CHROM 5529

On the variation between individual operators and techniques in quantitative sample application in thin-layer chromatography

Although direct *in situ* quantitation of thin-layer chromatograms has been greatly refined in the last two to three years the application of the samples remains a problem. The methods used by workers differ greatly and FAIRBAIRN AND RELPH[1] showed considerable variation between different techniques in the hands of certain experienced operators. They did not however investigate the variation between different workers using the same technique. BRIDGER AND RELPH[2] designed a machine to remove the influence of the operator. This complex apparatus is available commercially(Burkard Chromaplot) but the cost is high. BRAIN AND HARDMAN[3] have described a relatively simple and cheap semiautomatic apparatus which gave a coefficient of variation of 2.8 % for the delivery of 5 μl of chloroform. If *in situ* quantitation in thin-layer chromatography (TLC) is to become an accepted routine analytical procedure then it is essential that a simple and cheap method is devised for the application of small sample volumes in a manner which is unaffected by a particular operator.

The primary purpose of this investigation was to examine certain simple application procedures and to determine the extent to which the particular operator could influence the precision. In addition the effect of modifications in the chromatographic system, such as change in layer type or sample solvent, which are often under the control of the operator, were examined.

Experimental

Two types of application procedure were used. The first used a simple capillary disposable micropipette (Drummond microcap) and the second a modification of the semi-automatic apparatus described previously[3].

The sample volume was estimated on the layer, as an applied spot, as preliminary experiments had shown that the coefficient of variation of the applied sample was usually greater than that of a volume delivered into a solution. In all the experiments pre-coated plastic films (Macherey and Nagel) were used as they had greater layer coherence and uniformity than self-spread plates. Two sample solutions of different polarity were used (0.1 % Sudan Red in benzene and 0.1 % amaranth in methanol) and the applied spots scanned directly by transmission on a Vitatron TLD 100 flying spot densitometer (log mode, 525 (green) filter, 0.25 mm aperture). Chromatography before measurement would have resulted in a more even distribution of the material through the spots but this was practically impossible due to the number of estimations involved. However, the flying spot scanning system minimises the effect of local variations in homogeneity. Instrumental precision was better than 1 %.

There are considerable differences between workers as to the best method of using the disposable micropipettes and therefore four procedures were tested. Initially the tubes were filled by dipping into the sample and tilting them until they filled completely. Any residue which remained on the outside of the tube was carefully blotted off. The four procedures were as follows;

(1). *Simple capillation.* The lower end of the filled capillary was touched gently

onto the layer surface and the entire sample (2 μl) allowed to drain out at once by capillary attraction.

(2) *Repeated capillation.* The volume was delivered as in 1 except that it was applied as 5 μl by approximately 0.4 μl volumes by repeatedly lifting the capillary from the layer and returning it. This resulted in a slightly smaller spot.

(3) *Rinsed capillation.* The volume was delivered as in 1. After delivery the tube was refilled with the sample solvent and this solvent applied on top of the sample to wash out any residual sample. This resulted in a ring spot.

(4) *Bulb expulsion.* This was the method recommended by the manufacturers and involved the use of the teat device supplied. The tube was inserted through the lower bung and filled as usual. The bulb was held between two fingers, a third finger used to close the hole in the bulb, and the sample expelled, from a height of approximately 1 mm, by squeezing the bulb.

Seventeen different operators each applied five volumes of each test sample using each application procedure, and their personal preferences and characters were noted.

The size and shape of the spots vary with the layer and sample type as well as with the application procedure. Rinsing produced ring spots which persisted when the samples were run in solvent systems which gave low R_F values. In two cases Sudan Red in benzene gave larger spots than amaranth in methanol but this was reversed on silica gel.

Calculation of the coefficients of variation for each set of five deliveries by one operator under the same conditions showed a wide scatter (1.2–53.4 %) although the mean of 9.5 % was the same as that reported previously for a single worker[3]. All the coefficients obtained by a process involving a particular factor were taken and the mean and standard deviation of these calculated (Table I). The mean value gave a comparative statement on the overall precision in this case whilst the standard deviation of the coefficients (the "range") indicated the extent of variation from this mean figure. Of the different application procedures repeated capillation was significantly inferior to the other methods. Most operators found the bulb expulsion technique difficult and tedious and all thought simple or repeated capillation easiest (Table II). Most obtained their lowest individual variation with simple capillation but it is noteworthy that the next most popular procedure was the least accurate. There was no significant difference between application to silica gel and to aluminium oxide but application to the cellulose layer was much more variable. The difference between the two samples was not significant and neither was that between the sexes. An unexpected result was that the group with "normal" vision was significantly worse than those with corrected vision defects. The normal vision group contained a number of highly inaccurate deliveries, perhaps due to uncorrected vision defects.

A second series of experiments was run using a modified version of the semi-automatic syringe assembly[3]. A Hamilton 250 μl 725N microsyringe was fitted with a special brass collar at the base, together with a short, square cut, needle (Fig. 1). This replaced the long standard needle used previously and made the unit easier to manipulate. A metal template replaced the perspex one which was not solvent-resistant. In addition the bridge piece was made movable in a vertical plane so that it rested on the plate surface at each edge and thus preset the needle tip at fixed height from the layer surface. To use the equipment for repetitive spotting the collar was

TABLE I

PRECISION OF DELIVERY FROM DISPOSABLE MICROPIPETTE

	Mean coeff. of variation[a]	*range*[b]
Variations in technique		
Application method		
(1) Simple capillation	6.9	5.5
(2) Repeated capillation	13.0	10.4
(3) Rinsed capillation	9.4	7.3
(4) Bulb expulsion	9.9	8.8
Layer type		
Silica gel	6.8	4.8
Aluminium oxide	8.5	6.0
Polyethylimine cellulose	13.7	12.5
Solvent		
Benzene	9.9	7.7
Methanol	9.5	13.9
Operator characters		
Sex		
Male	10.0	8.7
Female	8.5	7.4
Vision		
"Normal"	11.8	13.9
Corrected defective	8.1	5.7

[a] Mean of all coefficients of variation obtained by a process involving a particular factor.
[b] Standard deviation of the coefficients of variation from this mean.

TABLE II

OPERATOR PREFERENCE WITH DISPOSABLE MICROPIPETTE

Application method	*First choice* (%)	*Most precise* (%)
(1) Simple capillation	64	82
(2) Repeated capillation	36	18
(3) Rinsed capillation	0	0
(4) Bulb expulsion	0	0

located in each template hole in turn and the button of the PB 600 repeating dispenser depressed to eject one-fiftieth of the total capacity. The application was thus to a large extent automatic.

Fourteen different operators, who were not familiar with the apparatus, each applied twenty-four spots, each of 5 μl, of the solution of Sudan Red in benzene onto silica gel layers. Mean coefficient of variation was 5.6% with a range of 1.6. As a comparison another operator, used to using the equipment, applied 12 × 24 spots in the same manner. The mean coefficient of variation was 3.5% and the range 0.7 suggesting that an element of skill remained. The most obvious variable was the manner in which the operating button was depressed, a sudden sharp pressure tended to spray the sample whilst a very gentle depression allowed creepback. The magnitude of the

creepback effect should be related to the physical properties of the sample solvent and when the single operator repeated the series with the amaranth in methanol solution both the mean coefficient of variation (4.6%) and the range (1.0) were significantly higher.

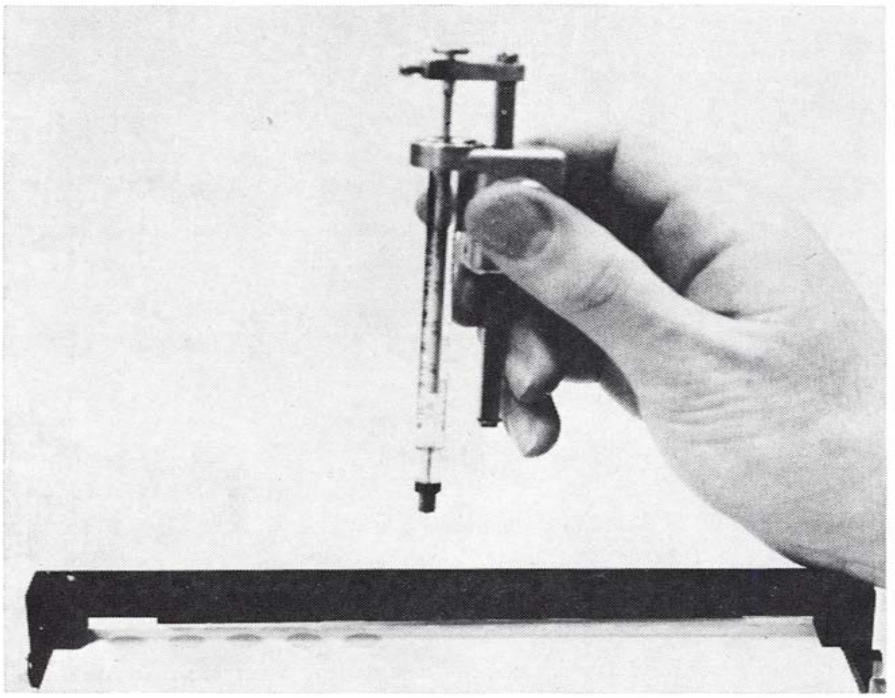

Fig. 1. A Hamilton 250 μl 725N microsyringe fitted with a special brass collar at the base together with a short, square cut, needle.

The error of any method decreases by $\frac{1}{\sqrt{\text{number of estimations}}}$ and hence the application error should be decreased if the total volume is applied as a series of smaller volumes. To test this experimentally the 250 μl syringe was replaced by one of 50 μl capacity which deliverd 1 μl at each stroke, and 5×24 spots, of 1 μl, of 3×1 μl, and of 5×1 μl, were applied to silica gel layers. Coefficients of variation were calculated as before and in addition the expected theoretical errors for three and five deliveries were estimated. Although the variation dropped rapidly with replication (Fig. 2) there was no overall improvement as the initial error with the smaller volume was much greater. In fact the experimental replicate values fell below those calculated but the creepback effect is not entirely random as the residue left by one delivery is likely to be taken up by a near subsequent delivery. A benefit with the replication was a more uniform spot shape and it can be seen that replication is of greatest value when the initial error is high.

Discussion

With the simple micropipette system there was a wide variation in the precision and this could be attributed to the exact technique used, personal factors such as vision, and to the experimental conditions (which are often controlled by the operator). However, in some cases extremely precise deliveries were made.

With the semi-automatic procedure the coefficient of variation was halved but

more important the range was drastically reduced so that the precision could be accurately estimated. Replication of results is always desirable and movement of the replication to the application stage would save on material and labour but as larger volumes are needed to obtain small variations the initial spots must be large, putting more strain on the chromatographic system.

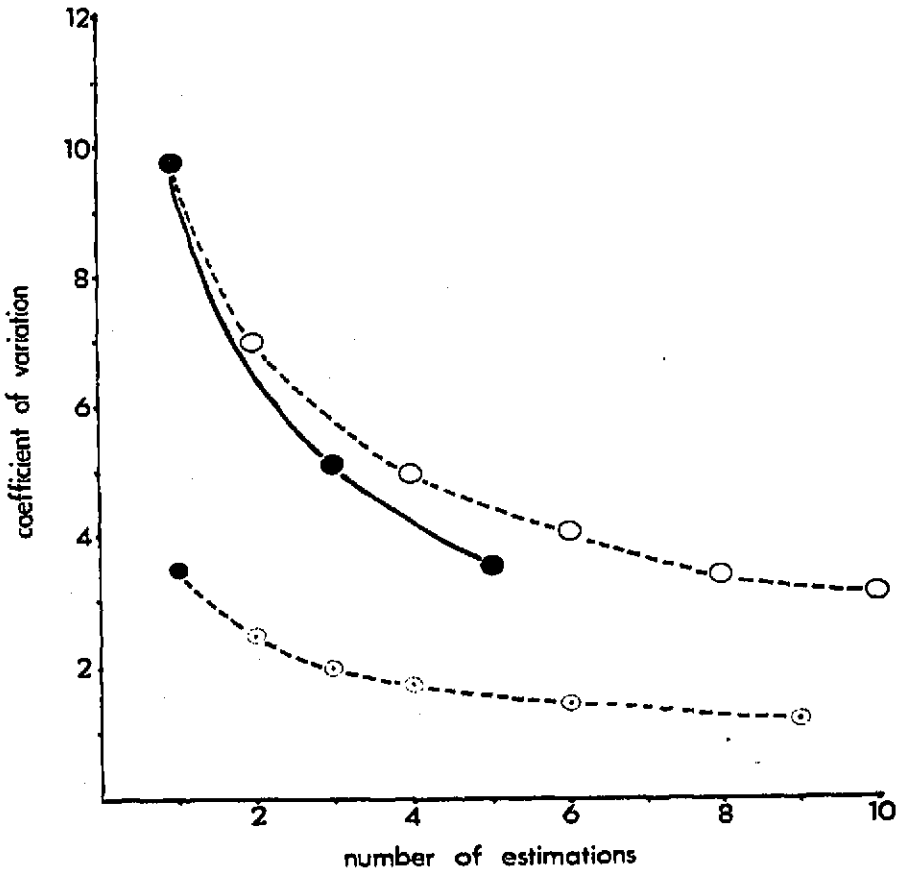

Fig. 2. Relationship between coefficient of variation and number of estimations. ● — ●, Experimental 1 μl and multiples; ○---○, multiples estimated from 1 μl value; ●, experimental 5 μl; ⊙---⊙, multiples estimated from 5 μl value.

The most important conclusion from this work is that any person requiring quantitative sample application should check carefully the personal errors involved using their own experimental conditions and not accept the published figures of other workers. Finally it must be remembered that the problem of quantitative application can be removed by the use of an internal standard[4] although this raises other problems, such as contamination of the sample.

Pharmacognosy Group, Welsh School of Pharmacy, UWIST, Cardiff CF 3NU (Great Britain)

K. R. BRAIN
T. D. TURNER

1 J. W. FAIRBAIRN AND S. J. RELPH, *J. Chromatogr.*, 33 (1968) 494.
2 Y. E. BRIDGER AND S. J. RELPH, *Brit. Pat. Appl.*, 54215/65 and 41036/66.
3 K. R. BRAIN AND R. HARDMAN, *J. Chromatogr.*, 38 (1968) 355.
4 R. KLAUS, *J. Chromatogr.*, 40 (1969) 235.

Received May 24th, 1971