Journal of Chromatography, 133 (1977) 49–57

CHROM. 9690

FLUORESCENCE PHOTOMETRY OF THIN-LAYER CHROMATOGRAMS AND ELECTROPHEROGRAMS

V. POLLAK

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

(Received September 16th, 1976)

SUMMARY

The paper brings a theoretical analysis of the performance of photometric methods for the quantitative evaluation of thin-layer chromatograms using fluorescence. Fluorescence quenching is considered as an extension of straight photodensitometry into the UV without requiring UV secondary optics nor UV sensitive photodetectors. For quantitative analysis quenching is not recommended.

The principal advantages of fluorescence photometry are good accuracy and high sensitivity without the need for costly and sophisticated instrumentation. These advantages are due mainly to a steady and in the ideal case altogether noise-free baseline and to the inherently linear relationship between photometric response and concentration. Intermediate integration on photographic film permits a further considerable increase in sensitivity. When evaluating the film recording, it may be necessary to compensate for the non-linearity of the gradation curve.

When a zone is encountered, optical noise begins to appear. The signal-to-noise ratio is constant, independent of amplitude, as in double-beam densitometers. Its magnitude is also of the same order. A serious drawback of the fluorometer is the dependence of the readings upon the intensity of the exciting radiation. It appears feasible to apply double-beam scanning also to fluorescence measurements. The benefits to be expected are mainly increased accuracy and elimination of the influence of the intensity of illumination. The sensitivity may become limited by the lower light intensity available in the fluorescence transmission mode, which seems best suited for the double-beam approach, because of the relative prominence of electrical noise. In most cases, however, double-beam fluorometers should be able to produce better results than double-beam densitometers. Single-beam fluorescence methods have a performance which is much superior to single-beam densitometry but inferior to double-beam densitometry.

INTRODUCTION

Photometric methods are today the favorite tool for the quantitative evaluation of separations on thin-media substrates. By and large these methods can be divided into two large groups. Straight densitometric methods are used for measuring

the change in transmittance or reflectance of the chromatogram caused by the presence of a zone of separated substance. The range of wavelengths over which these measurements are commonly carried out extends from the red end of the visible spectrum to the medium UV and even beyond that. In the context of this paper the characteristic feature of these methods is that no wavelength conversion takes place; the measurements are thus carried out at the same suitably selected wavelength which is used for illumination. The methods of the second group are comprehensively labeled "fluorescence measurements", though other kinds of induced radiation are occasionally used as well. Measurements take place at a wavelength longer than that of the illuminating light, which in this application almost always lies in the UV. It can thus be said that the characteristic feature of these methods is wavelength conversion and measurement at the converted wavelength.

Fluorescence measurements can again be divided into direct methods and methods based upon quenching. The first method uses the natural fluorescence of the investigated substance alone or, more often, with a strongly fluorescing compound coupled to it. The other method uses a uniformly fluorescing layer which actually produces the visible negative of the UV light distribution. The conversion layer is mostly built into the medium but can also be arranged in the form of a separate fluorescent filter in front of the photodetector.

FLUORESCENCE QUENCHING

Fluorescence quenching can in a sense be considered as an extension of direct photometry into the UV without the need for photodetectors sensitized to UV radiation and not requiring UV transparent optics on the secondary side of the device. Nearly all the rules applicable to direct measurements can thus be applied to quenching with few modifications. The results of quenching techniques tend, however, to be inferior for two principal reasons. The fluorescing layer acts as a source of additional optical noise, which is responsible for a larger error and a reduced sensitivity threshold. Double-beam scanning, usually the best way for combatting the effects of optical noise[1], is difficult to apply. The conversion sensitivity of the fluorescent layer is in general not constant and calibration becomes critical.

It should also be noted that the amplitude of the useful signal obtained by quenching is in most cases a non-linear function of concentration in the same way as with densitometric transmittance or reflectance. Approximate linearization can be carried out, if desired, by the same methods as used for direct measurements[2]. Now that solid-state and photomultiplier-type photodetectors with extended UV response are available at reasonable cost, it would appear that quenching offers little advantage and for demanding quantitative applications had better be avoided.

BASELINE

Direct fluorescence measurements have long been a favorite tool for many qualitative and quantitative determinations, mainly when very low concentrations are to be measured. The principal reason for this is that the fluorescing substance stands out as a bright zone on a dark background. When evaluated on a photometric scanning device the dark background produces a nearly ideally flat baseline at a

constant zero level. Any remaining jitter is partly due to electrical noise[3] and partly due to residual fluorescence of the chromatogram even in the absence of separated substance. The effects of electrical noise can be substantially reduced by integrating the output signal over a sufficiently long period of time. Noise due to background fluorescence —which is essentially time invariant— is, however, not diminished in this way.

When very low intensities of fluorescence are to be measured, very long periods of integration may become necessary. In these cases integration on photographic film becomes attractive, which permits in a simple way to obtain integration times of almost arbitrary duration. The exposed and developed film can then be evaluated by standard densitometry. For quantitative interpretation it is necessary to compensate for the non-linear gradation characteristic of the photographic material.

Intermediate photographic processing is also a possibility in direct chromatography, when extreme sensitivity levels are to be obtained. However, it would seem that the gains which might be obtained in this way are substantially smaller than in fluorescence. The method comes thus into consideration only for a few exceptional applications. It is well known that at very low light levels the so-called reciprocity law of photography breaks down. This law states that the degree of blackening of photographic material is proportional to the product of exposure time and illuminating intensity. At very low intensities it is, therefore, necessary to increase the exposure time more than proportionately to the reciprocal of the available light intensity. The increase over and above the regular product valid for higher light levels depends upon the type of material used and the way it is processed. It has to be considered for quantitative determinations, but this does not *a priori* preclude the applicability of the method.

Another method to measure very low intensities of fluorescence, which seems to hold promise for the future, is photon counting with subsequent digital integration. Today the method seems to have found little application in chromatography. However, if successful, it could extend the detection margin to quantities orders of magnitude below present thresholds.

LINEARITY OF RESPONSE

For small to medium concentrations of separated substance the intensity of the emitted fluorescence is almost ideally proportional to the quantity of fluorogen present. With single-beam devices the response is of course also dependent upon the intensity of the exciting beam. The approximate expressions below demonstrate this clearly[4].

$$I_{FT} = \frac{Fc}{2} \cdot e^{-\gamma_F} \cdot (1 - \varrho_E^2) \cdot (1 + \varrho_F) \cdot E_0$$

$$I_{FR} = \frac{Fc}{2} \cdot \frac{(1 - \varrho_E^2)(1 + \varrho_F)}{\gamma_E + \gamma_F} \cdot E_0 \qquad (1)$$

The indices E and F used in conjunction with the parameters ϱ and γ above refer to the values of these parameters for the exciting and excited radiation, respectively. I_{FT} and I_{FR} are the intensities of the fluorescent radiation emitted by a surface element

of the chromatogram and observed from the far and near (illuminated) sides, respectively. c is the concentration of fluorescing substance in the illuminated area; E_0 is the intensity of the exciting beam and F the coefficient of fluorescence of the investigated material. ϱ is the coefficient of reflectance of a sheet of medium thick enough so that its transmission can be disregarded. γ is the natural logarithm of the transmittance (2.3 times the optical density) of a very thin sheet of medium, which consequently has negligible reflectance. In the approximate form of eqn. 1 both are assumed to be independent of wavelength. An important property of ϱ is that it does not vary when the thickness or density of the medium changes, as long as its microscopic structure remains preserved. γ, on the other hand, varies linearly with thickness and/or density changes. For noise considerations, therefore, the latter parameter usually prevails. In many cases ϱ^2 is so small that chance variations of its magnitude can be neglected. The influence of the terms containing ϱ can then be accounted for by a constant correction factor b, which permits to reduce eqn. 1 to the form

$$A_{FT} = \frac{I_{FT}}{E_0} = \frac{Fcb}{2} \cdot e^{-\gamma_E}$$
$$A_{FR} = \frac{I_{FR}}{E_0} = \frac{Fcb}{2} \cdot \frac{1}{\gamma_E + \gamma_F} \approx \frac{Fcb}{2} \cdot \frac{1}{2\gamma} \qquad (1a)$$

It is interesting to note that A_{FT} depends only upon the value of γ for the excited fluorescence, whilst A_{FR} is also affected by that for the exciting radiation. It is easy to see that γ_E depends also upon the concentration c of the fluorogen. From eqn. 1a it can be concluded that A_{FT} is a linear function of c over a wide range of concentrations. The relationship between c and A_{FR} is linear too but due to the influence of γ_E over a more limited range.

As a consequence of the inherent linearity of fluorescence measurements no special steps are required to linearize the photometric response. Flying spot scanning is thus redundant, though it has no detrimental effects. The results are independent of the spatial concentration distribution and of the geometry of the zones encountered.

OPTICAL NOISE IN ZONE REGIONS

As mentioned above, fluorescence methods offer even with single-beam instrumentation a near noise-free baseline. However, when a zone of separated substance is scanned, optical noise begins to make its appearance.

The different mechanisms which are responsible for the generation of optical noise are discussed in ref. 5. When applying the conclusions obtained there to eqn. 1 it turns out that the optical noise of fluorescent measurements from either side is comparable to the noise encountered with straight transmittance measurements. The principle factors responsible for the optical noise are thus variations in thickness and/or density of the chromatogram and a fluctuating coefficient of specular reflection for the exciting radiation. Instability of the emission of the light source is another potential source of noise, especially when gas discharge lamps are used. However, the characteristics of the latter much more resemble electrical noise than optical noise. Frequently, it is erroneously concluded that because the baseline of fluorescence measurements is virtually noise free, the measurement as such is noise free as well.

This is obviously not the case. A closer inspection of eqn. 1 based upon the arguments presented in ref. 5, shows that the all important signal-to-noise ratio has a definite value which does not depend upon the useful amplitude, that is, upon the coefficients F and c. This result is obtained already with unsophisticated single-beam instruments; in densitometry it can also be obtained, but only with involved double-beam devices.

An estimate of the signal-to-noise ratio can be derived from the simplified expressions 1a. Obviously the only factor of which fluctuations can produce optical noise are the coefficients γ. Designating the r.m.s. value of their fluctuations by $\delta\gamma$ we can write for the first and second cases, respectively

$$\begin{aligned} e^{-(\gamma+\delta\gamma)} &\approx e^{-\gamma}\cdot(1-\delta\gamma) \\ \frac{1}{2(\gamma+\delta\gamma)} &\approx \frac{1}{2\gamma}\left(1-\frac{\delta\gamma}{\gamma}\right) \end{aligned} \tag{2}$$

In either case, the signal-to-noise ratio is determined by the ratio of the constant term to the fluctuating one*

$$\begin{aligned} \left(\frac{S}{N}\right)_{FT} &\simeq \frac{1}{\delta\gamma} \\ \left(\frac{S}{N}\right)_{FR} &\simeq \frac{\gamma}{\delta\gamma} \end{aligned} \tag{3}$$

It follows that the signal-to-noise ratio is indeed in both cases constant and independent of concentration. The dependence of the output signal upon the intensity of the illuminating light is, however, not removed and good long-term stability of the latter is therefore necessary. Variable surface reflection remains as a possible source of error. On the other hand, direct pick-up of specularly reflected light, which is a matter of concern with densitometric reflectance measurements, can here easily be removed by optical filtering of the light entering the photodetector.

When noise reduction measures, *e.g.* electrical or spatial filtering or integration, are employed, the coefficient $\delta\gamma$ refers to the noise level after processing.

The baseline noise of a fluorogram should ideally be zero, though in practice some spurious background fluorescence may be present, which prevents the noise from vanishing altogether. The fluorescent noise signal can obviously never become negative, whilst with densitometry optical noise amplitudes which are positive and negative with respect to the mean baseline level occur with equal probability. Exploitation of this fact should permit restoration of the true baseline level with a higher degree of accuracy and reliability. The limits are then given by the electrical noise of the photometer. The smallest quantity which can be determined will then be that which produces a fluorescent signal about three times stronger than the r.m.s. value of the electrical noise**. With intermediate photographic techniques, noise introduced by the photographic process may become the limiting factor. When the spurious

* These ratios are of the same order as the ones which are obtained with double-beam densitometry.

** The factor 3 is based upon the assumption that the residual noise has a near normal amplitude distribution. In this case the probability that amplitudes larger than three times the r.m.s. value occur becomes so small that it can be neglected.

background fluorescence is large, it may become the main factor determining the sensitivity threshold. Its masking effect can be reduced by making the mean zone area smaller. It may also be worth noting that with fluorescence always more light becomes available when the zone concentration increases; this is exactly the opposite of what occurs with densitometric methods. In some marginal cases this difference may become important.

The sensitivity of fluorometric methods is largely determined by the residual baseline noise. Accuracy, however, depends upon the combined effects of baseline noise and zone noise. As a consequence, quantitative determinations near the sensitivity threshold are rather inaccurate.

INTENSITY OF EXCITATION

Increasing the intensity of excitation by itself does not increase the sensitivity. The increased output of fluorescent light reduces, however, the masking effects of electrical and/or photographic noise and thus indirectly leads up to lower sensitivity thresholds and better accuracy at low concentrations.

Many substances when exposed to high radiation loads at elevated quantum energies (*e.g.*, UV) have a tendency to decompose. Sometimes it is possible to reduce the danger of photodecomposition without unduely decreasing the fluorescence output by concentrating the exciting radiation into a narrow band centered around the wavelength of maximum excitability. When tunable UV lasers become available at reasonable cost, they may well represent the ideal light source for these purposes. With lasers it might also be possible to use excitation by extremely narrow pulses followed by subsequent time gating of the response signal. This should permit to use the relaxation time of the fluorescent material as criterium for improved identification of the separated solute.

DOUBLE-BEAM SCANNING ALSO FOR FLUORESCENCE?

What has been said in the preceding sections of this paper can be summed up in the conclusion that fluorescent methods used in conjunction with relatively unsophisticated and inexpensive photometric equipment provide results which are largely superior to those which can be obtained on similar devices in the densitometric mode. They are, however, in a number of respects inferior to the performance of involved double-beam densitometers. In view of the dramatic performance improvement obtained in densitometry by the double-beam principle, it becomes tempting to investigate its potential applicability also for fluorometry.

Before going any further, it has to be understood that the goal of double-beam scanning in fluorometry is very much different from that which leads to its application in densitometry. In the latter case, it mainly serves to produce a smooth baseline. Incremental zone noise, when separated substance is present, is not reduced.

With fluorescence, baseline noise is inherently almost non-existent and the main concern becomes reduction of the (incremental) zone noise, which in densitometry is not diminished by double-beam scanning. It turns out that this is possible and the double-beam principle can be employed to reduce zone noise and to produce some other benefits inherent in this approach. The relative improvement in perfor-

mance, though, is much less than in densitometry. Still it appears that the advantages to be expected are sufficient to warrant efforts in this direction. Eqns. 4 below show the theoretical expressions for the transmittance A_T and reflectance A_R of a turbid medium, derived from the well known Kubelka and Munk equations[6]. The symbols employed have the same meaning as in eqn. 1 of this paper or in the references quoted.

$$A_T = e^{-\gamma} \frac{1 - \varrho^2}{1 - \varrho^2 \cdot e^{-2\gamma}}$$
$$A_R = \varrho \cdot \frac{1 - e^{-2\gamma}}{1 - \varrho^2 \cdot e^{-2\gamma}} \tag{4}$$

From comparison of eqns. 1 and 4 it appears that the expression for A_T in eqn. 4 is very similar to that obtained for fluorescence observed from the far (non-illuminated) side of the chromatogram. If the wavelength of the reference beam is close to that of the excited fluorescence, it can be assumed that the parameters ϱ and γ for both are nearly equal. Forming the ratio of both expressions then yields

$$\gamma = \gamma_F; \ \varrho = \varrho_F$$
$$A_{FT} = I_{FT}/E_0$$
$$\frac{A_{FT}}{A_T} = \frac{Fc}{2} \cdot \frac{1 - \varrho^2_E}{1 - \varrho^2} \cdot (1 + \varrho_F)(1 - \varrho^2 \cdot e^{-2\gamma}) \tag{5}$$

Most chromatographic media have ϱ values of the order of 0.3 and less. The terms containing ϱ^2 can then be neglected, so that

$$\frac{A_{FT}}{A_T} \simeq \frac{Fc}{2} \cdot (1 + \varrho_F) \tag{5a}$$

Obviously the only parameter in this expression fluctuations of which could produce optical noise is the coefficient ϱ_F. The resulting signal-to-noise ratio can be written down immediately

$$\left(\frac{S}{N}\right)_{FD} \approx \frac{(1 + \varrho_F)}{\delta\varrho_F} \tag{6}$$

Comparing this value with expressions 3 shows that the double-beam system is able to improve the signal-to-noise ratio in zone areas. The improvement obtainable depends upon the magnitude of the remission factor ϱ_F: the smaller the latter, the better. A numerical example will help to illustrate this point: assume $\varrho_F = 0.35$ and $\delta\varrho_F$ / 0.035. Both values are fairly typical. The zone signal-to-noise ratio encountered in single-beam fluorometry is than about 10. A double-beam system, however, will yield 1.35/0.035 $\simeq$ 40, a four times improvement. In the first case an accuracy of about $\pm 10\%$ can be expected against one of $\pm$ 2.5% in the second.

Ratio forming of the two beam signals ensures that the baseline remains flat

and free of optical noise. It also makes the output signal independent of minor changes in the intensity of illumination including the effect of a variable coefficient of surface reflectance[7]. The signal of the measuring beam has to be placed into the numerator of the ratio. For best noise compensation the wavelength of the reference beam should be chosen close to that of the excited fluorescence, preferably towards the UV.

The double-beam system does, however, not eliminate noise due to spurious background fluorescence. Electrical noise is even increased by approx. $\sqrt{2}$ because the electrical noise contributions of the two beams add. To the author's knowledge, no double-beam system for fluorescence is yet in operation. The advantages discussed above still await, therefore, experimental verification. However, even if only moderately successful, the system might well prove advantageous. The reason is that most double-beam densitometers are also used as fluorometers. Adapting them for double-beam fluorometry might well be easier and less costly than the modifications which are presently needed for single-beam use.

MEASUREMENTS FROM THE NEAR AND FAR SIDES OF THE CHROMATOGRAM

Fluorescence measured at the far (non-illuminated) side of the chromatogram has the important advantage that the measured results are near independent of the distribution of the fluorogen with depth. This is not the case when the measurements are carried out from the near (illuminated) side[8]. In either case it is necessary that the coefficient of fluorescence F be always proportional to concentration c. Otherwise serious errors may be incurred. This danger exists mainly when fluorescence is induced by possibly non-stochiometric coupling of the separated material to strongly fluorescent compounds.

The principal disadvantage of fluorescence measured from the far side is that the light intensity available is small. Electrical noise is thus bound to play a much larger role than if the measurements were performed from the near side. The disadvantage of the latter in conjunction with double-beam scanning is a substantially smaller improvement in optical zone noise; independence of the output from the illuminating intensity is, however, preserved. The lower intensity of light available at the far side is especially serious because the intensity of the emitted fluorescence is always much smaller then the intensity available at the exciting wavelength. The reason is a twofold one: the quantum efficiency of fluorescence is smaller than unity, so that the number of emitted photons is always smaller than the number of exciting photons. And the energy of the emitted photons is also substantially smaller than that of the exciting photons. The result is that with equal illuminating intensity less light energy is generally available for fluorescence measurements than for comparable densitometric methods. The lower light intensity available on the far side has of course a negative effect upon the improvement in sensitivity brought about by the double-beam approach.

ACKNOWLEDGEMENTS

The study upon which this paper is based was carried out during the author's stay as visiting professor at COPPE, the Coordinating Center for Graduate Studies in the Engineering Sciences of the Federal University of Rio de Janeiro, Brazil. The

author is indebted to prof. Flávio Grynszpan, then head of the Department of Biomedical Engineering, for putting the facilities of the Department at his disposal.

REFERENCES

1 V. Pollak and A. A. Boulton, *J. Chromatogr.*, 45 (1969) 200.
2 V. Pollak, *J. Chromatogr.*, 105 (1975) 279.
3 V. Pollak and A. A. Boulton, *J. Chromatogr.*, 46 (1970) 247.
4 V. Pollak, *Opt. Acta*, 21 (1974) 51.
5 V. Pollak, *Opt. Acta*, 23 (1976) 25.
6 V. Pollak, *Trans. BME*, October (1970) 288.
7 V. Pollak and A. A. Boulton, *J. Chromatogr.*, 72 (1972) 231.
8 V. Pollak, *J. Chromatogr.*, 133 (1977) 195.