

This article was downloaded by:

On: 24 January 2011

Access details: *Access Details: Free Access*

Publisher *Taylor & Francis*

Informa Ltd Registered in England and Wales Registered Number: 1072954 Registered office: Mortimer House, 37-41 Mortimer Street, London W1T 3JH, UK



Journal of Liquid Chromatography & Related Technologies

Publication details, including instructions for authors and subscription information:

<http://www.informaworld.com/smpp/title~content=t713597273>

Integration Errors in the Quantitative Evaluation of Flat Bed Separations

V. A. Pollak^a

^a Division of Biomedical Engineering Department of Electrical Engineering, University of Saskatchewan, Saskatoon, Saskatchewan, Canada

To cite this Article Pollak, V. A.(1988) 'Integration Errors in the Quantitative Evaluation of Flat Bed Separations', Journal of Liquid Chromatography & Related Technologies, 11: 7, 1403 — 1413

To link to this Article: DOI: 10.1080/01483918808067182

URL: <http://dx.doi.org/10.1080/01483918808067182>

PLEASE SCROLL DOWN FOR ARTICLE

Full terms and conditions of use: <http://www.informaworld.com/terms-and-conditions-of-access.pdf>

This article may be used for research, teaching and private study purposes. Any substantial or systematic reproduction, re-distribution, re-selling, loan or sub-licensing, systematic supply or distribution in any form to anyone is expressly forbidden.

The publisher does not give any warranty express or implied or make any representation that the contents will be complete or accurate or up to date. The accuracy of any instructions, formulae and drug doses should be independently verified with primary sources. The publisher shall not be liable for any loss, actions, claims, proceedings, demand or costs or damages whatsoever or howsoever caused arising directly or indirectly in connection with or arising out of the use of this material.

INTEGRATION ERRORS IN THE QUANTITATIVE EVALUATION OF FLAT BED SEPARATIONS

V. A. Pollak

*Division of Biomedical Engineering
Department of Electrical Engineering
University of Saskatchewan
Saskatoon, Saskatchewan
Canada S7N 0W0*

ABSTRACT

The paper discusses sources of errors which affect the accuracy of optical densitometry for the quantitative evaluation of TL chromatograms and flat bed electropherograms. Both 1D and 2D separations are considered. Relevant error sources fall into two classes. One which is deterministic in nature and one which has stochastic character. The main source of deterministic errors is the non-linear relation between densitometric response and concentration distribution. Linearizing transforms of the response signal are available which significantly reduce the magnitude of the error. They are most effective when "point" scanning is used but much less so with slit scanning. Stochastic sources produce noise which affects the measured value of the signal amplitude. Most important is generally the "optical" noise of the blank medium. In most cases it is this factor, which ultimately determines sensitivity and accuracy of the method. Efforts to reduce optical noise are, therefore, an important design feature for high performance instrument. Most effective in this regard is two wave lengths scanning. The technique is, however, applicable only if the response characteristic of the medium is spectrally flat over a range which is significantly wider than the spectral absorbance characteristic of the separate.

INTRODUCTION

Optical densitometry is today the most common approach to the quantitative evaluation of flat bed separations. The term refers here to separations obtained by thin layer chromatography (TLC) or flat medium electrophoresis (EP). TLC uses mostly only one-dimensional separations, whilst in electrophoresis two-dimensional techniques for certain applications are becoming increasingly common. The reasoning employed in the following applies mutatis mutandis to both TLC and EP and to one- and two-dimensional versions of the two methods. It is also applicable to other disciplines, where densitometry is used for the quantitative determination of substances on a flat support. Determination of the DNA content in cell preparations may be quoted as illustrative example. Quantitative measurements on photographic recordings (film or paper) also belong largely to this class. The information retrieved from the separation consists in principle of data concerning the spatial concentration distribution in the individual spots or zones of separated material. It is obtained indirectly by measuring the change ΔR in optical response (transmittance or remittance) caused by the presence of separate with concentration $c(x,y)$. This change is caused by a local increase of absorbance ΔK over the absorbance value K_0 of the blank separating layer.

$$\Delta K(x,y) = \alpha \cdot c(x,y) \quad (1)$$

The symbol α designates the coefficient of extinction of the separate. It is substance specific and wave length dependent. Expression (1) shows that ΔK is a linear function of c . This does not apply to the change in response ΔR which is a nonlinear function not only of ΔK but also of the optical parameters of the medium. Only under special conditions, e.g. at very low values of ΔK , can ΔR with reasonable accuracy be approximated by a linear dependence.

$$\Delta R \approx \theta \cdot \Delta K \approx \theta \cdot \alpha c \quad (2)$$

The raw acquired data are further processed to yield the wanted information which consists generally of two components. Identification of the separated substance in a zone is one and

determination of its quantity Q the other. Identification is usually based upon geometric considerations supplemented if necessary by spectral data. High accuracy of the pertinent data is not required. The situation is different with quantitation where good accuracy is in the majority of cases an absolute requirement.

FLUCTUATIONS OF THE OPTICAL RESPONSE

ΔR as well as the response R_0 of the blank medium are proportional to the sensitivity δ of the sensor and the intensity I of illumination. With opto-mechanical sensing [1] only a single photo-multiplier tube or solid state photo-diode is employed. δ is then spatially constant. This is not the case when electronic scanning (by camera tube or solid state array) is employed. The sensitivity is here spatially variable, a feature which can be expressed by writing $\delta(x,y)$. Since $\delta(x,y)$ is at least for moderate periods of time stable, any error caused by the spatial variability of δ can be compensated for during processing of the raw data and does not need further consideration here.

A similar argument applies to the intensity of illumination, which can also be spatially variable, but temporally stable. With proper design the variability is small and compensation generally not necessary. If desired it can as before be implemented in software.

The media used for TLC or EP can from an optical point of view be divided into transparent ones with negligible amount of scatter and translucent ones where scatter cannot be disregarded. Transparent media can densitometrically be examined only in transmission. Translucent ones with high transmittance can also be measured in transmission. Most of them are, however, nearly opaque and remission (back scatter) is then the appropriate mode.

Consider transparent media first. Information about the presence of separate and its amount is conveyed only by light which traverses the medium. Part of the light coming from the light source is, however, specularly reflected from the illuminated (near) surface and for measurement purposes lost.

This would be unimportant if the specular reflectance μ was constant. In reality, however, is $\mu(x,y)$ a spatially random function, which gives rise to a noise component (r.m.s.) $\overline{\mu(x,y)}.I$. For good accuracy at low values of αc steps should be taken to make $\mu(x,y)$ small and as constant as possible.

Specular reflection plays a similar role when translucent media are measured. There, however, another factor comes into play. Part v of the light entering the medium may experience only little scatter and the length of its path in the interior of the medium is then much shorter than the path length of a strongly scattered ray. The degree of interaction with the separate is, however, proportional to the path length. It follows that the unscattered portion of light exiting from the medium is for the measurement mostly a loss. $v(x,y)$ is similar to the coefficient μ of specular reflection a random spatial variable resulting in a noise component $\overline{v(x,y)}.I$. The twin bars indicate as above the r.m.s. (root mean square) value.

INTEGRATION ERRORS

For quantitative results it is necessary to integrate $c(x,y)$ over the area S of a particular spot. Immediately available from the measurement is, however, not $c(x,y)$ but $\Delta R(c(x,y))$. $\Delta R(c(x,y))$ is, as already remarked, a non-linear function of $c(x,y)$. It is then easy to see that generally

$$Q_s = \iint_S c(x,y) dx dy \neq k \iint_S \Delta R(x,y) dx dy \quad (3)$$

The symbol k with or without subscript is everywhere in this paper used to designate a scale constant which may have different values in different expressions. Inequality (3) becomes very nearly an equality at very low values of αc [2]. In all other cases $\Delta R(\alpha c)$ must first be inverted to the form $c(\Delta R)$. This is easiest done by using an approximate expression for $\Delta R(\alpha c)$ which can be easily inverted. A number of approximate relations for this purpose are in wide spread use. Some of them are derived heuristically [3], [4], other ones analytically from the Kubelka and Munk (K&M) equations [5]. The K&M theory [6] describes in

somewhat simplified form the optical response of a sheet shaped scattering medium, is, however, still too complicated for most practical applications.

A good approximation based upon the K&M theory for $\Delta R(\alpha c)$ measured in the transmission mode is logarithmic conversion. For media with negligible scatter the approximation becomes even an exact expression (The law of Beer and Lambert). The theoretically best linearization in the remission mode is obtained by summing the optical response R and its reciprocal value [2]. For optically dense media the reciprocal values of R alone is generally satisfactory [5]. Reference [2] brings a detailed discussion of linearization techniques for both transmission and remission based upon simplified forms of the basic K&M expressions.

In all cases it must be considered that a linearizing approximation is by definition just that. Its use for integration entails, therefore, an error in the determination of Q . With a good approximation the resulting error E_Q is usually small when compared to errors from other sources.

The linearization error, though deterministic in nature, cannot easily be corrected in software. Also reduction by averaging, low pass filtering or integration which are potent tools against stochastic errors, are against deterministic errors largely ineffective.

Another peculiarity of the linearization error is that its magnitude increases with increasing value of ΔR . The signal to noise ratio from this source changes, therefore, only little over the measuring range.

The reasoning above is based upon the assumption that the data acquisition system employs point scanning [1]. Point scanning is a must for two-dimensional (2D) separations. For one-dimensional chromatograms or electropherograms slit scanning is frequently used. The scanning aperture is here narrow in the y direction, but transversely covers the whole width of the scanned track. ΔR is with slit scanning optically integrated over the width of the slit. From equation (3) follows, that the value obtained will

generally differ from the integral of $c(x,y)$ over the same area. The result is an additional error. An exception are cases where it can be assumed that ΔR is practically constant over the width of the track.

$$\Delta R(x,y) = \Delta R(y) \quad (4)$$

The integration error caused by slit scanning can in part be corrected by making the scale factor k in (3) a function of the left side integral. The values of k for a sequence of values of the integral are determined by calibration. For good accuracy a fairly large number of calibration points must be used, which may use up a not insignificant proportion of the total area of the separating sheet. The procedure is cumbersome and the accuracy of the results is generally inferior to that which can be obtained by point scanning.

Another source of error which affects integration is determination of the spot boundaries $B(x,y)$ and, consequently, of the spot area S . It can be shown that $c(x,y)$ declines near the boundary of S approximately like a Gaussian function unless confined by artificial barriers [6]. Ideally the boundary is defined by the condition.

$$\Delta K(x,y) \epsilon S \approx k, \bar{n} \quad (5)$$

\bar{n} is the r.m.s. value of the optical noise of the blank parts of the medium. The area S^* encircled by the boundary $B(x,y)$ defined by condition (5) is obviously smaller than the true area S . The result is a negative error of Q . The error is reduced if \bar{n} is small. It will be seen later, that a small value of \bar{n} , i.e. a low optical background noise, is also for other reasons an important prerequisite to obtain accurate quantitative results. For \bar{n} to be small the medium should have uniform thickness and be as homogeneous as possible. Equally important is good technique and careful work during development.

FURTHER ROLES OF OPTICAL BACKGROUND NOISE

ΔR is always negative so that

$$R = R_0 + \Delta R \leq R \quad (6)$$

R_0 is here the optical response value of the blank medium in the absence of separate.

Even with the best of media R_o is never a constant, but fluctuates spatially in a random fashion. This is conveniently expressed by writing.

$$R_o = \bar{R}_o + r_o(x,y) \tag{7}$$

where $r_o(x,y)$ is a random variable in x and y . \bar{R}_o is the mean value of R_o obtained by averaging R_o over the total area of the medium. $r_o(x,y)$ has therefore no d.c. component. The spatial fluctuations of $r_o(x,y)$ result in another noise with r.m.s. amplitude $\overline{r_o(x,y)}$. $(1-\bar{\mu}) \cdot I$. The total optical noise is given by the addition of the powers of the three noise components mentioned.

$$\bar{n} = I \cdot [\bar{\mu}^2 + \bar{v}^2 + \overline{r_o}^2]^{1/2} (1-\bar{\mu}) \tag{8}$$

In many situations, though not always, $\overline{r_o}$ dominates and expression (8) reduces then to the simpler form.

$$\bar{n} \approx I \cdot \overline{r_o} \tag{8a}$$

It is worth noting that all three noise components in (8) have multiplicative character. The signal to noise ratio can, therefore, not be improved by increasing the sensitivity of the photodetector or the intensity of illumination provided that both magnitudes are sufficiently large so that electronic noise (which is additive) can be disregarded.

The optical noise as defined by expressions (8) or (8a) is generally the most important factor which limits sensitivity and accuracy of densitometric measurements. In the interest of good performance it is, therefore desirable to reduce it as much as practical, if possible to the level of electronic noise, which is the ultimate limitation.

ΔR is generally much smaller than R_o . It can then safely be assumed that $\overline{r_o}$ is independent of ΔR . R_o is the base line (surface) response with respect to which ΔR is measured. $\overline{r_o}$ is, therefore, also the error which affects ΔR and the relative error (the signal to noise ratio) from this source is $\Delta R/\overline{r_o}$.

PROCESSING METHODS TO REDUCE OPTICAL NOISE

An approach aiming at improving the accuracy with which ΔR can be determined was suggested by S. Ebel and his collaborators [8].

The method approximates the curve defined by the line averages $\overline{r_0(x,y)}$ by a sequence of straight line segments. The technique is intended for 1D separations. Extension to 2D work should be possible but does not seem to be in use.

A method developed and tested in our laboratory for 2D applications approximates the random surface $r_0(x,y)$ by a smooth surface defined by a 2D polynomial expression of order m . The coefficients of the defining equation are determined by an appropriate number of values of R_0 evenly distributed over the scanned area. The proportion of that area occupied by spots is generally low. The value of $r_0(x,y)$ at support points within a spot area can, therefore, in most cases be easily obtained by interpolation between neighboring accessible points.

Experimental tests with a fourth order approximation carried out on a 2D electrophoretic protein separation produced an about 70% decrease of the optical noise [9]. Higher order approximations appeared to produce very little further improvement, but increased significantly the computational overhead.

The situation is best understood by examining the spatial Fourier transform of $r_0(x,y)$. It is found that magnitude and frequency distribution of $r_0(x,y)$ are for different types of media significantly different. Also development and other steps in the separating process have considerable influence. Still there is a degree of similarity between the spatial (power) spectra of $r_0(x,y)$ from most separations. The spectral energy distribution seems to have generally two peaks separated by a fairly deep valley. The high frequency peak reflects mostly small localized irregularities of the optical parameters (thickness, absorbance and, with turbid media, scatter) of the medium. Spatial frequency has the dimension of inverse length. The beginning of the rise of the high frequency part of the spectrum at a frequency ω_s determines consequently the smallest diameter of spots which can still be resolved at low values of ΔK . In this context it is important to note that spot diameter increases with the peak value of concentration [6].

The high frequency components of the optical noise can be partly suppressed by spatial low pass filtering with cut-off at ω_s . In practice a Laplacian matrix is a convenient method [10]. Experimental work on 2D protein separations showed that a 3 x 3 or 5 x 5 matrix gave adequate results. A median filter based upon a 5 x 5 matrix appeared to be still superior [11]. The low frequency peak of the optical noise spectrum seems to be mainly due to imperfections of the development process. Non-uniform solvent evaporation, undesired material carried along by the flow of solvent and similar factors seem to be mainly involved. Sometimes the manufacturing process of the medium is responsible. Experiments to remove the low frequency part of the noise by high pass filtering fell short of expectations.

Better results were achieved by an approach which is in principle similar to the analytical simulation described above. Instead of simulating the surface $r_o(x,y)$ by a m -th order equation, $r_o(x,y)$ is approximated by a truncated Fourier series. The coefficients of the series are obtained from the appropriate number of uniformly distributed support points. The values of $r_o(x,y)$ at the support points are obtained in the already described way. It can be seen, that the approach represents a different implementation of a high pass filter.

TWO WAVE-LENGTHS SCANNING

The most effective method for the reduction of optical background noise is according to present experience two wave lengths (2λ) scanning. The technique appears to be able to reduce optical noise almost to the level of the electronic noise. Complete elimination down to zero is of course not possible by any method.

2λ densitometry is practical only under certain conditions. The medium employed ought to be "gray", that is to have an absorbance which is over a wide range independent of wave length. The separated material, on the other hand, should have a reasonably narrow absorbance spectrum.

The name indicates that the separation is scanned at two different wave lengths λ_1 and λ_2 . λ_1 should be at or near the absorbance maximum of the analyzed material, λ_2 well outside its absorbance band. With opto-mechanical scanning two detector systems are generally necessary. One of these is tuned to λ_1 , the other one to λ_2 . The ratio of the output signals yield ΔR almost completely free of optical noise.

With electronic array scanners only one detector system is necessary [12]. The separation is scanned twice, once at the wave length λ_1 and once at λ_2 . The values of both scans are digitized and kept in an intermediate memory. The data of the two recordings are later simultaneously read out pixel by pixel and divided by one another. The ratio obtained in this way yields again ΔR almost completely uncontaminated by optical noise.

ACKNOWLEDGEMENT

The work presented in this paper is part of a larger project concerned with the applicability of electronic scanning methods in the densitometry of flat bed separations and the factors affecting the accuracy of determinations. The project was supported by the National Science and Engineering Research Council of Canada (NERC).

REFERENCES

- [1] Pollak, V.A., "Electronic Scanning For The Densitometric Analysis Of Flat Bed Separations". "Advances In Chromatography", ed. J.C. Giddings, et. al., Marcel Dekker, N.Y., 1988, in Press.
- [2] Pollak, V.A., "Linearity Considerations In Quantitative Thin-layer Chromatography", J. Chromat. Vol. 393, 1987, pp. 143-153.
- [3] Ebel, S., "Evaluation And Calibration In Quantitative T.L. Chromatography", Topics in Current Chemistry, Vol. 126, 1984, pp. 70-94.
- [4] Treiber L.R. and Treiber, R., J. Chromatogr. Vol. 100, 1974, pp. 123-128.

- [5] Pollak, V.A., "Progress In Photometric Methods Of Quantitative Evaluation In TLC", "Advances In Chromatography", ed. J.C. Giddings, et. al., Marcel Dekker, N.Y. 1979.
- [6] Kubelka P. and Munk, F., "Beitrag zur Optik der Farbenstriche", in German. Zeitschr. f. Techn. Physik, Vol. 12, 1931, pg. 531.
- [7] Pollak, V.A., "The Concentration Profile Of Substances Separated On Thin-media Chromatograms", J. Chromatogr., Vol. 77, 1973, pp. 245-254.
- [8] Ebel, S., Alert D. and Schaefer, U., "Calibration In TLC/HPTC Using The Michaelis - Menten Function", Chromatographia, Vol. 18, 1984, pp. 23-27.
- [9] Pollak V.A. and Zhu, Y.H., unpublished results.
- [10] Pratt, W.K., "Digital Image Processing", Wiley and Sons, Interscience Series, N.Y., 1978.
- [11] Winkler, W., Inst. f. Measuring Technique, Techn, Univ. of Aachen, personal communication.
- [12] Pollak V.A. and Schulze Clewing, J., "Electronic Scanning For Flat Bed Densitometry", J. Liquid Chromat. submitted.