# Programmed Multiple Development in Thin-Layer Chromatography

Thomas H. Jupille and John A. Perry

The recent development of high performance thin-layer chromatography (TLC) techniques is transforming TLC into a new analytical and preparative tool (*1*). Two approaches to increasing the efficiency of TLC have been taken. One approach, the use of smaller, more closely sized particles in the thin layer (*2*), represents the application of general chromatographic principles to TLC. The second, Programmed Multiple Development (PMD) (*3, 4*) represents a novel exploitation of TLC phenomena.

## Factors Affecting Resolution in Chromatography

As a sample is carried through a chromatographic system, its different components travel at different average speeds that depend on the degree to which the components are retarded by the stationary phase. The sample thus becomes divided into different zones separated by distance (along a column or bed) or time (of emergence from a column outlet). The separation between zone centers depends on the selectivity of the system and on the length of column or bed traversed (*5*). As zones migrate, however, they also broaden. The width of a zone depends on the efficiency of the system and on the square root of the column or bed length.

The resolution between adjacent zones is the ratio of separation to average zone width. Inadequate resolution can be improved by (i) lengthening the column or bed, (ii) increasing the selectivity, or (iii) increasing the efficiency. Only limited improvements can be made by lengthening the column or bed. Resolution is directly proportional only to the square root of bed length. Thus, a threefold improvement in resolution requires a ninefold increase in bed length (*6*). Extremely long beds tend to require high inlet pressures for acceptable operation, result in drastically increased separation times, and produce highly diffuse, and therefore hard to detect, component zones.

A more subtle approach to improving resolution is to increase selectivity (*7*). As chromatographic techniques are improved, however, the limitations imposed by low efficiency become increasingly obvious (*8*) and the emphasis shifts toward improved efficiency. The development of higher efficiency then removes the burden of resolution from selectivity alone. The term "high performance" is used to describe a technique that emphasizes efficiency.

## PMD Spot Reconcentration

Programmed Multiple Development is defined as the repeated development of a TLC plate with the same solvent in the same direction for gradually increasing distances. Between developments, the plate is dried by controlled evaporation while it remains in contact with the solvent reservoir (*9, 10*). The evaporation is accomplished by heating the back of the TLC plate or by passing a stream of inert gas across the front surface of the plate.

The technique of PMD exploits the existence of an interface, the solvent front, which moves through the stationary bed. In TLC, the width of a spot on the stationary bed is usually large compared to the diameter of the stationary phase particles so that the lower edge of the spot is carried forward while the upper edge is still high and dry. Width throughout this article is defined as the distance between the leading and trailing edges of a spot. Because sample molecules travel at an average rate that is $R_F$ times the solvent velocity (*11*), the lower edge of the spot travels $R_F$ times the spot width before the upper edge begins to move (Fig. 1). The final spot width thus becomes, to a first approximation, $(1 - R_F)$ times the initial width.

Spot reconcentration occurs once in conventional TLC, at the beginning of development. If, however, the development is repeated $n$ times, the final width is expected to decrease to $(1 - R_F)^n$ times the initial width (*12*). In practice, observed spot widths do decrease with successive development, but the effect is less dramatic than the expression $(1 - R_F)^n$ would indicate. The difference results from spot broadening during development. The lower limit on spot width is set by the efficiency of the TLC system.

Most studies of multiple development have focused not on spot width but on separation. It has been shown, for instance, that in Unidimensional Multiple Chromatography the center-to-center separation between close spots increases, passes through a maximum, and then begins to decrease with successive developments (*10*).

It is instructive to examine spot width and separation simultaneously. Figure 2 shows the expected behavior of two spots with $R_F$ values of 0.25 and 0.29, that is, moving at 25 percent and 29 percent, respectively, of the solvent velocity. The spots are assumed to be initially 1 cm in diameter, and to undergo a series of developments in which the solvent front travels 10 cm from the origin. Conventional multiple development practice would dictate that the process be stopped after three such developments. This would provide maximum separation, but would not allow effective spot reconcentration.

A different picture would emerge if successive developments were carried out to increasing distances from the origin (*13*). Figure 3 shows the expected behavior of the same spots discussed above during a series of developments to progressively longer distances (a 1-cm development, then a 2-cm development, then a 3-cm development, and so on). A salient feature of this type of multiple development is that it allows effective reconcentration with minimum sacrifice of separation. Narrower spots at a given separation mean increased efficiency.

While spot reconcentration during solvent advance occurs in all types of multiple development, PMD allows an additional spot reconcentration during solvent removal (see Fig. 4).

Figure 4 demonstrates that spot widths in PMD rapidly approach a steady state minimum at which broadening and recon-

Mr. Jupille is director of analytical chemistry at Regis Chemical Company, Morton Grove, Illinois 60053, and Dr. Perry is a consultant in analytical chemistry in Chicago, Illinois.

centration are roughly balanced. As a result, spot widths on PMD chromatograms reflect the inherent efficiency of the TLC system used. A comparison of PMD separations of steroids on different brands of commercial precoated TLC plates reveals significant variations in performance. The separation of androsterone from etiocholanone, for example, is easily attainable on some brands of TLC plates but not on others under identical conditions (*14*).

In PMD, the plate remains in contact with the solvent reservoir during both solvent advance (development) and solvent removal (drying) (*9*). Drying of the plate is accomplished by increasing the rate of solvent evaporation. The solvent front then recedes through the bed until only enough wetted area remains to evaporate all of the solvent which enters the thin-layer bed. During the recession of the solvent front, the solvent continues to flow upward. As the solvent front recedes through a spot on the thin layer, the lower edge of the spot moves forward while the upper edge is dry. The result is reconcentration of the spot.

The magnitude of this spot reconcentration varies with the solvent front recession velocity and with the $R_F$ of the spot. To a first approximation, however, it is as effective as the reconcentration which occurs during solvent advance. For spots with low $R_F$ values, reconcentration during solvent removal is more effective than is reconcentration during solvent advance. The two effects are thus complementary (*15*).

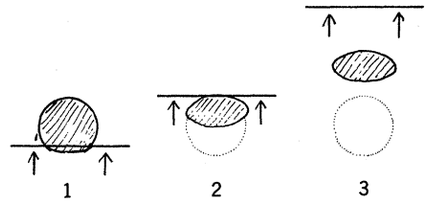



Fig. 1 (left). Spot reconcentration in TLC. (1) The advancing solvent front contacts the lower edge of the spot while the upper edge is still dry. (2) By the time the upper edge begins to move, the lower edge has traveled $R_F$ times the initial spot width. (3) The spot width has thus been reduced to $(1 - R_F)$ times its initial value at the beginning of the development.

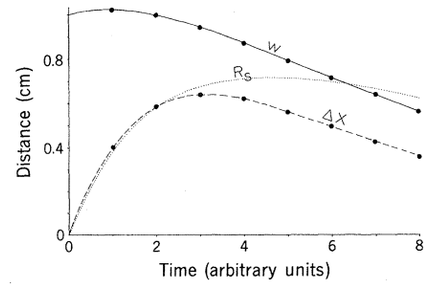



Fig. 2 (right). Expected spot behavior in Unidimensional Multiple Chromatography. The expected separation (solid line), spot width (dashed line), and resolution (dotted line) for spots of $R_F$ 0.25 and 0.29 through a series of 10-cm developments (the values at the end of each development are shown as circles). The spots are assumed to have an initial width of 1.0 cm. The plate height of the system is assumed constant at 0.002 cm. The time required for a single 10-cm development is taken as unity.

oxidative or thermal degradation of sensitive samples associated with the use of heat (Fig. 7) (*17*).

The on-off sequence of gas flow or heat, or both, which constitutes a PMD program is carried out automatically by the electronic PMD programmer. Program parameters such as the number, duration, and progression of development cycles can be preset on the programmer for a specific separation (Table 1). Typical PMD programs last about 1 hour, although programs as short as 9 minutes and as long as 72 hours have been used (*18*). The programmer can execute programs of from 1 to 99 development cycles lasting from less than a minute to as long as 10 days.

Thin-layer plates are prepared for PMD as they would be for conventional sandwich chamber TLC (*19*). A solution of the sample is applied as a spot 2.5 to 4.0 cm from the bottom edge of the TLC plate (*20*). The plate is put in the sandwich chamber and is then positioned in the PMD developer. Once the solvent front has reached the origin, the chromatographer pushes the "start" button and the program is then carried out automatically.

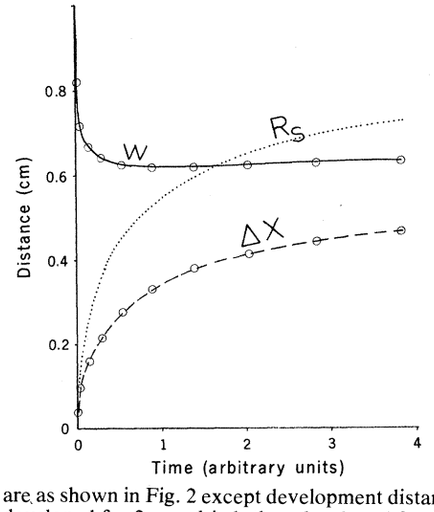
A PMD separation procedure requires a stationary phase, a mobile phase, and appropriate program parameters.

Silica gel is the most commonly used stationary phase in PMD, as it is in conventional TLC (*21*). Although different brands of commercial TLC plates differ somewhat in selectivity and flow rate, the most striking differences are those in efficiency mentioned earlier. We have found the newer "hard layer" TLC plates, which use polymer binders, to be

## PMD Procedures and Hardware

The original paper on PMD (*4*) described an apparatus for carrying out PMD that has undergone only minor change in the past 3 years. Figure 5 shows the TLC plate enclosed in a sandwich chamber and held in a frame which allows the back of the plate to be heated by infrared radiation. Development procedes normally as long as the heater is off. When the heater is turned on, however, the solvent front recedes to a steady state position. When the heater is turned off, the plate begins to cool, and development begins again.

A modification to the sandwich chamber (*16*) permits solvent removal by means of a stream of nitrogen (or other gas) down the front surface of the TLC plate, either with or without additional heat input (Fig. 6). The use of nitrogen for solvent removal avoids the risk of



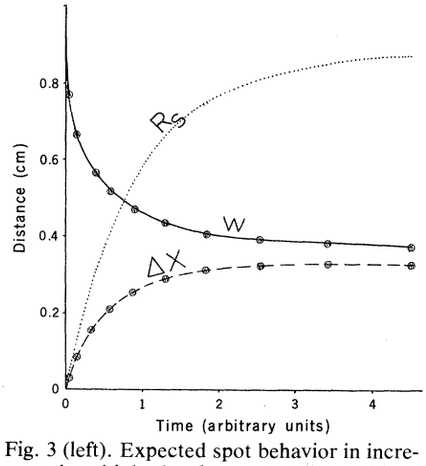




Fig. 3 (left). Expected spot behavior in incremental multiple development. All conditions are as shown in Fig. 2 except development distance. The plate is developed for 1 cm, dried, then developed for 2 cm, dried, then developed for 3 cm, and so on through a 10-cm final development.

Fig. 4 (right). Expected spot behavior in PMD. All conditions are as shown in Fig. 3 except the drying procedure. The plate is left in contact with the solvent reservoir while the solvent front is made to recede at 0.1 cm per second. The solvent flow velocity is assumed to be 0.067 cm per second at the reservoir level and to decrease linearly to zero at the solvent front during solvent removal.

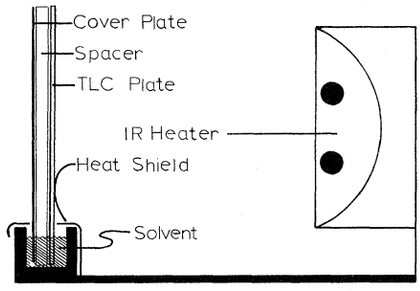



Fig. 5. Schematic diagram of PMD developer unit. The PMD developer holds a TLC plate in a sandwich chamber in such a position that it can be periodically heated with an infrared source.

generally superior in this respect to the softer silica gel G (gypsum binder) plates.

The optimum PMD solvent for a given separation is usually considerably different from its conventional TLC counterpart. Although PMD solvent selection cannot be divorced completely from a consideration of program parameters, a few generalizations can be made.

The requirement that PMD solvent systems be highly volatile is not ironclad; we have, for example, used such relatively nonvolatile solvent systems as dimethyl formamide and water successfully. However, solvent systems incorporating nonvolatile buffers or exceedingly tenacious solvents such as dimethyl sulfoxide cannot be used. Volatility becomes a consideration primarily in choosing between otherwise similar solvent components. Thus, benzene is preferable to toluene and isopropanol to butanol in most applications.

Solvent mixtures are not excluded from use in PMD. Surprisingly wide boiling ranges can be tolerated. Prudence, however, dictates that the boiling points of solvent components should be matched when possible. Thus, ethylene dichloride is often used rather than chloroform in mixtures with ethyl acetate (*22*).

Contrary to early fears, changes in solvent composition during the course of a PMD program do not generally severely affect resolution. In most PMD programs, the volume of solvent lost by evaporation is relatively small. A typical program lasting 1 hour, for example, might incorporate only 10 minutes or so of solvent removal and use 10 to 15 ml of solvent (this for a mixture of ethylene dichloride and ethyl acetate). Changes in composition that do occur are normally taken into account in choosing the initial solvent proportions.

The choice of program parameters can have a startling effect on the appearance of the final chromatogram. Of these parameters, the most fundamental is the mode, which represents the dependence of development time on cycle number. The PMD programmer allows a choice of three modes (Table 1). Mode 1 sets development time so that it is directly proportional to cycle number, that is, the second advance is twice as long as the first, the third advance is three times as long, and so on. Since development distance is proportional to the square root of development time in TLC, the effects of PMD mode 1 approach those of conventional multiple development after a large number of cycles. Mode 3 sets development time so that it is proportional to the square of the cycle number (that is, the second advance is four times as long as the first, the third advance is nine times as long, and so on). This has the effect of setting development distance so that it is directly proportional to cycle number, and gives rise to spot behavior like that shown in Fig. 4. Mode 2 is the arithmetic mean of modes 1 and 3, that is, the second cycle is three times as long as the first, the third cycle is six times as long as the first, and so on.

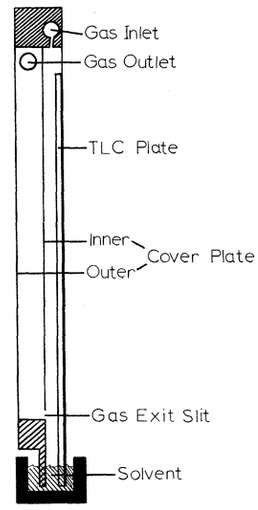



Fig. 6. Schematic diagram of PMD gas-flush chamber. This modified sandwich chamber allows the plate to be dried by a stream of nitrogen flowing down across the front surface of the plate. The double chamber design minimizes loss of solvent vapor to the outside during development.

The successful application of PMD to new problems is not difficult. It does,

Table 1. Parameters for PMD. The parameters are entered into the PMD programmer by means of thumbwheel switches on the front panel. They determine the sequence of development and drying which constitutes a PMD program.

| Parameter | Symbol | Limits | Function |
|---|---|---|---|
| Cycles | $n$ | 1 to 99 | Sets the number of developments and dryings in the program |
| Mode | Mode | 1,2, or 3 | Establishes the dependence of development time, $T_n$, on cycle number, $n$. Mode 1, $T_n \alpha n$; mode 2, $T_n \alpha n\,(n + 1)/2$; mode 3, $T_n \alpha n^2$ |
| Solvent removal | $F$ or $S$ | | $F$ is the fixed (constant) drying time between developments; $S$ is the scheduled (proportional to development) drying time between developments |
| Advance time | $t_a$ | 10 to 100 seconds | Sets time for first development; subsequent development times are multiples of $t_a$ |
| Advance power | $p_a$ | 0 to 12.5 percent | Sets the power level of the infrared heaters (100 percent equals 400 watts) during development |
| Removal time | $t_r$ | 0 to 100 seconds | Sets time for drying after the first development. Drying time after subsequent developments is either equal to (fixed) or a multiple of (scheduled) $t_r$ |
| Removal power | $p_r$ | 0 to 100 percent ($F$) or 0 to 50 percent ($S$) | Sets power level of the infrared heaters during drying |
| Preheat time | $t_{pr}$ | 0 to 100 seconds | Sets a preconditioning time before the first development during which the heater is controlled by the removal power level |
| Interim power | $p_{int}$ | 0 to 100 seconds | Sets the power level of the infrared heaters before, after, and during pauses in the program |

however, demand of the chromatographer an understanding of two processes which occur whenever multicomponent solvent systems are used in TLC: solvent demixing and vapor phase transfer.

Solvent demixing arises, particularly in adsorption chromatography, from the preferential retention of polar solvent components by the stationary phase (*23*). As a result, the area of the bed immediately behind the solvent front is depleted of the more polar components of the solvent. A binary solvent system will therefore give rise to two zones of constant polarity (nonpolar near the solvent front, polar near the origin) separated by a transition region (*24*). The transition may be gradual or it may be so abrupt as to constitute a secondary solvent front. In general, abrupt transitions are associated with solvent systems whose components differ greatly in polarity.

Separations occurring entirely within zones of constant polarity generally exhibit easily predictable PMD behavior. Reconcentration of spots in the lower zone, however, occurs when the spot is traversed by the secondary front rather than by the primary front.

Separations occurring in a transition region pose a more complex problem. Spots are typically narrow (the lower edge of the spot is in contact with a more polar solvent than is the upper edge). Improved resolution then requires increased separation, which is not usually the primary function of PMD. The best general approach to such a problem is the use of a homogeneous solvent which separates the components of interest, but produces broad spots. These spots can then be reconcentrated by PMD to improve resolution.

Vapor phase transfer from the solvent to the dry portion of the bed occurs in any thin-layer system in which the plate, solvent, and chamber atmosphere have not been extensively equilibrated. Such layer conditioning can significantly affect selectivity. Sandwich chambers, however, are prone to uneven saturation; this can give rise to variations in layer activity across the plate. The effect is particularly pronounced with solvent systems which contain a small percentage of a polar component in a nonpolar carrier (*25*). The result in PMD has been called the "necklace effect" because of the characteristic curvature of the spot positions (Fig. 8). The necklace effect in PMD can be reduced or eliminated by decreasing the polarity difference between the solvent components or by deactivating the plate surface.

Constant plate deactivation is readily and conveniently maintained in PMD by using vapor-saturated nitrogen for solvent removal. The nitrogen can be saturated with water (or any other) vapor by being bubbled through an appropriate solution before it enters the sandwich chamber (*26*). Saturation of the nitrogen in this manner is the recommended way of introducing traces of acid or base into a PMD system. The use of vapor phase transfer rather than addition to the solvent minimizes the adverse effects of solvent demixing.

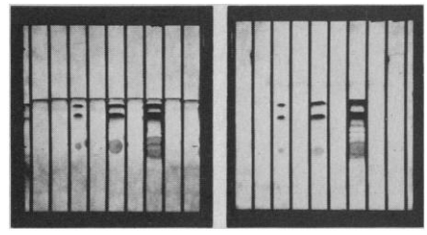
A feature of PMD that is shared, although less dramatically, by other types of multiple development is the position reproducibility of separated spots, regardless of origin location (*27*). Spot alignment shares the mechanism of spot reconcentration: lower spots move while upper spots do not (*28*). The opportunity for alignment in PMD occurs during both solvent advance and solvent removal. One result of spot alignment is the recommended use of spot position rather than $R_F$ value to characterize sample components in PMD (*16, 29*).

The combination of spot reconcentration and alignment allows the formation of an "intrinsic grid" for qualitative sample component identification (*30*). The grid is a series of fine horizontal lines on the developed TLC plate. The lines consist of standards of the components of interest in the samples. They unequivocally establish the locations of these components on the TLC plate. The grid is generated by applying a dilute solution of the standard mixture as a broad streak (extending horizontally across the plate) at or below the origin. Samples for qualitative analysis are then spotted in the normal manner and the plate is developed by PMD. Spot reconcentration compresses the initial broad streak of standards as it is separated into its components. Merger of a standard line with a spot in the sample confirms the presence of that component (Fig. 9) (*31*).

The spot reconcentration which we have discussed so far occurs only in the direction of spot migration. As a result, the typical spot on a PMD chromatogram is an elliptical band; the (reconcentrated) top-to-bottom width is considerably smaller than the side-to-side width (which has increased unchecked). The side-to-side width can, however, be limited. Two approaches have been taken.

The more straightforward approach involves the use of narrow (typically 2-mm wide) channels in the TLC plate to physically contain horizontal spread during development. The vertical spreading that occurs during development is, of course, counteracted by PMD spot reconcentration (*32*).

A more elegant approach, called centered PMD, brings about a dynamic horizontal spot reconcentration during solvent removal (*33*). This is accomplished by heating or by passing nitrogen along only the center line of the chromatogram. In either case, the effect is to increase the rate of evaporation at the center line over that of the surrounding plate. This causes a horizontal flow of solvent toward the center line and thus a horizontal compression of the spots. Circular spots 2 mm or less in diameter can be maintained with this technique.



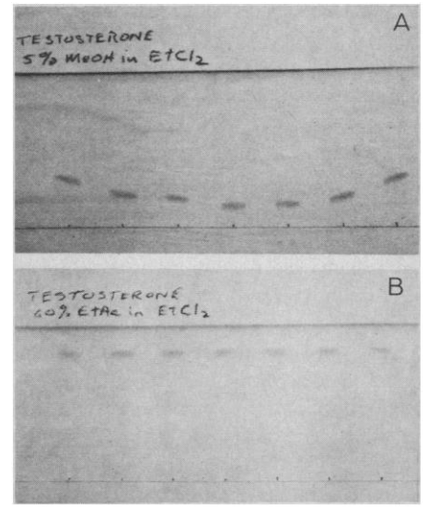



Fig. 7 (left). Effect of solvent removal on sample stability. The plate on the right was dried by heat; the plate on the left was dried with nitrogen. Note the effect on the formation of ghost spots below estradiol. The sample spots are (top to bottom): estrone, estradiol, and estriol. The solvent was a mixture of ethylene dichloride and ethyl acetate (10 : 1). The PMD programs required approximately 40 minutes. Fig. 8 (right). The "necklace effect." Uneven saturation of a sandwich chamber can produce variations in plate activity. The effect is particularly pronounced when solvent system components differ widely in polarity. Plate A was developed with an ethylene dichloride–methanol (19:1) solvent system. Plate B was developed in an ethylene dichloride–ethyl acetate (4:6) solvent system. PMD programs of six development cycles required 37 minutes. The sample was testosterone.

## Applications of PMD

High performance techniques are generally more complicated and more expensive than their conventional counterparts. At a minimum, they require the learning of new, and often the unlearning of old, procedures. As a result, the early applications of a high performance technique are likely to be those in which its advantages are necessary to the desired result. Only later, when the new technique is more generally available and widely known, does it displace conventional methods in routine use.

The applications of PMD in the first 3 years have been those in which high performance TLC has been necessary rather than merely convenient. Most fall into one of three categories: (i) applications requiring high sensitivity (*27*); (ii) applications requiring resolution of closely related molecules (*34*); and (iii) applications requiring resolution of complex mixtures (*18*).

Regardless of the detection or quantitation method used, sensitivity in TLC depends on the number of molecules per unit area of spot surface (*35*). Concentration of a given sample size onto a smaller area makes it easier to detect. Because PMD, as it is usually carried out, reconcentrates spots in only one dimension, that is, parallel to the direction of migration, the increase in sensitivity is directly proportional to the degree of spot reconcentration, or reduction in spot width (*27, 32, 36*). In the quantitative analysis of caffeine in cola beverages, for example (*37*), PMD followed by densitometry allowed the measurement of as little as 50 ng of caffeine. By way of comparison, the detection limit for caffeine by conventional TLC followed by densitometry is 250 ng. We found the detection limit for caffeine by high performance liquid chromatography (HPLC) to be in the 50 to 100 ng range (*38*). Thus PMD followed by densitometry allows sensitivity comparable to that of high performance liquid chromatography while preserving the TLC advantages of ease of sample preparation and high throughput. In the analyses the cola was spotted directly onto the TLC plate with no preliminary extraction or cleanup and 12 samples per plate could be run. The combination of sensitivity and throughput also led to the use of PMD for the analysis of buquinolate in animal feed (*39*) and in beef tissue (*40*) at a level of less than one part per million. These procedures are noteworthy for combining the spot reconcentration of PMD with the formation and detection of fluorescent derivatives.

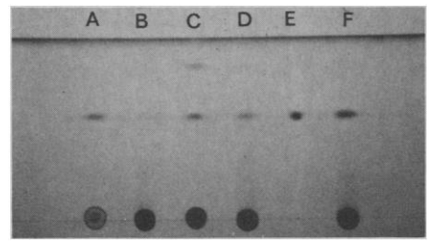



Fig. 9. A PMD intrinsic grid. The positions of spots of interest on the plate can be unequivocally marked by generating a grid of dilute standard all across the plate. Merger of a grid line with a sample spot confirms the presence of that component in the sample. The single grid line shown here was generated by applying a broad streak of a dilute caffeine solution across the origin of the TLC plate before spotting the samples: *A*, tea; *B*, Sanka; *C*, cola; *D*, decaffeinated coffee; *E*, standard; *F*, coffee. The solvent was a mixture of ethylene dichloride and ethyl acetate (4:6). The PMD program of seven development cycles required 55 minutes. Water vapor–saturated nitrogen was used for solvent removal.

The sensitivity of fluorescence detection in TLC has long been recognized and applied to the detection of naturally fluorescent compounds such as aflatoxins (*41*). The use of PMD in such analyses can greatly simplify sample preparation. We have, for example, analyzed aflatoxins in peanut butter without sample cleanup (Fig. 10); 150 $\mu$l of a chloroform extract is simply spotted directly onto the TLC plate. A development with benzene followed by a PMD program under the parameters shown in Fig. 10 separates the aflatoxins from other fluorescing substances.

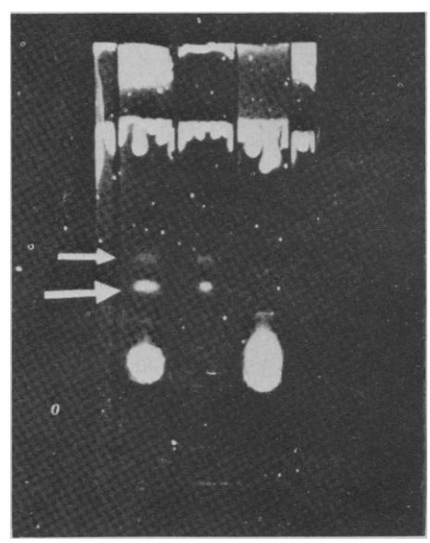


Fig. 10. The PMD of peanut butter extract. The PMD allows the analysis of aflatoxins without preliminary sample cleanup. The crude extract (150 $\mu$l) was spotted directly onto the TLC plate. Left, peanut butter extract to which aflatoxins $B_1$ and $G_1$ were added; center, standards (aflatoxins $B_1$ and $G_1$); right, control extract. The solvent was a mixture of chloroform, tetrahydrofuran, and ethyl acetate (18:1:1). The PMD program of seven development cycles required 55 minutes. Nitrogen was used for solvent removal.

Like TLC, PMD offers advantages over high performance liquid chromatography in the analysis of compounds which do not exhibit native fluorescence at room temperature: fluorescent chemical derivatives of compounds of interest can easily be formed on the TLC plate after separation with little or no loss of resolution.

Sensitivity can be improved by PMD both directly, by spot reconcentration, and indirectly, as the result of the greater tolerance of the method for sample overloading (*27, 36*). Large quantities of sample can be applied directly to the TLC plate for the analysis of trace components without danger of plate overloading and degradation of resolution. This has been demonstrated in the analysis of aflatoxins referred to above, in the detection of quinine in human urine (*42*), and in the analysis of limonin (a bitter flavor principle) in citrus juice (*43*).

Drawing direct comparisons between PMD and conventional TLC in the resolution of closely related compounds is difficult; the same solvent system is rarely optimum for both techniques. PMD has, nevertheless, demonstrated excellent resolution of such pairs as androsterone and etiocholanone (*14*) or testosterone and epitestosterone (*34*). The common thread in these separations is the observation that improved resolution is not the result of increased separation but rather of decreased spot width due to spot reconcentration.

Perhaps the most promising area of application of PMD is the analysis of complex mixtures. The relationship between top-to-bottom spot width and the number of components which can be resolved and identified by position in a single TLC system has been pointed out (*28*). In conventional TLC, two-dimensional development is typically resorted to when more than, say, 10 to 15 components must be identified in a mixture. Two-dimensional TLC is a flexible and useful technique; it does, however, give away one of the major advantages of TLC: the capability of separating a number of samples simultaneously. The reduction in spot width produced by PMD spot reconcentration allows the resolution of more components in a single dimension, and hence restores the parallel processing capability of TLC in many cases.

A suggestion of the capabilities of PMD is provided by the recent analysis

of alky ethoxylate surfactants by PMD followed by densitometry (*44*). The separation is based on the number of ethylene oxide groups in the molecule. After separation, the plate is charred with sulfuric acid, and the absorbance of the charred spots measured with a densitometer. The first 13 bands (from 0 through 12 ethylene oxide groups) are quantitated in this manner.

Even more complex mixtures than that described above can be resolved. This has been demonstrated by a number of commercial applications in dye and natural products research as well as by Perry's study of extended developments (*18*).

## Future Directions

We expect the expanded application of PMD to the separation of extremely complex mixtures (as, for example, in metabolic profiling) to be a major direction of future progress. This progress will be eased by continuing efforts to understand and explain the dynamics of PMD. Improvements in existing models for PMD spot behavior will allow easier and more rapid optimization of PMD program parameters as well as easier transfer of conventional TLC separation systems to PMD.

### References and Notes


1. The history of chromatography is traced by E. Heftmann, in *Chromatography: A Laboratory Handbook of Chromatographic and Electrophoretic Methods*, E. Heftmann, Ed. (Van Nostrand Reinhold, New York, ed. 3, 1975), pp. 1–13.
2. J. Riphahn and H. Halpaap, *J. Chromatogr.* **112**, 81 (1976).
3. J. A. Perry, U.S. Patent 3 864 250 (1975).
4. ———, K. W. Haag, L. J. Glunz, *J. Chromatogr. Sci.* **11**, 447 (1973).
5. The selectivity of a chromatographic system with respect to two different components is determined by the distribution factors, $k'$, of those components; $k'$ is the ratio of the quantity of sample in the stationary phase to the quantity of sample in the mobile phase. The most widely used measure of selectivity is the separation factor, $\alpha$. The separation factor between two components A and B is given by

$$\alpha_{AB} = (k_B'/k_A')(1 + k_A')/(1 + k_B')$$

6. This comparison is even more dramatic for the specific case of TLC, in which flow rate is difficult to control. Since TLC separation time is proportional to the square of the migration distance, the desired threefold improvement in resolution would require an 81-fold increase in separation time.
7. B. L. Karger, L. R. Snyder, C. Horvath, *An Introduction to Separation Science* (Wiley-Interscience, New York, 1973), pp. 12–33.
8. An increase in selectivity merely improves the resolution between two given components of the sample, and may decrease resolution between others. An increase in efficiency not only improves resolution, but also increases sensitivity, by concentrating sample components into narrower zones, and allows the resolution of more complex mixtures.
9. One can thus distinguish PMD from other forms of multiple development TLC, such as: Unidimensional Multiple Chromatography (UMC) described by Thoma (*10*), in which successive developments are carried out for the same distance, and Incremental Multiple Development (IMD), in which successive developments are carried out to gradually increasing distances, but in which the TLC plate is removed from contact from the solvent reservoir for drying between developments.
10. J. A. Thoma, *Anal. Chem.* **35**, 214 (1963); in *Advances in Chromatography*, R. A. Keller and J. C. Giddings, Eds. (Dekker, New York, 1968), vol. 6, pp. 106–113.
11. This is a crude but useful approximation. $R_F$, the relative migration distance, is the ratio of spot migration to solvent front migration distance. $R$, the relative migration velocity, is the ratio of spot migration velocity to sample migration velocity. $R$ equals $R_F$ only if the solvent velocity at any time is constant over the entire length of the bed. This is seldom exactly true. In practice, $R$ is greater than $R_F$ by as much as 15 percent [J. C. Giddings, *Dynamics of Chromatography* (Dekker, New York, 1968), part 1, p. 3].
12. Spot reconcentration and spot alignment share the same mechanism. The $(1 - R_F)^n$ pattern has been confirmed for the alignment of spots initially several centimeters apart. These are equivalent to single spots of the same width (and for which broadening during development can be neglected) [T. H. Jupille and J. A. Perry, *J. Chromatogr. Sci.* **13**, 163 (1975)].
13. T. H. Jupille and J. A. Perry, *J. Chromatogr.* **99**, 231 (1974).
14. ———, paper No. 213 presented at the 27th Pittsburgh Conference on Analytical Chemistry and Applied Spectroscopy, March 1976.
15. J. A. Perry, T. H. Jupille, A. B. Curtice, *Sep. Sci.* **10**, 571 (1975).
16. T. H. Jupille and A. B. Curtice, *Chromatographia* **8**, 193 (1975).
17. An additional advantage of gas-flush solvent removal is the maintenance of near-isothermal conditions during the entire program. Because $R_F$ values are temperature-dependent, isothermal operation makes for easier extrapolation of conventional TLC data to PMD systems.
18. J. A. Perry, *J. Chromatogr.* **113**, 267 (1975).
19. A sandwich chamber (S-chamber) is merely a glass plate which covers the thin layer but is separated from it by an air gap maintained by a spacer around the plate edge. The resulting TLC plate, spacer, and cover plate sandwich is dipped into an external solvent reservoir for development [E. Stahl, *Thin Layer Chromatography, A Laboratory Handbook* (Springer-Verlag, New York, ed. 2, 1969), pp. 69–71].
20. The point of sample application (origin) should be at or above the steady state position of the solvent front. This position depends on the heat input and gas flow across the plate as well as on the viscosity and boiling point of the solvent and on the permeability of the layer.
21. S. G. Perry, R. Amos, P. I. Brewer, in *Practical Liquid Chromatography* (Plenum, New York, 1972).
22. See, for example, the steroid separations reported in *PMD News* Nos. 9 (November 1974), 10 (February 1975), and 11 (May 1975) (Regis Chemical Company, Morton Grove, Ill.).
23. A. Niederwieser and C. C. Honegger, in *Advances in Chromatography*, R. A. Keller and J. C. Giddings, Eds. (Dekker, New York, 1966), vol. 2, pp. 123–167.
24. We depend here on an intuitive understanding of what is meant by solvent polarity. This topic is discussed by L. R. Snyder, *Principles of Adsorption Chromatography* (Dekker, New York, 1968), pp. 87–89.
25. E. Stahl, *Thin Layer Chromatography, A Laboratory Handbook* (Springer-Verlag, New York, ed. 2, 1969), p. 66.
26. Pure water can be used when the plate is to be developed with a water-immiscible solvent system. Water-immiscible systems, however, tend to pick up water from the thin layer and to carry it forward as a highly polar band immediately behind the solvent front. Partially saturated nitrogen (produced by bubbling the gas through calcium chloride or sulfuric acid solutions of appropriate concentration) can be used with such solvents.
27. J. A. Perry and L. J. Glunz, *J. Assoc. Off. Anal. Chem.* **57**, 832 (1974); J. A. Perry, T. H. Jupille, L. J. Glunz, *Ind. Res.* **16**, 2, 55 (1974).
28. T. H. Jupille and J. A. Perry, *J. Chromatogr. Sci.* **13**, 163 (1975).
29. Part of the reason for the utility of $R_F$ value in conventional TLC is that it is (usually) independent of the placement of the origin. This is not true in PMD (*16*).
30. J. A. Perry and T. H. Jupille, paper No. 214 presented at the 27th Pittsburgh Conference on Analytical Chemistry and Applied Spectroscopy, March 1976.
31. Position on a TLC plate does not prove (although it can disprove) identity [see K. A. Connors, *Anal. Chem.* **46**, 53 (1975)].
32. T. H. Jupille, paper No. 264 presented at the 26th Pittsburgh Conference on Analytical Chemistry and Applied Spectroscopy, March 1975.
33. J. A. Perry, *J. Chromatogr.* **110**, 27 (1975).
34. See, for example, *PMD News* No. 9 (November, 1974) (Regis Chemical Company, Morton Grove, Ill.).
35. T. H. Jupille and H. M. McNair, *Am. Lab.* **6**, 8, 54 (1974).
36. See, for example, *PMD News* No. 3 (February 1974) (Regis Chemical Company, Morton Grove, Ill.); see also (*27, 32*).
37. *PMD News* No. 14 (May 1976) (Regis Chemical Company, Morton Grove, Ill.).
38. The limiting factor in this case was pump noise. The detection limits for compounds of similar absorptivity by ultraviolet absorbance in high performance liquid chromatography is typically in the 10 ng range [T. H. Jupille, *Am. Lab.* **8**, 5, 85 (1976)].
39. J. P. Heotis, J. R. Diaz, D. C. Van Hart, J. L. Mertz, paper presented at the Kontes Symposium on Quantitative Thin Layer Chromatography held at the 27th Pittsburgh Conference on Analytical Chemistry and Applied Spectroscopy, March 1976.
40. J. P. Heotis, J. L. Mertz, D. V. Van Hart, J. R. Diaz, C. E. Benjamin, *ibid.*
41. L. Stoloff, in *Quantitative Thin Layer Chromatography*, J. C. Touchstone, Ed. (Wiley-Interscience, New York, 1973), pp. 95–112.
42. *PMD News* No. 6 (July 1974) (Regis Chemical Company, Morton Grove, Ill.).
43. T. H. Jupille, paper No. 2 presented at the 67th Annual Spring Meeting of the American Oil Chemists' Society, April 1976.
44. G. J. Fischesser and M. D. Seymour, paper No. 238 presented at the 27th Pittsburgh Conference on Analytical Chemistry and Applied Spectroscopy, March 1976.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