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CONTACT SPOTTING

A NEW APPROACH TO SAMPLE APPLICATION IN THIN-LAYER CHROMATOGRAPHY

D. C. FENIMORE and C. J. MEYER
Texas Research Institute of Mental Sciences, 1300 Moursund Avenue, Houston, Texas 77030 (U.S.A.)

SUMMARY

The introduction of high-performance adsorbent layers to thin-layer chromatography has necessitated improved methods of sample application in order to realize fully the resolution, sensitivity and speed achievable with this quantitative analytical technique. Systems utilizing microcapillary pipettes are capable of maintaining minimal sample spot diameters; but when the solution viscosity is high or the concentration of analyte is small, this method of delivery may be impractical or may require a large number of serial applications to the same spot. The process described in this paper enables rapid concentration of sample solutions of up to 50 μl with transfer of the residue directly to the plate resulting in spot diameters as low as 0.1 mm located with excellent precision. Multiple samples are applied simultaneously, and the densitometric measurement of separated components is highly reproducible. The method is particularly valuable in dealing with samples of biological origin where concentrated extracts are too viscous to permit delivery by micropipettes.

INTRODUCTION

Sample application is perhaps the most critical and often the most time-consuming step in thin-layer chromatography. If optimum resolution and sensitivity are to be achieved, the spot diameter must be held to a minimum which usually requires the deposit of a small volume of concentrated sample or repeated applications of dilute solution to the thin-layer plate. Concentrated solutions, particularly those encountered as extracts from biological materials, may be too viscous to permit the use of micropipettes or syringes, and a succession of applications of more easily managed dilute solutions is tedious at best and may even result in damage to the surface of the adsorbent layer through repeated contact with the tip of the pipette or syringe needle. Alternatively a solvent may be used in which the components of the sample exhibit a very low R_F. This may indeed minimize spreading of the spot, but such a solvent is precisely the opposite from considerations of polarity and solubility than would normally be chosen for quantitative delivery of sample material.

If the major impediment to efficient and quantitative application of sample is the sample solvent, then it would appear that removal of solvent prior to spotting would offer a significant improvement in preserving small spot diameters. A technique and apparatus for accomplishing solventless sample application has been developed which permits quantitative transfer of residues of several samples simultaneously to precise locations on a thin-layer plate.

DESCRIPTION OF THE TECHNIQUE

This method of sample application, referred to here as "contact spotting", consists essentially of evaporation of measured volumes of sample solution in depressions formed on a non-wetting, polymer film. Once the solvent is removed, the residues are transferred from the film to the adsorbent layer of the thin-layer plate by bringing them into contact. The process itself is thus quite simple, but an appropriate surface must have certain requisite properties. Obviously an inert surface is necessary which will not contaminate or irreversibly absorb the sample material. The surface must also be non-wettable by the common application solvents in order for the deposited solution to form a droplet which will remain coherent and symmetrical during evaporation of the solvent. In addition, the sample residue must not adhere to the surface in order for transfer to the thin-layer plate to be quantitative.

A fluorocarbon polymer film with a thin coating of a perfluoroalkane fluid, which is insoluble or sparingly soluble in most common solvents, provides a unique surface which embodies all of these requirements for the contact spotting process. In addition, the pliability of such a film allows formation of depressions which determine the final position of the sample residues and thus permits transfer to the thin-layer plate at precise locations.

APPARATUS

A number of preliminary designs for the spotting apparatus were investigated incorporating various geometries for fixing accurately the position of concentrated sample residue. This was not an insignificant problem, because a drop of liquid on a surface of minimal friction is easily disturbed by the gentlest of external stimuli. However, an arrangement which proved to be quite reliable in practice for the application of samples to 10 × 10 cm high-performance thin-layer chromatographic (HPTLC) plates is shown in Figs. 1 and 2.

A 2.54 cm wide strip of fluorinated ethylene–propylene polymer (FEP) film (0.025 mm thickness) coated with perfluorokerosene or a similar perfluorinated fluid is placed over a series of cylindrical pistons inserted in a flat plate. Each piston is capped with a silicone polymer septum 8 mm in diameter in which there is a central orifice 0.8 mm in diameter. With the septa flush with the surface of the plate vacuum is applied to the orifices of the septa which form small, well-defined dimples in the film. When the pistons are lowered slightly below the surface of the plate, the film is deformed into shallow depressions in which the small dimples are maintained. Each depression is of sufficient size to receive at least 50 μl of sample solution.

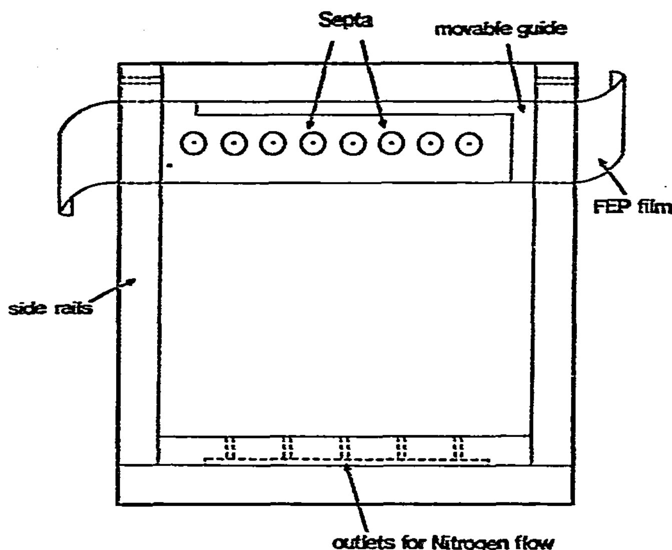

Fig. 1. Contact spotting apparatus for 10 × 10 cm HPTLC plates.

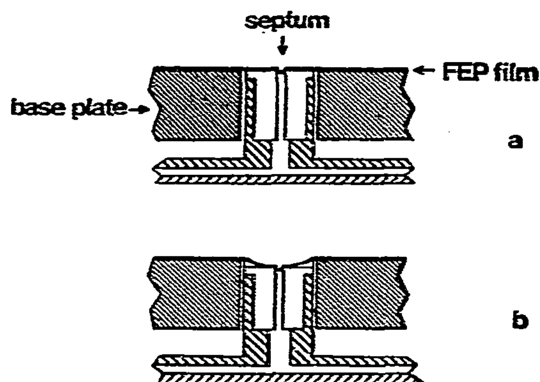

Fig. 2. Diagram of piston arrangement for forming depressions in film surface. (a) Vacuum applied to septum creates small central dimple. (b) Lowering of piston forms larger depression to hold sample.

With the apparatus covered, a gentle flow of nitrogen is passed over the samples facilitating evaporation of solvent. Once the solvent is removed, the residues are located in the central dimples as shown in Fig. 3. A thin-layer plate is then placed adsorbent side down over the sample positions and held firmly in place. The vacuum is replaced with nitrogen pressure of approximately 1.5 atm which forces the dimples upward and all samples are transferred to the adsorbent layer simultaneously.

The septum orifices are located on 1.2-cm centers which will yield 8 linear spots on a 10-cm plate with the outer spots 8 mm from the edges. A movable guide allows the plate to be offset by 4, 6, or 8 mm from the starting position allowing additional samples to be spotted between the original samples to give 15 or 22 spots on one edge of the plate.

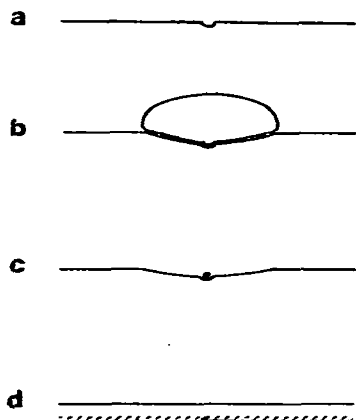

Fig. 3. (a) Film surface with central dimple. (b) Sample droplet located in depression. (c) Sample residue after solvent evaporation. (d) Residue transfer to TLC plate.

EVALUATION AND DISCUSSION

Although HPTLC plates (Silica gel 60, E. Merck, Darmstadt, G.F.R.) were used throughout these initial evaluations, it should be emphasized that contact spotting is applicable to any process requiring deposition of sample on an adsorbent surface. HPTLC, however, is particularly restrictive with respect to the size of the original sample spot, and it therefore furnishes an excellent model for testing methods of sample application.

It is generally accepted that the starting spot diameter in HPTLC should be no greater than 1.0 mm[1] and Kaiser[2] suggested that values for b_0, the starting peak width at half-height plus diffusion widening which would occur during the chromatographic run, should be on the order of 0.1 mm. Upon calculating the dosage volumes required for such low values of b_0 on HPTLC plates of 0.19 mm adsorbent thickness, he concluded that only volumes below 10 nl were compatible with HPTLC. If the sample solvent could be selected to produce an R_F of less than 0.1 for the least polar component of the sample, then dosage volumes of 100 nl might be tolerated. If larger volumes are required, as would be the case when dilution is needed to lower the viscosity of the sample solution to a point permitting delivery from a 100-nl pipette, then a proportionately greater number of applications must be made to the same spot. A volume of 1 μl would thus require 10 successive applications of sample solution, a number that is not impractical but would certainly increase the possibility of sampling error.

In contrast to deposition of samples in solution the spot diameter attainable with the contact process is determined solely by the volume of non-volatile sample components. It should be mentioned, however, that there may exist limits to the minimum spot size depending on the particular sample material. Some compounds tend to crystallize out as the solution becomes increasingly concentrated, and transfer of these solids may not be completely reliable. This problem may be solved by adding to the solution a minute amount of a non-volatile liquid in which the sample component is soluble. For example, precipitation of crystals of Sudan Red G

was prevented by adding acetophenone (1 part per 10,000) to a chloroform solution of the dye. Evaporation of 10 μl of dye solution containing 2.5 ng/μl followed by contact spotting on the plate produced spot diameters which were uniformly 0.31 mm in diameter ($n = 15$, S.D. = 0.014) as measured by an optical micrometer scale. The plate was developed in benzene to a distance of 1.5 cm with the Sudan Red G at an R_F of 0.27, and a densitometric scan of the plate perpendicular to the direction of development using a Zeiss KM-3 chromatogram spectrophotometer is shown in Fig. 4.

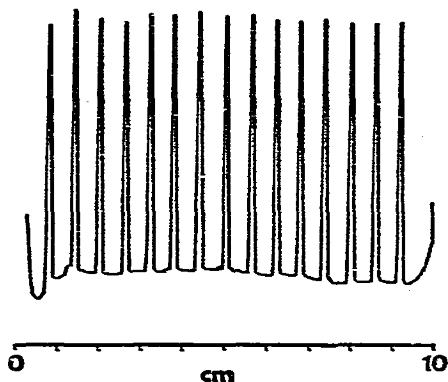

Fig. 4. Densitometric scan of 25-ng amounts of Sudan Red G applied to HPTLC plate by contact spotting. Plate developed in benzene to 3.5 cm. Scan is perpendicular to direction of development. Wavelength, 500 nm; slit, 0.05 mm; aperture, 3.5 mm.

The examination of sample spot size was extended using 10-μl volumes of chloroform solutions of Sudan Red G (0.5 ng/μl) containing methyl myristate at 1:100,000 and 1:200,000 dilutions. The resulting spot diameters were 0.135 mm (S.D. = 0.008) and 0.104 (S.D. = 0.011) respectively. These values are consistent with dosage volumes of 0.1 nl and 0.05 nl considering that volumes below 2 nl will not penetrate the adsorbent layer to the glass surface and hence the sample volume is hemispherical in shape. The diameters would therefore vary with the cube root of the volume ratio, in this case $\sqrt[3]{2}$ or 1.26, compared to a measured ratio of 1.29.

The reproducibility of peak height observed in scans such as that shown in the above example would indicate that transfer is quantitative. This was confirmed by examining sample volumes of 10, 20, and 50 μl of a tritium-labelled drug, desmethylimipramine (New England Nuclear, Boston, Mass., U.S.A.) in ethanol containing 1:10,000 octanol. The concentrations were selected to yield equal amounts of compound for transfer, 40 pg calculated from specific activity, after evaporation of the ethanol. The labelled compound was transferred to absorbent paper which could be divided easily for scintillation counting without the losses that can occur in attempting to recover adsorbent scraped from a thin-layer plate. The results of this experiment are shown in Table I. The recovery of 97% or above is well within the experimental error of the sampling and counting procedure, and transfer could thus be considered complete.

TABLE I

RECOVERY OF RADIO LABELLED COMPOUNDS BY CONTACT SPOTTING.

Sample volume (μl)*	*Standard** (DPM ± S.D.)*	*Transfer*** (DPM ± S.D.)*	*Percent of standard*
10	12,226 ± 544	11,904 ± 358	97.4
20	11,234 ± 275	11,072 ± 458	98.6
50	11,532 ± 217	11,574 ± 200	100.4

* Solutions prepared to contain equal amounts of [^{3}H]desmethylimipramine HCl (40 pg) per total volume.
** Disintegrations per minute of sample applied directly to absorbent.
*** Disintegrations per minute of sample applied to absorbent by dry-transfer.

Addition of non-volatile solvent is rarely necessary when dealing with materials of biological origin, because extracts of these samples usually contain relatively large amounts of residue. Although such components are a limiting factor in achieving minimal diameters, spot sizes experienced in assays of blood plasma for therapeutic drugs, for example, were quite compatible with HPTLC techniques.

Table II summarizes data obtained from blood plasma samples containing 1, 5, and 10 ng/ml concentrations of the phenothiazine drug, chlorpromazine. Extracts of 1-ml volumes of plasma containing 10 ng methyltrimeprazine as internal standard were dissolved in 100-μl volumes of heptane–5% isoamyl alcohol, and 50-μl aliquots were pipetted into the depressions of the contact-spotting apparatus. After solvent evaporation and sample transfer to the HPTLC plate the resulting spots were on the order of 1 mm diameter. The plate was developed in ethyl acetate–acetone–diethylamine (30:20:0.1) to a distance of 3.5 cm and after drying was exposed to nitric oxide vapors which form pale yellow derivatives with phenothiazine drugs. Densitometry was performed in the reflectance mode at a wavelength of 380 nm.

Peak height ratios of the chlorpromazine to the internal standard exhibited excellent reproducibility at the three concentrations. Attempts to perform this assay at these low concentrations utilizing micropipette sample spotting were for the most

TABLE II

PEAK HEIGHT RATIOS* IN HPTLC ANALYSIS OF CHLORPROMAZINE IN BLOOD PLASMA

	Chlorpromazine concentration (ng/ml)		
	*1.0***	*5.0*	*10.0*
	0.134	0.528	0.989
	0.137	0.547	1.011
	0.116	0.573	0.971
	0.114	0.550	0.958
	0.112	0.538	0.973
	0.111	0.541	0.936
Average	0.121	0.546	0.973
S.D.	0.012	0.015	0.026
C.V. (%)	9.7	2.8	2.6

* Ratios of chlorpromazine peak height to methyltrimeprazine (internal standard) peak height.
** Linear regression analysis of 18 samples yielded a correlation coefficient of 0.997.

part unsuccessful because of unacceptable variability of the observed peak height ratios due in part to the viscosity of the sample extract preventing reproducible delivery.

CONCLUSIONS

The advantages of contact spotting can be summarized as follows:

(1) Large volumes of sample solution (50 μl or more) may be employed.

(2) Many samples may be spotted simultaneously.

(3) Spotting is quantitative and therefore highly reproducible.

(4) Spot diameters are sufficiently small to be compatible with HPTLC techniques.

(5) Sample transfer by the contact process distributes pressure over a large enough area to prevent damage to the surface of the adsorbent layer.

Perhaps the most important feature of this process, particularly in those areas of analysis requiring routine examination of large numbers of samples, is the speed with which the samples can be applied to the thin-layer plates. The evaporation of the solvent, which is for the most part an unattended operation, requires only a few minutes, and the actual transfer of the spots is accomplished in seconds. The rapidity with which TLC and especially HPTLC can separate multiple samples simultaneously is well known, thus the potential of this analytical technique can only be further enhanced by accurate, fast sample introduction.

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