Programmed Multiple Development: High Performance Thin Layer Chromatography

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ABSTRACT AND SUMMARY

Poor resolution and sensitivity are not inherent in thin layer chromatography (TLC). They merely reflect the fact that TLC, as normally practiced, is not a high performance (efficient) technique. When efforts are made to improve the efficiency of TLC, the resulting improvements in resolution and sensitivity make high performance TLC fully comparable to high performance liquid chromatography (HPLC). Two approaches to improved TLC performance are the use of small-particle TLC plates and the technique of Programmed Multiple Development (PMD). Preliminary PMD methods for limonin in citrus juice and for caffeine in cola beverages clearly illustrate the use of PMD.

INTRODUCTION

Chromatographic techniques evolve. One facet of this evolution is a gradual shift in emphasis from selectivity to efficiency (1). The process is dramatically illustrated by the evolution in liquid chromatography (LC) packing materials. The large, totally porous particles (100 μm or so in diameter) in use before 1968 which allowed efficiencies of perhaps 200 theoretical plates gave way to solid-core, superficially porous materials (typically of 30-40 μm diameter) giving 750 plates. These, in turn, are being superceded by totally porous microparticulate packings (5-10 μm diameter) capable of 3000 plates (2).

The term "high performance" is typically applied to a mature or maturing technique; it reflects the emphasis on efficiency. We can make a good case for the following definition: a high performance chromatographic technique is one which is capable of generating the equivalent of 5000 or more theroretical plates. We stress the word "capable"; efficiency is often traded for faster separation or larger sample capacity. The 5000-plus theoretical plate definition of high performance applies to gas chromatography (GC) (capillary columns and some packed columns), to LC (microparticulate columns), and to evolving new techniques in thin layer chromatography (TLC).

The evolution in TLC is propelled by the growing awareness that TLC is capable of considerably higher efficiency than the 1500 or so theoretical plates predicted in 1968 (3). The development of high performance TLC is proceeding along two distinct (and complementary) lines.

One line of development is characterized by the use of smaller particles (and narrower size ranges) on TLC plates. A recent paper on "micro-HPTLC" (4) suggests a bed efficiency of some 4200 theoretical plates in only 16 min. This number is particularly impressive because the measurement system used is likely to understate the efficiency obtained. Micro-HPTLC plates are now becoming commercially available (E.M. Laboratories, Elmsford, NY). They are capable of great resolution and sensitivity. Their chief limitation, in fact, seems to be the small (nanoliter) sample volumes required for optimum results.

The second line of evolution exploits the spot reconcentration which occurs whenever the solvent front crosses a spot on the TLC plate (5,6). Loosely speaking, the lower edge of the spot has an opportunity to "catch up" with the upper edge (Fig. 1). The resulting reduction in top-to-bottom spot width to $(1\text{-}R_f)$ times its initial value has been demonstrated (7). Spot reconcentration normally occurs once in TLC. Its effect (and even its existence) are generally ignored.

In Unidimensional Multiple Chromatography (UMC)—the repeated, same-direction, same-solvent, same-distance development of a TLC plate—spot reconcentration occurs repeatedly. The spot width after n developments is, to a first approximateion, reduced to $(1\text{-}R_f)^n$ times its initial width (6). Separation between spots in UMC first increases, then passes through a maximum value and begins to decrease as developments continue (8). Typically, n is chosen for maximum separation rather than for effective reconcentration (Fig. 2). This means that n is typically smaller than required for effective reconcentration.

This is not true of Programmed Multiple Development (PMD). PMD is the repeated, same-solvent, same-direction development of a TLC plate to gradually increasing dis-

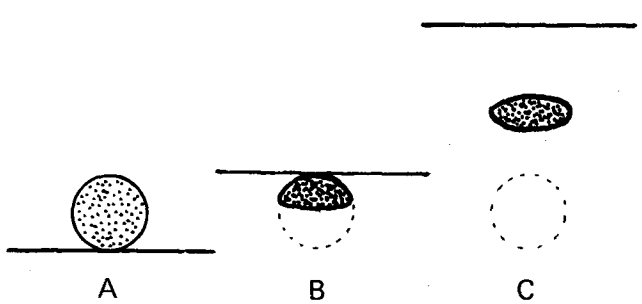

FIG. 1. Spot Reconcentration. A: Advancing solvent front contacts the lower edge of the spot while the upper edge is still stationary. B. By the time the solvent front reaches the upper edge of the spot, the lower edge has travelled R_f times the initial spot width. C: Spot now moves as a unit, having been reconcentrated to $(1\text{-}R_f)$ times its initial width.

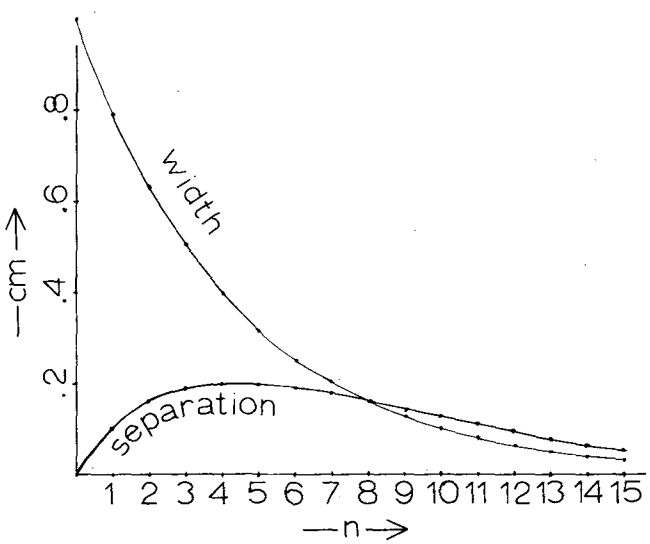

FIG. 2. Unidimensional Multiple Chromatography. Plot of spot width and separation between spots as a function of number of developments, n. Assumptions: spots of R_f 0.20 and 0.21 initially 1 cm wide; a series of 10 cm developments. Maximum separation occurs after four developments.

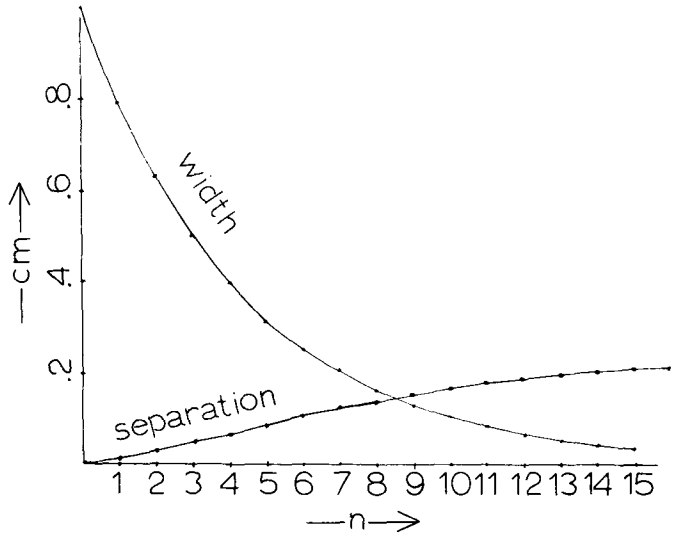

FIG. 3. Programmed Multiple Development (PMD). Plot of spot width and separation between spots as a function of the number of developments, n. Assumptions: spots of R_f 0.20 and 0.21 initially 1 cm wide; a PMD program of 1 cm, then 2 cm, then 3 cm, and so on, developments. Note that ten developments of this type require less time and provide better resolution than four 10 cm developments in unidimensional multiple chromatography (UMC).

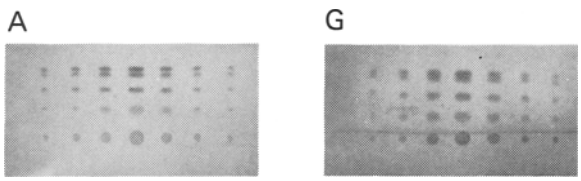

FIG. 4. Final spot width in programmed multiple development (PMD) reflects the efficiency of the plate. A: Best of a series of commercial plates tested, G: Worst. The spots are (from top to bottom) androsterone, etiocholanone, dehydroepiandrosterone, pregnanediol, and pregnanetriol. Both plates were developed by PMD for seven cycles in mode 2 in an ethyl acetate/ethylene dichloride (1:10) solvent with water saturated nitrogen used for solvent removal.

tances. Between developments, the TLC plate remains in contact with the solvent reservoir as it is dried by controlled evaporation (heat or gas flow) (9,10).

The salient feature of PMD is the ability to carry out a sufficient number of developments to assure compact spots while maintaining separation between spots (Fig. 3).

In practice, of course, spot reconcentration does not proceed indefinitely. Eventually a limiting, steady-state spot width is approached at which reconcentration (which is a function of spot width) and subsequent broadening (which is not a function of spot width) are balanced. This limiting width can be as low as 1 to 2 mm, but it is dependent on the sample loading and on the quality of the TLC plate used (Fig. 4). For this reason, we look forward to the combination of PMD and the new HPTLC plates.

APPLICATIONS

PMD is finding application in areas as diverse as dyes and drugs, air pollution and food. We wish to consider briefly a few such applications generated in our laboratory. These were chosen primarily to illustrate some features of PMD procedures in general.

The first is the analysis for a bitter flavor principle in citrus juice: limonin. Although limonin itself is not normally present in fresh, intact citrus fruits, the acidity of the juice can convert a nonbitter precursor, limonoate A-ring lactone, into limonin after squeezing.

In this procedure, boiled citrus juice (11) is diluted with

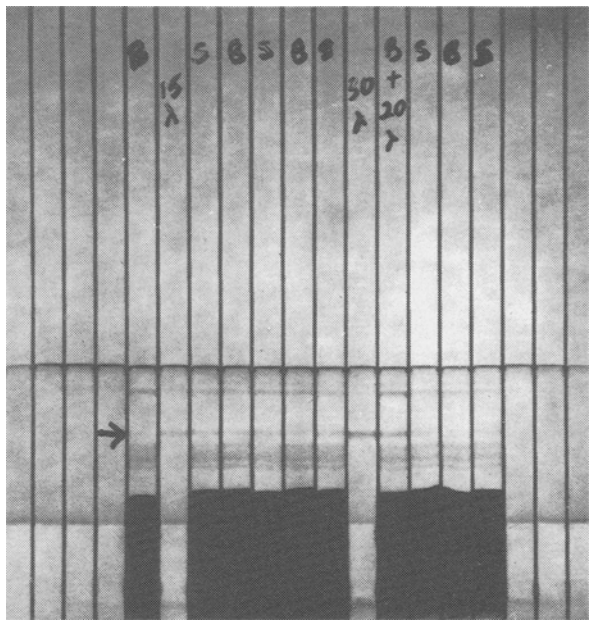

FIG. 5. Typical separation of limonin (arrow) from orange juice. Standards were 0.33 μg/μl.

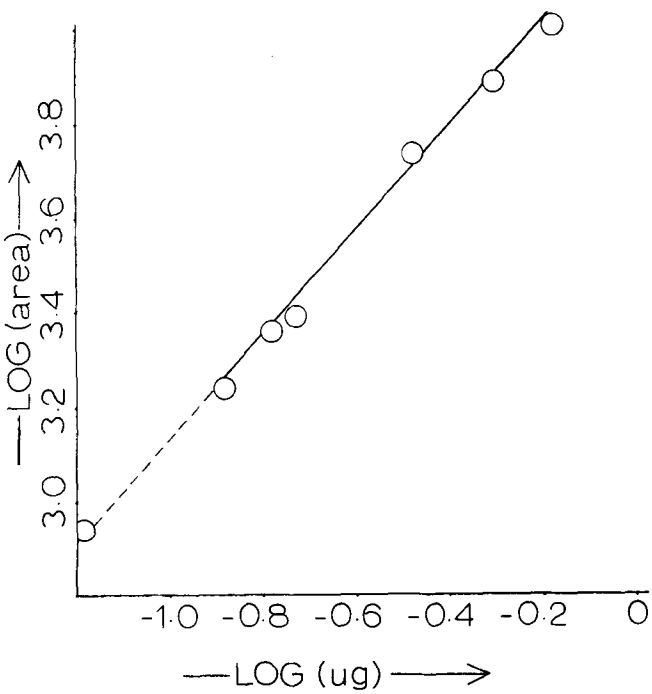

FIG. 6. Calibration Curve for Limonin. Plot of the logarithm of peak area versus logarithm of sample mass for limonin standards. The plot is linear from 0.066 μg to 0.660 μg of limonin. The slope of the line is 1.01, confirming a linear relationship.

an equal volume of methanol and then centrifuged. 200 μl of the supernatant are applied to a preadsorbent TLC plate (Quantum Industries; Fairfield, NJ). PMD development for six cycles in mode 3 (this yields developments of 20, 80, 180, 320, 500, and 720 sec duration) is carried out in an ethyl acetate/ethylene dichloride (1:10) solvent system. Solvent removal is accomplished with water-saturated nitrogen to ensure reproducible deactivation of the thin layer. Spraying with 1% p-(dimethylamino)benzaldehyde/1% H_2SO_4 in methanol followed by heating at 100 C for 10 min gives the chromatogram shown in Figure 5.

Quantitation with a Kontes densitometer and integrator (Kontes Glass Co.; Vineland, NJ) gives a linear relationship

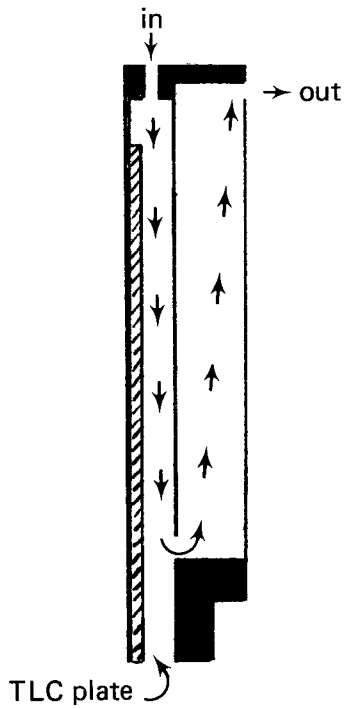

FIG. 7. Gas Flush Chamber. Programmed multiple development (PMD) may be carried out in this modified sandwich chamber using a stream of air or nitrogen to dry the plate either with or without additional heat input.

between peak area and sample size in the range from 66 ng to at least 660 ng (as high as we went) of limonin (Fig. 6). The peak areas were reproducible to 15% relative standard deviation.

This procedure and results illustrate a number of points about PMD:

(a) Solvent migration distances are shorter in PMD than in equal-time conventional TLC. The higher efficiency of PMD (apparently 4700 theoretical plates for limonin, which implies 8000 for the entire bed length used) is the result of spot reconcentration.

(b) Although much of the early work on PMD was done using heat-only solvent removal, this technique proved to be a limitation when working with heat-sensitive compounds. Most of the current work, as illustrated here, uses a "gas-flush" modified sandwich chamber (Fig. 7) which allows solvent removal at ambient temperature if desired.

(c) Water saturation of the drying gas is often done in PMD. It allows a reproducible deactivation of the adsorbent surface. The resulting suppression of tailing and increase in linear capacity is thoroughly discussed in Snyder's book on adsorption chromatography (3). Impregnation of the drying gas can also be used to introduce acid or base into the system.

(d) The solvent system used here is not one that is normally used for TLC of limonin, (11,12). Conventional solvent systems can be used in PMD, often with excellent results. In most cases, however, optimum PMD results require some solvent system modification.

A second example reinforces these points and make an interesting comment about sensitivity in high performance TLC.

Caffeine in coffee and cola beverages can be quantitated by spotting an aqueous sample directly onto the TLC plate. PMD development for six cycles in mode 3 is carried out in an ethyl acetate/ethylene dichloride (68:32) solvent system using water-saturated nitrogen for solvent removal. The caffeine is detectable by fluorescence quenching at 254 nm. A comparison of a PMD plate with a conventional TLC plate

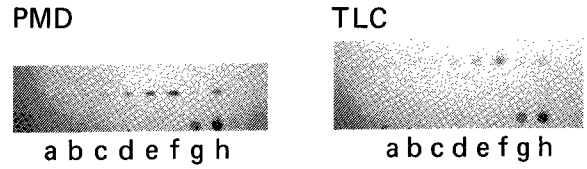

FIG. 8. Comparison of Programmed Multiple Development (PMD) and Conventional Thin Layer Chromatography (TLC) of Caffeine. a: 0.250 μg; b: 0.500 μg; c: 1.00 μg; d: 2.50 μg; e: 5.00 μg; f: 10.0 μg; g: 5 μl cola; h: 5 μl coffee.

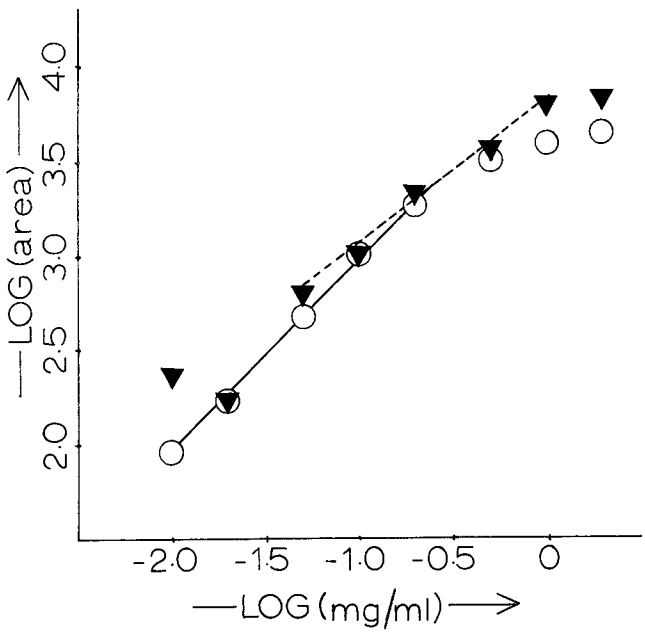

FIG. 9. Calibration Curves for Caffeine. Plot of the logarithm of peak area versus the logarithm of sample size for caffeine standards run by PMD/densitometry (circles) and by TLC/densitometry (triangles). Linear range of PMD/densitometry extends down to 50 ng compared to 250 ng for TLC/densitometry.

run in methyl acetate for 45 min is shown in Figure 8. Densitometry gives the area/sample size relationships shown in Figure 9. The minimum detectable quantity of caffeine by PMD/densitometry was 50 ng. The minimum detectable quantity of caffeine by TLC/densitometry was 250 ng. The minimum detectable quantity of caffeine by HPLC in our laboratory was 100 ng. PMD results were reproducible to 6.6% relative standard deviation.

Sensitivity in TLC is a function of spot size (13). Any quantitative TLC method ultimately measures the number of molecules per unit area of spot surface. Any technique which increases the efficiency of TLC (and hence produces more compact spots) is expected to improve sensitivity (4). In this case, PMD provides an improvement by a factor of five. The sensitivity of high performance TLC is, in fact, fully comparable to the sensitivity of HPLC.

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