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Note

Sensitivity and the dimensions of the separating medium in thin-layer chromatography

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There are many tasks, both in industry and research, where it is necessary or desired to analyze minute sample quantities by thin-layer chromatographic (TLC) methods. The smaller the sample, the smaller is of course also the amount of solute concentrated in a given zone. The presence of a zone is usually determined optically and optical means are also the ones most commonly used for quantitative evaluation of the separated material. Optical methods provide also the probably most sensitive technique for detecting very small amounts of separated substance. But even these fail when the observed concentration drops below a certain threshold. Indirectly this detection threshold poses also a limit to the sensitivity of the chromatographic method. Intuitively it can be expected that the dimensions of the medium will have some bearing upon this threshold and that further sensitivity increases might be possible by reducing them. It is this question which this paper sets out to investigate.

FUNDAMENTAL CONSIDERATIONS

Before going any further it appears appropriate to define the two principal terms around which this discussion will revolve. The first one is the sensitivity U, which shall here be defined as the smallest change of the optical signal which can be reliably identified as not being of purely random origin. It will be assumed that observation is effected via photoelectric conversion. Visual inspection without technical aids can be shown to have a sensitivity threshold which for most cases related to chromatographic applications is inferior to that which can be obtained photoelectrically. Besides that, visual methods by themselves are very subjective and do not lend themselves very well to quantitative characterization.

The sensitivity defined in the way above is an objective parameter of the devices and of the medium used, but is of little immediate significance to the chromatographer. For him the important quantity is the smallest amount of the substance he wishes to analyse which can be observed or quantitatively determined with acceptable error. This amount, Q_{min}, depends, however, on a number of extremely variable factors, *e.g.*, the extinction factor a or the coefficient of fluorescence, F, the spectral position of the absorbance peak, the mean concentration, c, the photostability of the substance, and others. It would thus not be suitable for general reference purposes.

The second quantity of importance is the concentration of the zone material.

It will be defined as the ratio of the amount of substance contained in the volume under a surface element Δz of the chromatogram to that element. This definition does not explicitly involve the thickness X of the medium and differs, therefore, from the volume concentration in the usual sense[1].

$$\int_Z c \, dz = Q \tag{1}$$

In the further discussion it will be assumed that either single-beam fluorometry or double-beam densitometry are used for observation. Single-beam densitometers have too low a sensitivity threshold to be of interest in this context.

The principal parameter which determines the sensitivity of the method is the level of noise which accompanies the useful signal. Fluorometry and double-beam densitometry exhibit virtually zero optical baseline noise and during zone areas the optical signal-to-noise ratio is independent of concentration[2,3]. It follows that for work with low concentrations the deciding factor is electrical noise.

ELECTRICAL NOISE

The electrical noise of photo(densito)meters is almost exclusively due to the photodetectors. It is constant and independent of the level of illumination. Statistically it has a normal (Gaussian) amplitude distribution and its r.m.s. (root mean square) level is proportional to the square root of the electrical bandwidth, W. Any residual optical baseline noise can, with good approximation, be also described by the same kind of mathematical model.

The noise bandwidth is determined by the minimum bandwith needed to process the useful signal. This parameter can be shown to be approx.

$$W \approx v/d \tag{2}$$

Here v is the scanning velocity and d the diameter (width) of the beam aperture.

INTEGRATION

To obtain the total amount of substance within a zone, the measured local concentration values have to be integrated over the zone area Z. Integration increases the useful signal amplitude by approx. Z/d^2. The r.m.s. noise amplitude increases too, but due to the random character of the noise only by $\sqrt{Z}/d$; the net result is an improvement in signal-to-noise ratio proportional to $\sqrt{Z}/d$.

DECREASING THE ZONE AREA

Making the zone area smaller by a factor p increases the noise after integration, other things equal, by $\sqrt{Z}/p$. The integrated signal amplitude remains, however, unchanged, provided the amount of separated substance Q remained the same.

$$\int_Z c(z) \, dz = \bar{c}Z = Q = \int_{Z_p} p \cdot c(z) \, dz = p \cdot \bar{c} \cdot \frac{Z}{p} \tag{2}$$

The signal-to-noise ratio improves thus by $\sqrt{p}$. Since the smallest quantity, Q_{min}, which can be detected is proportional to the noise level, decreasing the zone area p times increases the sensitivity by $\sqrt{p}$. It should be noted that this sensitivity improve-

ment applies only to the case where the original noise level has been constant and independent of the signal strength. (This is the case also with single-beam densitometers.) With fluorometry and double-beam densitometry the signal-to-noise ratio sufficiently above the sensitivity threshold is constant and independent of concentration. In this case reduction of the zone area does not bring any improvement; on the contrary, for best results the zone area should be large, but subject to the restriction that the optical noise prevails over the electrical noise. The reason for this seeming paradox is that with constant signal-to-noise ratio the smoothing effect of integration increases when the zone area is made larger.

REDUCING THE THICKNESS OF THE MEDIUM

Densitometry

Consider now the effect of a reduction of the thickness of the medium, first upon direct densitometry and then upon fluorometry. Transmittance A_T and reflectance A_R of a turbid sheet with absorption K_0 and scatter S can be expressed by the following relationships[3]

$$\gamma = X \cdot K_0 \sqrt{\left(1 + \frac{2S}{K_0}\right)}$$

$$\varrho = \frac{1 - \sqrt{\left(1 + \frac{2S}{K_0}\right)}}{1 + \sqrt{\left(1 + \frac{2S}{K_0}\right)}}$$

$$A_T = e^{-\gamma X} \frac{1 - \varrho^2}{1 - \varrho^2 \cdot e^{-2\gamma X}}$$

$$A_R = \varrho \frac{1 - e^{-2\gamma X}}{1 - \varrho^2 \cdot e^{-2\gamma X}} \tag{3}$$

When the thickness X is made very small, these expressions become

$$\begin{aligned} &X \to 0 \\ &e^{-\gamma X} \simeq 1 - \gamma X \\ &A_T \simeq (1 - \gamma X) \frac{1 - \varrho^2}{1 - \varrho^2 \cdot e^{-2\gamma X}} \simeq (1 - \gamma X) \frac{(1 - \varrho^2)}{(1 - \varrho^2)} = 1 - \gamma X \\ &A_R \simeq \varrho \frac{+2\gamma X}{1 - \varrho^2 \cdot e^{-2\gamma X}} \simeq 0 \end{aligned} \tag{4}$$

As to be expected, very thin layers are unsuitable for reflectance measurements. In the transmittance mode the useful increment of the response due to a concentration c is, perhaps somewhat surprisingly, independent of thickness.

$$\Delta K \simeq \alpha c$$

$$\Delta A_T \simeq X \left[\gamma(K_0) - \gamma\left(K_0 + \frac{\Delta K}{X}\right)\right] \simeq X \cdot \frac{\partial \gamma}{\partial K} \cdot \frac{\Delta K}{X} = \Delta K \cdot \gamma_K(K_0) \tag{5}$$

The result above tallies with the conclusions obtained in ref. 3 which demonstrated that double-beam transmittance measurements do not exhibit optical noise due to thickness fluctuations. In the reflectance mode the opposite is the case. Assuming

that the thickness X is large enough to make reflectance measurements at all possible we can write

$$A_R \approx \varrho$$
$$\Delta A_R \approx \varrho\left(K_0 + \frac{\Delta K}{X}\right) - \varrho\,(K_0) \simeq \frac{\partial \varrho\,(K = K_0)}{\partial K} \cdot \frac{\Delta K}{X} \tag{6}$$

This result can be interpreted as saying that the values obtained from double-beam reflectance measurements vary inversely proportional to the thickness of the medium. Since baseline noise is zero anyway, this conclusion applies only to zone regions. In other words, the incremental noise which appears during the scanning of zone regions is partly, and possibly mostly, caused by thickness fluctuations and proportional to their magnitude. Transmittance measurements are not sensitive to these and should, therefore, in many cases provide a lower signal-to-noise ratio.

Fluorometry

Eqn. 4 shows that the transmittance of a very thin medium tends towards unity. The intensity of fluorescence observed from either side is thus the same and proportional to the product cF, where F is the coefficient of fluorescence of the fluorogen. With finite thickness X the corresponding expressions for observation from the non-illuminated and illuminated sides are, respectively[4]

$$A_{TF} \sim \frac{cF}{2} \cdot \exp\,(-X\gamma)$$
$$A_{RF} \sim \frac{cF}{2} \cdot \frac{1}{2X\gamma} \tag{7}$$

It can be seen that in both cases the amplitude of the fluorescent signal increases if X becomes smaller. Both modes are, therefore, susceptible to optical noise due to thickness variations. In most cases the sensitivity threshold is determined by electrical noise; reducing X is then an efficient means to increase the sensitivity of the method and to improve the accuracy at near threshold concentrations. The measure has little effect at higher concentrations where optical noise dominates, which in the fluorescent mode is a constant proportion of the useful signal amplitude.

CONCLUSIONS

Two important conclusions can be drawn from the reasoning above. First, reducing zone spread, *e.g.* by narrowing the width of the separating strip or by development modifications, improves the sensitivity threshold only proportionally to the square root of the decrease in zone area, that is substantially less than what is generally assumed. And second, reducing the thickness of the separating layer to very small values has some advantage for fluorescence measurements, but has little to offer for densitometry.

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