THE QUANTITATIVE ASSESSMENT OF FLUORESCENCE ON THIN MEDIA CHROMATOGRAMS

A THEORETICAL STUDY

VICTOR POLLAK

Division of Biomedical Engineering, University of Saskatchewan, Saskatoon, Saskatchewan (Canada)

AND

ALAN A. BOULTON

Psychiatric Research Unit, University Hospital, Saskatoon, Saskatchewan (Canada)

SUMMARY

Owing to inherent wavelength conversion in fluorescence analysis, the measuring signal is much less affected by optical background noise than is the case in absorptiometric measurements by both transmittance or reflectance procedures. This explains the higher sensitivity and accuracy obtained in fluorescence analysis. A further advantage of fluorimetry is the much wider range of concentrations over which the response is nearly linear. Measurements from either side of the medium are shown to be nearly equivalent in this respect. More light is, however, available if the measurements are carried out from the illuminated side.

INTRODUCTION

In several recent papers[1-3], we discussed the theory of the transmission and reflection of light in a dispersive medium based upon the simplified theory of KUBELKA AND MUNK[4]. The treatment was oriented towards the requirements of quantitative thin media chromatography (and electrophoresis) by photodensitometric methods. Fluorescence measurements, which in this field are equally important, were not covered. The present paper, therefore, corrects that omission and concentrates upon an application of the general theory to fluorescence methods.

In the case of fluorescence analysis, measurements are carried out at a wavelength of light different from the illuminating wavelength; this represents the fundamental difference between conventional transmittance or reflectance measurements and fluorescence procedures. In a sense, there is an analogy between fluorescence techniques and the use of frequency conversion in the common superheterodyne radio receiver. In this type of receiver, now used almost universally, the frequency of the

received signal is, at the input of the receiver, converted to a different wavelength; further processing of the signal is achieved in the new spectral position. The main advantages of this technique are better selectivity and higher sensitivity, the latter due to a reduction in noise.

Similar causes are responsible for the advantages of fluorimetric methods compared with conventional photodensitometry. The sensitivity of all photometric methods is basically determined by the background noise[5-7]. In the common types of photometric instrumentation as used in chromatographic evaluation, this background noise consists of two components. One is electrical noise originating in the photo-detector element and in the input stages of the associated amplifier chain. The other is optical noise caused by random fluctuations of the optical transfer of the chromatographic medium. If sufficient light is available, the first component can be made negligibly small and so the sensitivity, accuracy and resolving power of the method are principally determined by the optical background noise.

The principal advantage of fluorescence procedures rests with the low level of optical background noise. Ideally, the medium itself should not fluoresce at all. As only the intensity of light at the wavelength of fluorescence is evaluated, the medium affects the received signal only in those areas where a fluorophore is present. Optical background noise is consequently generated only within the boundaries of the zone of separated fluorophore. Its intensity is, therefore, much smaller than in conventional photodensitometric evaluation because in this situation optical noise is generated all over the scanned area of the medium.

In practice, of course, this ideal situation is never achieved and some spurious fluorescence is encountered all over the medium, giving rise to an optical noise signal similar in character to that encountered in direct photodensitometry. Fortunately, however, the intensity of this signal is relatively weak in comparison with that encountered without wavelength conversion. It is this factor that makes fluorescence methods attractive as a simple and sensitive approach not requiring sophisticated instrumentation. This does not imply, of course, that the results obtained in fluorimetry could not be improved if more sophisticated instrumentation were to be used. It should be noted that the above argument applies only to direct fluorescence measurements and not to indirect methods such as quenching.

Recently, rather sophisticated and refined instruments for the photodensitometric evaluation of thin media chromatograms have been developed[8,9]. The basic principle in these cases has been to apply two light beams of different wavelengths, followed by a suitable combination of the two signals in such a way as to remove much of the background optical noise. Depending upon the amount of noise cancellation, these instruments produce results that are comparable with those obtained in simple fluorescence measurements.

Most of the versions of the double-beam principle as used in conventional photodensitometry are not very efficient when applied to fluorimetry because the reference beam tends to acquire noise information from parts of the medium outside the fluorescing zone. This information is not compensated for by a corresponding noise signal in the measuring beam and so, instead of an improvement in the signal to noise ratio, a deterioration often occurs. Moreover, noise due to spurious fluorescence outside the fluorescing zone is not affected at all. There are certain modifications of the double-beam principle, however, which will avoid the first of the above problems

while maintaining the principal advantages. Spurious fluorescence is, in this case, best reduced by computer processing based upon its statistical properties. On balance, therefore, an evaluation of the amount of a separated fluorophore in applicable cases, using proper instrumentation, still appears to offer the lowest obtainable sensitivity thresholds.

THE KUBELKA AND MUNK THEORY APPLIED TO FLUORIMETRY

When a beam of light impinges upon an optically turbid medium, part of it is absorbed and converted into heat, and part is scattered at the particle boundaries in the medium in all directions. To determine the optical transfer of such a medium is a complex and mathematically extremely difficult task. Fortunately, however, for many cases of technical importance a simplified theory developed by KUBELKA AND MUNK[4] gives adequate results. The basic assumption of this theory is that the light inside the medium propagates only in the forwards *and* in the backwards directions perpendicular to the boundary surfaces of the medium, which are assumed to be plane parallel.

When the conclusions obtained from the KUBELKA AND MUNK theory are verified experimentally by measuring the optical transfer of the medium, it has to be kept in mind that the above assumption applies only to points inside the medium. Surface points can, for most practical purposes, be considered as omnidirectional point sources of light. The KUBELKA AND MUNK theory gives with good approximation the total intensity of these fictitious light sources. The intensity of light radiated by them in a given direction is proportional to the cosine of the angle between that direction and the surface plane of the medium. The intensity decreases with the square of the distance from the radiating surface element. The measured optical transfer values, *e.g.*, optical density and coefficient of reflectance, are, therefore, strongly dependent upon the distance between medium and optical pick-up system and upon the angle of inclination of its optical axis against the surface plane of the medium (Fig. 1).

In the KUBELKA AND MUNK theory, two parameters are used to characterize the optical response of the medium. One is the coefficient of absorbance, K, and the
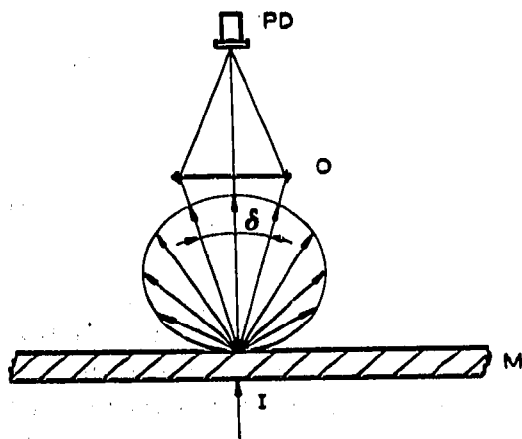

Fig. 1. Directional diagram of transmitted scattered radiation. M = medium; I = illuminating beam; O = optical pick-up system; PD = photo-detector.

other the coefficient of scattering, S. If the theory is to be applied quantitatively, the numerical values of these parameters have to be known or they must be determined independently.

The theory further assumes that the medium can be considered as a sheet with plane parallel surfaces and that the direction of illumination is perpendicular to the surface. Between the boundary surfaces the medium is assumed to be homogeneous. This means that neither K nor S is a function of the distance x from the illuminated surface.

In chromatography, the concentration of a separated substance is very frequently a function of the distance from the surface. It is then mainly the coefficient of absorption, K, that changes. The resulting error is usually negligible if the concentration of the separated substance is small. The error encountered at higher concentrations depends upon the non-uniformity of the concentration profile, the values of the optical constants of the medium and the mode of photometric determination used.

Part of the light impinging upon the surface of the medium is immediately reflected from the surface without entering the medium to any appreciable depth. This "specular" reflection is not affected by the possible presence of the separated substance in the medium and, therefore, does not convey any information about its interior. From a chromatographic point of view, specularly reflected light is a loss and has to be discounted from the total intensity of illuminating light.

In fluorescence measurements, the wavelength of the primary illuminating beam is usually in the ultraviolet region and is, therefore, invisible to the naked eye. When fluorescence is encountered, part of the primary radiation is converted to a different wavelength, usually in the visible range. This energy is lost to the primary beam. The loss is irreversible very much like the loss by absorption. The proportion of primary energy converted at any given point inside the medium to a different wavelength can be designated the coefficient of fluorescence, F, which is, of course, proportional to the local concentration, C, of fluorescing substance:

$$F = \alpha \cdot C \tag{1}$$

The KUBELKA AND MUNK theory assumes that the medium is homogeneous; F is, therefore, considered to be independent of the distance from the surface. This assumption is, of course, a simplification, as the concentration profile of the separated substance will usually vary with depth.

The fundamental equations of the KUBELKA AND MUNK theory can be written in the following form (see ref. 1 for further details):

$$\frac{\mathrm{d}^2 j(x)}{\mathrm{d}x^2} = \gamma^2 j(x)$$

$$\frac{\mathrm{d}^2 r(x)}{\mathrm{d}x^2} = \gamma^2 r(x) \tag{2}$$

Here $j(x)$ is the intensity of light travelling in the forwards direction and $r(x)$ that of the backwards travelling component, and x is the distance from the illuminated surface (see Fig. 2). For convenience, it is usual to assume that the thickness of the medium is equal to unity:
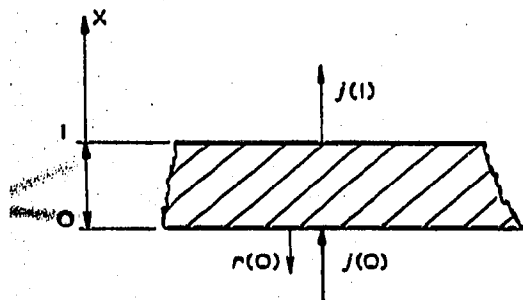
$$0 \leqslant x \leqslant 1 \tag{3}$$

Fig. 2. Schematic representation of the KUBELKA AND MUNK hypothesis. $j(o)$ = incident light intensity (specular reflection discounted); $j(1)$ = transmitted light intensity leaving the medium with the angular distribution shown in Fig. 1; $r(o)$ = backscattered (reflected) light intensity.

The coefficient γ in eqn. 2 is very approximately equal to the optical density of the medium provided that the mentioned distance effects are discounted. It is, however, expressed in natural logarithmic units whereas conventional optical density is measured in logarithmic units to the base ten. In the solution of the above equations as derived in ref. 1, another coefficient, designated ϱ, appears. ϱ is the coefficient of re-reflexion for a medium with very large (theoretically infinite) optical density, and is measured on a linear, that is non-logarithmic, scale. It can be shown that

$$-1 \leqslant \varrho \leqslant 0 \qquad (4)$$

Both γ and ϱ can be determined from the optical constants, K and S, of the medium:

$$\gamma = \sqrt{K(2S + K)} \qquad (5)$$

$$\varrho = \frac{K - \gamma}{K + \gamma} \qquad (6)$$

From eqn. 6, it can be seen that ϱ does not depend upon the thickness of the medium.

The effect of fluorescence upon the propagation of the primary radiation is tantamount to an increase in absorbance, that is, an increase in energy loss of the exciting radiation:

$$\gamma_p = \sqrt{(K + F)(2S + F + K)} \qquad (7)$$

$$\varrho_p = \frac{K + F - \gamma_p}{K + F + \gamma_p} \qquad (8)$$

The total energy density of the exciting radiation at any point x in the interior of the medium is equal to the sum of the forwards and backwards travelling components:

$$v(x) = r_p(x) + j_p(x) \qquad (9)$$

Addition of eqn. 2 gives:

$$\frac{d^2v(x)}{dx^2} = \frac{d^2}{dx^2}[r_p(x) + j_p(x)] = \gamma_p^2[r_p(x) + j_p(x)] = \gamma_p^2 v(x) \qquad (10)$$

Eqn. 10 is essentially identical to the well known equation describing an electrical transmission line with purely resistive parameters. A transmission line of

this type is easily implemented and can, therefore, serve as a convenient model to simulate the optical behaviour of turbid media[1]. The general solution to eqn. 10 is well known (see ref. 10 for details) and consequently it has not been listed here.

The intensity of fluorescence $f(x)$ excited at any particular point in the interior of the medium is proportional to the total energy there and, of course, to the coefficient of fluorescence F, which was assumed to be constant throughout the medium. This yields

$$f(x) = F\,v(x) \tag{11}$$

The propagation of the light created by fluorescence inside the medium can be described by a set of equations identical with those illustrated in eqns. 2. These two equations can again be combined into a single second-order differential equation of the same type as that listed in eqn. 10. The constants γ and ϱ appearing in the solutions are defined by eqns. 5 and 6, and they therefore do not contain the coefficient F. No index will be used to designate them.

It should be noted that the coefficients of absorbance and scattering are, in general, wavelength dependent. For chromatographic purposes, however, it is desirable to use a medium which is "gray" throughout the range of wavelengths employed, that is, a medium where the values of K and S change very little within this range. If F is then small compared with K, the coefficients γ and ϱ are essentially the same for both the primary and the excited radiations and the subscript p can, therefore, be deleted:

$$\begin{aligned}\gamma &\approx \gamma_p\\ \varrho &\approx \varrho_p\end{aligned} \tag{12}$$

The length of the optical path, which has to be traversed by the excited fluorescence before reaching the surface, is obviously dependent upon the coordinate x of the point where it was generated. There is no obvious reason why any particular direction of propagation should have preference over another. It can, therefore, be assumed that half of the generated intensity of fluorescent light travels in the forwards direction and the other half in the backwards direction. It should be noted that these are the only two directions permitted by the KUBELKA AND MUNK theory.

Let the transmittance of a sheet of medium thickness x now be $A_T(x)$. The fluorescent light generated at point x arrives, therefore, at the two surfaces with the intensities (see Fig. 3)

$$i(x)_{far} = \frac{F\,v(x)}{2} \cdot A_T(1 - x) \tag{13}$$

$$i(x)_{near} = \frac{F\,v(x)}{2} \cdot A_T(x) \tag{14}$$

The subscript "near" designates the illuminated surface and "far" the opposite one. The total intensity of fluorescence generated at point x is, of course, determined by eqn. 11.

Now, each point along the axis x contributes its share to the total intensity of fluorescence observed at the surface. This intensity is, therefore, the result of the
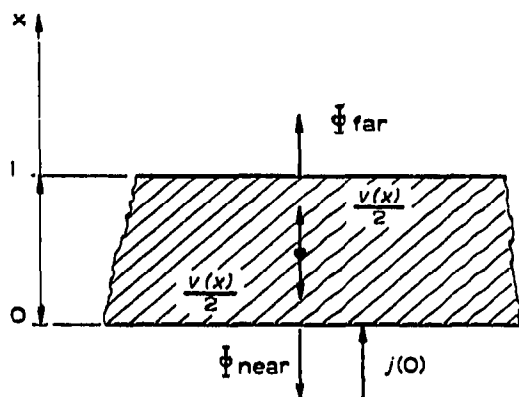
Fig. 3. The propagation of fluorescence radiation excited in the bulk of the medium. $v(x)$ = total energy density of primary radiation at x; Φ = total amount of fluorescence reaching the surface of the medium for $j(0) = 1$.

superposition of all elementary contributions for values $0 \leqslant x \leqslant 1$. Mathematically, this amounts to integration:

$$\Phi_{far} = \int_0^1 i(x)_{far}dx = \int_0^1 \frac{F\,v(x)}{2} \cdot A_T(1-x)dx \qquad (15)$$

$$\Phi_{near} = \int_0^1 i(x)_{near}\,dx = \int_0^1 \frac{F\,v(x)}{2} \cdot A_T(x)dx \qquad (16)$$

The mathematics for solving eqns. 15 and 16 will be published in a separate paper. The complete solutions are much too complex for interpretation in practical terms. However, for several cases of practical importance, considerable simplifications are possible. The results obtained for these specific cases will be discussed in the following sections.

THE CASE OF BEER'S LAW

The first case to be considered is that of a medium with very little scattering, where Beer's exponential law holds with reasonable accuracy. With such a medium, the back-scattered component of the incident light is too weak to permit useful measurements in the straight "reflectance" mode. Fluorescence measurements can, however, still be carried out from either side of the medium.

In an as yet unpublished theoretical study, the intensity of fluorescence measured at the illuminated (*i.e.*, "near") side of the medium is determined by the expression:

$$\Phi_{near} = \frac{F}{2K+F}\{1 - e^{-(2K+F)}\} \qquad (17)$$

At dilute concentrations of fluorophore it can usually be assumed that $F \ll K$, and eqn. 17 can then be simplified to:

$$\Phi_{near} \approx \frac{F}{2K}(1 - e^{-2K}) \qquad (17a)$$

If, in addition, the optical density of the medium is very low, a further simplification is possible:

$$1 - e^{-2K} \approx 2K; \qquad K \ll 1$$
$$\Phi_{near} \approx F \tag{17b}$$

In the opposite case, when the concentration of a strongly fluorescing substance is large, eqn. 17 becomes:

$$F \gg K$$
$$\Phi_{near} \approx [1 - e^{-F} \cdot e^{-2K}] \tag{17c}$$

For measurements from the far side of the medium, the expression is:

$$\Phi_{far} = e^{-K}(1 - e^{-F}) \tag{18}$$

At low concentrations of fluorophore, eqn. 18 reduces to:

$$F \ll 1$$
$$\Phi_{far} \approx F \cdot e^{-K} \tag{18a}$$

Comparison of eqns. 17 and 18 shows that both methods give, in most cases, comparable results. Measuring from the near side has the obvious advantage that the available intensity of light is larger. This factor only becomes important, however, in the case of media with very high optical density or when detecting equipment of low sensitivity is used.

TURBID MEDIUM WITH MEDIUM TO LARGE OPTICAL DENSITY

The next case to be considered is much more general and covers most media of practical importance. It is assumed that the blank medium has appreciable scattering and at the same time a transmittance lower than approximately 0.5 optical density units. Another simplifying assumption is that the optical parameters of the medium are very nearly the same for the exciting wavelength and the fluorescent response. In other words it is assumed that the medium is "gray" until far into the ultraviolet region and that the coefficient of fluorescence F is small compared with K (see eqn. 12).

The above assumptions are, of course, only approximately valid. They are, however, justified because of the considerable simplification to the resulting relations without producing excessive errors.

For the intensity measured at the near end of the medium the expression obtained (theoretical studies to be published) is:

$$\Phi_{near} \approx F \cdot \frac{1 - \varrho^2}{2\gamma} \tag{19}$$

Under the conditions assumed above, $e^{-\gamma}$ is very nearly equal to the transmittance of the medium for the incident radiation. $0.4\,\gamma$ is approximately equal to the optical density of the medium, if the distance effects mentioned earlier in this paper are discounted.

At the far side of the medium, the intensity of fluorescent light is:

$$\Phi_{far} \approx F \cdot \frac{e^{-\gamma}}{2\gamma} \cdot (1 - \varrho^2)(1 + \varrho) \tag{20}$$

In most practical cases, $\varrho^2 \ll 1$ so that the term $1 - \varrho^2$ in eqns. 19 and 20 can usually be neglected. It can also be shown (see eqn. 6) that the coefficient ϱ is independent of the thickness of the medium and, therefore, does not carry noise produced by random changes in the thickness of the medium.

Comparison of eqns. 19 and 20 shows that the intensity of fluorescent light is, in general, much higher at the near side than at the far side. The former is also less influenced by thickness variations of the medium and, therefore, less noisy.

A decided advantage of fluorescence measurements compared with absorptiometry is the extremely good linearity of response in terms of concentrations. Eqns. 15 and 20 show that the range of linearity for fluorescence measurements extends to much higher concentrations than is the case for conventional transmittance or reflectance measurements. This fact is, of course, empirically well known to chromatographic workers. Only at fairly high concentrations do deviations from linearity begin to appear. Fluorescence measurements can be made from either side of the medium without affecting the linearity.

Eqns. 19 and 20 also indicate why conventional double-beam systems are not very effective in further reducing the residual optical noise encountered when scanning a fluorescing zone. The expressions for conventional transmittance and reflectance measurements, as derived in ref. 1, differ considerably from the corresponding expressions developed in this paper for fluorescence intensity. A nonconverted reference beam is, therefore, influenced by the optical background noise of the medium in a different way and noise cancellation by either difference or ratio forming of the two beam signals is possible only to a limited extent.

REFERENCES

1 V. POLLAK, Trans. I.E.E.E. on B.M.E., 17, No. 4 (1970) 287.
2 V. POLLAK AND A. A. BOULTON, J. Chromatogr., 50 (1970) 19.
3 V. POLLAK AND A. A. BOULTON, J. Chromatogr., 50 (1970) 39.
4 P. KUBELKA AND F. MUNK, Z. Tech. Phys., 12 (1931) 593.
5 A. A. BOULTON AND V. POLLAK, J. Chromatogr., 45 (1969) 189.
6 V. POLLAK AND A. A. BOULTON, J. Chromatogr., 45 (1969) 200.
7 V. POLLAK AND A. A. BOULTON, J. Chromatogr., 46 (1970) 247.
8 L. SALGANICOFF, M. KRAYBILL, D. MAYER AND V. LEGALLAIS, J. Chromatogr., 26 (1967) 434.
9 V. POLLAK AND A. A. BOULTON, J. Chromatogr., 63 (1971) 87.
10 R. K. MOORE, Travelling Wave Engineering, McGraw-Hill, New York, 1960.