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PROGRAMMED MULTIPLE DEVELOPMENT IN THIN LAYER CHROMATOGRAPHY

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Programmed multiple development (PMD)[1-11] is a method of using thin layer chromatography(TLC)[12,13]. We shall first briefly characterize conventional TLC.

CONVENTIONAL THIN LAYER CHROMATOGRAPHY

The basic operations of conventional TLC are sample deposition, plate development, and solvent removal. First, a spot (or, for preparative work, a streak) of a mixture to be separated is deposited from solution near the edge of a thin layer plate. This plate is usually a 0.250 mm-deep layer of silica gel supported by glass. After the spot has dried, the plate is developed: the edge of the plate below the spot is immersed in solvent, which moves by capillary action into the thin layer. During development, the vapor space over the thin layer is enclosed. With a

suitable solvent, the spotted components move with the solvent and separate as they move. When the solvent has almost crossed the plate, the plate is removed and dried.

The separated components remain on the thin layer. They are then identified and either assayed or recovered, or both.

These operations and elements of equipment are easily used, widely adaptable, and effective. As a result, TLC has grown phenomenally. Any acceptable form of TLC must combine every one of the following characteristics (all of which have been carefully retained in PMD):

--Chemically wide applicability.

--Microanalytic capability.

--The thin layer plate.

--Spot recoverability--of the separated spots, the unmoved residue, and the unseparated components in the solvent front.

--Solvent volatility.

--Development without attention.

--Adaptability to parallel processing, that is, to multiple, simultaneous, similar separations.

Conventional TLC has grown despite handicaps inherent in and imposed by each basic operation: deposition, development, and drying. Deposition and

development prevent the full potential of the thin layer plate from becoming manifest. Also, the tedium and impractical duration of repeated long developments and interposed dryings place a powerful variation of multiple development--which we shall be describing--effectively out of reach.

In conventional TLC, the first operation, sample deposition, immediately limits resolution (resolution is defined as the center-to-center separation of two spots, divided by the average top-to-bottom width of these spots). A well-deposited spot, "which should be as small as possible"[13], measures perhaps 3 mm in diameter. But the minimum spot width that the thin layer plate can produce equals perhaps 20-50 particle diameters--less than 1 mm even with coarse-particled commercial plates[8]. Thus sample deposition, however skillful, both obscures how narrow a spot or streak can be and dissipates possible sensitivity.

Then, during development in conventional TLC, the effects of diffusion proceed unchecked. Diffusion is necessary: to become separated, molecules of different types must move--and can move only by diffusion--back and forth between the solvent and the particles of the thin layer. But because of diffusion, conventional TLC spots spread.

Within a spreading spot, the molecular density steadily decreases; and with it, sensitivity decreases again. Also, diffuse, spread-out spots cannot be readily located, and so the nominal locations of these spots cannot be used with much confidence for qualitative analysis.

Thus in conventional TLC, the flow of solution into the surrounding bed during sample deposition and the unchecked diffusional spreading of spots during development effectively limit and mask several capabilities of the thin layer plate. TLC resolution potential suffers particularly. The thin layer plate, which can offer a great many resolved spots per unit distance along the chromatogram, is usually used as though it can offer only a few. Conventional TLC spots, at once too wide, spread constantly.

The top-to-bottom width of conventional TLC spots and streaks is generally beyond control.

Polyzonal TLC

In conventional TLC, two techniques can affect the top-to-bottom width of some spots, but not all. These two techniques are polyzonal TLC [14] (considered next) and unidimensional multiple chromatography [15] (which we shall consider presently). The simplest case of polyzonal TLC involves a two-component solvent in

which the components differ appreciably in solvent strength. As the solvent moves into the thin layer, the stronger solvent is selectively retarded while the weaker flows ahead. An internal solvent front results. A solute that is strongly desorbed by the stronger solvent, but not by the weaker, is found in the internal solvent front of the stronger solvent.

The spot from such a solute is no wider, top-to-bottom, than the internal solvent front: "...individual substances may migrate with a solvent front and, by displacement, form small spots parallel to the front. The high concentration of the substance makes their detection especially easy. Therefore, polyzonal TLC is often more sensitive than the standard technique and is (suitable for) not only analytical but also...micropreparative work."[14]

For a given sample with a number of components, solvent gradients increase the range of solute adsorptivities that can be accommodated, but not the number of components of similar adsorptivity that can be resolved: "...polyzonal TLC...is rather suited for a survey of the different substance classes present in a mixture but is not satisfactory for the separation of isomers."[14]

Two-dimensional TLC

There are several types of multiple development in conventional TLC. In two-dimensional multiple chromatography, the sample is applied to one corner of the plate. The plate is then developed, producing a chromatogram along one edge. After the plate is dried, it is rotated. The edge nearest the chromatogram is then immersed in the same or another solvent. The resultant development produces a two-dimensional array of spots. This use of multiple development merely elaborates the conventional treatment of thin layer chromatography. Spot width is considered given, and only the center-to-center separation of the spots is sought, achieved, or improved. (Nevertheless, only thin layer chromatography lends itself to this convenient and powerful technique.)

Successive solvents

In multiple development by successive solvents, the plate is repeatedly developed in the same direction by successively weaker solvents for successively greater distances. For separating groups of components in which the groups differ substantially in adsorptivity, multiple development by successive solvents is easier to apply than polyzonal TLC (indeed

it is generally easier to apply, although it may not succeed so well in particularly recalcitrant separations). Polyzonal TLC tends to require a specific solvent composition for each specific sample composition. Moreover, solvent components that may be theoretically indicated for polyzonal TLC solvents may also turn out to be immiscible: polyzonal TLC presents more than one problem.

Compared to polyzonal TLC, multiple development by successive solvents allows less attention to be paid to the specific characteristics of both the given sample and the corresponding solvent for development. It enables the thin layer plate to accommodate solutes with a greater range of adsorptivities than could be developed by any single one of the successive solvents. Nevertheless, similarly adsorptive components cannot be resolved any better with this approach than with the others already described.

Nor does multiple development by successive solvents affect spot top-to-bottom width, unless the plate is repeatedly developed with each successive solvent. For each such solvent, such multiple development then becomes a form of unidimensional multiple chromatography.

Unidimensional multiple chromatography

The one conventional TLC technique par excellence for separating similar components is unidimensional multiple chromatography (UMC). In UMC, the plate is repeatedly developed in the same direction by the same solvent for the same distance. Of its ability, Truter remarked, "...it (UMC) can be very useful because it enables closely related compounds, such as isomers, to be separated from one another"[13]. Thoma[15] not only spoke of the"superb resolving power" of UMC, but also showed that that power can be increased, at least theoretically, without limit (see the later discussion on Thoma's UMC theory).

However, UMC has not been used much.

One very good reason for the neglect of UMC is that, as a form of conventional TLC, it is forbiddingly tedious and time-consuming. The plate must be repeatedly developed for a certain time, and then removed and dried after each development. Truter, for instance, showed a small thin layer plate on which a dye mixture had been developed 36 times for 5 cm[13]. Despite the mere 5 cm distance, this meant that someone had had to develop that plate for about 10 minutes, and then remove it, and then dry it--36 times! This must not only have taken at least the whole of one very tedious day but

also have precluded, meanwhile, any other activities. Also, the developed chromatogram showed no particular effect with regard to spot top-to-bottom width. However, UMC has a very definite effect on spot width.

Thoma[15] mentioned that UMC decreases the top-to-bottom width of a spot: "...on the second and subsequent passes, the solvent flows over the trailing edge of the spot before reaching the leading edge. This effect aids in sharpening the bands." (Whereas, continuous development affects only the center-to-center separations: "During continuous chromatography, the magnitude of the spot constantly increases, since there are no sharpening forces at play.")

Jupille has shown how UMC tends to decrease spot top-to-bottom width[5, 6]. This treatment neglects diffusional spreading. Two identical-R_f molecules (each behaving as a statistical aggregate: although diffusional spreading is neglected, the following equation describes without qualification the <u>centers</u> of these two aggregates), originally separated along the direction of development by a distance X_1, become after n UMC developments separated by a smaller distance X_f, as follows:

$$X_f = (1 - R_f)^n X_1 . \qquad (1)$$

Suppose the molecules are originally separated by 10 mm, as by being at the top and bottom of a deposited spot 10 mm in diameter. After, say, 4 developments the two molecules will be separated--given an R_f of, say 0.9--by $(1 - 0.9)^4$ x 10 mm, or 0.001 mm. On the other hand, if their R_f is 0.1, they will still be separated by $(1 - 0.1)^4$ x 10 mm, or 6.5 mm. In other words, in UMC the top spots can become very narrow but the bottom spots do not change much. (We shall see in a moment that this conclusion is quite general.)

The repeated, equal developments of UMC tend to crowd the spots upward and into the solvent front. This was expressed by Thoma[15], who related the single-development R_f to the apparent R_f observed after n UMC developments, $R_{f,n}$:

$$(1 - R_f)^n = 1 - R_{f,n} . \qquad (2)$$

So, to keep the chromatogram as well spread out between origin and solvent front after n UMC developments as it was after one development, one uses a weaker solvent. But this, of course, depresses the R_f, and thus would seem--by equ. (1)--to make any decrease of spot top-to-bottom width still less obvious. However, this, though true, does not fully convey the final effect.

We can show--this does not seem to have been presented elsewhere--that the effect of any given

number of UMC developments on spot top-to-bottom width can be determined solely from the final spot position. Substituting equation (2) into equation (1) gives

$$X_f = (1 - R_{f,n}) X_i . \qquad (3)$$

The ratio that expresses the fractional multiplier of the original spot top-to-bottom width is X_f/X_i: $(X_f/X_i) X_i = X_f$. A rearrangement of equation (3), namely,

$$(X_f/X_i) = 1 - R_{f,n} , \qquad (4)$$

shows that the UMC-caused narrowing of the original spot top-to-bottom width is always exactly given by the final position of the spot in the multiply developed chromatogram, without regard to the preceding number of multiple developments. It also shows that all UMC chromatograms have the same appearance with respect to spot shape along the chromatogram--narrow spots at the top, changing to wide spots at the bottom--regardless of the number of developments.

For instance, for a spot very near the solvent front, the apparent R_f is near unity. Therefore (X_f/X_i) is near zero, and so is the final spot width. Or, for a spot with an apparent R_f of 0.1, the fraction (X_f/X_i) is 0.9: the final spot width is still 0.9 as large as the original, no matter its development history.

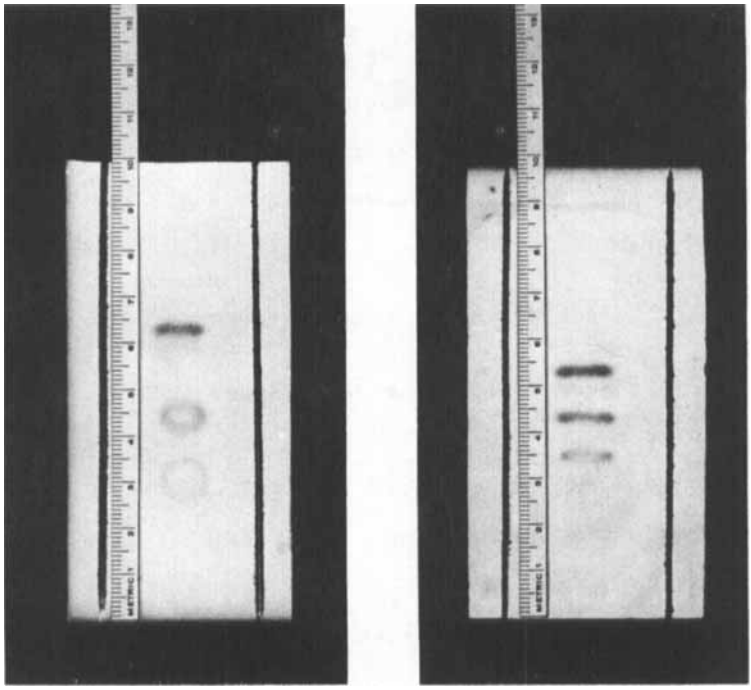
Figure 1 shows two chromatograms. To reveal more clearly the effect on spot shape of multiple development techniques, the spots in both were deposited as identical circular rings. Figure 1A shows a UMC chromatogram; Figure 1B shows the corresponding PMD chromatogram.

PROGRAMMED MULTIPLE DEVELOPMENT

Programmed multiple development (PMD) was introduced in 1973[1].

PMD removes emphasis from TLC technique while at the same time emphasizing and revealing the full potential of the thin layer plate. Deposited spots need not be either as small as possible or precisely located. Yet developed PMD spots show constantly minimized top-to-bottom widths and origin-independent, precise position.

Presently, we shall further document and illustrate these and various other aspects of PMD. First we shall, as with the other techniques, briefly indicate the nature of PMD. A description of PMD instrumentation and the use of this instrumentation then follows. The paper concludes with two application areas. The first treats the application of PMD to the present uses of TLC. The second suggests how PMD may expand a use of TLC that is presently essentially untouched.



A. FIGURE 1 B.

A. UMC spots are narrow at the top of the chromatogram changing to wide at the bottom, without regard to the number of developments. This UMC chromatogram was made with five 500-second developments. The spots were deposited as circular rings in order to show the effect of UMC on spot shape. Except for the manner of development, the chromatograms of Figures 1A and 1B were identical in all respects. (Courtesy of Separation Science (9).)

B. PMD spots tend to be uniformly narrow, top-to-bottom, throughout the chromatogram. These spots were deposited as circular rings in order to show the effect of PMD on spot shape. This PMD chromatogram was made with a 5-cycle, Mode 1 program having 100-second unit time for solvent advance and 100-second, fixed unit time for solvent removal, at power 10. Except for the manner of development, the chromatograms of Figures 1A and 1B were identical in all respects. (Courtesy of Separation Science (9).)

In PMD, a conventional TLC plate is automatically cycled through a preset number of developments. In each succeeding development, the solvent excursions extend farther into the plate. Following each development, automatically controlled evaporation causes the solvent front to recede, usually to the spot origin. After the last prescribed development, further development is prevented by controlled evaporation.

One crucial difference between PMD and any form of conventional TLC is that during PMD, the plate never leaves the solvent. Nor is the plate touched, or moved. Very early, the necessity of this facet of PMD design became apparent, and then later, the implications of it.

The necessary and experimentally convenient condition for the initially frequent oscillations of the solvent front is that neither the plate nor the solvent move. Under this condition, a great many exploratory programmed multiple developments were conducted manually. It became increasingly clear that these complex, variably extended, and precisely timed operations would have to be carried out automatically if they were to be carried out reliably. Unattended operation, always convenient, is in PMD also necessary.

Because in PMD the plate never leaves the solvent, the solvent flows at all times toward the solvent front. Whether the front is advancing or receding, any

solute molecules behind the front move toward any similar-R_f molecules immobilized beyond the front. Such immobilized molecules may be either those not yet reached by the advancing front, during solvent advance; or those just deposited from the receding front, during solvent removal. In these ways, spots are reconcentrated, top-to-bottom, twice per PMD cycle (Fig. 2). Spot reconcentration is a prime characteristic effect of PMD.

The same mechanisms that reconcentrate spots also align them. As a result, neither the location nor the area of the PMD spot depends on either the location or the area of the originally deposited spot. Both sensitivity[4] and qualitative analysis[7] benefit, as indeed does every aspect of TLC.

PMD instrumentation and programming

The primary elements of PMD instrumentation are the programmer and the developer (Fig. 3). One programmer can control any number of developers that are executing a given program in parallel.

The operator sets into the switches on the face of the programmer the characteristics of the program he desires. The switch labels include the terms cycle, segment, advance, removal, scheduled, fixed, and mode. These are more or less self-explanatory.

Each development cycle comprises a solvent advance segment and a solvent removal segment. The duration of these varies with the cycle number, the mode, and the condition for the solvent removal segment--fixed or scheduled. If the duration of the solvent removal segment is fixed, it is invariant; if it is scheduled, it conforms to the mode schedule used for solvent advance.

The development mode specifies segment duration and also the maximum cycle number. A segment duration T varies with the current cycle number and the unit time t in a manner specified by the mode:

TABLE I

Mode	Segment duration T	Maximum cycles available
1	$T = n\ t$	99
2	$T = (n(n + 1)/2)\ t$	28
3	$T = n^2\ t$	20

For solvent advance segments, the operator selects the unit time and the advance power. The unit time t varies in 10-second steps from 0 to 100 seconds. It is used by the programmer in accordance with the mode selected and the current cycle number, as just described.

The advance power varies in 11 steps from 0 to 10. Each step represents the number of increments of advance power being applied to the developer radiator

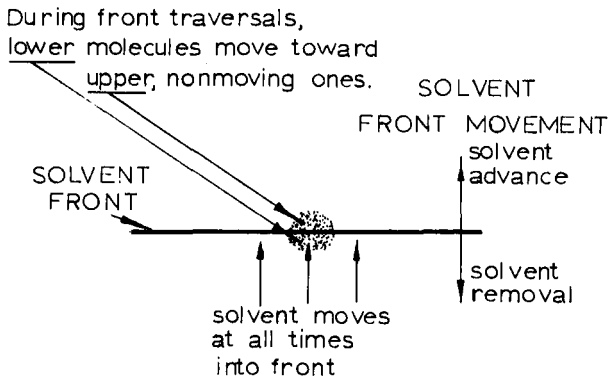


FIGURE 2

Spot reconcentration in PMD.

during solvent advance. Each increment of advance power equals 1.25% of the maximum power available to the developer radiator. (The "maximum power" is equivalent to that obtainable directly from the house--i.e., in the U.S., for example, 110 V ac--by the radiator.) Thus the advance power is limited to 12.5% of maximum power, at most.

For the solvent removal segments, the operator selects the removal unit time condition and the unit time, as well as the removal power. The unit time condition is either fixed or scheduled. (As mentioned earlier, fixed time does not change with cycle number, whereas scheduled time does change with cycle number according to the mode setting.) The unit time t for sol-

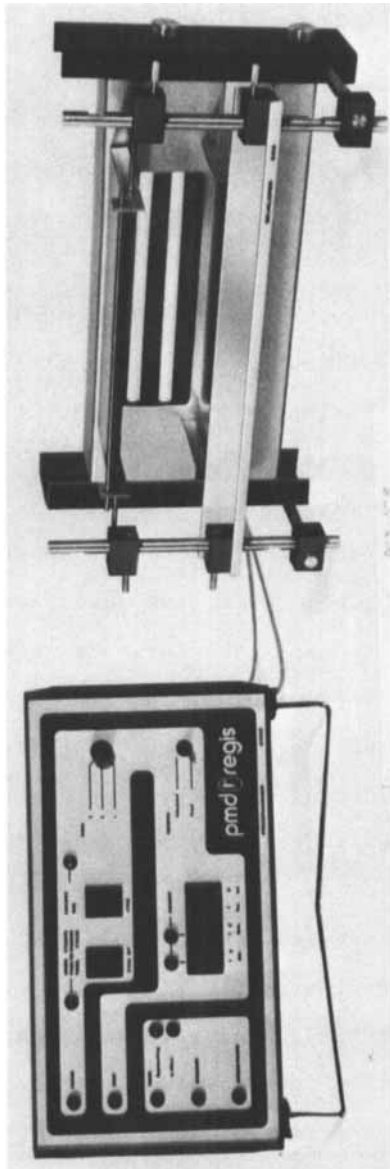


FIGURE 3. PMD instrumentation: Programmer (left); and developer (right).

vent removal also varies in 10-second steps from 0 to 100 seconds.

The removal power varies in 11 steps from 0 to 10. The incremental value of these steps depends on the removal time condition--fixed or scheduled. If the removal time is fixed, each increment represents 10% of maximum power, whereas in scheduled time, removal power is limited to 50% of the maximum power available.

The operator also selects the other two parameters: preheat time and interim power. Preheat time varies in 10-second steps from 0 to 100 seconds. It applies to that interval after the plate is first immersed in the solvent but before the program proper begins. During preheat time, the power level selected for removal power is applied. Thus when the program begins, the plate is in roughly the same thermal condition that obtains at the completion of each subsequent solvent removal. Preheat time is more pertinent for short than for longer programs, for which it is usually set at zero.

The time between the end of one program and the beginning of another, or during an interruption (PAUSE, to be described) of a given program, is called interim time. During interim time, interim power is applied. (A remote alarm actuator on the programmer also becomes energized, signalling program interruption

or termination.) Interim power varies in 11 steps, 0 to 10, from 0% to 100% of maximum power, by 10% increments. Whether interim power may be applied, if called for, is governed by another, on-off switch--the interim power can be turned on or off instantly, at any given power level.

Thus, aside from the power level settings, the operator sets into the programmer the timing information for a projected program: the number of cycles, the mode, whether removal time is to be fixed or scheduled, and the unit times for solvent advance and removal.

The durations of possible programs can vary widely, from less than a minute to over ten days. So that the operator can determine easily and quickly how long a projected program might take, the programmer carries an EVALUATE button. If this button is pushed before a program is started, the programmer displays in three digits how many minutes the program would take. If the button is pushed after the program has been started, the display shows how many minutes have elapsed. If either of these exceeds 999 minutes (16 hours), the display flashes 000 at 1-second intervals. Otherwise, during a program the display shows the number of cycles already completed.

Solvent removal may be conducted partly or wholly by gas, with or without radiant heating (as op-

posed to being conducted by radiant heating alone). A socket at the rear of each developer is activated by the house current at all times except during solvent advance. This can be used to actuate a solenoid valve in a gas supply to a "nitrogen facing plate." The nitrogen facing plate is an assembly that encloses the thin layer plate, is used on and in conjunction with the developer, and suitably shapes the solvent-removing gas stream. This gas stream passes downward over the thin layer as a uniformly flowing, thin gas sheet. As a result, the solvent front recedes uniformly. (Too high a flow rate produces not only too high a rate of solvent front recession but also a solvent front that dips in the middle--with consequent distortion of the chromatograms. This matter is considered again in the discussion on solute lability and solvent removal.)

The thin layer plates used in PMD are conventional. On receipt, they are usually cut to smaller sizes--PMD chromatograms are usually more compact than conventional. The developer accommodates plates 100 mm and 200 mm in height, and up to 200 mm in width.

If gas is to be used for solvent removal, the plate is simply spotted, usually 25 mm from the edge, and inserted in the nitrogen facing plate, which is then placed in the solvent trough on the developer trough support.

If heat alone is to be used for solvent removal, the thin layer is removed from the plate in two 1-mm wide scores parallel to the vertical edges of the plate and 10 mm from them. After the plate has been spotted, it is clamped to a matching glass facing plate but separated from that facing plate by a 3-mm thick spacer around the top and vertical edges (Fig. 4). The assembly is then placed in the solvent trough on the developer (Fig. 5).

If the program has already been set into the programmer, the operator presses the START button. The programmer then directs the developer in the execution of the program either until the program has been completed or until the operator presses the PAUSE button.

Actuating PAUSE allows the current cycle to be completed. On completion of solvent removal in that cycle, interim power is applied and the remote alarm socket is energized. Then, development is stopped but the current state of the program remains in the programmer. Later, when PAUSE is terminated, the program proceeds at the point at which it was interrupted.

PAUSE allows, for instance, an easy and convenient incorporation into PMD of multiple development by successive solvents, yielding the advantages of each. The usual use of PAUSE is the examination or photography

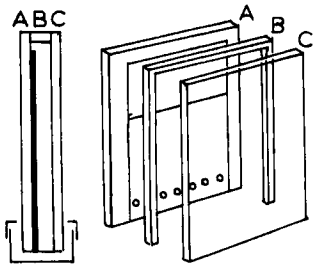


FIGURE 4

If solvent is to be removed by radiant heating alone, the scored and spotted thin layer plate is separated from a matching glass facing plate by a non-porous spacer around the top and edges.

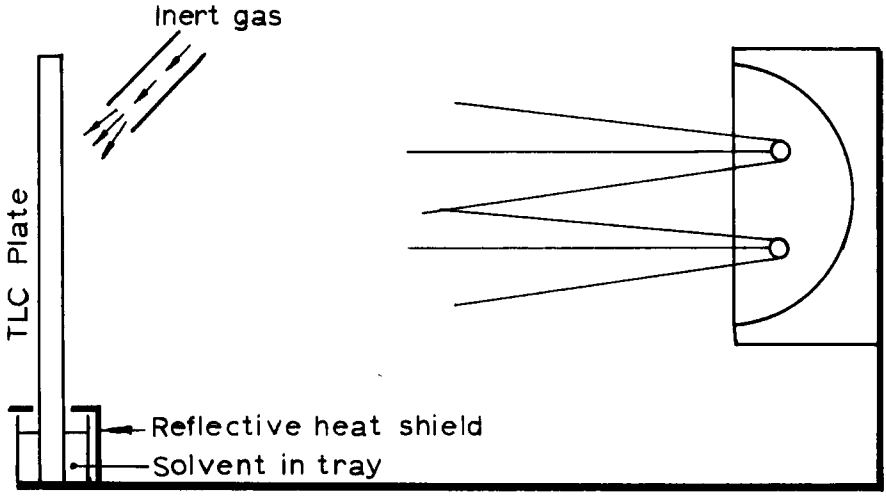


FIGURE 5

The developer holds the thin layer plate in its appropriate assembly during the programmed multiple development and executes the program. Each developer receives power directly from the house, therefore one programmer can control any number of developers executing a given program.

of chromatograms as experimental programs are in progress.

The power levels (and/or the gas flow rate) can be changed at any time. Therefore, the power level may be adjusted, if the current setting for it is not already known, while solvent removal is under way. Solvent removal is adjusted so that the solvent front recedes slowly and uniformly to a point at or just below the spot origin.

By Poiseuille's Law[16], the velocity of solvent advance into a uniform thin layer bed at ambient temperature is inversely proportional to the distance from the solvent level in the reservoir to the solvent front. Thus the distance of solvent advance varies as the square of the time allowed for that advance. Mode 3 allows for such increase of time. The solvent front nominally advances one increment of distance with each successive Mode 3 cycle.

Mode 1, on the other hand, allows only one additional time increment with each successive Mode 1 cycle. The additional distance of solvent advance decreases with each successive Mode 1 cycle. Mode 2 is intermediate between Modes 1 and 3.

Mode 1 produces the greatest number of multiple developments within a given overall time, for a given unit time. Mode 1 therefore also maximizes spot recon-

centration. In consequence, Mode 1 spots are narrowest, top-to-bottom, among the modes; and, for a given solvent, Mode 1 accents the development and resolution of spots that have a relatively low R_f for that solvent.

Mode 3, in contrast, produces the greatest center-to-center separations of spots but the least number of developments within a given overall time, for a given unit time. Spots with Mode 3 are the least reconcentrated, and therefore the broadest. For a given solvent, Mode 3 accents the development of the higher-R_f spots.

Solute lability and PMD solvent removal

Figure 6 presents in diagram the nominal relative distances of successive solvent advances among the three modes, for a given unit time. The actual distances depend very much on the method of solvent removal. The stronger the heating during solvent removal, the more compact is the resulting chromatogram. Also, at least to a first approximation, the greater is the spot reconcentration.

Solvent removal involves several interacting variables: solute lability, plate temperature, solvent flow rate, and removal duration. As we shall see, allowing for high solute lability entails either decreased plate efficiency--the degree to which molecules of different R_f's separate as they move--or slower separations.

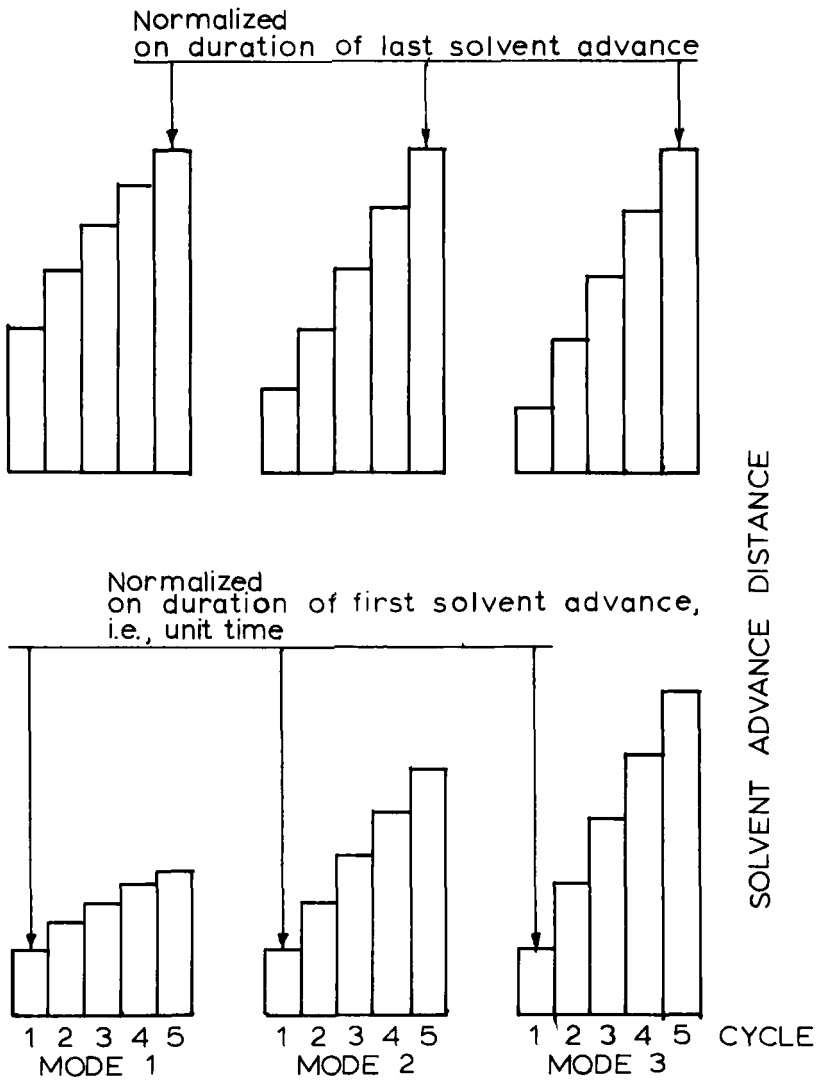


FIGURE 6

PMD Mode 3 programs produce an equal distance of solvent advance on successive cycles, greatest center-to-center separation, but broadest spots. Mode 1 produces most cycles per overall time and the narrowest spots, but the least center-to-center separation. Mode 2 is intermediate.

Solvent diffusivity limits the plate efficiency of adsorbent thin layer plates [17, 18]. Increasing the temperature exponentially increases solvent diffusivity [19]. Therefore, plate efficiency increases with increasing plate temperature [20].

However, solute lability may require that the plate temperature be held to or below ambient levels. In this case, the plate would be periodically cooled below ambient by solvent removal by gas sweep without heating. But decreasing the plate temperature decreases plate efficiency, unless the solute molecules are allowed additional time for diffusion through the solvent. This means still lower flow rates (normal flow rates are already too high).

(The plate efficiency of present commercial TLC plates would increase with lower flow rates. Even with solvents that show a usable compromise between high diffusivity and reasonably low volatility, solvent flow rates are too high. An optimal flow rate with such a solvent, at ambient temperature, would be about 0.001 cm/sec or about 4 cm/hour [17, 18]. The actual flow rate for such solvents used at ambient temperatures does not decrease to 4 cm/hr until the solvent front has risen some 10 cm from the solvent level in the reservoir.)

The spot reconcentrating mechanisms of PMD act against the spot spreading mechanisms present in TLC

generally. At an average optimal flow rate, a PMD spot still shows, for a given loading, a certain minimum top-to-bottom width; we can call this width, necessary. At flow rates higher than optimal, such a spot shows a width greater than necessary; we can call that increment of width, excess. Because practically all TLC flow rates are greater than optimal, practically all spots show excess width.

Excess width that too severely damages plate efficiency and resolution can be reduced by either decreasing the flow rate or increasing the solvent temperature, or both.

The value of the optimal flow rate changes sharply with temperature. If the solvent temperature increases, then the value of the optimal flow rate increases markedly. Conversely, a particular flow rate that may be optimal for ambient temperature quickly becomes too high as the solvent temperature decreases.

More to the point with regard to the method of solvent removal: an excess width that is tolerable with a solvent held at ambient and/or above-ambient temperatures may easily become intolerable if the solvent is chilled, as by evaporation into a moving stream of gas. Thus, allowances for high solute lability entails either decreased plate efficiency (increased excess width) or slower separations.

Separations can be conveniently and appropriately made slower in either of two ways: the spot can be deposited higher on the plate, or the plate can be scored horizontally below the spot. If the spot is deposited, say, 6 cm rather than 2 cm from the edge, and the solvent level is 1 cm from the edge, then the flow rate at the point of deposition will be (2 - 1)/(6 - 1) or 1/5 normal. (The separation will not take quite 5 times longer, however, because the flow rate will remain not only more nearly optimal but also more nearly linear. Nor need time be lost initially: the plate can be wetted below the point of deposition before being placed in contact with the solvent reservoir.)

Alternatively, the bed can be removed by a horizontal score, except just below the deposited spot, where a section of the bed is left untouched. The solvent gains access to the bed only through that section. The flow rate can be easily and reproducibly decreased in this way by factors up to 10, and will also become both more nearly optimal and more nearly linear.

In practice, however, the increased excess width is almost always accepted. The user seldom elects to slow a separation in either the ways just described or any manner whatsoever. It should be realized, however, that solvent removal by gas does not <u>per se</u> invalidate PMD spot recnncentration mechanisms. Rather, dif-

fusivity is exponentially related to temperature. Decreasing solvent temperature radically shifts the balance of spot reconcentrating versus spot spreading mechanisms in favor of spreading. The result is increased excess width. If the more reconcentrated, dense, and mutually aligned spots typical of PMD are desired despite solvent cooling, the balance can be restored only through slower separations.

Too fast a gas sweep can simply bypass spot reconcentration by solvent removal, and convert PMD into something of an inefficient UMC. Inefficient, because the solvent temperature and the plate efficiency then become especially low. UMC, because the spots would become like those of UMC: narrow and aligned at the top of the chromatogram, but wide and unaligned at the bottom.

If solvent removal is done by heating (with or without gas, so long as the plate temperature increases), the two spot reconcentrating mechanisms are not independent but complementary. Stronger heating decreases the efficiency of spot reconcentration by solvent removal, but simultaneously increases that from solvent advance; and conversely[9]. Whether heating is strong and brief, or gentle and prolonged, the solvent removal mechanism is more effective for spot reconcentration than the solvent advance mechanism[9].

The more gentle and prolonged the solvent removal, the more effective the resultant spot reconcentration[1]. This holds whether the solvent is removed by gas sweep or heating or both. Therefore the provisions for scheduled time, lower heating power during scheduled time, and independent control of solvent removal unit time were incorporated into the programmer. For effective spot reconcentration by solvent removal, the recession velocity of the solvent front should be small compared to the forward velocity of the spot being reconcentrated[1, 9]

PMD application areas outlined

The applications of PMD fall into two areas. The first area, treated next, corresponds to most current applications of TLC. In it are utilized those properties of PMD that are the most striking and the most quickly manifested--the narrow, uniform, aligned spots and streaks that are produced from possibly broad and/or unaligned origins.

The second area corresponds to a theoretically well established but practically untouched area of TLC: the separation of complex natural mixtures of similar-R_f components in one continuous and unattended operation comprising many--say, over 20 or over 50--multiple developments. With PMD, this becomes feasible and con-

venient. In the treatment of this subject, some of Thoma's theory of UMC[15] is discussed, along with some extended programmed multiple developments[11]

Also in the second area, centered PMD[10] is discussed. In centered PMD, the edges of spots are caused to move laterally into the centers of the spots. As a result, developing spots suffer no molecular loss by diffusion; sensitivity remains high, maximum, and constant.

APPLICATIONS OF SPOT RECONCENTRATION AND ALIGNMENT

The various distinguishing characteristics of PMD that are of use with respect to the current uses of TLC tend to be based on one phenomenon: spot reconcentration. On the other hand, spot reconcentration requires a properly slow solvent removal if both low-R_f and high R_f spots are to be reconcentrated; but on the other hand, spot reconcentration is fully manifested within a very few cycles. So if the spots to be developed are few, then solvent removal may be de-emphasized and quickened. The resulting spot reconcentration, if not maximal, is nevertheless usually quite satisfactory. The PMD programs that correspond to current TLC uses therefore tend to be short: few cycles, short solvent advances and removals, brief durations overall.

Although the various characteristics stemming from spot reconcentration are commonly rooted, nevertheless for clarity we illustrate the nature and advantages of them more or less separately, in the following order: precision of spot position; independence of spot position from origin position; broadened origins (but) narrow spots; (tolerance of) extraneous material in the origin; and sensitivity enhancement.

Precision of spot position

For a given program, spot position becomes a precise datum. To illustrate this, 12 7-cycle, acetone-solvent PMD chromatograms were made on Silica Gel G from (large) 5-microliter spots of 0.2 microgram/microliter solutions of amphetamine and methamphetamine. The spot positions were then measured as distance from the edge of the plate, with the following results[7]:

TABLE II

	Distance, cm		Relative
	Mean	Std. dev.	std. dev.
Amphetamine	6.16	0.16	2.6%
Methamphetamine	4.16	0.06	1.4%

Thus, PMD spot positions can be precisely measured, and PMD spot alignment is a precisely verifiable fact. Therefore spot identities can be precisely correlated with PMD spot positions.

Independence of spot position from origin position

The positions of PMD spots do not reflect the positions of the origins--see Figure 7, for instance. Figures 8A and 8B show corresponding conventional and

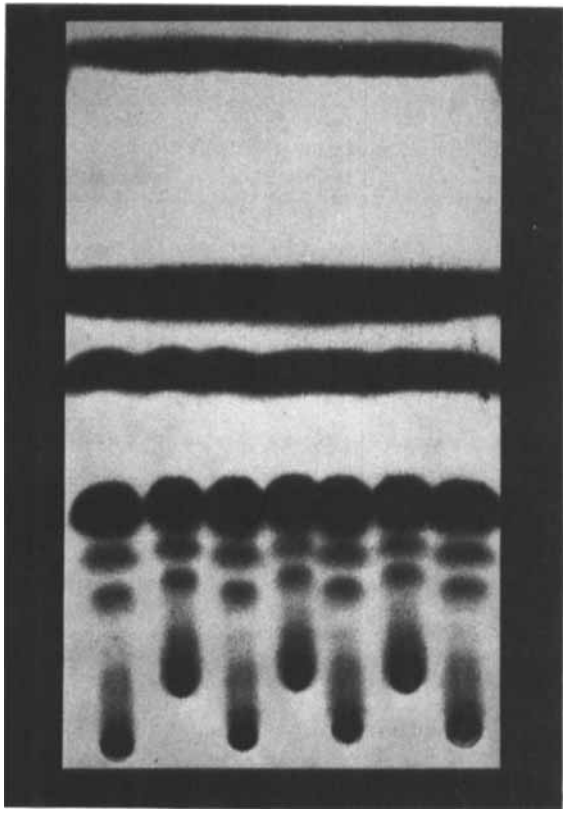


FIGURE 7

The positions of developed PMD spots do not reflect the positions of the spot origins.

The 5 mm radii of the original spots plus the 4 mm displacement of the two rows of spot centers along the direction of development produced an origin "streak" 14 mm wide, top-to-bottom, overall. The developed PMD streaks were, however, only 1 to 3 mm wide, depending primarily on the degree of overloading. (Original photograph courtesy of Industrial Research.)

PMD chromatograms made from intentionally staggered origins. The conventionally single-developed spots are displaced by origin displacement, but the PMD spots are not.

Broadened origins, narrow spots

The intentionally staggered origins of Figures 8A and 8B can be imagined superimposed, corresponding to an extremely broad origin. Figures 9A and 9B show corresponding conventional PMD chromatograms made from broad origins. Each origin became spread out by the single deposition of a larger-than-normal quantity of solution--as opposed to the repeated depositions and dryings of minimum liquid volumes necessary to increase sensitivity in conventional developments. Conventional chromatograms reflect a widened origin, but PMD spots do not.

Figure 10 shows a similar demonstration. Each of the four chromatograms in Figure 10 contained 100 nanograms of estrone and estradiol. The left PMD and the left conventional chromatograms were made from 1 microliter of concentrated solution (containing 100 ng estrogen/microliter); the right chromatograms were made from a single deposition of 100 microliters of dilute solution (containing 1 ng estrogen/microliter). PMD integrates the components nicely from the broadened

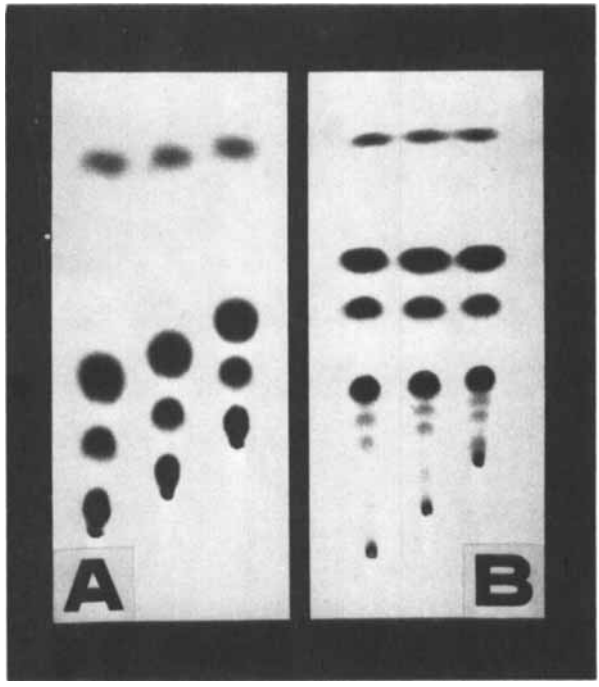
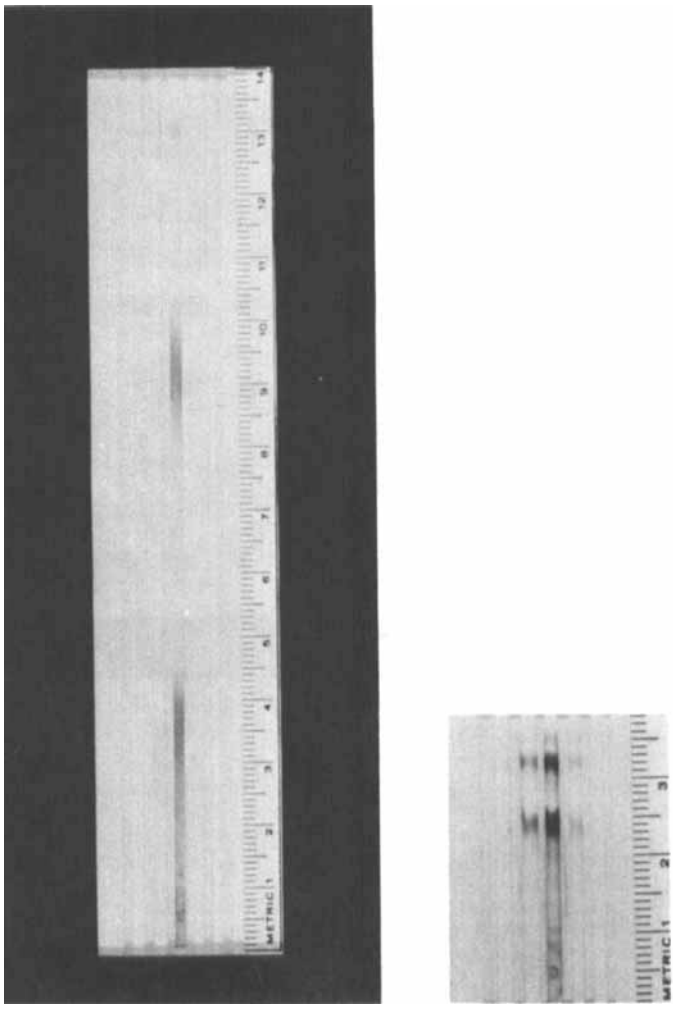


FIGURE 8

A. The final positions of conventionally developed TLC spots do reflect the positions of the spot origins, although one can see that the degree of the reflection is a function of R_f, even for a single development.

B. Even a very few PMD cycles nearly obliterates the developed spot-spot origin relationship, although a trace of this remains at very low R_f's.

origin, but such an origin renders the conventional chromatogram useless.



A. FIGURE 9 B.

A. Spreading during conventional development merely aggravates the spreading that occurred during deposition of 2 microliters on these 2 mm wide channels. The chromatograms of Figures 9A and 9B differ only in the manner of development. (Courtesy of the Journal of the Association of Official Analytical Chemists (3).)

B. PMD obliterates any effects from spreading during sample deposition--here, 2 microliters deposited on channels 2 mm wide. The chromatograms of Figures 9A and 9B differ only in the manner of development. (Courtesy of the Journal of the Association of Official Analytical Chemists (3).)

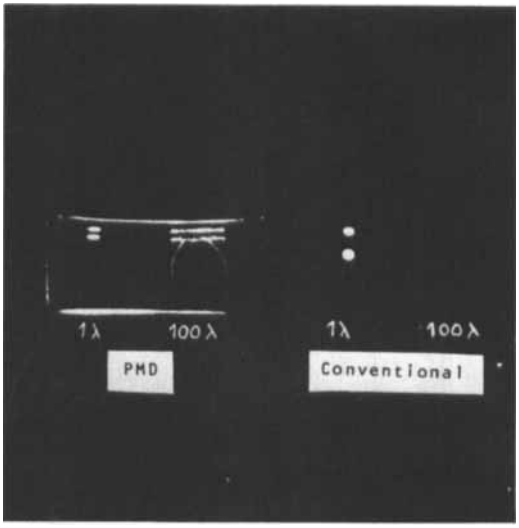


FIGURE 10

Each of these four chromatograms contained 100 nanograms of estrone and estradiol, deposited either from concentrated solutions (left) or dilute (right). The large spot from the dilute solution ruins the conventional chromatogram, but 7 PMD Mode 1 cycles yield the same resolution and sensitivity from the large origin as from the small. (Regis PMD News #4.)

Extraneous material in origin

The broadened origins that can be made usable by PMD include those having considerable material that is extraneous (some such origins look impossibly encrusted). Figure 11, as a first instance, shows PMD 5-cycle chromatograms made from quinine-spiked raw urine. The five arrow-indicated quinine spots correspond to quinine concentrations in urine of 5, 2.5, 1.25, 0.625, and zero micrograms per milliliter[4]. The

FIGURE 11

Each of these PMD chromatograms was made from a 50-microliter, quinine-spiked raw urine origin. The arrow-indicated, narrow quinine spot is nicely reconcentrated from the broadened, salt-filled origin. (Courtesy, American Laboratory (4).)

use of raw urine can eliminate preceding clean-up, thus simplifying urine screening (PMD intentionally accommodates parallel processing). The narrow (about 3 mm) quinine spots are well reconcentrated from the 20-mm wide origins. Sensitivity is good--an easily detectable 5 micrograms/milliliter.

Similar impossible-looking, broadened origins are shown in the outer two chromatograms of Figure 12. The origins of the left and right PMD chromatograms were made from single 150-microliter depositions of crude extract from peanut butter. The extract forming the left chromatogram was first spiked to represent 100 ppb afla-

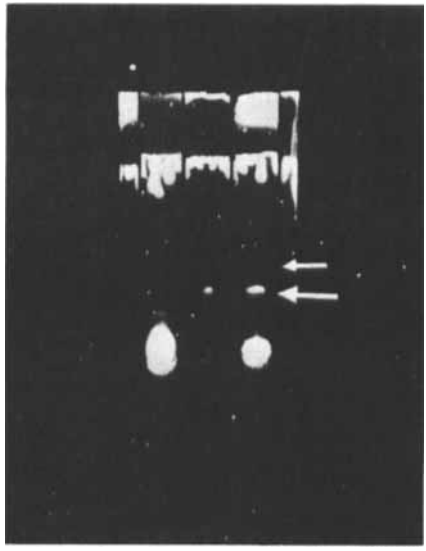


FIGURE 12

The origins to left and right were made from 150 microliters of spiked extract. After interferents were first washed ahead with benzene, the arrow-indicated aflatoxins B_1 and G_1 were Brought out by PMD. Standards are shown in the center chromatogram. Sensitivity corresponds to 100 ppb of each aflatoxin in the original sample. (Regis PMD News #8.)

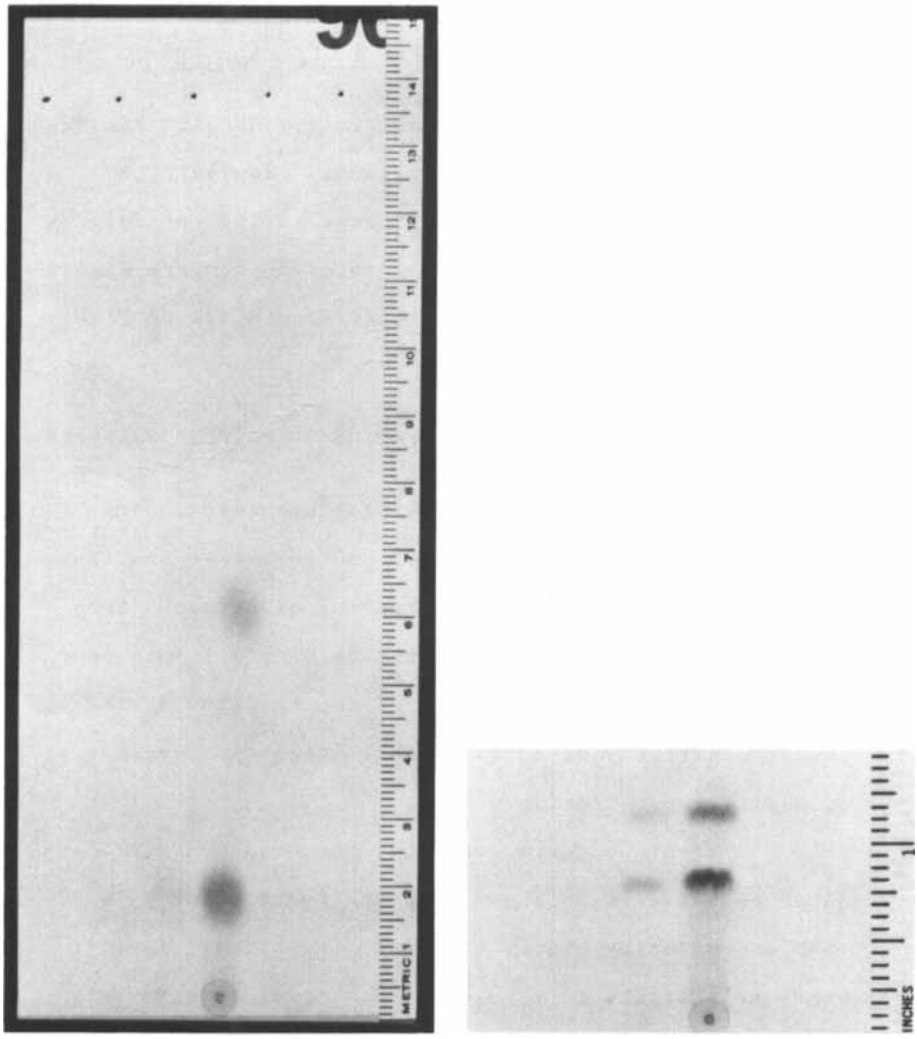
toxins B_1 and G_1. The chromatograms were then developed, first with benzene to wash away interfering matter, then with 7 Mode 3 PMD cycles using $CHCl_3$/THF/EtOAc 18/1/1, and nitrogen for solvent removal[21]. The developed aflatoxin spots, despite the origin, are narrow and well resolved, and show requisite sensitivity.

Sensitivity enhancement

The "sensitivity" of a spot, for any given method of detection, varies as the molecular density within the spot. Thus, again for a given method of detection, sensitivity is enhanced if spot molecular density is increased--a direct result of spot reconcentration. (As mentioned, we show later how sensitivity can be even further enhanced by causing the lateral edges of the PMD spots to move toward the centers, producing a spot of maximum molecular density that does not thereafter decrease by loss due to diffusion. This is called centered PMD; it is an experimentally simple and easy variation of PMD[10].)

In Figures 13A and 13B, five chromatograms were made on each plate from 2-microliter volumes of solutions of a dye. From left to right on each plate, the relative concentrations of the deposited solutions were 1, 10, 100, 2, and 0.2%. On the conventional chromatograms of Figure 13A, the major spots could be seen on the 10% chromatogram but not on the 2%. However, in Figure 13B the major spots on the 2% and 1% PMD chromatograms could be seen. Thus the PMD sensitivity enhancement for the major spots seems to be a factor of about 10 in Figure 13.

In Figures 9A and 9B, the 2-microliter volumes were deposited onto 2-mm wide strips. The narrow strips



A. B.

FIGURE 13

A. Five chromatograms were conventionally developon this unchanneled plate. The relative amounts of sample, left to right, were 1, 10, 100, 2, and 0.2. The plates shown in Figures 13A and 13B were identical in all respects, including the time of development--54 minutes--, except for the manner of development. (Courtesy of the Journal of the Association of Official Analytical Chemists (3).)

B. Both the absolute and the relative amounts of sample in the five chromatograms of this plate were the same as the plates shown in Figures 9A, 9B, and 13A. Compared to Figure 13A, this shows a sensitivity enhancement by PMD of a factor of about 10. Using narrow channels--see Figure 9B--yields an additional factor of about 5. (Courtesy of the Journal of the Association of Official Analytical Chemists (3).)

do not enhance conventional sensitivity--compare Figure 8A with Figure 13A, both conventional. However, the narrow strips do enhance the PMD sensitivity for major spots by an additional factor of about 5--compare Figure 9B, PMD, narrow strips, with either Figure 13A or Figure 9A, both conventional[3].

APPLICATION OF PMD TO THE SEPARATION OF COMPLEX MIXTURES

The following is an initial progress report on the demonstrative application of PMD to the separation of complex mixtures in one continuous operation. Such mixtures are assumed to be fractions of still more complex mixtures. The components of one fraction are assumed to differ substantially in R_f from the components of a different fraction.

Within a given fraction, the components are assumed to exist in profuse abundance and to show R_f's that are mutually highly similar. Selectivity improvements help little in resolving such components, merely shifting still-unresolved components around. Needed, rather, is the potential of the thin layer plate to display one resolved component every 20 to 50 particle diameters--even though random R_f distribution makes full resolution most improbable--, and the means to make this potential manifest.

Thoma's UMC theory

It may have been to the separation of the mutually similar components of such fractions that unidimensional multiple chromatography (UMC) was first applied[22]. The theory of UMC was developed and discussed by Thoma[15, 23], who considered the application of its "superb resolving power"[15] to the separation of homologs. He concluded, "There is no theoretical limit to the number of homologs which can be resolved by UMC if liquid-liquid partition is the only factor involved in resolution."

Thoma also commented that the guide lines suggested for selection of liquid-liquid operating conditions would apply as well to various other types of partitioning systems, and presented applications using paper and silica gel.

Before we examine the Thoma theory, we should consider to what degree its conclusions apply to PMD.

Thoma's theory (which antedated PMD by a decade) envisions only conventional solvent removals; the useful effects of UMC occur only during solvent advances. However, PMD solvent removals can and should develop spots and decrease spot widths at least as effectively as solvent advances. Whether PMD solvent removals actually provide these advantages can depend on the operator. If solvent removal is done poorly--

"poorly" usually means "with too fast a gas sweep"--, then useful PMD effects are also limited to solvent advances.

However, if PMD solvent removal is done well, so that the solvent front traverses every spot in the chromatogram and always recedes slowly compared to the forward movement of the spot being traversed, then one PMD cycle yields somewhat more resolution than two PMD solvent advances.

Thus, one reason Thoma's conclusions do not apply exactly to PMD is that the possibly useful effects of PMD solvent removal were not envisioned.

Thoma's conclusions were intended to apply only to UMC, wherein by definition the solvent advance distance remains constant. PMD Mode 1 approaches this. PMD Mode 1 allows one additional unit time to each successive solvent advance. At ambient temperature, the distance of solvent advance varies as the square root of the time allowed for that advance. Therefore the eleventh Mode 1 solvent advance nominally extends farther than the tenth by the square root of 11/10, or 5.5%; the twenty-first, 2.5% farther than the twentieth; the forty-first, 1.25% farther than the fortieth; and so on. PMD increasingly resembles UMC in distance of solvent advance, with increasing cycle number.

Thoma's conclusions for UMC apply fairly well to PMD solvent advances, particularly because his conclusions were stated for reduced separations. The reduced separation of two TLC spots is the center-to-center separation of these spots divided by the origin-to-front distance. (Thus, the single-development reduced separation of two identically developed spots equals the difference between the R_f's of these spots.) Having the conclusions so stated immediately erases the problem of correlating the usually sizeable, constant UMC solvent advances with the initially small but always increasing PMD solvent advances.

In sum, Thoma's conclusions can be applied to PMD with the following qualifications: The conclusions for reduced separation neglect spot width and thus resolution. Thoma's conclusions relating a given nominally achievable reduced separation to a corresponding required number of UMC developments can apply to PMD Mode 1: the required number of UMC developments should be divided by a PMD program-dependent factor. This factor should be about two; in producing reduced separations, PMD cycles should be at least twice as efficient as UMC developments. However, the factor could be less than one, in the following case.

If gas is used for solvent removal, and if the operator uses such a vigorous gas sweep that solvent re-

moval becomes practically instantaneous, then the benefits of solvent removal are simply discarded. Worse, in the process the PMD solvent becomes severely chilled and less efficient. In this case, one PMD cycle does not even equal one PMC development.

In his theoretical development, Thoma recognized and mathematically expressed the consequences of increasing the number of multiple developments while at the same time decreasing the solvent strength. A nice balance of the two keeps the chromatogram spread out between the origin and the solvent front. However, the expressions predict something else: not all the components become equally separated.

Certain components become better separated. The best-separated components are always found 0.632 (that is, $(1 - e^{-1})$) of the way from the origin to the solvent front. This is true no matter how many multiple developments have occurred. Furthermore, the reduced separation of these best-separated components increases as the number of multiple developments increases.

Thoma presented a family of curves showing the variation of reduced separation with the number of developments, for two components of an arbitrarily chosen degree of similarity. Each curve shows a maximum re-

duced separation. Each curve corresponds to the average single-development R_f of the two similar components.

The lower the single-development R_f, the higher the number of developments needed to bring the components to the 0.632 point. However, once the pair gets to that point, the greater their maximum reduced separation.

Thoma also showed that the number n_{opt} of multiple developments that produces the maximum reduced separation is related to the average single-development R_f as follows:

$$n_{opt} = \frac{-1}{\ln (1 - R_f)} . \qquad (6)$$

Thoma then showed--our Figure 14--the increase of maximum reduced separation with decrease of single-development R_f and corresponding increase of number of multiple developments (calculable from equation (6)), again for two components of arbitrarily chosen similarity. As we follow the curve on Figure 14 from right to left, we see that the maximum reduced separation increases more and more rapidly as the single-development R_f decreases and the corresponding optimum number of multiple developments increases. From Figure 14 and equation (6), we can find for the two arbitrarily similar components the average R_f's and the numbers of mul-

tiple developments required to achieve the reduced separation values we pick at will--here, 0.2, 0.3, and 0.4.

TABLE III

Optimum number of multiple developments	Average single-development R_f	Maximum reduced separation
16	0.06	0.2
50	0.02	0.3
133	0.007	0.4

This concept is singular in chromatography. It involves neither selectivity nor plate efficiency. It suggests that a purely mechanical action--repeated development--can open up an arbitrarily large proportion of the chromatographic bed to two components that though actually chromatographically different may appear inseparable.

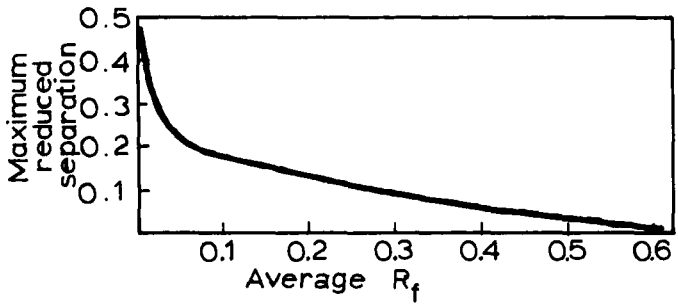


FIGURE 14

If the maximum reduced separation is plotted against the single-development R_f (and by inference of equation (6), the corresponding optimum number of multiple developments), the maximum reduced separation increases more and more sharply as the R_f approaches zero and the optimum number of multiple developments increases by equation (6). (15)

Theoretically, this concept holds without limit. Experimentally, one must find solvents of adequately low solvent strengths to produce the correspondingly, adequately low R_f's. Also, the spot loading must not cause overloading despite the low solvent strength. And the separation theoretically attained would certainly be vitiated by streaking. We shall not consider here whether practice can meet these demands of theory generally. It would seem necessary to demonstrate practical success in a given case. Given success, one would conclude that, for that case, the possible experimental obstacles had either not shown themselves or been overcome. But it would seem premature to conclude, before the fact, that experimental attempts generally would or would not succeed.

Also, Figure 14 and the just-cited table refer not to <u>any</u> two components but only to the two chosen for Figure 14. Two components, mutually more similar than these two, would show for each combination of R_f and number of multiple developments a smaller reduced separation than that indicated. The specific pair of components determines what reduced separation corresponds to what R_f-n_{opt} combination. Nevertheless, the reduced separation desired can be chosen at will.

(Nominally, continuous development can accomplish a better center-to-center separation than any alter-

native approach: in chromatography generally, components separate faster from each other than they spread into each other[24]. In practice, attempting a continuous development separation like this--deliberately selecting a solvent to produce such low R_f's in order eventually to realize a chosen separation--would make the already impractically slow continuous development, practically impossible (and the spots, completely undetectable).)

Making a set of demonstrative PMD separations of an arbitrarily complex mixture seemed like a good way to test the predictions of Thoma, to apply PMD in a new way, and thus also to extend the uses of TLC. Moreover, we could use these separations as a vehicle for trying out some other ideas and accomplishing some long-standing objectives with regard to both PMD and the TLC plate.

With regard to PMD, we wished to demonstrate the hitherto unused capability of the programmer to conduct long as well as short programs; to show lateral as well as longitudinal spot reconcentration on uniform non-channeled plates; and to move PMD spots apart without having them spread--to achieve arbitrarily large center-to-center separations of these spots without sacrificing their characteristic high molecular density.

With regard to the TLC plate, we wished to demonstrate the presently unused capability of the TLC

plate to achieve and display highly detailed separations; and to use the whole TLC plate with uniform efficiency--to place some non-special spot or spots an arbitrary but relatively large distance, say, 10 cm, from the origin.

To accomplish these objectives in the same set of experiments, we prepared a reproducibly complex mixture of dyes by mixing equal volumes of three commercial dye solutions that are mixtures themselves. Spots from the complex mixture were developed by a conventional single development and by a number of PMD Mode 1 programs. From these, we selected separations made by 9, 10, 32, and 68 cycles[11]. To make these programs as long as possible for a given cycle number, we used the maximum unit time--100 seconds--, thus causing the longest program to last 72 hours. To achieve lateral as well as longitudinal spot reconcentration, we applied a new technique.

Centered PMD

Achieving lateral as well as longitudinal spot reconcentration on ordinary non-channeled commercial plates is experimentally simple[10]. It should be useful in short as well as long PMD programs, because it maximizes molecular density and thus sensitivity. In any program, it prevents molecules from diffusing away from

the spot; sensitivity therefore becomes maximum and thereafter remains constant, at least with respect to molecular loss due to diffusion, no matter how long the program. The resulting spot sizes become functions of spot loading down to a certain minimum loading. The smallest spots are one to two millimeters in diameter--considerably narrower than the channels on commercially channeled plates.

Lateral spot reconcentration is achieved by causing selective evaporation along the line of the chromatogram. This is done most simply by interposing a slotted mask (Fig. 15) between the radiator and the plate. The mask is attached to the plate-spacer-facing plate assembly, on the back of the thin layer plate (Fig. 16).

With such a mask in place, direct radiation comes onto the thin layer plate only through the slots, which are directly over the chromatograms (during spotting, the mask is used as a spotting guide). Therefore evaporation is selectively increased along the chromatograms during solvent removal. Shortly after radiation begins, the solvent front dips, one dip per slot and thus per chromatogram. The dips recede toward the origin. Solvent, which always moves toward the front, moves from the sides of the chromatogram into the dipped front. The lateral components of the solvent movements

cause the laterally more outward spot molecules to move toward their respective spot centers; the spots become laterally reconcentrated.

Lateral spot reconcentration can be brought about in other ways--any way that will cause such lines

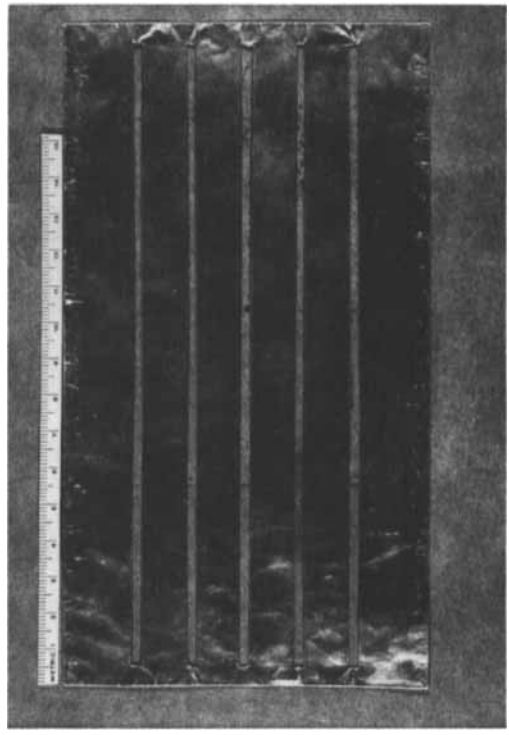


FIGURE 15

A simple reflective and thermally insulating slotted mask interposed between the thin layer plate and the radiator causes lateral spot reconcentration during solvent removal. (Courtesy of the Journal of Chromatography (10).)

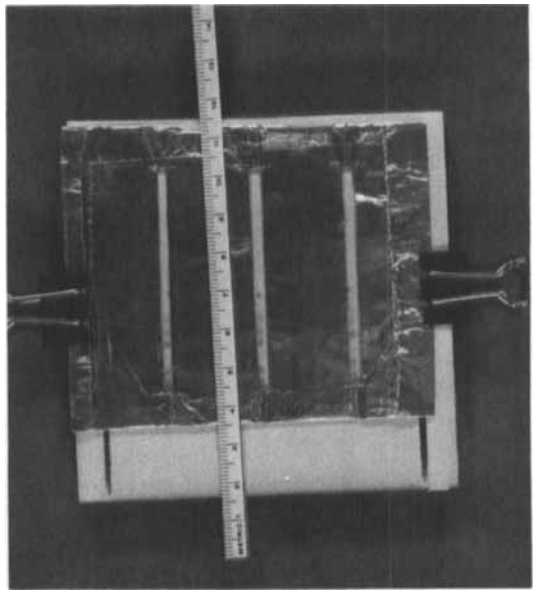


FIGURE 16

In use for lateral spot reconcentration, the mask is clamped to the thin layer plate assembly. (Courtesy of the Journal of Chromatography (10).)

of preferred evaporation. Grooving the TLC bed produces the effect by decreasing the thickness of the wetted bed (this has been done, but is not accurately reproducible at this writing, whereas masks are). Arranging a gas sweep so the gas emerges in thin strips close to--say, 1 mm distant from--and aligned with the chromatograms should produce the effect, although this has not been tried at this writing.

The requirement for effective lateral spot reconcentration is exactly the requirement for effective

longitudinal (top-to-bottom) spot reconcentration: the solvent front should traverse every spot in the chromatogram slowly with respect to the forward velocity of the spot being traversed.

We refer to that PMD that incorporates lateral spot reconcentration as centered PMD.

The sensitivity advantage of centered PMD over conventional single-development TLC is illustrated in Figures 17, 18, and 19[11]. Figure 17 shows three conventional single-development chromatograms. The center chromatogram was made from full-strength dye solution, whereas the outer two were made from solution one-tenth as strong. Spreading quickly ruined the outer two conventional chromatograms.

Figures 18 (9 cycles) was developed for 90 minutes overall, about the same time as Figure 17. The sensitivity advantage ranges from a factor of about four to a factor of about 20, increasing with R_f.

Figure 19 (32 cycles) took 17 hours. The spots are weaker than those of Figure 18 because they are less overlapped and also partly photo-decomposed, bleached. But they are still clearly visible. And, they are equally visible at all R_f's and have not spread.

Every one of our stated objectives is shown attained in the 68-cycle, 72-hour development of Figure 20. The long-program capability of the programmer is

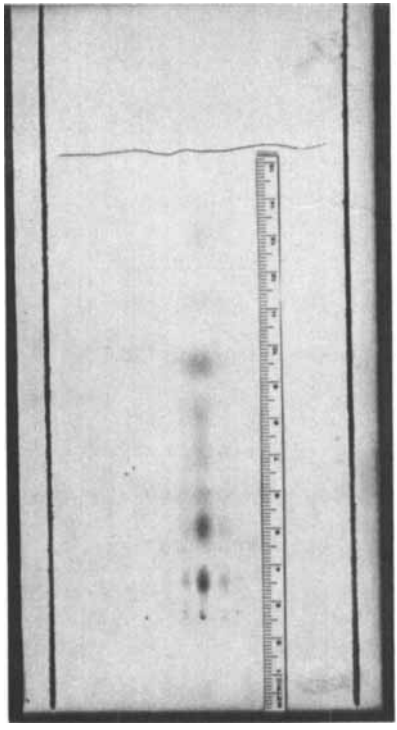


FIGURE 17

Three conventional single-development chromatograms. The outer chromatograms contain one tenth as much sample as the center chromatogram; the 80-minute development with its accompanying spreading quickly renders these outer chromatograms useless. (Courtest of the Journal of Chromatography (11).)

demonstrated. The spots are laterally reconcentrated and kept that way while being moved, in some cases, over 10 cm from the origin. The capability of the commercial TLC plate for separations of this detail is demonstrated; and of much greater detail, suggested. The plate

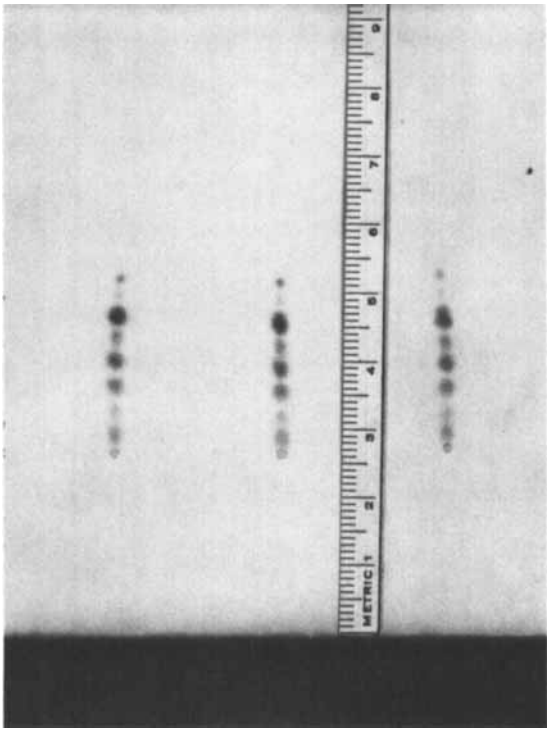


FIGURE 18

A 9-cycle PMD chromatogram of the same amount and composition of dye mixture as used for the outer two chromatograms of Figure 17. The sensitivity advantage increases with R_f to a factor of perhaps 20. This chromatogram shows the effects of both lateral spot reconcentration and gentle radiation during solvent advance, both of which increase sensitivity. (Courtesy of the Journal of Chromatography (11).)

was used with uniform spot-per-distance efficiency; spot sizes were determined solely by spot loading.

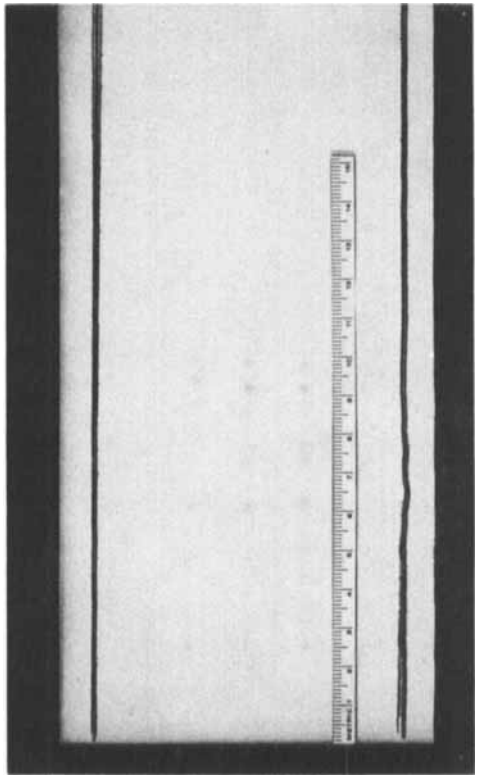


FIGURE 19

A 32-cycle centered-PMD chromatogram that lasted 17 hours. The sample corresponded in amount and composition to the samples of Figures 17 and 18. Some sensitivity has been lost to bleaching, but not to diffusion away from the spots. The additional developments have markedly improved resolution--see also Figures 21 and 22 for spot and chromatogram correlation. (Courtesy of the Journal of Chromatography (11).)

An experimental separation

Figures 19 and 20 complement Figure 18 in demonstrating the detailed-separation capability of the

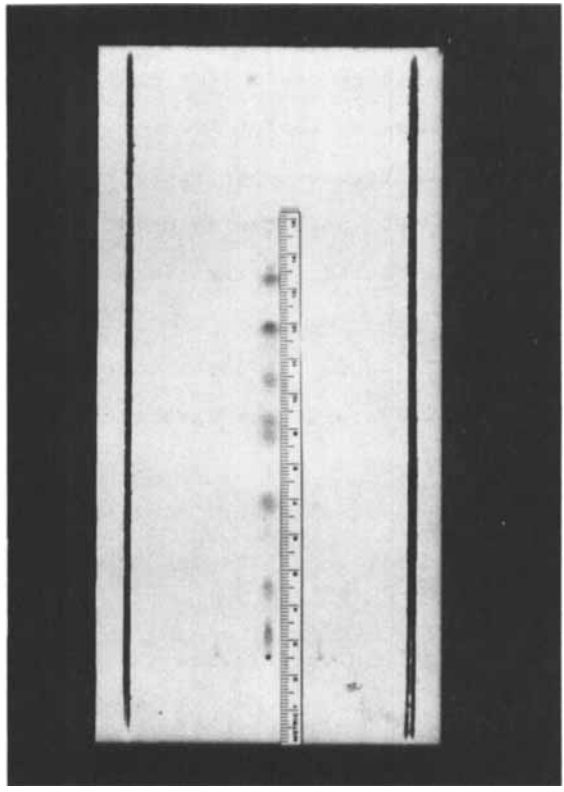


FIGURE 20

This 68-cycle, centered-PMD chromatogram lasted 72 hours without sensitivity loss due to diffusion away from the spots. Notice spots 1, 2, and 3 (see Figures 21 and 22 for spot identities) which have not been obliterated by the nearby major spot 4 or lost by spreading. Compare these spots to those in the outer two chromatograms of Figure 17. Notice also that the commercial TLC plate could have accommodated a still more complex mixture. (Courtesy of the Journal of Chromatography (11).)

TLC plate. Yet the particle size of the plates used for these chromatograms is about 10 times the 1-to-5 micro-

meter diameter considered optimal[25]; thus the presently demonstrated capability could (and can) be increased by at least the same factor of 10.

These two figures--particularly Figure 20--also demonstrate that PMD spots can be moved apart at will, without diffusion-caused decrease in spot molecular density.

Figure 21 shows the chromatograms redrawn to scale and roughly centered for ease of comparison. Chromatogram A (shown in Figure 17, center) came from a conventional single development. Chromatograms B (Fig. 18), C, D (Figure 19), and E (Figure 20) were all made by centered PMD.

Tables IV and V correlate the chromatograms and spots.

Figure 22 shows the chromatograms redrawn and normalized for the distance from the origin to spot 4. This approach does not reveal any change in center-to-center separation. It also covers most of each chromatogram. Figure 22 allows us to correlate these experimental results with the Thoma predictions.

Thoma's theory predicts an increase in the proportions of the bed allotted to certain components as the number of multiple developments increases. If the theory is invalid, all the spot-connecting lines on

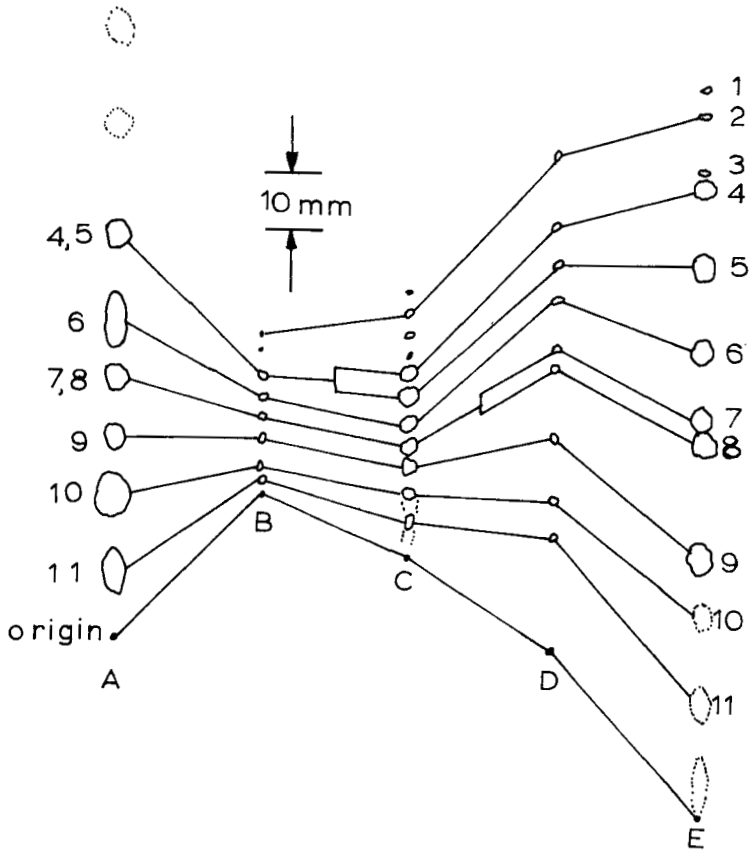


FIGURE 21

Chromatograms A (Fig. 17), B (Fig. 18), C, D (Fig. 19), and E (Fig. 20), redrawn to scale as shown (Courtesy of the Journal of Chromatography (11)).

Figure 22 between one chromatogram and the next should be roughly parallel. But if the theory is valid, then a sizeable increase in the number of multiple developments should cause the lines to diverge; this obtains.

TABLE IV

Chromatogram[a]	A	B	C	D	E
Figure	17	18		19	20
Cycles	1[b]	9[c]	10[d]	32[d]	68[d]
Hours overall	1.3	1.5	1.9	17.	72.
Dilution spotted[e]	1.0	0.1	1.0	0.1	1.0
Solvent strength[f]	0.32[g]	0.30[h]	0.30[h]	0.30[h]	0.27[h]

a The chromatograms are redrawn to scale in Figure 21 and redrawn normalized with respect to the origin-to-spot 4 distance in Figure 22.

b Conventional development.

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

d 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.

e One microliter per spot. Solution composition, equal volumes of Camag Dye Mixtures I, II, and IIN.

f Solvent strength determined from ref. 26.

g Benzene.

h Tuned solvents (Regis Chemical Company).

TABLE V

Spot number	Color	Spot position in mm, Chromatogram 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

Chromatograms C, D, and E correspond to 10, 32, and 68 cycles, respectively. As the number of cycles increases, the solvent strength should be decreased, to hold the chromatogram roughly centered between origin and solvent front. This was not done between chromatograms C and D; both were developed with the same solvent. However, the solvent strength was decreased for chromatogram E.

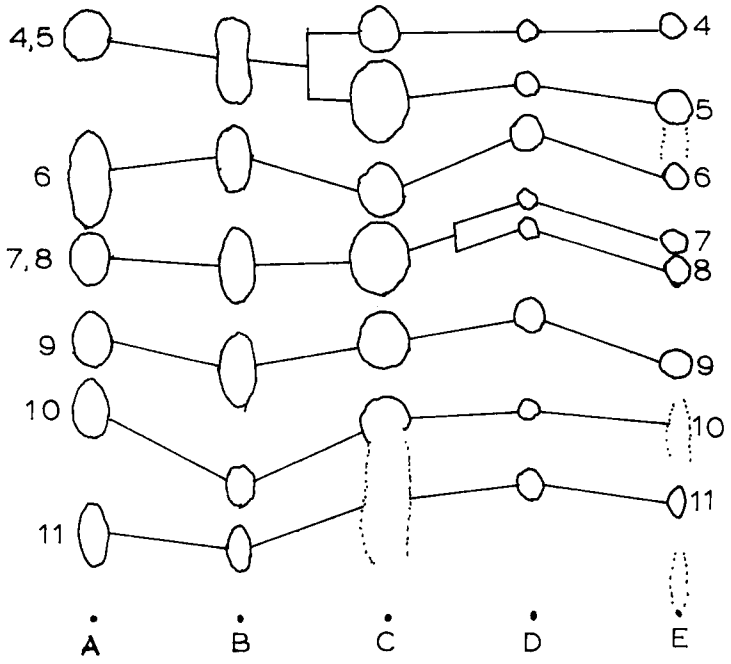


FIGURE 22

Chromatograms A (Fig. 17), B (Fig. 18), C, D (Fig. 19), and E (Fig. 20), redrawn normalized with respect to the distance from the origin to the center of spot 4 (Courtesy of the Journal of Chromatography (11)).

With the same solvent used for both chromatograms C and D, we can see the expected crowding of spots toward the front in chromatogram D. However, the movement and crowding is not uniform. The Thoma-predicted increase in allotted proportion of the bed, or gap, does seem to appear between spots 8 and 9. Otherwise, had the gap not appeared, the lines connecting spots 9, 10,

and 11 would be expected to slope upwards as much as those for spots 6, 7, and 8.

With a weaker solvent for chromatogram E than for chromatogram D, the chromatogram drops back toward the origin and the spots are again better distributed. However, by definition, spot 4 does not drop back. Thus the Thoma-predicted gap appears in this case between spots 4 and 5.

That the predicted gap should move from one location within the chromatogram to another is rather to be expected. Equation (6) shows a definite relationship between the optimum number of developments and R_f, which is to say, solvent strength. If the solvent strength is not that required by equation (6), then the reduced separation achieved is not the maximum but some other, and the position for the greatest reduced separation that has been achieved is not at a point 0.632 of the way from the origin to the front but at some other position. This agrees with what we see.

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