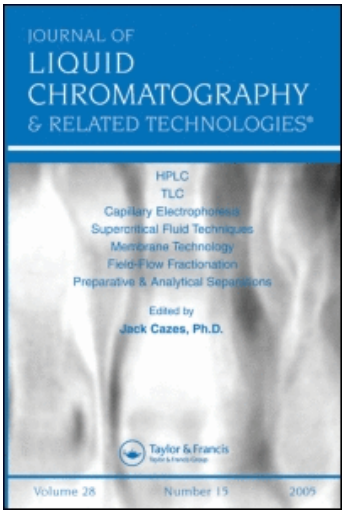

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NON-LINEAR CALIBRATION IN QUANTITATIVE ANALYSIS BY HPTLC UTILIZING A FIBRE OPTIC FLUORESCENCE DETECTOR

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ABSTRACT

The non-linear calibration in quantitative determinations by high performance thin-layer chromatography utilizing a fibre optic fluorescence detector is examined. Dansyl derivatives of polyamines, as model compounds, are separated in HPTLC silica-gel plates with chloroform:triethylamine (TEA):polyoxyethylene-10-lauryl ether (POLE) (8:4:5%) (v:v:m) as solvent. The obtained calibration graphs show a non-linear relationship between the fluorescence intensity and the quantity of the amine (0-150ng).

The study of the non-linearity shows that the best fits are obtained for the quadratic and cubic regressions. However, for concentrations lower than 50 ng, linear calibrations are obtained with R-squared > 0.99. Furthermore, the principal parameters used to characterize the methods of analysis by HPTLC with fluorescence detection are calculated: sensibility, detection limit, precision, and accuracy.

INTRODUCTION

High performance thin-layer chromatography (HPTLC) is undergoing a resurgence as a separation tool in many laboratories due to the availability of

high efficiency adsorbent materials, the minimal quantity of organic solvents needed, the simplicity of the analysis, and the overall low cost. High performance thin-layer chromatography (HPTLC) permits comparable separations to HPLC[1-5] and has a great utility for applications in which many samples, with minimal sample preparation, are analysed.

In the quantification of analytes separated by TLC, densitometry has proved most useful.[6-8] Recently, as an alternative, the use of fibre optic sensors has been suggested since it allows the measurement of fluorescence emitted by fluorophors at some distance from the source of excitation and the detector.[9-12] Moreover, a fibre optic sensor, in combination with a spectrofluorimeter, not only captures complete excitation and emission spectra, but routinely produces three-dimensional spectra.[9-11]

Data acquisition times are considerably better than in the conventional densitometry. However, the background signal is generally elevated due to the high amount of diffusely scattered radiation. Moreover, the use of a fibre optic to transport the radiation allows a certain amount of light from the surroundings to be added to the fluorescence emitted by the analyte, thus increasing the background signal. This signal may reduce the sensitivity and the dynamic range of quantitative HPTLC analysis.

This paper deals with the non-linearity of the calibration graphs for dansyl derivatives of putrescine, cadaverine, spermidine, and spermine after being eluted with chloroform:TEA:POLE (8:4:5%) (v:v:m) in HPTLC silica gel plates. The objective of this work was to know the extent of such non-linearity and to establish criteria that insures the reliability of the results. Furthermore, the principal parameters used to characterize the methods of analysis by HPTLC with fluorescence detection are calculated: sensibility, detection limit, precision, and accuracy.

EXPERIMENTAL

Apparatus

Fluorescence measurements and spectra were made with a Perkin Elmer LS-50 luminescence spectrometer equipped with a Perkin Elmer fluorescence plate reader accessory. A bifurcated fibre optic was used to transfer excitation and emission energy between the plate and the spectrometer. The spectrometer was connected via an RS232C interface to an Epson PCAX2e, containing Fluorescence Data Manager Software (FLDM) that controls the instrument.

Microwave dansylation system: CEM (Matthews, NC, USA) MDS 2000 microwave digestion system. The system delivers approximately 630 W

(100%) of microwave energy at a frequency of 2450 MHz at full power and provides constant feedback control of reaction conditions through continuous monitoring of pressure data on a control vessel. The system is provided with method and data storage capabilities and a printer.

Microwave dansylation vessels: CEM PFA Teflon lined advanced composite digestion vessels were used for sample dansylation. The vessels are constructed with a PFA Teflon liner and cover for high purity analysis and are capable of sustaining temperatures of 200°C and pressures of 13.9 bars.

Reagents

Putrescine dihydrochloride, cadaverine dihydrochloride, spermidine trihydrochloride, spermine tetrahydrochloride, and dansyl chloride were supplied by Aldrich Chemie (Beerse, Belgium) and polyoxyetylene-10-lauryl ether (POLE) by Sigma (St. Louis, MO, USA).

For chromatographic analysis, chloroform (Panreac, Barcelona, Spain) and triethylamine (TEA) (Fluka, Buchs, Switzerland) were used.

Silica gel HPTLC aluminium sheets (Merck), without fluorescent indicator and with a layer thickness of 0.1 mm, were used as the stationary phase.

All other chemicals used were of an analytical reagent grade and were used without further purification.

Microwave Assisted Dansylation Method

To 2 mL of solution of the amines, 2 mL of saturated sodium bicarbonate solution and 3 mL of dansyl chloride reagent (5 mg/mL) were added. The solution was mixed 1 min and transferred, quantitatively, to microwave dansylation vessels. The vessel was closed and introduced into the microwave cavity. The reaction was made at 40% (252 W) for 5 min, keeping a maximum pressure of 3.4 bars inside the reactor, according to the method described in a previous paper.[13] When the vessel was cold, the mixture was extracted with two portions of 2 mL of toluene and taken to 10 mL with this solvent.

Thin Layer Chromatography and Analytical Method

Sample application was by the spray on technique using a microprocessor controlled Camag Linomat IV device. Sample volumes of 1-10 μL (containing from 10 to 150 ng of each analyte) were applied to the plates at a rate of 10 sec μL^{-1}.

The dansyl amines were chromatographed on 10x10 silica gel HPTLC aluminium sheets, which were activated before use. The thin-layer plate was developed in chloroform:TEA:POLE (8:4:5%) (v:v:m), light protected, until the solvent migrated a distance of about 8 cm from the origin.

Once the spot corresponding to the analyte had been located, in situ quantitative scans were done at λ_{exc}= 338 nm and λ_{em}= 502 nm, using slits of 10 nm for excitation and emission. As a blank to correct fluorescence intensity measurements, the signal corresponding to the dry stationary phase, after elution with the above mentioned mobile phase was used.

RESULTS AND DISCUSSION

The Calibration in HPTLC

Quantitative determination of fluorescent compounds separated by HPTLC, measuring their radiation emitted when they are excited at appropriate wavelengths, is based on the measure of the peak size, area, or height. In our case, we have based this study on the measure of peak height.

Figure 1 shows the three dimensional chromatogram and its contour map for a mixture of dansyl derivatives of the polyamines putrescine, cadaverine, spermidine, and spermine over the range 10-100 ng, for each amine. As can be observed, the different derivatives show very similar peak widths and the concentration profiles of spots exhibit a roughly Gaussian profile (cylinder character).[14] The integral of the concentration density over the spot area was equal to the volume of the cylinder, which in turn is proportional to peak concentration value. In this way, we can substitute integration by measuring the maximum fluorescence intensity (or peak height) at the centre of the spot.

To establish relationship which exists between the response of the detector and the concentration of analyte on the chromatographic plate, seven samples, with different amounts of the mixture of dansyl derivatives, have been injected. Each one of the injections has been carried out by triplicate. After the plates were eluted with chloroform:TEA:POLE (8:4:5%) (v:v:m), the maximum fluorescence intensity for each one of the separated analytes was read.

Figure 2 shows the calibration graphs for the studied amines.

Linear Fits

Assuming a linear relationship between the signal supplied by the detector and the quantity of the amines, the chemometric characteristics of such regression have been established. The results are given in Table 1. The value of R^2 usually is considered a good indicator of the linearity of the calibration graphs.

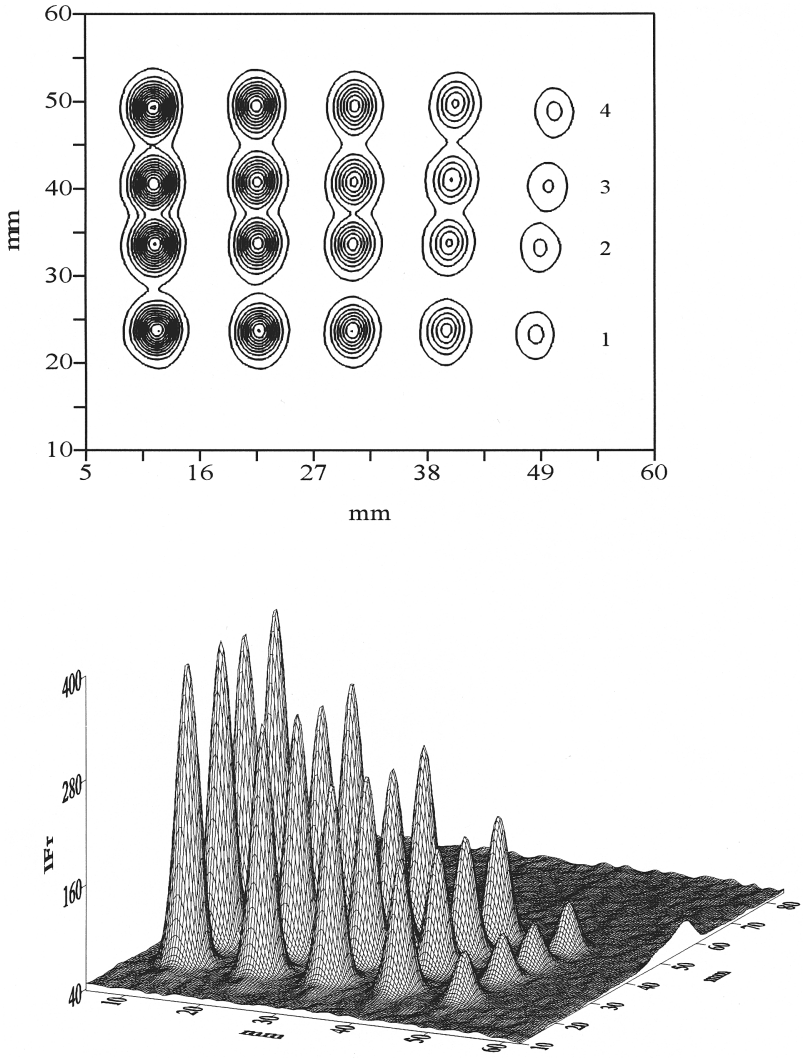

Figure 1. Three-dimensional chromatogram and its contour map scanned at λ_{exc}= 338 nm and λ_{em}= 502 nm for five mixtures of dansyl derivatives of the polyamines: (1) putrescine, (2) cadaverine, (3) spermidine, and (4) spermine, after they were eluted with chloroform:TEA:POLE (8:4:5%) (v:v:m).

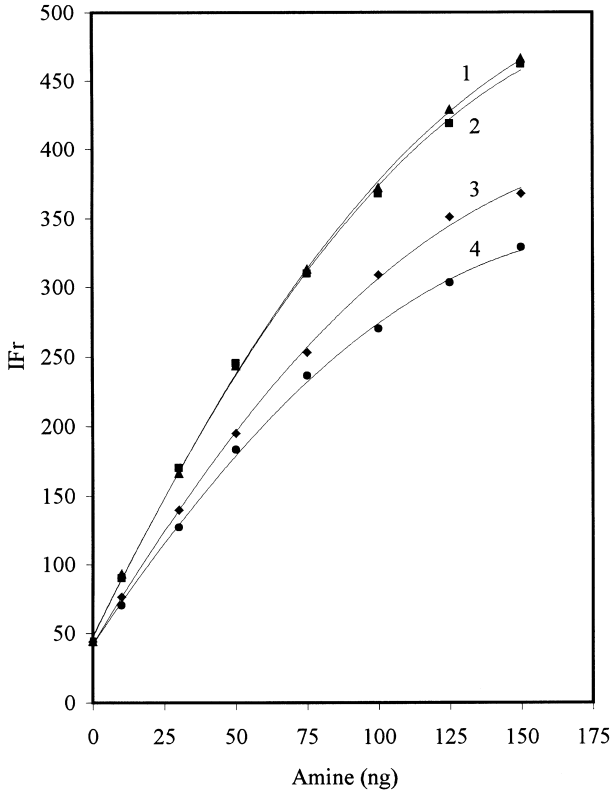

Figure 2. Calibration graphs of dansyl derivatives of the polyamines: (1) putrescine, (2) cadaverine, (3) spermidine, and (4) spermine, after they were separated by HPTLC with chloroform:TEA:POLE (8:4:5%) (v:v:m), as mobile phase, and silica-gel, as stationary phase.

In our case, the linear regression presents maximum values of 0.978, which cannot be considered high for the type of estimated fit. On the other hand, the standard errors of the estimate ($S_{y/x}$) are higher, in all the cases, than 20.

The linearity of the calibration graphs can also be expressed by the term (1-RSD)•100, where RSD is the relative standard deviation of the slope of the calibration line.[15] The thus obtained values, for each one of studied amines, are given in Table 1. As can be observed, the linearity calculated in this way presents values that oscillate between 92.7% and 93.9% for spermine and cadaverine respectively.

Table 1

Chemometric Characteristics of the Different Calibration Functions

	Putrescine				Cadaverine			
	Linear	**Quadratic**	**Cubic**	**Power**	**Linear**	**Quadratic**	**Cubic**	**Power**
a	76.30±15.24	47.93±4.03	43.45±2.17	37.23±8.69	75.08±14.74	47.45±3.02	45.61±3.33	37.95±9.18
b	2.77±0.18	4.30±0.14	4.89±0.15	12.82±2.60	2.82±0.17	4.32±0.10	4.56±0.23	12.07±2.61
c		-0.01±0.0009	-0.02±0.002	0.70±0.04		-0.01±0.0007	-0.01±0.004	0.72±0.04
d			$(4.71\pm1.09)\bullet10^{-5}$				$(1.93\pm1.68)\bullet10^{-5}$	
$\mathbf{R^2}$	0.975	0.999	0.999	0.997	0.978	0.999	0.999	0.997
$\mathbf{S_{y/x}}$	25.99	5.44	2.56	9.30	25.12	4.07	3.94	9.87
F	236.8	2763.0	8321.5	945.0	263.0	5131.2	3642.5	868.0
(1-RSD) •100	93.5				93.9			

	Spermidine				Spermine			
	Linear	**Quadratic**	**Cubic**	**Power**	**Linear**	**Quadratic**	**Cubic**	**Power**
a	66.16±12.84	42.18±3.03	44.82±2.71	36.83±11.26	64.96±12.25	42.08±2.93	40.79±3.49	36.43±10.28
b	2.23±0.15	3.53±0.10	3.19±0.19	9.51±3.19	1.93±0.14	3.17±0.10	3.33±0.24	9.39±3.18
c		$(-9\pm0.7).10^{-3}$	0.003±0.003	0.72±0.06		-0.008±0.0007	-0.01±0.004	96±0.06
d			$(-2.77\pm1.36)\bullet10^{-5}$				$(1.34\pm1.76)\bullet10^{-5}$	
$\mathbf{R^2}$	0.973	0.999	0.999	0.993	0.967	0.999	0.999	0.992
$\mathbf{S_{y/x}}$	21.89	4.08	3.20	12.11	20.89	3.95	4.12	10.97
F	216.8	3196.5	3460.4	361.0	177.6	2566.6	1568.3	330.3
(1-RSD) •100	93.3				92.7			

The residual values reach amounts of 70%, at low concentrations of analyte. The distribution obtained for residuals suggests a curvilinear fit in the relationship of the analytical signal with the quantity of polyamine.

However, for concentrations of amines lower than 50 ng, it is possible to obtain linear relationships between the fluorescence intensity and the quantity of the amine injected, with values of $R^2 > 0.99$.

Non-Linear Fits

The experimental data used upon commenting the linearity of the linear fits have been treated for their fit to quadratic ($y = a + bx + cx^2$), cubic ($y = a + bx + cx^2 + dx^3$), and power ($y = a + bx^c$) regressions. The values obtained for the coefficients, as well as the remaining chemometric characteristics, for each one of the studied compounds are given in Table 1.

If we consider the values of R^2 as a measure of the validity of the fit, it can be observed that, in all the cases, the equations explain, in more than a 99.2%, the relationship between the analytical signal and the quantity of the amine. This value becomes 99.9% in the case of the quadratic and cubic fits. Also, for these fits, the lowest values of the standard error of the estimate are obtained. On the other hand, the values of F become more than 10 times higher in the cubic and quadratic fits, as compared to the linear and power ones.

The maximum values of the obtained residuals, reach 17% for the power fit, in the case of spermine. However, for all the amines and upon using the quadratic and cubic fits, these values do not reach 10%.

Performance Characteristics

Independant of the problems of linearity studied in the previous heading, the calibration can obtain basic information about characteristic of analytical interest.

Sensibility

According to IUPAC,[16] the sensibility of an analytical method represents the variation of the detector signal originated per unit of analyte concentration. This relationship coincides with the slope of the calibration line and, therefore, it depends on the instrumental conditions used and it is known as calibration sensibility.

In addition, it is possible to establish the calibration sensibility as the relationship signal/concentration corresponding to each point of the calibration graphs. The Figure 3 shows the representation of the calibration sensibility ver-

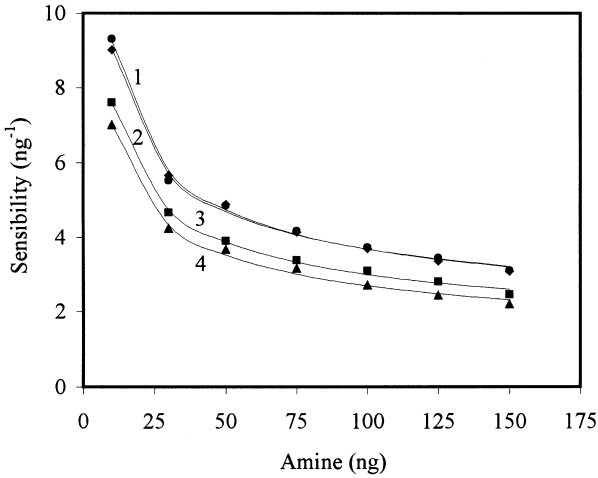

Figure 3. Calibration sensibility *versus* polyamine concentration: (1) putrescine, (2) cadaverine, (3) spermidine, and (4) spermine.

sus the amine concentration. It can be observed, as was to be expected, that the best calibration sensibilities are obtained for low quantities of analyte. These representations also show the lack of linearity of the calibrations graphs.

A number of authors[17,18] have suggested introducing the term analytical sensibility (SA). The analytical sensibility is calculated through the expression b/S_s, where b is the calibration slope and S_s indicates the standard deviation of the measurements of the analytical signal to a given concentration. The disadvantage of the analytical sensibility is that it must be evaluated for all the concentrations of interest. Even, for linear analytical graphs, where the calibration sensibility stays constant, the analytical sensibility varies with the analyte concentration.

A most appropriate expression for the analytical sensibility would be one which takes into account the regression analysis, whose value is characteristic of the instrumental calibration method and independent of the instrument used, and of the mathematical transformations carried out for readings of measurements: $SA= b/S_{y/x}$, where $S_{y/x}$ is the standard error of the estimate and b the slope of the regression line. Thus, the defined analytical sensibility results more appropriately in the characterizing of an analytical method.

The relationship between the peak height and the chromatographed analyte concentration is not a straight line, but it is fitted to an equation of second degree. Therefore, to determine the sensibility, we have to take into account only the linear section of the calibration graphs, which, as we have seen above, is obtained for polyamine concentrations lower than 50 ng. The studied analytes present SA next to one, except in the case of spermine, which presents a value of 2.8, due to the low value of the standard error of the estimate that presents its regression line.

Detection Limit

There are several ways to calculate the detection limit, although for instrumental methods, the IUPAC recommends establishing the detection limit as the concentration whose signal corresponds to the average of the blank signal (y_B) plus three times the standard deviation value of the analytical signal corresponding to the blank:

$$y = y_B + 3S_B \quad (1)$$

The value of a, the calculated intercept, can be used as a y_B estimation, its own blank signal:

$$y = a + 3S_B \quad (2)$$

Thus, a fundamental assumption of the least square method without weighting, is that each point, including the blank, has a variation which is normally distributed with a standard deviation estimated by $S_{y/x}$. Therefore, it is appropriate to use $S_{y/x}$, instead of S_B, in the detection limit estimation:[19]

$$y = a + 3S_{y/x} \quad (3)$$

However, these expressions do not take into account the imprecision of slope and intercept values obtained in the regression. The detection limit, according to Winefordner,[20] is calculated through the equation:

$$C_L = \frac{K\left[S_B^2 + S_a^2 + (a/b)^2 S_b^2\right]^{1/2}}{b} \quad (4)$$

where b and a are, respectively, the slope and the intercept of the regression line; S_B, S_a and S_b are the blank, the intercept, and the slope standard deviations, respectively, and K is a numerical factor chosen according to the desired confidence level.

In those techniques, as chromatographic ones, where it is not possible to carry out blank determinations, an approximate estimation of the statistic detection limit, could be calculated through equation:[15]

$$LOD = 3\frac{S_{y/x}}{b}\sqrt{\frac{(n-2)}{(n-1)}} \quad (5)$$

The expressions (4) and (5) to calculate the detection limit, require the existence of a linear relationship between the measured signal and the analyte concentration. As it was shown previously in this case, a linear relationship between the measured fluorescence and the analyte concentration injected in the plate, does not exist, therefore, in both equations we will consider only the linear section of the calibration graphs.

To calculate the detection limit seven scans of the chromatographic plate were made after they were eluted in the same experimental conditions as the ones used to obtain the calibration graphs. With the obtained results (y_B= 44.12 and S_B= 0.711), and assuming as recommendable, a value of K= 3, the detection limit values are obtained for each one of the studied analytes, and for the different fits carried out, except the linear.

From the values in Table 2, it can be observed that with the exception of the procedure recommended by the IUPAC, which leads, in some instances even to negative values, the other procedures with few exceptions, present good agreement for the different calculation and fit methods.

Precision and Accuracy

To calculate the precision of the determination, according to the three types of used fits: quadratic, cubic, and power, seven measurements of a standard solution of each one of the dansyl derivatives of the amines in study were made. The concentrations have been calculated by measuring peak height and determining the quantity of the amine directly from the established calibration. The parameters obtained upon carrying out the statistic evaluation of such results for each one of the amines, are shown in Table 3.

In accordance with the results shown, and taking a confidence level of 95% and six freedom degrees, we can conclude that for the studied analytes, the quadratic fit is the one which, in all the cases, leads to correct and sufficiently precise methods.

In accordance with these results, the proposed method presents good repeatability, which permits high precision in the analysis. However, to assure the accuracy, it is necessary to have strict external and internal quality controls.

Table 2

Comparison of LOD (ng) for the Different Calibration Functions

	Equation				
	1	**2**	**3**	**4**	**5**
Putrescine					
Linear Range	-0.3	0.5	2.9	2.4	2.5
Quadratic	-0.4	0.5	3.8		
Cubic	0.6	0.4	1.6		
Power	0.6	0.1	3.0		
Cadaverine					
Linear Range	-0.6	0.5	3.8	3.1	3.2
Quadratic	-0.3	0.5	2.8		
Cubic	0.1	0.5	2.6		
Power	0.6	0.1	3.5		
Spermidine					
Linear Range	0.2	0.7	3.1	2.5	2.7
Quadratic	1.2	0.6	3.5		
Cubic	0.4	0.7	3.0		
Power	1.0	0.1	6.5		
Spermine					
Linear Range	1.1	0.8	1.1	0.9	1.2
Quadratic	1.3	0.7	3.8		
Cubic	1.6	0.6	3.8		
Power	1.1	0.1	6.1		

In particular, the preparation of the calibration solutions, the used plates, the mobile phase, etc., are critical aspects. Even though control charts are used for different purposes, its interest for the analyst consists in that they constitute an indispensable way to monitor the precision and accuracy of the analytical process.

Control charts have been obtained from the chromatographic analysis of a sample of amines previously dansylated in accordance with the described procedure. Such control samples, preserved at 4°C, was analysed during six

Table 3

Parameters of the Statistic Evaluation for the Different Calibration Functions

	$\bar{x}$	S	RSD (%)	E (%)	t_{exp}	t'_{exp}
Putrescine (27.5 mg)						
Quadratic	27.7	0.3	1.2	1.1	0.6	1.7
Cubic	27.0	0.3	1.3	1.2	1.4	3.8
Power	27.7	0.4	1.4	1.3	0.5	1.2
Cadaverine (29.0 ng)						
Quadratic	29.0	0.1	0.4	0.4	0.1	0.1
Cubic	28.7	0.1	0.5	0.4	2.3	6.1
Power	29.0	0.2	0.5	0.5	0.1	0.3
Spermidine (28.5 ng)						
Quadratic	28.7	0.2	0.8	0.8	0.7	1.8
Cubic	29.2	0.2	0.8	0.8	2.8	7.5
Power	29.1	0.3	0.5	1.0	2.2	5.9
Spermine (29.0 ng)						
Quadratic	29.1	0.2	0.8	0.8	0.2	0.5
Cubic	28.8	0.2	0.9	0.8	1.0	2.6
Power	29.5	0.3	1.0	0.9	1.8	4.9

months. As a central value we have considered the mean, and as warning and control limits, the intervals defined by $\bar{x} \pm 2S$ and $\bar{x} \pm 3S$ have been used, respectively. The obtained results are shown in Figure 4. The charts appear to reflect a measurement process that is in control: there are no points outside the control limits and no extensive runs in the data.

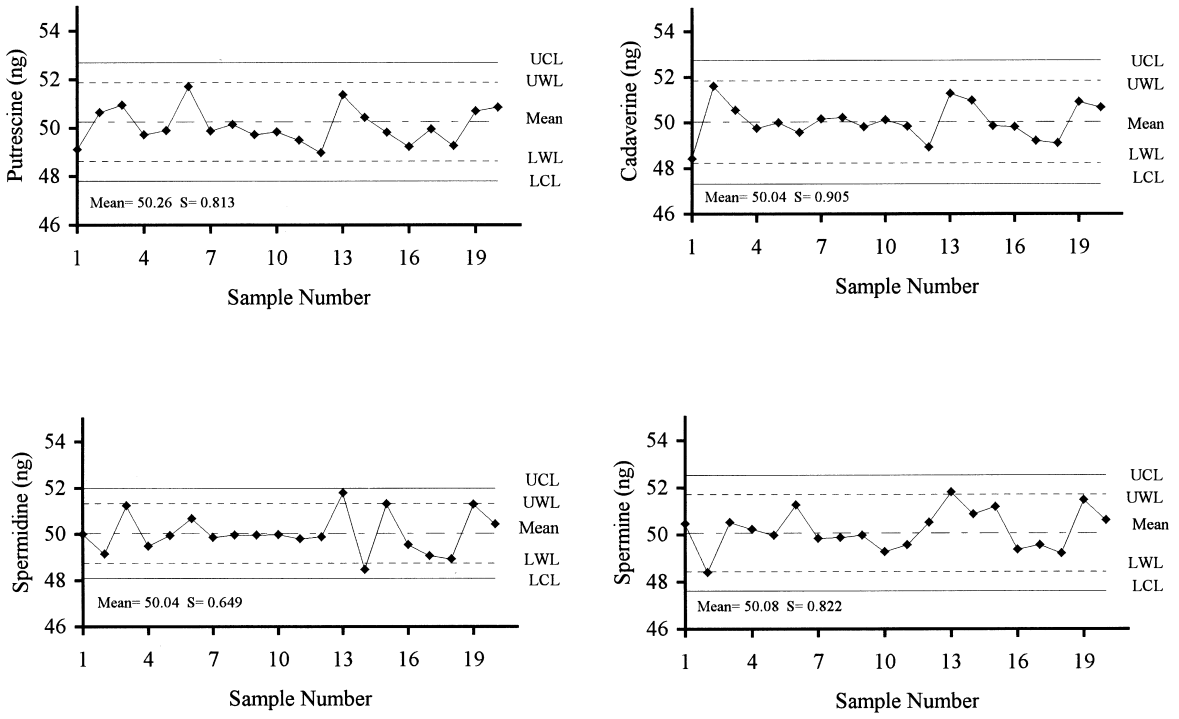

Figure 4. Control charts of the dansyl derivatives of the studied polyamines. UCL and LCL: upper and lower control limits ($\bar{x}\pm3S$). UWL and LWL: upper and lower warning limits ($\bar{x}\pm2S$).

CONCLUSION

The relationship which exists between the response of a fibre optic sensor and the amount of polyamines retained on a surface of silica gel is studied. The lack of linearity of these calibration curves is established, and it is shown that a quadratic fit is the one which better explains the relationship between the fluorescence intensity and the amounts of analyte.

The principal parameters of quality used to characterize the analysis methods by HPTLC with fluorescent detection are calculated. These parameters have been calculated using different models and the results have been compared. Furthermore, we used control charts to estimate the precision of the analysis method by HPTLC with fluorescence detection.

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