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Responses of different UV–visible detectors in high-performance liquid chromatographic measurements when the absolute number of moles of an analyte is measured

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ABSTRACT

A simple model for the determination of the absolute number of moles of an analyte in high-performance liquid chromatography with UV–VIS detectors was tested on five commercial instruments. The data show that three gave results in agreement with theory and the other two gave systematic errors of *ca.* 18 and 20%. The possibility of presenting the experimental results of a chromatographic analysis directly as the number of moles instead of peak area is briefly discussed.

INTRODUCTION

In a previous paper [1], a simple equation was derived for the absolute measurement of the number of moles of an analyte from peak-area data. The equation applies to measurements with flow-through non-destructive detectors. The experimental validation of the proposed model from which the above-mentioned equation was derived is particularly easy for UV–VIS detectors in high-performance liquid chromatography (HPLC) as all the quantities involved can be obtained in a straightforward way. A test with a Varian 2550 UV–VIS detector showed that there is a positive systematic error in the number of moles measured of *ca.* 18% independent of volume of sample injected, wavelength and type of analyte. The flow-rate had only a neglibible effect in the range investigated (0.5–5 cm^3 min^{-1}).

The error, even if significant, is not very high and, as fairly drastic assumptions were introduced in the model, it is possible that, with different optics and cell design, experimental conditions could be found with which the systematic error encountered is less severe than that obtained with the tested apparatus.

One of the assumptions made was parallelism of the light beam rays. However, it has been demonstrated experimentally that with concentration gradients, which are naturally present when an analyte is eluted, with a UV–VIS chromatographic cell the rays can be deflected out of the light sensor [2], simulating an absorbance in agreement with the positive error found. An attempt to take into account in the model the variation of the refractive index in a specific cell failed, owing to difficulties in deriving mathematical equations describing the concentration gradients in time and space.

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TABLE I

MAIN CHARACTERISTICS OF THE INSTRUMENTS USED

Specification	Varian 2550	Perkin-Elmer LC-95	Hewlett-Packard HP 1090 diode-array	Jasco 875 UV	Waters Assoc. Lambda Max Model 481
Spectral band width (nm)	8	5	4	8	5
Wavelength accuracy (nm)	±2	±1	±1	±2	±2
Wavelength reproducibility (nm)	±0.3	±0.5		±0.3	±0.5
Light source	D_2 lamp	D_2 lamp	D_2 lamp	D_2 lamp	D_2 lamp
Wavelength range (nm)	190–600	190–700	190–600	190–600	190–700
Flow-cell volume (μl)	8	4.5	4.5	8	14
Measured path length (mm)	9.96	9.85	5.9	9.68	10.1
Detector output (V/A.U.)	0.5	1.0	0.5	1.0	1.0
Detector output (measured) (V/A.U.)	0.485	0.912		1.012	
Integrator	Varian 4290	Varian 4290	HP Series 300 computer	Spectra Physics 4270	Model 730 Data Module
Injector	Rheodyne Model 7010	Rheodyne Model 7125	Rheodyne Model 7161	Rheodyne Model 7125	Model U6K Universal Chrom

For these reasons we considered it more convenient to test the simple model proposed previously [1] on different detectors to see if, at least for some of them, the systematic error is eliminated or reduced to such a level that only random errors are detected. This paper presents the experimental results obtained with five commercial UV–VIS detectors.

EXPERIMENTAL

The detectors used and their major characteristics are summarized in Table I. The preparation of the samples, the calibration procedures and the measuring sequence have already been described [1]. It was difficult, however, to follow the same procedure when measuring the numerical factor for transforming the area given by the Hewlett Packard and Waters Assoc. chromatographs to A min (where A is the absorbance) as required in our calculations, because in these instruments there is an integrated data acquisition and treatment system with no direct access to the various steps followed in reaching the final results. Both, however, give A *versus* time diagrams with areas of peaks.

It was therefore possible to integrate a peak manually from the hard copy of a chromatogram and obtain the desired value of A min. By comparison with the numerical value of the area given, the transformation factor was easily calculated. A statistical analysis has shown that the precision of values so obtained was better than 1% at the 95% confidence level.

RESULTS AND DISCUSSION

The experimental results are summarized in Table II, where the slopes and intercepts of the straight lines obtained with three analytes (paranitroaniline (PNA), toluene and chromate) and the detectors reported in Table I are shown. The data at the bottom ("Average") were obtained by using all the points available for all substances. Some data at low wavelength are missing because the experimental points were too scattered to be of any use. A possible explanation may be found an insufficient energy output of the source or in optical components with high UV absorption. The straight lines were obtained by plotting on the abscissa the number of moles injected and on the ordinate the number of moles found according to the equation

$$A(i) = 10^3 \, \varepsilon \, b \, N(0)/F \tag{1}$$

where $A(i)$ (A min) is the area of the peak, F (cm^3 min^{-1}) the mobile phase flow-rate, ε (cm^2) the molar absorptivity of the analyte at the given wavelength, b (cm) the cell thickness and $N(0)$ the total number of moles; 10^3 is a multiplying factor introduced to maintain the numerical value of ε given in the literature.

The ideal behaviour is found when the line has a slope of unity and passes through the origin. Table II indicates that three detectors show nearly ideal behaviour as at the 95% confidence level the slopes and the intercepts cannot be said to be different from the sought values. This implies that, with these instruments and under the specified experimental conditions, the systematic error is lower than the random error at the 95% confidence level. It is evident that a still better agreement is found

TABLE II

a = Intercept; b = slope; r = correlation coefficient.

Substance	Varian 2550	Perkin Elmer LC-95	Hewlett-Packard HP 1090 diode-array	Jasco 875 UV	Waters Assoc. Lambda Max Model 481
Toluene	$a=0.05\pm1.27$			$a=0.07\pm1.56$	$a=0.07\pm0.25$
($\lambda=206$ nm)	$b=1.19\pm0.13$			$b=0.99\pm0.10$	$b=1.17\pm0.05$
	$r=0.9944$			$r=0.9956$	$r=0.9985$
PNA	$a=0.10\pm0.32$		$a=0.14\pm0.44$	$a=0.77\pm1.95$	$a=0.01\pm0.13$
($\lambda=228$ nm)	$b=1.20\pm0.04$		$b=1.01\pm1.01$	$b=0.96\pm0.17$	$b=1.21\pm0.04$
	$r=0.9983$		$r=0.9998$	$r=0.9999$	$r=0.9974$
PNA	$a=0.06\pm0.19$	$a=0.05\pm0.15$	$a=0.05\pm0.27$	$a=0.02\pm0.85$	$a=0.03\pm0.19$
($\lambda=376$ nm)	$b=1.16\pm0.02$	$b=0.96\pm0.04$	$b=0.96\pm0.01$	$b=1.01\pm0.08$	$b=1.20\pm0.05$
	$r=0.9999$	$r=0.9987$	$r=0.9999$	$r=0.9999$	$r=0.9980$
Chromate	$a=0.10\pm0.23$	$a=0.004\pm0.13$	$a=0.08\pm0.024$	$a=0.08\pm0.05$	$a=0.03\pm0.29$
($\lambda=373$ nm)	$b=1.19\pm0.10$	$b=0.98\pm0.01$	$b=0.96\pm0.02$	$b=0.94\pm0.02$	$b=1.22\pm0.06$
	$r=0.9992$	$r=0.9998$	$r=0.9998$	$r=0.9999$	$r=0.9981$
Average	$a=0.001\pm0.18$	$a=0.091\pm0.09$	$a=0.013\pm0.13$	$a=0.13\pm0.48$	$a=0.02\pm0.09$
	$b=1.18\pm0.02$	$b=0.99\pm0.01$	$b=0.98\pm0.01$	$b=0.99\pm0.55$	$b=1.20\pm0.02$
	$r=0.9991$	$r=0.9997$	$r=0.9995$	$r=0.9995$	$r=0.9996$

when all points relevant to all the different substances (bottom entries in Table II) are pooled. This is to be expected because in this way the errors introduced in setting the instruments parameters such wavelength and in preparing the solutions are averaged.

The data relevant to the Varian spectrometer given in Table II were obtained with a different instrument located in a different laboratory. This choice was made on purpose to see if the systematic error was similar to that obtained previously [1]. Even if it is risky to draw conclusions from only two instruments tested, the practical coincidence of the values points toward a physical origin related to the optics and/or cell design.

With the Waters Assoc. instrument there is the problem of the cell form, which is tapered instead of cylindrical, so that one of the assumptions of the model (constant cell cross-section) is not fulfilled. However, the link between this type of cell and the systematic error encountered was not investigated.

Use of molar absorptivity values

From the data presented above, it is clear that it is possible to measure the absolute number of moles of an analyte in a given sample if the appropriate instrument is chosen. If we assume that calibrations such as wavelength and absorbance in such an instrument are made automatically, as in all modern spectrometers, and that the flow-rate is fed to the data-treatment system directly from the pump, then the data can be presented as number of moles instead of peak area if the value of εb for the specific peak is known.

In our calculations we used the product εb derived from calibration graphs (A *vs.* c) measured with the chromatographic system under investigation [1]. In this way, any systematic errors related to ε and b are removed at the cost of a calibration graph

for each analyte. The value of b can be determined in many ways with high accuracy once and for all for a given cell, thus leaving only ε as the last quantity needed. Values of ε can be found in the literature if the mobile phase is not too complex. However, it is well known that ε is constant only if the instrumental spectral band width (SBW) is at least ten times smaller than the natural band width (NBW) of the absorption band of the analyte under investigation [3–5]. Now, for obvious reasons, with a chromatographic microcell it is difficult to have a small SBW (see Table I), and consequently any use of ε values taken from the literature must be carefully considered. Recently a UV–VIS detector has been proposed also for gases or vapours [6]. In this instance an even more cautious approach must be made, as the absorption bands of gases and vapours are very sharp. Of course, fixed-wavelength detectors cannot be used for measurements of the absolute number of moles except when the molar absorptivity of the analyte is constant in the source line wavelength interval.

The experimental values of b given in Table I were obtained from PNA at 376 nm and K_2CrO_4 at 373 nm from the calibration graphs relevant to each instrument and the molar absorptivity given in the literature [7] for the chromate and measured on a Perkin-Elmer Model 554 spectrometer with 0.2 SBW for PNA. It was then possible to calculate a molar absorptivity for each compound on each instrument, which, as already shown [4], should be a function of the SBW/NBW ratio. The results are summarized in Table III. No systematic errors are evident even when the SBW/NBW ratio is fairly large, as with toluene. A possible explanation of the data shown in Table III may be the difficulty in setting the wavelength reproducibly on commercial instruments. It must be noted that this kind of error increases with increase in the SBW/NBW ratio.

TABLE III

ε VALUES (cm^2)

Instrument	Toluene (λ = 206 nm)	PNA (λ = 228 nm)	PNA (λ = 376 nm)	Chromate (λ = 373 nn
Perkin-Elmer PE 551 UV–VIS spectrophotometer	$7.20 \cdot 10^3$[a]	$5.80 \cdot 10^3$[a]	$1.56 \cdot 10^4$[a]	$4.82 \cdot 10^3$[b]
Varian 2550	$6.90 \cdot 10^3$	$5.81 \cdot 10^3$	$1.56 \cdot 10^4$	$4.81 \cdot 10^3$
Perkin-Elmer LC-95			$1.56 \cdot 10^4$	$4.74 \cdot 10^3$
Hewlett-Packard HP 1090 diode array		$6.03 \cdot 10^3$	$1.56 \cdot 10^4$	$4.84 \cdot 10^3$
Jasco 875 UV	$6.40 \cdot 10^3$	$6.20 \cdot 10^3$	$1.55 \cdot 10^4$	$4.70 \cdot 10^3$
Water Assoc. Lambda Max Model 481	$7.50 \cdot 10^3$	$5.94 \cdot 10^3$	$1.56 \cdot 10^4$	$4.83 \cdot 10^3$

[a] Obtained on PE Model 551 with 0.2-nm slit width.
[b] Ref. 7.

CONCLUSIONS

It is possible with well chosen commercial instruments to measure the absolute number of moles with flow-through detectors. However, one must know the flow-rate, the molar absorptivity of the analyte and the thickness of the cell. All these quantities except the molar absorptivity can be easily obtained and automatically acquired by a data-treatment station. A databank of ε values could be considered as a

first step toward calibrationless analysis in the linear region of the absorption–concentration law.

However, the data presented indicate that a reduction in the random errors is necessary with present-day instruments. We are now trying to assess the origin of these errors and the relevant limits of detection for real samples.

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