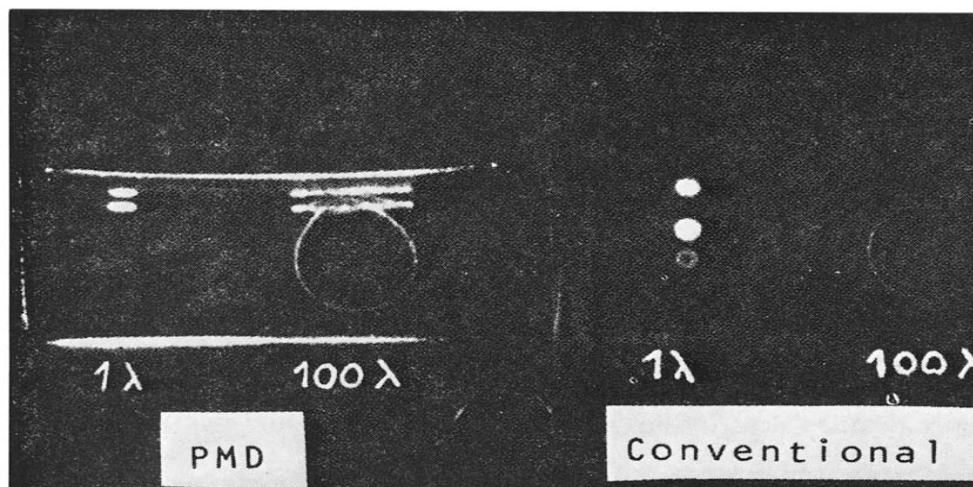| Compound	Distance (mm)		Relative S.D. (%)
	Mean	S.D.	
Amphetamine	61.6	1.6	2.6
Methamphetamine	41.6	0.6	1.4

Fig. 6. PMD spots are tight and, within wide limits, do not reflect the characteristics of the origin. Each of the four chromatograms contains 100 ng of estrone and estradiol deposited either from concentrated (left) or dilute (right) solutions[19].

elongated origins resulting from depositing normal microliter volumes of solution on to such channels do not matter with PMD. Sensitivity enhancements of about 50 over conventional TLC are found. Also, many more channels per plate can be accommodated, so that many more samples can be developed simultaneously on one plate.

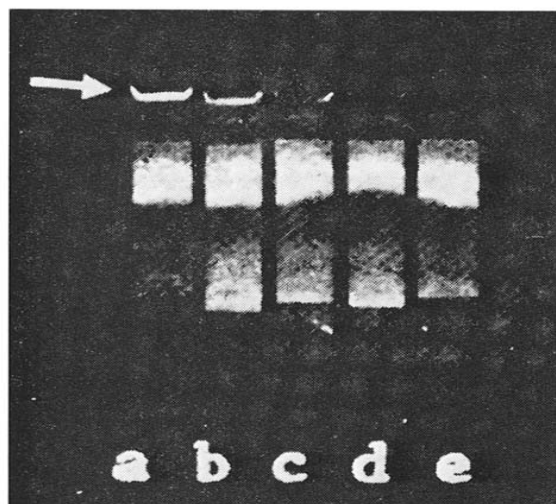Another approach that includes higher molecular density among its attributes

Fig. 7. PMD concentrates traces even from origins that are both broadened and contaminated. Here, quinine traces are retrieved with good sensitivity from 50-μl samples of raw urine[12,14]. Quinine (arrow) concentrations of 5, 2.5, 1.25, 0.625, and 0 μg/ml are shown in chromatograms a through e, respectively. Solvent: acetone. Seven mode 2 cycles.

is centered PMD[16]. Conventional non-channeled TLC plates are used. Evaporation is caused to occur preferentially along the line of the chromatogram. In consequence, the molecules of the solvent and, therefore, of the solutes move toward the center line of the chromatogram from each side. With this technique, both lateral and longitudinal diffusion are countered. As a result, spot molecular density soon reaches a certain level characteristic of the program and of the spot, once it is isolated, and thereafter remains constant. Trace components show spot diameters of 1–2 mm. The chromatograms shown in the Experimental section of this paper were made with centered PMD.

2. BRIEF OUTLINE OF PMD PROGRAMMING AND INSTRUMENTATION

Much more detailed descriptions of PMD programming and instrumentation than this have been published[7,9,14].

PMD solvent advances, initially very short, always increase. The manner of increase depends on the mode.

The duration of the first solvent advance is identical with the unit time for any succeeding solvent advance. Unit times range in 10-sec steps from 0 to 100 sec.

Each solvent advance is followed by a solvent removal. A solvent advance segment followed by a solvent removal segment makes up a cycle.

The segment duration T depends on the mode, the cycle number n, and the unit time t. The maximum cycle number also varies with the mode (Table 3).

TABLE 3

DEPENDENCE OF SEGMENT DURATION (T) ON MODE AND CYCLE NUMBER

Mode	Maximum cycle number	Total segment duration (T)
1	99	$T = n\,t$
2	28	$T = [n(n+1)/2]\,t$
3	20	$T = n^2\,t$

The duration of a solvent removal may be fixed or scheduled. If fixed, it is the unit time set for solvent removal, without regard for cycle number. If scheduled, it varies with the mode, cycle number, and solvent removal unit time as just shown.

PMD instrumentation includes primarily a programmer and a developer. The information needed for a PMD program is set into the face of the programmer by the operator. The developer then executes the program under the direction of the programmer.

A given PMD, once started, can be carried out automatically without further attention from the operator. It can also be altered while in progress.

3. PMD VARIABILITY

The durations of most programs to date have roughly equalled the durations of the corresponding conventional TLC separations. In such periods, relatively many

programmed multiple developments can be carried out. These usually suffice because spot reconcentration is so quickly effective. The program used for the PMD chromatogram in Fig. 1, for example, produced five multiple developments and lasted 33 min.

The durations of possible 5-cycle PMD programs, however, range from about 3 min to about 3 h. The durations of all possible PMD programs range from less than 1 min to over ten days.

4. EXTENDED PROGRAMS: PMD CAPABILITY, UMC THEORY, AND TESTS

As far as PMD instrumentation is concerned, programs can as easily have many cycles as few. Also, compared to conventional multiple developments, PMD cycles progress at very high frequency.

The many-cycle, high-frequency capability of PMD, certain related properties of the TLC plate, and the properties of the centered PMD spot are mutually complementary for application to extended, *i.e.* many-cycle, programs.

The conventional TLC plate lends itself well to extended PMD programs in two ways. First, the typical silica gel G precoated plate can easily support any currently programmable number of Mode 1 PMDs. (With a 10-sec unit time, for instance, even the last and farthest solvent advance, the 99th, would take only 990 sec, or about 17 min. Such an advance can almost be accommodated on a 10-cm plate even with solvents such as benzene or acetone, so 20-cm plates are more than adequate.) Secondly, when not overloaded, the current TLC plate can easily display one separated spot every millimeter or so. Both capabilities could, if necessary, easily be improved by using an adsorbent of smaller particle size, a simple change to make.

The centered PMD spot exhibits essentially constant molecular population and density. Such a spot can be moved, without changing its shape or density, for any relevant time and over any relevant distance. (For preparative separations, the whole plate can be used and the centered-spot aspect simply disregarded.)

In sum, thoroughly refining the separation of complex mixtures of similar-R_F components by using large, unprecedented numbers of multiple developments has now become feasible.

Increasing the number of multiple developments, it will be seen, yields the results generally found when chromatographic bed efficiency is sharply improved: two components come from what had been thought to be only one, a trace component emerges from under a major, and so forth.

In addition, there is another effect, predicted by Thoma[3,21] for UMC. It is singular in chromatography.

Consider two solutes mutually similar but not chromatographically identical. Thoma showed that in theory two such solutes can be separated to any desired extent by applying an adequate number of multiple developments.

One envisions a set of trial separations. In the set, the selectivities of both the stationary phase and the solvent are held constant. As solvent strength is reduced in each successive trial, the number of multiple developments in that trial is increased enough to move the component pair, on the average, a fraction $(1 - e^{-1})$ or 0.632 of the distance across the bed of stationary phase.
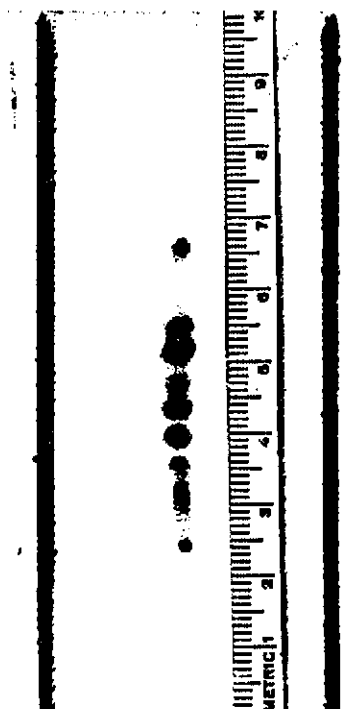
Fig. 8. Centered-PMD chromatogram, 10 cycles, 1.9 h, 1.0-strength dye solution, solvent strength 0.30.

Under this regime, the proportion of the total bed devoted solely to the center-to-center separation of the two components increases with the number of multiple developments required for the 0.632 fractional traverse, and can approach unity. Thus the eventual proportion becomes simply a matter of choice.

The set of related separations shown and discussed in the balance of this paper was prepared as a demonstration and test. We wished to demonstrate: (i) that extended programs are now feasible; (ii) that quite complex mixtures of similar-R_F components can now be cleanly and conveniently separated by TLC in one continuous operation; (iii) that the present conventional TLC plate is adequate for such separations; and (iv) that centered-PMD spots can be moved at will and without spreading over relatively large distances and during relatively long programs. We also wished to test the predictions of the Thoma theory.

5. EXPERIMENTAL

Except for the solvent trough, the equipment, materials and procedures have previously been described[16]. The solvent trough used for the longer developments was fitted with a solvent reservoir arranged to maintain a constant liquid level in the trough.

The development conditions and a guide to Figs. 8–11, as well as the other relevant chromatograms already published[16], are presented in Table 4.
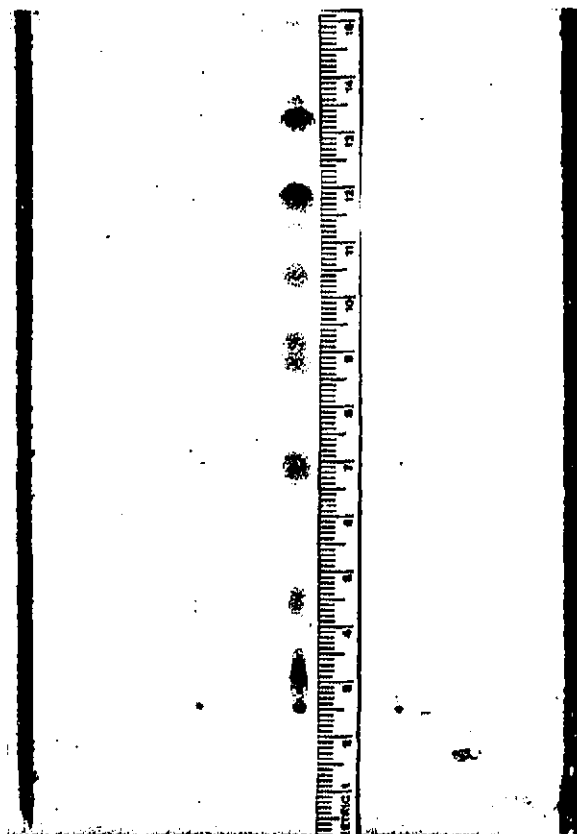
Fig. 9. Centered-PMD chromatograms, 68 cycles, 72 h, solvent strength 0.28. Center chromatogram made from full-strength dye solution, outer chromatograms made from 0.1-strength dye solution.

Figs. 10 and 11 show the five relevant chromatograms redrawn to scale (Fig. 10) and normalized with respect to spot 4 (Fig. 11). In each of these two figures the chromatograms are correlated for spot identification.

The colors and positions of the spots numbered in Figs. 10 and 11 are given in Table 5.

6. RESULTS AND DISCUSSION

The two longest chromatograms —the 32 cycle–17 h chromatogram described earlier[16] and the 68 cycle–72 h chromatogram shown in Fig. 9— were programmed for maximum duration in Mode 1. They establish that such long-lasting programs are feasible. They show that centered-PMD spots do not spread during either movement or duration on the plate. (On the other hand, it should be realized that such numbers of Mode 1 cycles need not take so long. A 32-cycle program can be run in about 2 h, a 68-cycle program in about 9 h.)

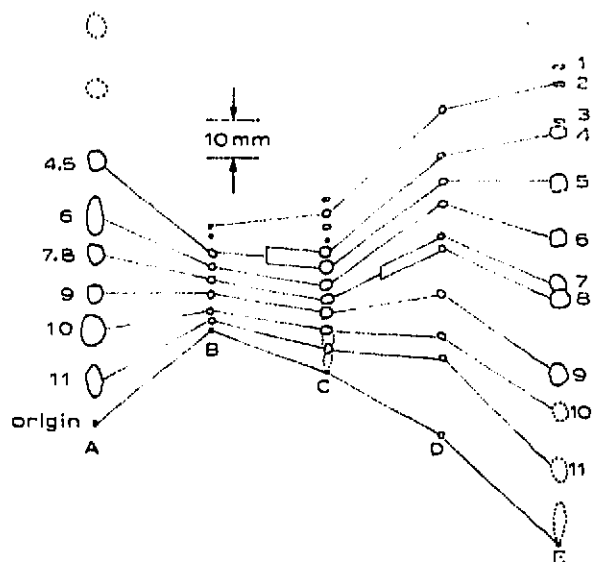Continuously increasing the number of programmed multiple developments

Fig. 10. Test set of extended-program, centered-PMD chromatograms redrawn to scale and displaced vertically for approximate centering.

can continuously improve resolution. Spot [4, 5], for instance, becomes cleanly separated into spots 4 and 5 only after 10 cycles. Also, spot [7, 8] becomes cleanly separated into spots 7 and 8 only after 32 cycles. Spot 4, still exactly superimposed on spot 5 after one development, shows after 68 cycles a center-to-center separation from spot 5 of 14 mm.

Because the successive linear increments of successive Mode 1 solvent advances continually decrease, Mode 1 solvent advance distances increasingly resemble those of UMC. Fig. 11, which presents an approximation to reduced separations (the given
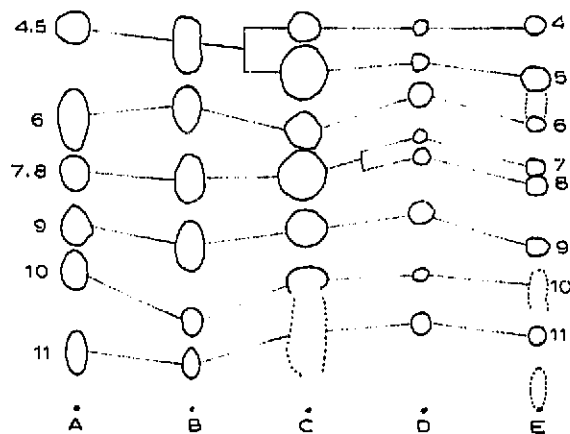

Fig. 11. Test set of extended-program, centered-PMD chromatograms redrawn normalized with respect to spot 4.

TABLE 4

DESCRIPTION OF CHROMATOGRAMS REDRAWN IN FIGS. 10 AND 11

	Chromatogram[a]				
	A	*B*	*C*	*D*	*E*
Figure	—[h]	—[c]	8	—[d]	9
Cycles	1[e]	9[f]	10[g]	32[g]	68[g]
Hours overall	1.3	1.5	1.9	17	72
Dilution spotted[h]	1.0	0.1	1.0	0.1	1.0
Solvent strength[i]	0.32[j]	0.30[k]	0.30[k]	0.30[k]	0.28[k,l]

[a] The chromatograms are redrawn to scale in Fig. 10 and normalized with respect to spot 4 in Fig. 11.

[h] See Fig. 3 in ref. 16.

[c] See Fig. 4 in ref. 16.

[d] See Fig. 5 in ref. 16.

[e] Conventional development.

[f] PMD, Mode 1. Unit times: solvent advance, 100 sec; solvent removal, 100 sec, fixed. Powers: advance, 1; removal, 10.

[g] PMD, Mode 1. Unit times: solvent advance, 100 sec; solvent removal, scheduled, in sec — C, 40; D, 20; E, 30 for 30 cycles, 10 thereafter. Powers: advance, 0; removal, 10. (For explanation of terms, see ref. 16).

[h] One microliter per spot. (For solution composition, see ref. 16).

[i] Solvent strength determined from ref. 24.

[j] Benzene.

[k] Tuned solvents (Regis, Chicago., Ill., U.S.A.).

[l] Tuned solvent mixture, 0.30:0.25, 1:1.

center-to-center separation distances divided by the chromatographic bed length), can therefore be used as an approximate check of the Thoma theory. If the reduced separation for a given spot-pair increases as the number of multiple developments increases, then the lines connecting the spots of that pair should diverge from left to right.

TABLE 5

CORRELATION OF SPOTS SHOWN IN FIGS. 10 AND 11

A–E refer to chromatograms A–E.

Spot No.	Color	Spot position (mm)				
		A	*B*	*C*	*D*	*E*
1	Yellow					150
2	Maroon		52	66	99	146
3	Gold					136
4	Orange	96	46	55	98	133
5	Light orange	96	46	52	92	119
6	Rose	81	42	47	86	104
7	Coral	70	39	44	77	92
8	Pale coral	70	39	44	74	88
9	Yellow	60	35	40	62	69
10	Gray	50	32	36	51	60 ?
11	Brown	38	29	31	44	45 ?
Origin		25	26	25	25	25

(For this use of Fig. 11, however, chromatogram B is not well suited, because the plate was being heated during solvent advance whereas the plates of the other chromatograms were not. Heating the plate compresses the chromatogram. Therefore, in using Fig. 11, chromatogram B should be ignored and instead, the corresponding spots of chromatograms A and C should be mentally connected.)

Chromatograms A (one conventional development) and C (10 cycles) were made with an increasing number of cycles but a decreasing strength of solvents —from 0.32 to 0.30. The (mental) lines from the A-spots to the corresponding C-spots are generally parallel. Thus they show no change in reduced separations.

Chromatograms C (10 cycles) and D (32 cycles) were made with the same solvent strength. Therefore the spots of D should all be displaced toward the solvent front with respect to the spots of C, and the position of increase for the reduced separation should occur at a comparatively low apparent $R_{F,n}$. Spots 5–8 do show the expected displacement toward the front, but more so than does spot 9. Thus the reduced separation between spots 8 and 9 does increase from chromatogram C to D, and the increase corresponds to a comparatively low apparent $R_{F,n}$.

Chromatograms D (32 cycles) and E (68 cycles) were made with an increasing cycle number but a decreasing solvent strength, from 0.30 to 0.28. Therefore, the position of increased reduced separation, if such is to be seen, should be higher in chromatogram E than D. The reduced separation between spots 4 and 5 is indeed seen to increase from chromatogram D to E, and the position of that increase has moved to a higher apparent $R_{F,n}$.

Overall, these observations agree with the predictions of Thoma concerning changes in reduced separation as a function of the number of multiple developments. Increases in reduced separation do occur and do change position as might be expected. However, the increases, which are less than striking, must be sought.

The evidence presented here indicates that any changes in reduced separation produced even by dozens of multiple developments certainly do not dominate the chromatogram. The maximum reduced separation does not by any means usurp the body of the chromatogram, nor do the reduced separations far from the maximum diminish unwontedly.

So long as the chromatogram can be, and is kept, roughly centered on the chromatographic bed by such relatively small alterations in solvent strength as those used here, increasing the number of PMDs merely increases resolution throughout the chromatogram.

Resolution is proportional to the square root of the number of theoretical plates. In conventional TLC, the number of theoretical plates is calculated as $16(x/y)^2$, where x is the distance of the spot from the point of deposition and y is the top-to-bottom spot "width". Because of spot reconcentration, theoretical plates may not be calculated on any multiply developed chromatogram. Spot reconcentration either curtails (UMC) or obliterates (PMD) the normal effects of diffusion. Nevertheless a number of equivalent plates may be calculated from the same measurements and procedure.

We define the number of equivalent plates to be the number of theoretical plates a conventional development would have to produce to generate the spot characteristics being measured. Calculation of the numbers of equivalent plates with these chromatograms throws a new light on the potential of TLC.

The maximum number of theoretical plates attainable in conventional TLC has been discussed by Snyder[22]. For a single 150-mm solvent advance and a spot having the optimal R_F of 0.33, Snyder concluded that 333 theoretical plates could be realized in 30–60 min, or 667 in two directions in 1–2 h. If the spot were carried across the plate by continuous development, as many as 1500 plates could be realized, but 10–20 h would be required.

In comparison, consider spot 2 on chromatogram B[16]. Displaced about 25 mm from the origin and having a "width" of about 1.5 mm, spot 2 shows about 4,500 equivalent plates, produced in 90 min.

(We continue in the next paragraph to cite spot 2 rather than the somewhat more displaced and nominally more favourable spot 1. The presence of spot 1 above spot 2 establishes that spot 2 was not in the solvent front. The top-to-bottom "width" of spot 2 was not adventitious.)

On each chromatogram, spot 2 has a top-to-bottom "width" of 1–2 mm or, as a compromise, 1.5 mm. On chromatogram D, in which it was displaced about 75 mm from the origin, spot 2 shows about 40,000 equivalent plates. On chromatogram E, displaced about 120 mm from the origin, spot 2 shows a little over 100,000 equivalent plates.

These chromatograms were produced on commercial pre-coated plates made with relatively coarse particles that allow faster solvent flow. The minimum spot top-to-bottom "width" found with PMD on such plates equals 25–50 particle diameters[14]. With PMD, as with conventional TLC[22,23], the minimum spot size can be expected to be directly proportional to the particle size. The minimum spot size to be expected on plates made with the 1- to 5-μm-diameter particles known to be more nearly optimum for resolution should be about one-tenth the minimum spot size found here.

A conventional 20-mm TLC plate, made with particles 1–5 μm in diameter and multiply developed with effective R_F-independent spot reconcentration should be able to present over 10^6 equivalent plates throughout the upper half of the plate for spots that are not overloaded.

7. SUMMARY

Introduced in 1951, multiple development by repeated solvent advances improves the resolution available from a given chromatographic system. Repeated solvent advances compress the top-to-bottom "width" of a thin-layer chromatographic spot by the factor $(1 - R_{F,n})$, where $R_{F,n}$ expresses the final location of the spot between the origin and the solvent front. Spots near the front become almost lines drawn parallel to the solvent front, but near the origin spot shapes change little.

In contrast, all spots developed by programmed multiple development (PMD) are line-like. PMD, introduced in 1973, adds solvent removals to solvent advances while, throughout, solvent flows constantly towards the front. Both advances and removals are carried out automatically and relatively rapidly. Used in thin-layer chromatography, PMD spots tend to be uniformly tight and do not reflect the characteristics of the origin. Therefore, PMD is currently used mainly to improve the separability and molecular density of spots.

The form of PMD that is called centered PMD compresses spots laterally as well as longitudinally, so that the molecules of a given spot are brought and held together during successive developments. This further improves trace detectability. It also allows separations to continue without spot spreading as long as necessary or desired.

Thoma predicted that continued multiple development will change the proportion of the chromatographic bed devoted to components ultimately found slightly more than halfway (0.6) toward the solvent front. Tests support the prediction but show that the effect is minor if only some dozens of multiple developments are involved. The principal result of extended programs is a steady improvement of resolution throughout the chromatogram.

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