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Journal of Chromatography A, 1089 (2005) 105–110
JOURNAL OF CHROMATOGRAPHY A
www.elsevier.com/locate/chroma

Calibration of a detector for nonlinear responses

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Received 26 April 2005; received in revised form 14 June 2005; accepted 22 June 2005

Abstract

A calibration curve is often needed to derive from the record of the detector signal the actual concentration profile of the eluate in many studies of the thermodynamic and kinetic of adsorption by chromatography. The calibration task is complicated in the frequent cases in which the detector response is nonlinear. The simplest approach consists in preparing a series of solutions of known concentrations, in flushing them successively through the detector cell, and in recording the height of the plateau response obtained. However, this method requires relatively large amounts of the pure solutes studied. These are not always available, may be most costly, and could be applied to better uses. An alternative procedure consists of deriving this calibration curve from a series of peaks recorded upon the injection of increasingly large pulses of the studied compound. We validated this new method in HPLC with a UV detector. Questions concerning the reproducibility and accuracy of the method are discussed.

Keywords: Nonlinear calibration curve; HPLC; UV-detector; Reproducibility

1. Introduction

Detector calibration is an important step in nearly all investigations involving the quantitative measurements of physico-chemical parameters. It is often critical in nonlinear chromatography which involves the use of concentrated solutions. Detector responses are most often nonlinear in the wide concentration ranges of interest. An absolute calibration curve relates the concentration of a compound (C) in a detector cell and the detector response (h). Commonly, an absolute calibration curve is derived from the signals acquired by flushing the detector cell with a series of solutions of known concentrations and measuring the steady-state response. This "steady-state" method, however, has its own drawbacks. It needs a significant amount of time and, even when automatize, it immobilizes a valuable instrument and is highly consuming of chemicals. If the absolute calibration curve is needed to transform a recorded chromatogram (signal versus time) into an elution band profiles (concentration, C, versus time), this procedure cannot be applied during the acquisition of the chromatograms but before or after that, which may explain the relative lack of reproducibility of the method. If detector response is linear, the detector response factor is a constant that can be easily derived from an elution chromatogram [1]. However, this is not a case when the detector response is nonlinear.

Recently, a method was proposed to derive directly an absolute calibration curve from a series of data acquired from pulse injection experiments [2]. The calibration curve is the relationship written

$$C = k_1 h + k_{2,n} h^n \tag{1}$$

where the coefficients k_1, $k_{2,n}$, and n characterize the analytical calibration curve relating the injected amount (q) and the area under the chromatographic peak (S). They can be derived from sets of data, i.e., of amount of compound injected and areas of the peaks recorded, following the equation

$$q = F_v k_1 S + b_n S^n \tag{2}$$

where F_v is the mobile phase flow rate, k_1, b_n, and n are fitting parameters. The coefficient $k_{2,n}$ in Eq. (1) is related to the coefficient b_n and to σ, the standard deviation of the peak

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doi:10.1016/j.chroma.2005.06.064

assumed to have a Gaussian profile through the relationship

$$k_{2,n} = F_v^{-1} b_n n^{0.5} \pi^{0.5(n-1)} 2^{0.5(n-1)} \sigma^{n-1} \quad (3)$$

This equation results from the assumption made that the column is not overloaded, hence gives nearly Gaussian peak profiles, and that the response of the detector exhibits a mildly nonlinear behavior, hence Eq. (1) with a single power term. As a result, Eq. (3) relates the properties of the Gaussian profiles and some experimental conditions. The validation of the proposed method was discussed using the example of the analysis of toluene with a DAD detector, under rather conventional HPLC conditions [2]. It was shown that the error made in the determination of the injected amount of toluene was less than 14% for samples between 18 and 35 μg and less than 4% between 35 and 163 μg.

A satisfactory agreement was found between this method and a numerical indirect method [3]. At the same time, significant discrepancies were observed between calibration curves obtained using these indirect methods and the steady-state method consisting of measuring the signal after filling the detector cell with solutions of known concentrations. The differences were around 10%, which gave discrepancies in the estimates of the injected amounts of 20%. This surprising result induced us to extend our study to the calibration errors made with other types of detectors and to investigate the calibration errors encountered with detector responses exhibiting different degrees of nonlinear behavior. In this work we report on the calibration of a UV detector using the various injection techniques available. The reproducibility of the calibration experiments and some particular conclusions of our earlier work [2] concerning the new calibration procedure are discussed.

2. Experimental

2.1. Equipment and materials

All chromatographic measurements were carried out using a HP 1100 liquid chromatograph (Agilent Technologies, Palo Alto, CA) equipped with a binary pump module, a column oven, a variable UV detector (Table 1), a data acquisition system, a computer controller, and an autosampler.

Table 1
Characteristics of the UV detector used in the present work and DAD detector used in work [2])

	UV (G1314A)	DAD (G1315A)
Detection type	Double beam photometer	1024-Element photodiode array
Light source	Deuterium lamp	Deuterium lamp
Linear absorbance range	>2 AU	>2 AU
Band width (nm)	6.5	
Wavelength accuracy (nm)	±1	±1
Cell		
Path length (mm)	10	1.7
Volume (μl)	14	6

The mobile phase was a methanol-water solution (80:20, v/v). Both methanol and water were HPLC grade solvents purchased from Fisher Scientific (Fair Lawn, NJ, USA). The sample component was toluene, also from Fisher Scientific. All chemicals were used as supplied. The column used was a 150 mm × 4 mm Luna C18 from Phenomenex (Torrance, CA, USA).

2.2. Procedures

2.2.1. Steady-state method

Calibration of a detector using the steady-state or frontal analysis method consists of measuring the detector response after filling successively the detector cell with solutions of the studied compound at different concentrations. The concentrations of the sample solutions were increased stepwise, by mixing a stream of the pure mobile phase and a stream of a solution of toluene in the mobile phase (3.2466 g/l for 270 nm and 3.2182 g/l for 275 nm), using the binary pump. The sample concentration is adjusted by changing the ratio of these two flow rate. Duplicate measurements were carried out the same day and the average values were taken. These measurements were repeated several times, after a 2–3 day interruption. Preliminary experiments showed that the detector signal is the same whether there is a column between the pump and the detector or merely a length of a narrow-diameter connecting tubing that causes the significant pressure drop needed for the accurate operation of the pump. The time needed to reach steady state is longer in the former case. Therefore, the steady-state experiments were carried out without the column.

The calibration was carried out at two wavelength, 270 and 275 nm. Both plots of the peak areas versus the sample size are nonlinear.

2.2.2. Pulse injection method

The calibration using the pulse technique was made at the same wavelengths, using two different methods, by means of the injection of fixed sample volumes (20 μl) of solutions of increasing concentrations (0.59 to 75.6 g/l) or by means of the injection of various sample volumes (5–100 μl) of solutions at a fixed concentration (34.43 g/l). Each sample was injected in triplicate and the averaged values are reported. The standard deviation of the peak area serves as a measure of the repeatability (intra-day precision). To investigate the longer term reproducibility (inter-day precision) of the results, series of pulse injections of increasing concentrations were made during an interval of continuous work of the detector and were repeated twice, before and after switching off the detector for a 1-week period.

2.3. Calculations

Standard methods of nonlinear regression analysis included in the SigmaPlot 6.0 software (SPSS Inc., Chicago,

IL) were used in order to calculate the relationships between the injected amount to sample and the area of the peak or the detector response to changes in the concentration of the feed solution in steady-state experiments. The determination of the calibration curves based on the actual shape of the chromatographic peaks was done using the indirect numerical technique [3], as described in details earlier [2].

Calibration curves were used to calculate the amount of analyte under a chromatographic peak, using the following equation

$$\hat{q} = \int_{V_1}^{V_2} C(h[V])\,\mathrm{d}V \tag{4}$$

where V_1 and V_2 are the beginning and the end points of a peak in volume scale, $C(h[V])$ is the concentration corresponding to the detector response, $h[V]$, as given by the calibration curve. This integral was determined using the method of trapezoids. Because the discretized record of each peak contains more than 500 data points, such evaluation was quite precise. The mass $\hat{q}$ is supposed to be equal to the injected amount. That requirement was used to validate the calibration curves.

3. Results and discussion

3.1. Repeatability and reproducibility

There is much information in the literature that is devoted to the problem of the reproducibility of analytical data in HPLC ([4–8] and references therein). As a rule, these papers deal with the case of a linear detector response. Frequently, such investigations are devoted to the analysis of complex, multi-component systems, in which the degradation of an analyte and/or the influence of the sample matrix are the essential sources of errors [5–8]. In contrast, we deal here with a stable compound that is available in pure form. This choice was made to avoid the sort of troubles just listed and to estimate the errors that result only from the equipment characteristics. The reproducibility of nonlinear calibration curves is of particular interest in the case of UV detection, which is poorly covered in the literature, especially now that the availability of computers allows the convenient use of nonlinear calibration curves.

The reproducibility of the UV-detector response depends on several factors, mainly the stability of the detector parameters (the voltage and current applied to the lamp, the aging of the lamp), the detector wavelength ruggedness [4], and the robustness to changes in the environmental conditions (i.e., the temperature, the atmosphere pressure, and the ambient electromagnetic field). When UV-detection is applied to HPLC, the repeatability and the reproducibility of the detector response depends also on the stability of the chromatographic peak parameters, e.g., the reproducibility and the stability of the flow-rate, mainly a function of the characteristics of the pump. Besides, the accuracy and the reproducibility of the injection characteristics have a considerable influence. However, when a modern automatic sampling system is used, these performance are quite satisfactory. When the detector response becomes nonlinear, the reproducibility of the peak shape becomes important for the accuracy of the method. This is because a larger fraction of the injected amount is detected under conditions of a linear detector response if the peak becomes broader, hence shorter, by virtue of some additional axial or apparent dispersion than when the peak shape remains unchanged. Frequently, the absolute calibration curve ($C(h)$) of a UV-detector is convex downward. Then, for a given amount of compound injected, the area of the peak recorded will be lower for the narrow, tall peak given by a highly efficient column than for the more diffuse peak given by a poorly efficient column, in spite of the fact that the injected amount is the same. Thus, different calibration curves must correspond to peaks of different shapes, even for a given compound.

The differences between two consecutively recorded series of stepwise steady-state chromatograms were less than 0.4% within the entire concentration range, at both 275 and 270 nm. The reproducibility of the detector response was better than 1.3% at 275 nm but it increased to 8% with increasing concentration at 270 nm. Probably, the higher sensitivity of the detector response at 270 nm was the source of this relative lack of reproducibility, due to stronger deviations from Beer–Lambert's law at that wavelength. Because the $C(h)$ curves are not linear but are convex toward the detector response axis, an error in the response in the nonlinear part of the curve leads to a higher relative error in the concentration at high concentrations. So, these different errors at high detector signals correspond to the differences observed in the response factors, the concentration for a given response being approximately 7.5 times lower at 275 nm than at 270 nm. It was also found that changing the flow rate from 1 to 0.5 ml/min did not change the detector response.

The relative standard deviation of the value of the peak area for three consecutive measurements characterizing the repeatability of the pulse injections at the two wavelengths chosen was smaller than 0.5% within the entire range of sample amount investigated. Data on the "day-to-day" reproducibility of the pulse injections are listed in Table 2. The values of the standard deviations are 2–12 times larger than those corresponding to the repeatability. The relative error observed at 270 nm appears to be independent on the injected amount. This observation is in agreement with the increase of the error of reproducibility of the detector response with increasing concentration that was found in frontal analysis experiments.

It is worth noting now that, during the acquisition of the series of data previously published [2], the frontal analysis and the elution experiments were carried out on different days. It is quite possible that this circumstance has played a role in the difference that was observed then between the calibration curves directly and indirectly measured.

Table 2
Long term reproducibility of peak area as a function of injection amount

Injected amount (10^{-5} g)	SD (%) $\lambda = 275$ nm	SD (%) $\lambda = 270$ nm
1.40	4.7	3.7
8.56	5.4	4.4
29.11	6.3	4.7
111.36	4.1	5.1
124.99	0.9	5.0
145.47	2.3	5.0

3.2. Comparison of direct and indirect methods of calibrating detector responses

In order to avoid the reproducibility problems or rather, to limit their consequences, the pulse injections and the steady-state experiments were carried out at each wavelength during a single period of continuous work of the detector (i.e., without switching it off). Fig. 1 shows the two plots of S versus q. The detector sensitivity is much higher at 270 nm than at 275 nm and the corresponding graph is more curved. The parameters of Eq. (2) for the two data sets are reported in Table 3. The calibration data at 275 nm were obtained in two different ways, as described in Section 2.2. The two series of data are in good agreement, proving that there are no significant systematic error inherent in the autosampler. Therefore, only the data obtained with the injection of a constant volume sample and variable sample concentrations were included in later calculations. Table 4 lists the coefficients of the calibration equation, Eq. (1), calculated by the numerical method [3], using Eq. (3), and derived from the results of the steady-state experiments. The theoretical method involving Eq. (3) requires that the standard deviation of the peaks of the compound studied remain constant when the sample size increases [2]. Since the peak standard deviation depends on the sample concentration (as will be discussed later), we calculated the coefficients of the nonlinear terms of the calibration curve for two extreme values of the standard deviation, 0.208 and 0.224 at 275 nm, and 0.205 and 0.246

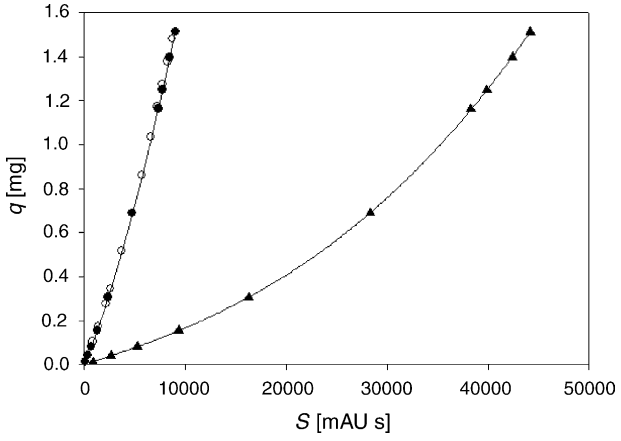

Fig. 1. Plots of the injected amount vs. the peak area, at wavelengths of 270 nm (△), and 275 nm ((●), fixed sample volume and variable concentration; (○), fixed concentration and variable sample volume). The solid lines are fittings of the data to Eq. (2) with best coefficients reported in Table 3.

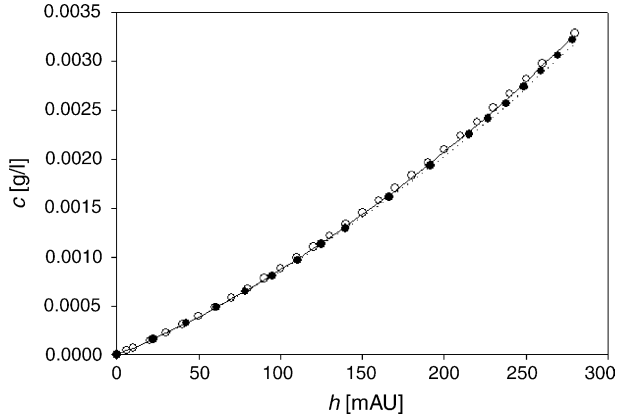

Fig. 2. Comparison of the steady-state calibration data (●), calibration curves determined by the numerical indirect method (○), and curves calculated with $\sigma = 0.208$ (dotted line) and $\sigma = 0.224$ (solid line). Detector wavelength = 275 nm.

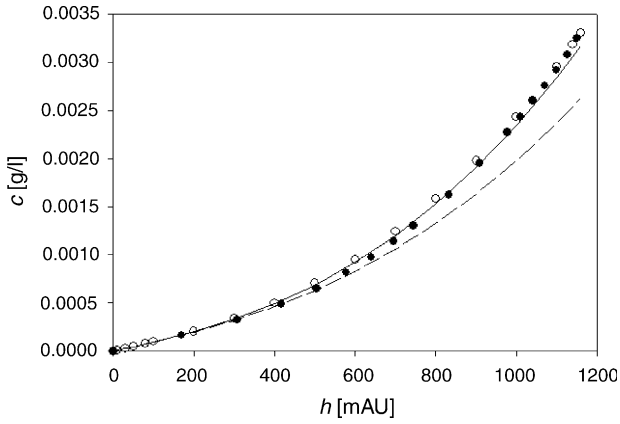

Fig. 3. Comparison of the steady-state calibration data (●), calibration curves determined by the numerical indirect method (○), and curves calculated with $\sigma = 0.205$ (solid line), $\sigma = 0.246$ (dashed line), and detector wavelength = 270 nm.

at 270 nm. The corresponding calibration curves are plotted in Figs. 2 and 3. There is a good agreement between the calibration curve directly measured and the one calculated at 275 nm. In the case of the measurements made at 270 nm, there is some divergence between the direct and the calculated calibration curves at intermediate sample amounts, the direct steady-state calibration curve being somewhat lower than the one reconstructed from the peak shape, using the numerical method.

Table 3
Experimental coefficient of Eq. (2) for the two detector wavelengths

	$F_v k_1$	b_n	n
275 nm			
Coefficient	1.083×10^{-7}	3.12×10^{-11}	1.829
SD	0.022×10^{-7}	1.4×10^{-11}	0.046
270 nm			
Coefficient	1.482×10^{-8}	7.09×10^{-16}	2.601
SD	0.021×10^{-8}	2.5×10^{-16}	0.032

$R^2(275\,\text{nm}) = 0.99999$; $R^2(270\,\text{nm}) = 0.99999$.

Table 4
Coefficients of the absolute calibration curves (Eq. (1)) determined from the shape of a chromatographic peak, directly, by frontal chromatography, and calculated by Eqs. (2) and (3)

	Indirect method	Proposed theoretical method		FA	
		$\sigma = 0.208$	$\sigma = 0.224$	Parameter	SD
Detector wavelength 275 nm					
k_1	6.654×10^{-6}	6.564×10^{-6}	6.564×10^{-6}	6.912×10^{-6}	0.029×10^{-6}
$k_{2,n}$	4.75×10^{-8}	4.425×10^{-8}	4.705×10^{-8}	1.654×10^{-8}	0.013×10^{-8}
n	1.829	1.829	1.829	2[a]	
	Indirect method	Proposed theoretical method		FA	
		$\sigma = 0.205$	$\sigma = 0.246$	Parameter	SD
Detector wavelength 270 nm					
k_1	8.982×10^{-7}	8.982×10^{-7}	8.982×10^{-7}	8.534×10^{-7}	0.2×10^{-7}
$k_{2,n}$	2.417×10^{-7}	1.698×10^{-11}	2.273×10^{-11}	5.476×10^{-12}	1.3×10^{-12}
n	2.601	2.601	2.601	2.815	0.032

[a] A parabolic function with fixed $n = 2$ was used to fit.

Table 5
Material balance for peaks estimated with calibration curves measured by numerical indirect and direct (FA) methods at the two detector wavelengths

Injected amount (10^{-5} g)	275 nm				270 nm			
	Numerical method		FA method		Numerical method		FA method	
	q (10^{-5} g)	δ (%)	q (10^{-5} g)	δ (%)	q (10^{-5} g)	δ (%)	q (10^{-5} g)	δ (%)
1.18	1.24	4.7	1.28	8.3	1.21	2.7	1.29	9.3
4.07	4.06	−0.1	4.07	−0.02	3.83	−5.8	4.19	3.1
8.15	8.14	−0.1	8.28	1.6	7.73	−5.2	8.28	1.7
15.54	15.59	0.3	15.93	2.5	14.69	−5.5	15.73	1.2
30.49	30.60	0.3	32.16	5.5	29.47	−3.3	30.48	−0.1
68.86	69.30	0.6	72.23	4.9	67.18	−2.4	68.25	−0.9
116.11	116.21	0.1	117.92	1.6	112.15	−3.4	114.32	−1.5
124.81	124.78	−0.03	126.54	1.4	120.83	−3.2	122.81	−1.6
139.61	139.13	−0.4	140.66	0.8	135.16	−3.2	137.04	−1.8
151.15	151.10	−0.03	151.13	−0.01	145.88	−3.5	148.97	−1.4

In Tables 5 and 6, the amounts actually injected are compared with those calculated using the different calibration curves. Regarding the mass balance of the elution chromatograms, the indirect numerical calibration method shows excellent results at both wavelengths. Applying the calibration curve determined by the steady-state method also gives good agreement between actually injected and estimated sample amounts, except for very low amounts. At the same time, one can see the systematic variation of the results. So, at low injected amounts the calculated mass of toluene is larger than that actually injected into the column while, for samples larger than 31 μg, it is smaller, although by less than 2%. This systematic downward shift is more pronounced at 270 nm but it remains between 2 and 6%.

Table 6
Material balance of the experimental peaks using theoretical calibration curves

Injected amount (10^{-5} g)	275 nm				270 nm			
	$\sigma = 0.208$		$\sigma = 0.224$		$\sigma = 0.205$		$\sigma = 0.246$	
	q (10^{-5} g)	δ (%)	q (10^{-5} g)	δ (%)	q (10^{-5} g)	δ (%)	q (10^{-5} g)	δ (%)
1.18	1.23	4.6	1.24	4.7	1.28	8.2	1.28	8.3
4.07	4.05	−0.4	4.06	−0.2	4.04	−0.6	4.06	−0.1
8.15	8.11	−0.5	8.14	−0.1	8.14	−0.1	8.25	1.3
15.54	15.48	−0.4	15.57	0.2	15.32	−1.4	15.81	1.7
30.49	30.27	−0.7	30.55	0.2	29.77	−2.4	31.68	3.9
68.86	68.12	−1.1	69.13	0.4	63.22	−8.2	70.43	2.3
116.11	113.70	−2.1	115.86	−0.2	99.68	−14.2	114.28	−1.6
124.81	122.01	−2.2	124.40	−0.3	106.44	−14.7	122.53	−1.8
139.61	135.88	−2.7	138.68	−0.7	117.46	−15.9	136.03	−2.6
151.15	147.46	−2.4	150.60	−0.4	125.58	−16.9	146.03	−3.4

Table 7
Dependence of the caracteristics of the toluene peak on injected amount

Injected amount (10^{-5} g)	275 nm			270 nm		
	Height (mAU)	σ (min)	$2.355\sigma/W_{0.5}$	Height (mAU)	σ (min)	$2.355\sigma/W_{0.5}$
1.18	3.53	0.208	0.9828	27.70	0.205	1.0031
4.07	11.35	0.210	0.9842	85.63	0.207	0.9870
8.15	21.90	0.211	0.9766	165.2	0.209	0.9737
15.54	39.80	0.212	0.9736	285.7	0.212	0.9501
30.49	72.04	0.214	0.9596	470.8	0.219	0.9195
68.86	141.7	0.219	0.9517	750.0	0.232	0.8667
116.11	211.5	0.222	0.9396	964.1	0.241	0.8397
124.81	223.2	0.223	0.9415	998.7	0.242	0.8397
139.61	242.0	0.223	0.9381	1052	0.244	0.8309
151.15	257.2	0.224	0.9347	1090	0.246	0.8345

Applying the theoretical calibration curve (Eqs. (2) and (3)) to the data acquired at a wavelength of 275 nm gives satisfactory results. With $\sigma = 0.208$, the mass of toluene is underestimated by 0.3–2.7%. With $\sigma = 0.224$, the error made is less than 0.7%, except for the lowest amounts. The results obtained at 270 nm are less good. With the small standard deviation, the error reaches 17%. But for the broadest peak dispersion, the error remains less than 4%, except for the lowest injected amount (error, 8.3%). In general, the calibration curves calculated for the largest values of σ give better estimates of the injected amount.

These results show that the convergence of the mass balance is not very sensitive to the choice of the value of σ used to determine the coefficient $k_{2,n}$ when the calibration is done at 275 nm, but that it is for the calibration done at 270 nm. There are two reasons for that. Firstly, the range of values of σ is 2.6 times smaller for the peaks recorded at 275 nm than for those recorded at 270 nm. Because the power n in Eq. (3) is of the order of 2 (Table 3), the increase of σ causes an increase of $k_{2,n}$ by only 6% at 275 nm but by 25% at 270 nm. Secondly, the calibration curve at 270 nm is more strongly curved, hence it depends more on the nonlinear term of the calibration equation than at 275 nm. So, the contributions of the power term to the total signal at the apex of the highest peak are 41 and 63% at 275 and 270 nm, respectively.

The derivation of Eq. (3) is based on the assumption of a Gaussian profile for chromatographic peaks [2]. Deviation of the peak shape from Gaussian can be evaluated using the ratio $2.355\sigma/W_{0.5}$ (with $W_{0.5}$ being the peak width at half-height), which is equal to 1 for a Gaussian peak. It is seen from the data of Table 7 that there are only moderate deviations from the shape of a Gaussian distribution (the ratio $2.355\sigma/W_{0.5}$ deviates by 10–15% from 1). These variations do not result in a great deterioration of the accuracy of the calibration curves. A similar situation was observed in an earlier work [2]. Thus, one can assert that the proposed method is applicable for peaks that have a non-Gaussian profile provided that their shape does not deviate too much from this profile.

4. Conclusions

The proposed method allows the determination of the calibration curve of a detector based on the relationship between the amount of a compound injected and the area of its peak, when the peaks have a Gaussian or a nearly Gaussian profile. This method is applicable even when the q versus S function is strongly curved but better results are obtained if the detector response is only slightly nonlinear. The standard deviation of the largest peak should be used in the calculations. The method is useful in chromatographic studies when the eluate concentration in each point of a band profile must be determined. The method is based on simple mathematics. Its use saves cost and time. Its drawbacks are that its accuracy depends on all the factors that influence the accuracy and precision of elution profiles in chromatography, i.e., the injection profile, the flow rate, the band broadening, besides the detector characteristics.

Acknowledgements

This work was supported in part by grant CHE-02-44693 of the National Science Foundation and by the cooperative agreement between the University of Tennessee and the Oak Ridge National Laboratory.

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