Journal of Chromatography, 482 (1989) 207–214
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 21 826

DETERMINATION OF THE ABSOLUTE NUMBER OF MOLES OF AN ANALYTE IN A FLOW-THROUGH SYSTEM FROM PEAK-AREA MEASUREMENTS

G. TORSI*, G. CHIAVARI, C. LAGHI and A. M. ASMUNDSDOTTIR[a]
Dipartimento di Chimica G. Ciamician, Università di Bologna, Via Selmi 2, 40126 Bologna (Italy)
and
F. FAGIOLI and R. VECCHIETTI
Dipartimento di Chimica, Università di Ferrara, Via Borsari 46, 44100 Ferrara (Italy)
(First received November 22nd, 1988; revised manuscript received July 26th, 1989)

SUMMARY

A simple model is proposed for the determination of the absolute amount of an analyte when non-destructive detection systems are used in high-performance liquid chromatography and flow-injection analysis. The method requires only a knowledge of the cell thickness, molar absorptivity of the analyte and flow-rate if absorption is measured. The data obtained with a commercial apparatus are consistent with the model both for a compound with well known spectroscopic characteristics (K_2CrO_4) and for common organic substances such as toluene and *p*-nitroaniline. A systematic error of *ca.* 18% is present with the detector used for all analytes. The possible origin of this error is discussed.

INTRODUCTION

Quantitative results in high-performance liquid chromatography (HPLC) and flow-injection analysis (FIA) are generally based on peak-height or peak-area measurements using calibration graphs or other calibration methods. However, no information about the absolute amount of the analyte present in the sample is obtained, as both the analyte and the standard undergo the same physical and chemical processes with possible losses through various mechanisms. In this paper, we present a method for determining the absolute number of moles of an analyte from peak-area measurements in flow-through systems such as HPLC and FIA when the analytical signal is obtained through a detector which measures a physical property of intensive nature such as light absorption, fluorescence, electrical conductivity, electrode potential or refractive index. An amperometric sensor can be included if the current passing through the elctrodes is so low that mass-transfer phenomena can

[a] On leave from the University of Iceland.

0021-9673/89/$03.50

practically be ignored. These detectors can be classified as non-destructive[1] and related equations for chromatographic analysis can be found in a recent publication[2].

In this paper, the theoretical treatment will be restricted to light absorption detectors because the derivation is similar to that given for atomic absorption spectrometry with electrothermal atomization[3,4,7–9] and because the experimental verification of the model is very easy. The extension of the equations derived here to other types of detectors is generally trivial.

In order to determine the absolute number of moles in a flow-through absorption system, one has to measure the flow-rate of the mobile phase, the cell thickness and the molar absorptivity of the analyte at the selected wavelength. The cell thickness is obtained from the manufacturer of the detector, the molar absorptivity from the litterature or by measuring it in the normal way and the flow-rate from the selected value in the HPLC system.

As in the final equation new parameters are introduced (ε, b and F), the accuracy and precision of the analysis will be lower. However, the often tedious and sometimes expensive calibration procedures are eliminated. In addition, data obtained under widely different experimental conditions can be compared. Of course, it is always possible to follow the usual calibration methods. In this instance, if one is sure that no analyte has been lost, the product εb can be obtained, from which either one can be calculated if the other is known.

The final equation derived and tested in this paper has already been obtained for chromatographic measurements[2,5]. However, it has always been associated with peaks of gaussian shape and has never been used for measuring the absolute amount of an analyte as proposed here. As we are interested in the analytical aspects of the problem, the form of the peak is immaterial because in the theoretical treatment no assumptions are made about the form of the peak. We are concerned only with peak overlapping because, in such an event, the measurement of the area is less precise.

The same considerations can be made about the influence of cell design on the form of the peak[6]. For this reason, the large body of literature dealing with chromatographic peak generation and cell design is not considered.

In fact, peak-area data have always been used in everyday measurements because it is considered to be the most dependable parameter in calibration procedures, independent of the peak shape. In this paper we show that, at least with the detector used, it is possible to make absolute measurements. The model was verified by using an RP C_{18} column with an ion of well known spectroscopic characteristics (CrO_4^{2-}) which shows no interaction with the stationary phase. For this reason, the peaks are skewed gaussian, similar to those obtained in FIA measurements. Two other substances were analysed to test the model, more like those encountered in normal HPLC measurements, *viz.*, toluene and *p*-nitroaniline (PNA), chosen for their availability in sufficiently pure form and, with PNA, for the presence in its spectrum of peaks at different wavelengths.

THEORETICAL

The starting equation for absorption measurements is the Lambert–Beer law:

$$A = \varepsilon bc = 10^3 \varepsilon N \tag{1}$$

where A is the absorbance, c (mol/l) the concentration, b (cm) the cell thickness, N the number of moles/cm^2 in the light path and ε the molar absorptivity. A and ε are referred to a fixed wavelength. The multiplying factor 10^3 is used to maintain the numerical value of ε. The assumptions made in deriving the Lambert–Beer law are also valid for eqn. 1. However, it is not an essential requirement that the analyte molecules are uniformly distributed along the cell light path; it is sufficient that they are uniformly distributed in planes perpendicular to the optical beam[3]. In this event, only the final part of eqn. 1 is meaningful.

As in a flow-through system N, and therefore A, are time dependent, eqn. 1 is rewritten as

$$A(t) = 10^3 \varepsilon N(t) \tag{2}$$

The function $N(t)$, as already mentioned, has been extensively studied for HPLC and FIA systems. It can be defined by a convolution integral[3,4] of the type

$$N(t) = 1/S_c \int_0^{t'} S(t')R(t - t')\, dt' \tag{3}$$

where S_c (cm^2) is the cross-section (assumed constant) of the cell, t is a dummy variable and $S(t)$ and $R(t)$ are the number of moles of analyte entering and leaving the cell per unit time, respectively. If we take the integral of both sides between the limits 0 and ∞, we obtain

$$\int_0^{\infty} N(t)\, dt = 1/S_c \int_0^{\infty} S(t)\, dt \int_0^{\infty} R(t)\, dt \tag{4}$$

or

$$\int_0^{\infty} N(t)\, dt = N(0)\tau(r)/S_c \tag{4a}$$

where $N(0)$ and $\tau(r)$ are the total number of moles passed through the cell and the equivalent time constant of the removal function, more directly perceivable as the average time spent by a mole of the analyte in the cell, respectively. If we now return to eqn. 2 and take the integral, we obtain

$$\int_0^{\infty} N(t)\, dt = \frac{1}{10^3 \varepsilon} \int_0^{\infty} A(t)\, dt = \frac{1}{10^3 \varepsilon} A(i) \tag{5}$$

where $A(i)$ (min) is the area of the peak of the analyte under study. Then, by combining eqns. 4a and 5, we obtain

$$A(i) = 10^3 \varepsilon N(0) \tau(r) / S_c \tag{6}$$

In this equation, all quantities, except $\tau(r)$ can easily be measured. In HPLC and FIA, $\tau(r)$ can be expressed as the ratio of the cell colume, V (cm^3), to the flow-rate, F (cm^3/min), which is assumed to be constant. Then we have

$$A(i) = 10^3 \varepsilon N(0) V / F S_c \tag{7}$$

By expressing V as the product of b and S_c, we arrive at the final equation:

$$A(i)\ F = 10^3 \varepsilon b N(0) \tag{8}$$

The experimental validation of eqn. 8 is very easy as all the terms can be obtained by direct measurements. Its limits lie in the non-linearity of the absorption measurements and in the deviation of the experimental conditions from those assumed in the model. The most important deviations are that the light beam is not composed of rays parallel to each other and homogeneously distributed in the planes perpendicular to the beam, that the analyte is not homogeneously distributed in planes perpendicular to the beam and that the flow-rate is not constant. The limits of the Lambert–Beer law are well known. As the bandwidth of the instrument used is large (see below), care must be taken to use concentrations low enough to obtain a linear response.

With the present trend toward miniaturization, the physical limitations on the beam and the parallelism of its rays inside the cell are certainly difficult to implement. The second deviation is equivalent to invoking a plug-flow regime. As the flow is assumed to be laminar under the experimental conditions in this work, distorsions due to different velocities in the planes perpendicular to the flow and the beam are unavoidable. No problems should be encountered in controlling the flow-rate with modern pumping systems.

The time constant of the detector electronics has not been considered in deriving eqn. 8, because peak-area measurements are independent of the detector response rate.

EXPERIMENTAL

All experiments were carried out with the same Varian 2510 liquid chromatograph, equipped with a Varian 2550 spectrophotometric detector, the monochromator of which has a bandwidth of 8 nm, a Rheodyne 7125 injector with 10-, 20- and 50-μl loops and a Perkin-Elmer LC-100 integrator. An Erbasil C_{18} (10 μm) analytical column (250 × 4.6 mm I.D.) was used. Absorption measurements were carried out with a Perkin-Elmer Model 551 instrument with a 2-nm bandwidth. K_2CrO_4 was dissolved in the aqueous mobile phase containing 0.05 M Na_2HPO_4. Toluene and PNA were also dissolved in the mobile phase, which was water–methanol (25:75, v/v).

The chemicals, purchased from Carlo Erba, were of analytical-reagent grade and were recrystallized from deionized or distilled water before use. Methanol (HPLC grade) was used as received. Doubly distilled water was used throughout. All measurements were carried out at room temperature (20–25°C).

Calibration of the system

Before starting the designed measurements, a series of calibrations were made in order to reduce the systematic errors as much as possible. Such calibrations are essential for absolute measurements. In our case, a cautiosus approach was necessary because the chromatographic instruments are not designed for such calibrations.

The calibration of the Perkin-Elmer spectrometer was performed by measuring the molar absorptivity of K_2CrO_4, which is well known[10,11]. Calibration graphs for all the analytes were obtained in the usual way. The spectrometric system of the chromatograph was checked by circulating the analyte solutions for a sufficiently long time until no change in absorbance was detected. As absorbance is not dependent on flow-rate, and as the cell thickness was the same (1.0 cm), no difference should have been observed between the two sets of measurements. However, it was found that the slopes of the calibration graphs obtained with the chromatographic detector were low up to 3%. As the standard deviation of the slopes of the calibration graphs was *ca.* 0.5%, it was concluded that the difference was significant. The observed systematic error presumably arises from different bandwidths of the instruments and/or from the cell thickness being different from the given value. As we measure the product εb, the two effects can be separated by measuring the actual value of b. This measurement is not necessary, however, because by using the experimental value of εb the relevant systematic error is corrected. The flow-rate was obtained by measuring the time needed to deliver a known volume of eluent at the exit of the cell.

The output of the detector and its linearity were controlled with a high-impedence voltmeter by checking the correspondence between the digital output used in the calibration graphs and the output voltage. The integrator was controlled by feeding a known voltage for a known time.

The injection loops of 10, 20 and 50 μl were calibrated as follows: about 100-fold concentrated solutions of the analyte used for normal chromatographic separation were injected using different loops. A 10-ml volume of mobile phase was then collected to ensure that all the sample was eluted. The concentrations were measured on the Perkin-Elmer instrument by making use of the calibration graphs obtained previously. The actual volumes found were 12, 22 and 51 μl, respectively. The systematic errors found are probably due to an imperfect fit of the loops in the injector.

RESULTS AND DISCUSSION

Figs. 1 and 2 show the influence of injection volume and flow-rate obtained with K_2CrO_4 solutions. The analyte concentration, flow-rate and volume injected varied between $0.8 \cdot 10^{-4}$ and $4 \cdot 10^{-4}$ M, 0.5 and 5 cm^3/min and 10 and 50 μl, respectively.

Only part of the data are shown here because they are all clustered together. One can see that the ratio $A(i)F/10^3\varepsilon bN(0)$ is higher than the theoretically expected value. It is not significantly influenced by the injection volume and increases slightly with increasing flow-rate. The dependence of peak area on the number of moles of K_2CrO_4 at constant flow-rate is shown in Fig. 3. Similar data obtained at constant flow-rate and with a 10-μl loop for toluene and PNA are shown in Fig. 4.

In Figs. 3 and 4, the experimental data lie on a straight line as expected, as the peak area changes linearly with the amount of analyte. Table I shows the numerical values of the slope and intercept of these lines. They are fairly good except for toluene.

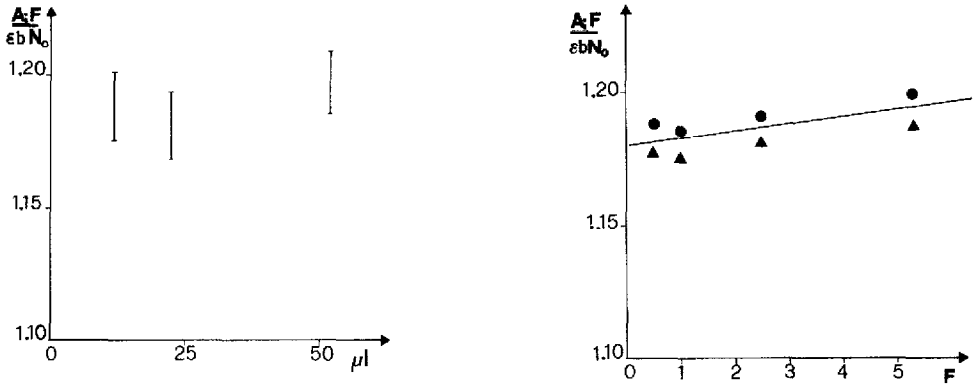

Fig. 1. Influence of volume injected at constant flow-rate. Analyte, K_2CrO_4; flow-rate, 1.01 cm^3/min; concentration $2 \cdot 10^{-4}$ *M*. The bars represent 95% confidence intervals.

Fig. 2. Influence of flow-rate. Analyte, K_2CrO_4; nominal volume of loop, (●) 10 and (▲) 50; concentration, $2 \cdot 10^{-4}$ *M*. The straight line is the regression line of the experimental points.

The reason for the latter discrepancy is the low energy available at such low wavelengths and the sharpness of the peak with associated problems with peak-area measurements due to baseline instability and wavelength irreproducibility.

As already seen, the slope shows a systematic error of about 18% independent of analyte and wavelength. The origin of this error is unknown and the answer must wait

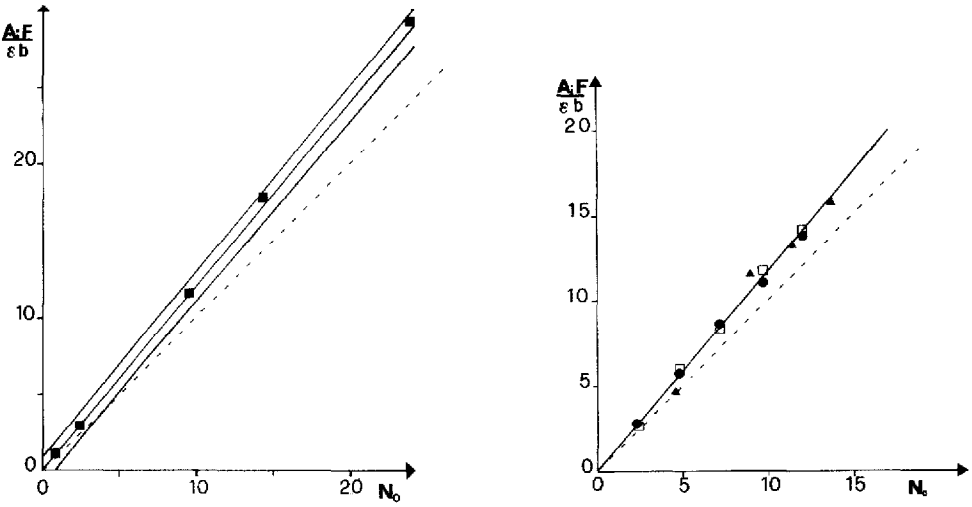

Fig. 3. Calibration graph at constant flow-rate (1.01 cm^3/min) for K_2CrO_4 for different loops and concentrations. λ_{max} = 373 nm. The dashed line is the ideal line. The outer lines represent the 95% confidence limits.

Fig. 4. Calibration graph for toluene [(▲) λ_{max} = 206 nm] and PNA [λ_{max} = (●) 376 and (□) 228 nm]. Flow-rate, 1.01 cm^3/min; volume of loop, 12 μl.

TABLE I

PARAMETERS OF THE STRAIGHT LINES SHOWN IN FIGS. 3 AND 4

Substance	λ_{max} *(nm)*	*r*	*Slope*	*Intercept*
PNA	376	0.9999	1.16 ± 0.02	$+0.06 \pm 0.19$
PNA	228	0.9983	1.20 ± 0.04	-0.10 ± 0.32
Toluene	206	0.9874	1.19 ± 0.13	$+0.04 \pm 1.27$
K_2CrO_4	373	0.9992	1.19 ± 0.10	$+0.10 \pm 0.23$

further investigation. The presence of the same error for different analytes and different wavelengths indicates a physical effect. The first part of the chromatographic system that must be checked is the microcell. In a microcell, as already noted, the beam rays are not parallel, as required by the model. We have seen, however, that when the cell is filled with a homogeneous solution the error, if present, is very small. Therefore, the systematic error found here should be linked to the passage of analyze through the cell at variable concentration. Our suggestion is a combined effect of non-parallelism of the beam and non-homogeneity of the analyte in the planes perpendicular to the beam, which we know is present as a consequence of the laminar flow. This hypothesis was confirmed by direct measurements of deviations of light beams in chromatographic cells when concentration gradients are present[12]. A model that takes into account such deviations is, of course, much more complex and outside the scope of this paper.

CONCLUSION

The data presented, seem to follow the model proposed fairly closely. The systematic error of 18% presumably originates from instrumental deviations between the requirements of the model and actual experimental conditions. In spite of this high systematic error, which is independent of wavelength and analyte, the utility of the method from an analytical point of view is good. We are now extending the work to other systems to investigate the origin of the above error. Experiments will also be carried out on real mixtures to establish the advantages of absolute calibrations.

ACKNOWLEDGEMENTS

Financial support from CNR (Contributo 8800088.03 and 0905/6-861725) is gratefully acknowledged. One of us, A.M.A., expresses her thanks to the Ministero degli Esteri, for a scholarship in Italy. We also thank Dr. Mustafa Ozcimder (University of Samsun, Turkey) for useful discussions.

REFERENCES

1 S. Dal Nogare and R. S. Juvet, *Gas–Liquid Chromatography*, Wiley-Interscience, New York, 1962, p. 188.
2 J. P. Novak, *Quantitative Analysis by Gas Chromatography*, Marcel Dekker, New York, 2nd ed., 1988, Ch. 3.
3 S. L. Paveri-Fontana, G. Tessari and G. Torsi, *Anal. Chem.*, 46 (1974) 1032.
4 M. G. T. van der Broek and L. de Galan, *Anal. Chem.*, 49 (1977) 2176.

5 L. M. MacDowell, W. E. Barber and P. W. Carr, *Anal. Chem.*, 53 (1981) 1373.
6 D. C. Stone and J. F. Tyson, *Anal. Chim. Acta*, 179 (1986) 427.
7 B. V. L'vov, *Spectrochim. Acta*, Part B, 33 (1978) 153.
8 D. C. Baxter and W. Frech, *Spectrochim. Acta*, Part B, 42 (1978) 1005.
9 R. Vecchietti, F. Fagioli, C. Locatelli and G. Torsi, *Talanta*, 36 (1989) 743.
10 R. W. Burke, E. R. Deardogg and O. Menis, *J. Res. Natl. Bur. Stand.*, (1972) 76A469.
11 E. A. Johnson, *Photoelectron Spectrosc. Group Bull.*, 17 (1967) 505.
12 K. Peck and M. D. Morris, *J. Chromatogr.*, 448 (1988) 193.