RAPID QUANTITATIVE SPOTTING OF SUBMICROLITER VOLUMES FOR THIN-LAYER CHROMATOGRAPHY

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SUMMARY

Simple, inexpensive applicators for thin-layer chromatography can be easily constructed to reproducibly deliver 40 nl or less. They are appropriate for quantitative work in the 10^{-10} molar range with a coefficient of variation of less than 5 %. Although on calibration the variability appears to be much greater, in incremental spotting the deviations from the mean are additive and the pluses and minuses cancel out. Two spotter designs are described one of which is made from Nichrome wire and the other from ordinary sewing needles.

INTRODUCTION

In an earlier communication[1] the use of Nichrome wire loops for thin-layer chromatography (TLC) spotting was described. The inexpensive devices were easily constructed to deliver 250–500 nl. They could be quickly and accurately calibrated by spotting an aqueous dye solution onto filter paper and obtaining the product of the two diameters of a series of elliptical (or circular) spots. It was reported that the variability of delivery of these loops was close to $\pm$ 10 % and, therefore, they were not recommended for quantitative use. It has since been determined that by simplifying the design of the loops they can be made to reproducibly deliver 40 nl or less and that they are in fact well suited for quantitative work.

METHODS

The loops were fashioned from B & S Gauge No. 24 or 30 Nichrome wire by means of a small hemostat, jewelers' pliers and a magnifier. The design, as shown in Fig. 1, consists of a simple double bend of wire which functions as a surface capillary. The first 180° bend is made a few millimeters from one end of the wire (Fig. 1A). The second 180° bend (Fig. 1B) is made close to the first as possible and the loop tightly and firmly compressed (Fig. 1C). The excess wire is then cut and ground down by means of a small hand grinder with a fine abrasive wheel so that it is exactly even with the bend.
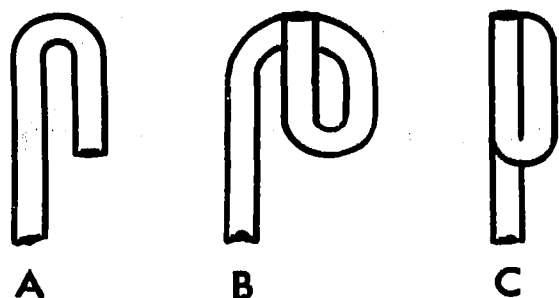
Fig. 1. Construction of the Nichrome wire loops: (A) first 180° bend; (B) second 180° bend; (C) appearance of the completed loop after compression.

For use, the long end of the wire is cut to a convenient length and mounted in a commercial needle holder or similar homemade device. A simpler applicator design can be fashioned by cutting perpendicularly through the eye of a small steel sewing needle and grinding the resulting spurs so that they are exactly even and pointed (Fig. 2).
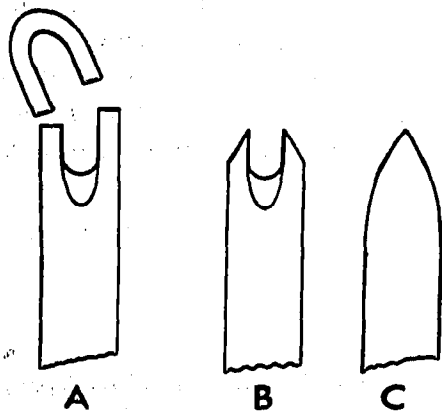

Fig. 2. Construction of the sewing needle applicators: (A) cut through the eye; (B) appearance after grinding; (C) side view after grinding.

The delivery volume of the spotters was determined by applying a 1 % aqueous solution of Crystal Violet (C.I. No. 42555) as a series of ten spots on Whatman No. 40 filter paper. The diameters of the elliptical (or circular) spots were measured by means of a Bausch & Lomb measuring magnifier (No. 81-34-38) with metric scale and an effective linear magnification of 4.5 times. The product of the two diameters $(D \cdot d)$ is directly proportional to the volume delivered[1]. The mean volume (L), standard deviation (S_D) and coefficient of variation $(V_D = S_D/L \times 100)$ were calcu lated for each spotter. Ten spots from each of four 1-μl Drummond micropipets were used as the reference standard (mean $D \cdot d = 21.56$ mm^2, $S_D = 0.79$, $V_D = 3.66 \%$).

A 5 mM solution of glucose was applied to a 7.5 × 7.5 cm Eastman 6064 cellulose Chromagram with a loop calibrated to deliver 126 nl. The five-point curve was spotted incrementally (two through six loops) with careful drying between additions. The chromatographic solvent was ethyl acetate–pyridine–water (12:5:4) (ref. 2).

The spots were detected with a silver nitrate, potassium hydroxide, sodium thiosulfate spray sequence[3]. The chromatogram was quantified by the area-absorbance

product method $(A \cdot D \cdot d)$ as described in another paper[3] and the correlation coefficien (r), standard error of estimate $(S_E)^*$ and coefficient of variation $(V_E)^{**}$ were calcu lated[4].

RESULTS

Calculation of the glucose data yielded a correlation coefficient of 0.993, : standard error of estimate of 0.067 nmoles, and a coefficient of variation (V_E) o 4.2 %. A graph (Fig. 3) revealed that this V_E value reflected the deviation of one poin
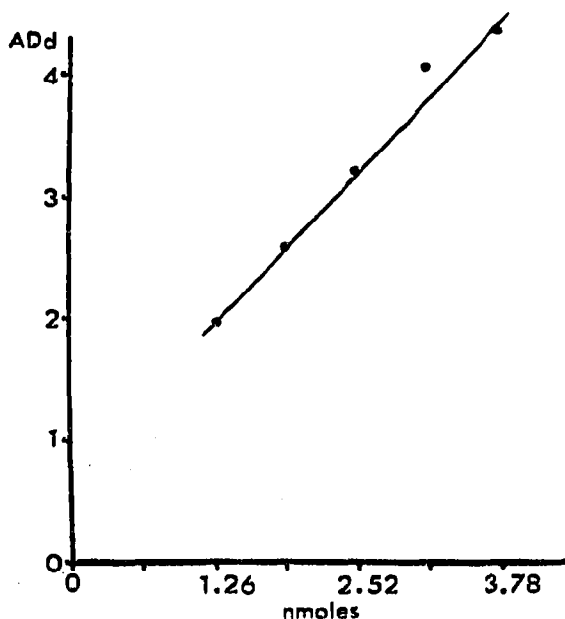

Fig. 3. Quantitative TLC analysis of a loop spotted glucose standard.

(3.15 nmoles) from an otherwise near perfect correlation. Omission of this point gav an r of 1000 and V_E of 0.76 %. The 2.52 nmoles point gave a calculated value o 2.49 nmoles based on the five-point curve and 2.56 nmoles according to the four-poin curve data. The line as drawn is based on the slope and intercept from the four-poin data. Calibration of the 126-nl loop had given $S_D = 7.2$ nl and $V_D = 5.7$ %.

The wire loops have been used for the quantitative TLC of a variety of sub stances in aqueous solution[3] with V_E values ranging from 1.2–4.2 %. The coefficien of variation of an incrementally spotted standard curve (V_E) is invariably less tha: that observed when calibrating the particular loop that was used. To better under stand this phenomenon, a mathematical model was used. The ten-point calibratio: data from one of the more variable loops $(V_D = 9.75 \%)$ were added cumulatively s as to simulate incremental spotting on a chromatogram (i.e., 1, 1 + 2, 1 + 2 + : etc.; Table I) and the regression of the cumulative volume on the number of increment was calculated. To reduce the averaging effect of the ten point data, groups of fiv figures were treated in the same manner (Table II). S_D, S_E, V_D and V_E values ar given in the tables.

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* $S_E = \sqrt{(\Sigma(Q - Q_o)^2)/N}$.
Where $(Q - Q_o)$ is the deviation of the observed quantity from the regression equation, $Q_o = \alpha(A \cdot D \cdot d) + \beta$. Quantity can be expressed either in nanomoles or number of loops of material.
** $V_E = S_E/\text{mean} \times 100$.

TABLE I

CALIBRATION DATA OF A 204-nl LOOP WITH THE CALCULATED VOLUMES EXPRESSED INDIVIDUALLY AND ADDED CUMULATIVELY IN ORDER TO ASSESS THE EFFECT OF THE LOOP VARIABILITY ON IN-CREMENTAL SPOTTING

No.	Calculated volume (nl)	Cumulative volume (nl)
1	184	184
2	195	379
3	213	592
4	205	797
5	176	973
6	204	1177
7	245	1422
8	223	1645
9	195	1840
10	195	2035

Mean (x) 203.5 nl r 1.000
S_D 19.9 nl S_E 16.8 nl
V_D 9.75% V_E 1.5%

TABLE II

CALIBRATION DATA OF TABLE I ADDED CUMULATIVELY IN GROUPS OF FIVE AND TREATED STATISTICALLY

The S_D and V_D were also calculated for each of the sets and are given for comparison.

No.	Volume (nl)	No.	Volume (nl)	No.	Volume (nl)	No.	Volume (nl)
1	184	6	204	1	184	2	195
2	379	7	449	3	397	4	400
3	592	8	672	5	573	6	604
4	797	9	867	7	818	8	827
5	973	10	1062	9	1013	10	1022
r	1.000		0.999		0.999		1.000
S_E	8.9 nl		16.0 nl		13.2 nl		5.89 nl
V_E	1.5%		2.5%		2.2%		0.95%
S_D	15.0 nl		21.5 nl		27.5 nl		11.4 nl
V_D	7.7%		10.1%		13.6%		5.6%

A spotting device fashioned from a small sewing needle delivered a mean volume of 68.9 nl ($S_D = 4.7$ nl, $V_D = 6.82$ %). The variability was somewhat greater than has been generally obtained with wire loops of approximately the same size.

DISCUSSION

Both types of sample applicators have a number of advantages especially in the range of 10^{-8} l: They are inexpensive, relatively easy to construct and simple to calibrate. They have ample precision for quantitative work, are simple to use — giving a very small origin spot — and can be flamed to prevent any carry over of sample.
The more rigid the loop design, the less chance that it will deform during use

and become altered in volume. Heavier wire size and smaller volume capacity contribute to rigidity. While the sewing needle applicators inherently have a greater rigidity, they appear to be more variable than wire loops of the same size.

The spotting devices are calibrated by measuring the diameters of a series of sample spots. Since the limit of measurement is fixed at $\pm$ 0.05 mm, the accuracy of calibration varies inversely with the volume. Knowledge of the true volume is not essential, however, if the same spotter is used to deliver both the unknown and standard solutions since:

$$\text{Unknown concn.} = \text{standard concn.} \times \frac{\text{Equiv. No. of loops of standard}}{\text{No. of loops of unknown}}.$$

Both types of applicator can be used on either cellulose or silica gel layers, though they must be very lightly touched to the silica gel surface to prevent lifting of the adsorbent. Since the devices are unsuitable for use with solvents as volatile as chloroform, they have been used almost exclusively for water soluble substances on cellulose layers.

At the present time, the practical lower limit that can be constructed is approximately 36 nl for the wire loops and about 63 nl for the needle applicators. With practice one can easily make several of either design in an hour in the desired size range. Calibration takes less than 5 min each if a high-speed calculator is available.

Currently, the largest loop size in use is 557 nl. If ten-volume increments or 5.57 μl is taken as the reasonable upper limit for a standard curve, and the lowest measurable amount of a given substance is 0.2 nmoles, then the lower limiting concentration for that substance is $3.6 \times 10^{-5} M$. Ordinarily, it is not the concentration that is the limiting factor and loops 50 nl or less are preferred in order to restrict the size of the origin spot, improve the chromatographic resolution and reduce the required sample volume.

Although on calibration the spotters have been found to have coefficients of variation (V_D) as high as 10 %, they have been shown to be quite suitable for quantitative work, routinely yielding coefficients of variations (V_E) of less than 5 % and frequently less than 2 %. The data in Table I show that the S_D and S_E values (expressed in nanoliters) are similar.

In the calculation of S_D, the divisor, is $(N-1)$ in contrast to (N) in the calculation of S_E. If S_D is recalculated using (N) in the divisor, the value is decreased to about 90 % of the original but $S_D>S_E$ nevertheless. This is because in incremental spotting the deviations are additive and the pluses and minuses cancel out or, in mathematical terms, the algebraic sum of the deviations from a mean equal zero. This is more obvious in Table II. Since the coefficient of variation equals S_D or S_E expressed as a percentage of the mean, it varies inversely with the mean value. In incremental spotting in chromatography or in the kind of mathematical exercise presented in Tables I and II, the mean value must necessarily be greater than that obtained in the calibration procedure and the coefficient of variation therefore appreciably lower.

The variability of the spotters is of two types: how much liquid they pick up and how much they deliver. Unlike a micropipet or syringe, excess liquid obviously cannot be wiped off. In practice, however, if reasonable care is taken to immerse the applicators to about the same depth they will pick up essentially the same volume of solution. It is important that the surface capillary be completely covered but that

little of the straight section be immersed. In this way the capillary is completely filled, but little or no excess liquid adheres to the shaft of the spotter to later run down onto the chromatogram. Attempts to siliconize the applicators have proven of no benefit and it is not really necessary. If the spotters are carefully made so that the projecting surfaces both touch the chromatogram, delivery will be complete and reproducible. Wire loops that are particularly variable can be seen under the magnifier to have projections of uneven length so that the meniscus may or may not contact the surface when the loop is touched to the medium.

Incremental spotting where the origin may be less than 2 mm in diameter makes it critically important that the applicator touch down in *precisely the same place* each time otherwise a doubling of the chromatogram will result. Good precision can be achieved with little effort and can be helped by first making a light pencil mark at the origin and/or practising with a colored solution.

Since the loops are relatively simple to make and quite inexpensive, they can be used in the teaching laboratory as a means of introducing students to qualitative[5] and quantitative[3] analysis in the nanomolar range. Their speed and reliability recommend their use for routine screening of urine amino acids which can be quantified simply by running appropriate standards alongside the unknowns.

ACKNOWLEDGEMENTS

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