The calibration of plate differences in the thin-layer chromatographic determination of R_M values

The partition coefficient is one of the important factors in studies of the dependence of biological activity on the chemical and physical properties of a group of compounds. BOYCE AND MILBORROW[1] have suggested that reversed phase thin-layer chromatography can be used as a simple method for determining comparable partition values for a group of compounds, the actual parameter of interest in such studies being the R_M value, where $R_M = \log_{10}(1/R_F-1)$.

In practice it is found that although R_M values are quite consistent on any one chromatographic plate, much larger variation occurs between plates even when the requirements for reproducibility, which have been much discussed in the literature, are followed. When comparable R_M values are required for more than about 20 compounds it is not possible to run all of the compounds on any one plate, and it becomes desirable to adjust the observed R_M values for plate differences. This paper discusses a method of calibrating plate differences by using two compounds as standards.

Discussion and results

Several writers have discussed the use of a single reference compound. DHONT AND DE ROOY[2] advocated using the ratio (R_F of compound)/(R_F of standard), and BRENNER[3] has discussed using the difference between the R_M values of a compound and the standard compound. The validity of all such methods using a single standard compound to adjust for plate differences rests on the assumption that the plate to plate variations of the R_F or R_M values of any two compounds are positively correlated.

In order to investigate this assumption, R_M data for some molluscicidal compounds determined in two experiments have been studied. In the first experiment thirteen N-*n*-alkyltritylamines (the homologous series from C_1–C_8 and some of the α- and β-methylated series) were run on each of nine plates, in the second experiment sixteen *m*- and *p*-substituted N-tritylmorpholines were run on each of ten plates. In both cases silica gel impregnated with liquid paraffin was used as the stationary phase, and aqueous acetone as the mobile phase. The experimental details are discussed by BOYCE AND MILBORROW[1], and BOYCE AND WILLIAMS[4].

For each pair of compounds the correlation between the variations in R_M value from plate to plate was calculated. For compounds of similar mean R_M these correlations were positive, but where the mean R_M values differed to any extent the correlations were non significant or negative. This result implies that a single standard compound can not be used to adjust for plate differences except where the mean R_M values are similar.

A graphical study of the data from the two experiments suggested that the observations can be described by the equation:

$$R_{cp} = R_c + \alpha_p + \beta_p R_c + \varepsilon \tag{1}$$

where

R_{cp} is the observed R_M of compound c on plate p,

α_p and β_p are constants characterizing plate p,

ε is the residual variation, and

R_c is the "ideal" R_M value of compound c in the hypothetical system which we try to reproduce in the plates.

The statistical techniques discussed by E. J. WILLIAMS[5] were used to fit eq. (1) to the two sets of data. It was found that both sets of coefficients α_p and β_p contributed significantly (P<0.01) to a considerable reduction in residual variation. It is therefore proposed that (1) be used as an empirical model for calibrating plate differences.

An equation similar to (1), but using R_F instead of R_M, has been suggested for paper absorption chromatography by GALANOS AND KAPOULAS[6]. As R_M is approximately a linear function of R_F for $0.15 \leqq R_F \leqq 0.85$ the two models are for most practical purposes identical. However the use of the R_M value in eqn. (1) is preferable, not only because this is the parameter we wish to estimate in activity studies, but also on theoretical grounds. For, if the compounds of a homologous series are run on each of several plates, the plot of R_M against the number of homologous structural elements for each plate will be linear following the Martin equation. The variation in both slope and position of these lines is described exactly by eqn. (1), but this will not be so if R_F is substituted for R_M.

Practical application

Although the most efficient estimation of the plate constants α_p and β_p in eqn. (1) would be achieved by using statistical incomplete block designs, these are not sufficiently flexible for practical application. In order to estimate α_p and β_p two standard compounds should be selected, with R_F values of about 0.25 and 0.75, from the compounds whose R_M values are to be determined, and these two standards run in at least two positions on each plate, the remaining compounds being allocated to the vacant positions on the plates. In order to obtain estimates of the standard errors of the R_M estimates each compound should be replicated and the allocation of compounds to plates and to position on the plate should be at random.

If we define the hypothetical system as that in which, for each of the two standards, R_c is equal to the observed mean R_M over all plates, so that $\sum_p \alpha_p = \sum_p \beta_p = 0$, we can calculate α_p and β_p as follows:

Let x_p, y_p be the mean R_M of the two standards on plate p, and let $\bar{x}$, $\bar{y}$ be the mean of x_p and y_p over all plates,

then:

$$\alpha_p = (\bar{x}y_p - \bar{y}x_p)/(\bar{x} - \bar{y})$$

$$1 + \beta_p = (x_p - y_p)/(\bar{x} - \bar{y})$$

If compound c is present on plate p with observed R_M equal to R_{cp}, then the ideal R_M value is estimated by

$$R_c = \frac{\sum_p (1 + \beta_p)(R_{cp} - \alpha_p)}{\sum_p (1 + \beta_p)^2}$$

where $\sum_p$ represents summation over all plates on which compound c is run.

An estimate of the residual variance, assumed homogenous, is given by:

$$s^2 = \frac{1}{n} \sum_{c,p} (R_{cp} - \alpha_p - (1 + \beta_p)R_c)^2$$

where n is the number of error degrees of freedom.

The standard error of the estimate R_c is:

$$\frac{s}{\sqrt{(\sum_p (1 + \beta_p)^2)}}$$

This can be approximated by s/n_c where n_c is the replication of compound c.

This method of calibrating plate differences using two standard compounds has subsequently been used in these laboratories and has allowed an increase in the accuracy of R_M determinations equivalent to a threefold increase in replication.

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