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OPTICAL NOISE IN PHOTOMETRIC SCANNING OF THIN MEDIA CHROMATOGRAMS

II. DOUBLE-BEAM DIFFERENCE SYSTEMS

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SUMMARY

In this the second part of a theoretical treatment of the quantitative analysis of thin media chromatograms the double-beam difference system of scanning has been investigated. This system is much more sensitive than any single-beam arrangement. The incorporation of a "flying-spot" system as opposed to fixed slits permits the quantitative analysis regardless of zone geometry. Perfect balance between both scanning beams and a high degree of stabilization of the light source are essential for good performance. The limits in sensitivity are obtained when the optical noise approaches the electrical noise.

INTRODUCTION

In the preceding paper[1] the concepts of "signal" and "noise" as they are used in communication theory have been applied to single-beam transmission photodensitometers. It was shown that for the quantitative analysis of very small amounts of absorbing substances a linear relationship may be assumed between the resultant electrical signal and concentration and that in this case the parameter of greatest importance is $\bar{A}/\bar{A}$ which refers to the relative non-uniformity of the medium itself.

In this paper the double-beam difference system[2-8] first utilized by SALGANICOFF *et al.*[2] is investigated. In this device (see Fig. 1) in addition to the principal measuring beam arranged to have a wavelength corresponding to that of the peak absorption of the substance of interest there is also a reference beam. The wavelength of this reference beam is selected so as to be virtually unabsorbed by the zones of interest. In practice there may be some difficulty in achieving this at high concentration levels. In order to cancel out the optical noise arising from the irregularities of the paper background the difference between the electrical output signals of both beams is formed and recorded.

The double-beam method appears to possess important advantages in comparison with any presently conceivable single-beam arrangement. This paper is intended, therefore, to extend the results obtained for the single-beam device to the double-beam difference system. The meaning of the symbols is the same as in the preceding paper and in addition the general theoretical relationships given there will be used here again. Those equations developed in the preceding paper and used here are denoted by adding [I] to their number.

BALANCE OF BEAMS

For efficient noise rejection it is evidently necessary that both beams should ideally possess equal spectral energy density ε_0 and ε_R, and also an equal spectral bandwidth and equal spatial cross section $F = F_R$ (the index R will here always refer to the reference beam).

In practice, of course, all those conditions cannot ideally be met and a finite difference in radiant flux I_0 remains.

$$I_{0R} = I_0(1 + \beta) \qquad (\beta \ll 1) \tag{1}$$

In addition to the differences in the radiant flux between the two beams there is also a certain difference in transmission for these two wavelengths.

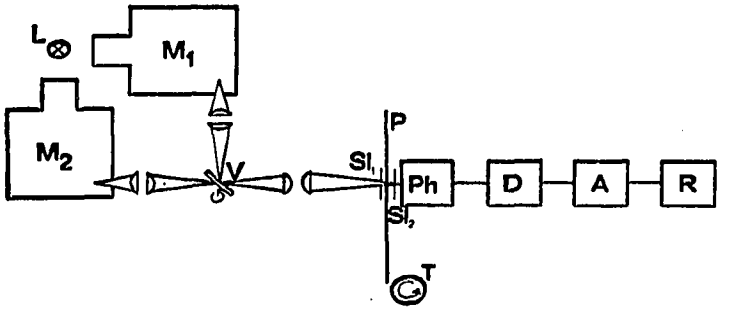

Fig. 1. Schematic representation of a dual beam chromatogram scanner with difference system. (according to SALGANICOFF *et al.*[2]). L = light source; M_1, M_2 = monochromators; V = vibrating mirror; Sl_1 = slit 1; P = chromatogram; Sl_2 = slit 2; Ph = photodevice; T = chromatogram transport mechanism; A = amplifier; R = recorder; D = device for obtaining difference signal.

In order to keep this difference small, the two beams should be spectrally as close together as the width of the absorption band permits. In agreement with eqn. 5 [I] we may now write

$$\begin{aligned} g(\lambda_0 - \lambda_R) &= g_0 + \gamma \\ A_R &= A(1 + g_0 + \gamma) \\ \bar{A}_R &= \bar{A}(1 + g_0) \qquad (g_0 \ll 1) \end{aligned} \tag{2}$$

$$\begin{aligned} I_{0R}A_{Rs} &= I_0A_s(1 + \beta)(1 + g_0) \\ &\approx I_0A_s(1 + \beta + g_0) \end{aligned} \tag{3}$$

In order to cancel out as much of the background optical noise as possible, it is desirable that in the average both sides of eqn. 3 should be equal. For this purpose a

mechanically adjustable diaphragm or two polaroids may be used. This permits the adjustment of the relative intensity of both beams so that

$$I_{0R}\bar{A}_R - I_0\bar{A} = I_0(\beta + g_0 + C) = I_0\Delta\beta \to 0 \qquad (4)$$

The coefficient C takes care of the adjustment of the diaphragm.

In order to avoid the stability problems which might occur when d.c. amplification of the output signal is used, the two illuminating beams are generally chopped. SALGANICOFF *et al.*[2] use a vibrating mirror and a common photodetector for this purpose. The adjustable diaphragm in this case, however, as well as other minor differences in the optical pathways of the two beams tend to introduce a phase difference into the two signals reaching the photodetector. The result of this phase shift is that even with ideal balancing the output signal does not become zero (see Fig. 2).

$$|I_0| = |I_{0R}| \text{ but } \hat{I}_0 - \hat{I}_{0R} \neq 0$$

This effect may be abolished by introducing a phase-sensitive (synchronous) detector circuit which only responds to the in-phase component I_0' of both signals. The diaphragm is then adjusted to make $I_0' = I_{0R}$.

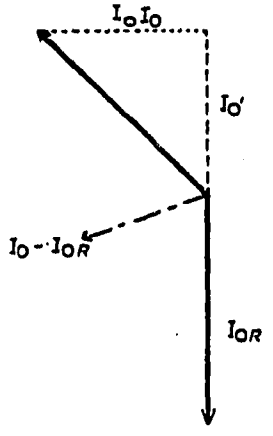

Fig. 2. Effect of a phase shift on the difference of two signals.

However, even with synchronous detection a complete equalization of both signals over any extended period of time is not possible. One of the reasons is inconstancy in time of the parameters involved in the term β. Another reason is the difficulty in adjusting in practice to the true value of g_0, which may vary to a certain degree from one chromatogram to another, even if samples from the same batch are used. As a consequence $\Delta\beta$ behaves to a certain degree as a chance variable and the value used in the equations should be understood as the most unfavourable value, both with regard to amplitude and sign, which may be expected with some reasonable probability.

To minimize $\Delta\beta$, the two beams should be spectrally close together and their optical pathways nearly identical. A common photodetector, serving both beams on a time-sharing basis, as schematically shown in Fig. 1 causes both beams to pass slightly different areas of the chromatogram. The difference is dependent upon the alternating frequency f and the speed u with which the illuminated area is moving.

$$\xi = \frac{u}{f}/\Delta W_c \tag{5}$$

The result is equivalent, at least as far as noise cancellation is concerned, to a minimum value of $\Delta\beta \simeq \xi$. To avoid this two independent photodetectors with simultaneous chopping of both beams can be used. Another possibility is of course to make f sufficiently large. A minimum condition is evidently:

$$f \geq u/\Delta W_c \tag{5a}$$

If eqn. 5a is violated, both beams pass different areas of the medium, jeopardizing altogether the advantage of the double-beam method. For all these reasons a complete equalization of the two output signals is not feasible; and it, therefore, becomes necessary to consider a finite difference in the final output signal.

$$I_0\bar{A}\Delta\beta = |(I_{0R}\bar{A}_R - I_0\bar{A})|_{\min} \tag{6}$$

The minimum value of this expression has to be found by suitably adjusting the intensity of one of the beams [*e.g.* by changing the diaphragm (co-efficient C in eqn. 4)].

THE OPTICAL SIGNAL TO NOISE RATIO

The overall noise is also here essentially determined by expression 7[1] except that $g_0\cdot(\lambda-\lambda_0)$ is replaced by $\Delta\beta$. Further we have to consider the additional local unbalance caused by the useful signal I_c. In this way we obtain:

$$I_0 = I_0\frac{1}{\sqrt{F}}\left[\bar{\bar{A}}^2(\Delta\lambda)\left\{\Delta\beta + \bar{\alpha}_c\cdot\frac{F_c}{F}\cdot\frac{\Delta\lambda_c}{\Delta\lambda}\right\}^2 + 2\bar{\bar{\gamma}}^2(\Delta\lambda)\bar{A}^2(\lambda_0)\right]^{1/2} \tag{7}$$

As a consequence of the definition of $\Delta\beta$ given earlier, here and in all the following formulae that sign of $\Delta\beta$ has to be considered which gives the most unfavourable result. As already mentioned in connection with eqn. 7[1] usually one of the terms in 7 will prevail and then only this term need be considered. This permits a considerable simplification of the expressions involved.

In most practical cases it will be the first term which dominates. The straight addition of the two terms in the brace is justified if $F_c/F \simeq 1$, which is the most important case. If $F_c/F \ll 1$ the square root of the sum of the squared terms would be more appropriate. It should be kept in mind that $\bar{A}(\Delta\lambda)$ decreases to a certain degree with increasing spectral bandwidth $\Delta\lambda$. If the illuminated region does not contain any absorbent, the second term in the brace becomes zero. In order to obtain minimum noise under this condition, $\Delta\beta$ should be as small as possible, as is of course expected. The total differential output signal of the optical system is:

$$It = I_s - I_c = I_0\bar{A}\left[\Delta\beta - \bar{a}_c\cdot\frac{F_c}{F}\cdot\frac{\Delta\lambda_c}{\Delta\lambda}\right] \tag{8}$$

and the signal to noise ratio σ_1, with the term containing $\bar{\bar{\gamma}}$ being neglected, becomes:

$$\sigma_1 \simeq \frac{I_c}{I_\nu} = \frac{\bar{A}}{\bar{A}(\Delta\lambda)}\cdot\frac{a_c\cdot\dfrac{F_c}{F}\cdot\dfrac{\Delta\lambda_c}{\Delta\lambda}}{\Delta\beta + a_c\cdot\dfrac{F_c}{F}\cdot\dfrac{\Delta\lambda_c}{\Delta\lambda}}\cdot\sqrt{F} \tag{9}$$

To maximize this expression we put as before $F_c/F = \Delta\lambda_c/\Delta\lambda = 1$. All further expressions will refer to this condition. Using expression 2a[1] we obtain

$$\sigma_1 = \frac{\bar{a}_c}{\bar{\bar{a}}} \cdot \frac{\sqrt{F}}{\Delta\beta + \bar{a}_c} \tag{9a}$$

The way in which this can be achieved was explained in the discussion section of the preceding paper. Either the solute has to be applied in bands which extend across the full width of the chromatogram after development or the fixed illuminating slit has to be replaced (by a flying-spot arrangement with subsequent integration) over the whole zone area.

The two procedures are, however, equivalent only if a sufficiently large amount of investigated substance is available. If very small samples are to be analyzed, a small spot-shaped zone may provide a higher value of average concentration and consequently a better signal to noise ratio if flying-spot scanning is employed. The reason is of course that the useful signal is—with a limited amount of analyzed substance Q_s available—proportional to Q_s, regardless of the area over which Q_s is spread, whilst the optical noise increases proportionally with the square root of the area $\sqrt{F}$. A closer inspection shows that there is no contradiction to expression 9, since the latter is based upon the assumption that there is sufficient solute available, so that spreading does not change the concentration.

In a double-beam arrangement, as opposed to the single-beam system it is relatively easy to discriminate between zone and non-zone parts of the chromatogram. From eqn. 8 it is apparent that It is mainly dependent upon α_c so long as $\Delta\beta$ is small enough. To obtain the signal to noise ratio with external integration, $\sqrt{F}$ in formula 9a has to be replaced by $\sqrt{S}$, where S is the area of integration. There is no necessity to emphasize that when logarithmic forming of the output signal is employed this has to be done before averaging is carried out.

With very low concentrations, that is for small values of α_c, the second term in the denominator may be neglected; this gives:

$$\sigma_1 \text{ (low)} \simeq \frac{\bar{a}_c}{\bar{\bar{a}}} \cdot \frac{\sqrt{S}}{\Delta\beta} \tag{10}$$

For larger concentrations $\Delta\beta$ may be neglected, resulting in

$$\sigma_1 \text{ (high)} \simeq \frac{\sqrt{S}}{\bar{\bar{a}}} \tag{11}$$

Inspection of the original expression 9 shows that in this case the fraction F_c/F cancels out; this means that bandzones or flying-spot scanning do not produce here any significant improvement in σ. Further it is interesting to note that the signal to noise ratio at high concentrations appears to be independent of the amplitude of the useful signal. The explanation of this fact is, of course, that the chromatogram background noise affects the useful signal in a multiplicative rather than an additive way. As a consequence of this the noise signal is proportional to the useful signal. At the same time the noise contribution from the rest of the chromatogram virtually cancels out provided $\Delta\beta$ is sufficiently small. The noise produced by the irregular transmission of I_c within the absorbing area, however, is not affected at all by the difference forming

procedure. A qualitative illustration of the dependence of the signal to noise ratio upon the ratio $\bar{\alpha}_c/\Delta\beta$ is shown in Fig. 3.

In some cases the second term in eqn. 7 will dominate; we then obtain a signal to noise ratio:

$$\sigma_2 \simeq \frac{\alpha_c}{2\bar{\bar{\gamma}}(\Delta\lambda)}\sqrt{S} \qquad (12)$$

It should be noted that $\bar{\bar{\gamma}}$ can be reduced by the same means used to make $\Delta\beta$ small and by using the maximum spectral width $\Delta\lambda$ compatible with the linearity requirements.

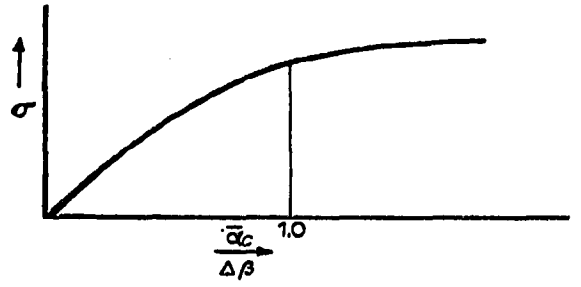

Fig. 3. Signal to noise ratio (σ) in dependence upon the ratio of concentration to beam equalization.

OTHER SOURCES OF ERRORS

Another possible source of error is the varying average transmittance of the medium, which may show considerable fluctuation from one chromatogram to another (see eqn. 8). These deviations will produce proportional errors in the optical output signal similar to those produced by variations in the illuminating light density. With present chromatographic media such as the current qualities of papers, loaded papers, coated sheets etc., it appears that the calibration should be repeated whenever a new paper is inserted into the scanning device. As the changing value of transmittance appears as a multiplicative factor in the useful output signal I_c the percentage of error introduced in the measurement of A_c is independent of concentration. With higher concentrations where the linear approximation in eqn. 2[1] is no longer valid the error in the result from this source tends to become smaller.

A further factor to be considered is the surface reflection factor ψ (see eqn. 4[1]), which may change from one chromatogram to another. It results in a change in the proportion of light entering into and transmitted by the medium; it is therefore equivalent to a variation in the intensity of the light source. Suitable calibration procedures at the beginning of each measurement are the best remedy.

STABILITY REQUIREMENTS FOR THE LIGHT SOURCE

From the arguments developed above it appears that the optical signal to noise ratio deteriorates rather rapidly if α_c becomes small against $\Delta\beta$. $\Delta\beta$ may therefore in a certain sense be considered as a threshold value, which imposes a limit upon the sensitivity that may be obtained in quantitative scanning, using the double-beam difference method.

To ensure good stability of $\Delta\beta$, a few precautions have to be taken: the voltage supplying the light source should be highly stabilized. Care should be taken to derive both beams from the same area of the lamp, because the temperature distribution and emission density within the lamp are not constant and may vary with time, supply voltage, etc. The lamp should be replaced in advance of obvious aging effects. An important source of error is the instability of the illuminating light source I_0. A change ΔI_0 in I_0 produces a change in detector output ΔIt (eqn. 8) proportional to the change ΔI_0. The observer, however, attributes this change to an apparent change in useful signal output ΔI_c. For ΔI_c we obtain the relation

$$\Delta It = \Delta I_0 \bar{A}(\Delta\beta - a_c) = \Delta I_c$$

$$\frac{\Delta I_c}{I_c} = \frac{\Delta I_0}{I_0}\left(\frac{\Delta\beta - a_c}{a_c}\right)$$

$$= \frac{\Delta I_0}{I_0}\left(\frac{\Delta\beta}{a_c} - 1\right) \qquad (13)$$

From this expression it follows that the percentage error in the measured output will in general be larger than the percentage change in I_0, depending upon the ratio $\Delta\beta/a_c$. Again the consequences will be more serious with weaker concentrations.

For a very crude estimate of the value of ΔI_0 let us assume an incandescent lamp where most of the energy supplied is emitted as radiant energy. If the supply voltage changes by e % the emitted radiant flux I_0 changes approximately by $2e$ %. This underlines the importance of good stabilization of the supply voltages.

NUMERICAL EXAMPLE

The best way to illustrate the results obtained above is probably a numerical example. The first value we have to consider in this context is the optical noise value of the medium itself. According to our own measurements as well as data obtained from the literature[2,9] the value of $\bar{\bar{\alpha}}$ with Whatman No. 3 paper is of the order of 0.05 optical density units. In natural units this is about 0.15. The spatial fundamental F^* of the noise appears to be approximately 2 × 2 mm. The optical density of this type of paper is according to SALGANICOFF *et al.*[2] of the order of 3.4 units; this corresponds to a transmittance $\bar{A} \simeq 3 \cdot 10^{-4}$.

Let us now assume a single-beam instrument with an illuminated slit area 2 × 50 mm. The solute is assumed to be applied in bands, so that $F_c/F = 1$ and the spectral width of the beam shall be sufficiently narrow, so that $\Delta\lambda_c/\Delta\lambda = 1$. Using eqn. 12[1] we obtain a signal to noise ratio.

$$\frac{\sigma}{\sqrt{25}} \geq 2 \simeq \frac{\bar{a}_c}{0.15} \qquad (\sigma \text{ min} \simeq 10) \qquad (14)$$

Assuming that for reasonable accuracy a minimum signal to noise ratio of 10 is required, we obtain the value of the smallest signal which can still be measured to about 0.30, that is $\approx$0.13 optical density units.

Passing now to a double-beam difference forming device with the same area of illumination we obtain from eqn. 9a.

$$\sigma_1 = \frac{\bar{a}_c}{0.15} \cdot \frac{\sqrt{25}}{\Delta\beta + a_c}$$

$$\simeq 33 \cdot \frac{1}{1 + \frac{\Delta\beta}{\bar{a}_c}} \qquad (15)$$

According to SALGANICOFF *et al.*[2] it seems that values of $\Delta\beta \approx 0.02$ may be obtained in practical operation. For a minimum value of $\sigma_1 \gtrsim 10$ we obtain a minimum value of $\bar{\alpha}_c \approx 1 \cdot 10^{-2}$, that is about 0.004 optical density units, as the weakest signal to be processed. Realization of this value, however, requires very careful optical design and an electrical arrangement with a low enough noise figure.

Subsequent integration over the area of the zone could improve those values by a factor of 2 to 3, depending upon the area of the zone; this applies of course to single-beam devices as well. Against the single-beam instrument the double-beam method offers an improvement of about 32 times. The error in determining a signal of this intensity will be about $\pm 1/\sigma \approx 10$ % due to optical noise plus a certain amount due to surface reflection, instability of the light source, etc., disregarding both $\bar{\bar{\gamma}}$ and the electrical noise. To obtain a value of $\Delta\beta$ of the order mentioned ξ (see eqn. 5) has to be well below this value. Again assuming $\Delta W_c = 2$ mm and a paper velocity of 1 mm/sec, we obtain for a single photodetector arrangement a chopping frequency

$$\xi \leq 0.01$$

$$f = 200$$

To obtain improved accuracies at the same sensitivity or a higher sensitivity at the same accuracy, $\Delta\beta$ has to be decreased. Further improvement can be obtained by making use of the fact that the optical noise is affecting the useful signal in a multiplicative way. The residual noise in eqn. 15 can then be decreased by replacing the difference signal at the output by a ratio signal and using different chopping techniques. A planned device incorporating these features will be described shortly. If the optical and electrical noises are of comparable amplitude, their powers have to be added; this amounts to reducing the signal to noise ratio in eqn. 15 by a factor of $\sqrt{2}$. By the same factor, of course, accuracy is decreased and the minimum amount of investigated substance is increased. In general a design with both noise components equal will give the best trade off between performance and cost.

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REFERENCES

1 A. A. BOULTON AND V. POLLAK, *J. Chromatog.*, 45 (1969) 189.
2 L. SALGANICOFF, M. KRAYBILL, D. MAYER AND V. LEGALLAIS, *J. Chromatog.*, 26 (1967) 434.
3 B. CHANCE, *Rev. Sci. Instr.*, 22 (1951) 619.
4 B. CHANCE, *Methods Enzymol.*, 12 (1957) 273.
5 R. RIKMENSPOEL, *Appl. Opt.*, 3 (1964) 351.
6 R. RIKMENSPOEL, *Rev. Sci. Instr.*, 36 (1965) 497.
7 J. C. COWLES, *J. Opt. Soc. Am.*, 55 (1965) 690.
8 I. E. BUSH, *Methods Biochem. Anal.*, 11 (1963) 149.
9 R. B. INGLE AND E. MINSHALL, *J. Chromatog.*, 8 (1962) 369.