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STUDY OF THE BEHAVIOUR OF THE ABSORBENT BLANKS IN ANALYTICAL PROCEDURES BY USING THE H-POINT STANDARD ADDITIONS METHOD (HPSAM)

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Summary—This paper studies the behaviour of reagent blank in different extractive-colorimetric procedures (determination of sympathomimetic amines with NQS reagent) by using the H-Point Standard Additions Method (HPSAM) in order to study and characterize the different possibilities that the blank can introduce in an analytical procedure. We define two kinds of blanks: *the external blank* (from reagent alone solutions data) and the *internal blank* (from extrapolation of reagent plus analyte solutions data). Comparison between both gives the information about the reproducibility of the behaviour of the reagent blank. A procedure to evaluate, and characterize, errors (if they exist) is described, and a guide for optimizing the measuring procedure is presented.

In spectrophotometry it is common to use, for the determination of an analyte, the formation of an absorbent complex by reaction between the analyte and an external reagent. The absorbance of the formed complex is calculated by subtracting from the measured absorbance value, the one corresponding to the "reagent blank", added in the initial concentration, if it absorbs at the measurement wavelength.

In this context, a calibration line obtained as an addition of absorbances (reagent plus added analyte-formed complex-) can be considered as a standard addition line with an added sample portion equal to zero. Its intercept will be a function of the absorbance of the "reagent blank" at the selected wavelength. This allows us to use the H-Point Standard Addition Method (HPSAM)[1–5] in order to study the different behaviour observed in different calibration lines, with the purpose of clarifying the significance of the "reagent blank" and choosing it adequately in a particular analytical procedure.

For the theoretical development of the method, we have defined two kinds of blank.

(a) The external blank, obtained from reagent solutions without analyte, or from reagent plus placebo, which has been processed through the analytical method. It is the usually named "reagent blank" or "reference solution".

(b) The internal blank, obtained by extrapolation from the calibration data of the analyte in presence of the reagent, and it corresponds with the intercept of the analyte calibration line.

In this paper we examine the possibilities of the HPSAM in extractive-colorimetric procedures that uses an absorbent blank, as it is the determination of sympathomimetic amines with NQS reagent (the sodium salt of 1,2-naphtoquinone-β-sulphonic acid), in order to study and characterize the different possibilities that a reagent blank can introduce in an analytical procedure.

THEORETICAL BACKGROUND

According with the fundamentals of the HPSAM applied to analytical procedures that uses a reagent blank,[5] two wavelengths, λ_1 and λ_2, are selected for the external blank, lying on both sides of its absorption maximum, and where it shows the same absorbance against solvent blank.

The calibration lines obtained at λ_1 and λ_2 (Fig. 1(a)) will be described by the equations:

$$A(\lambda_1) = b'_i + b_0 + M(\lambda_1) \times C_i; \quad i = 0,1,\ldots,n \quad (1)$$

$$A(\lambda_2) = A'_i + A_0 + M(\lambda_2) \times C_i; \quad i = 0,1,\ldots,n \quad (2)$$

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where the subscript i denotes the different solutions for n additions of analyte; C_i is the analyte added concentration; b_0 and A_0 are the absorbance values of the complex formed at λ_1 and λ_2, measured when $i = 0$ (when the analyte added concentration is zero, and therefore, the formed complex concentration is also zero, so $b_0 = A_0 = 0$); b_i' and A_i' are the absorbance values for the excess of free reagent at λ_1 and λ_2 in the different solutions prepared to apply the method; and $M(\lambda_1)$ and $M(\lambda_2)$ are the slopes of the calibration lines at λ_1 and λ_2.

The use of $\Delta A_{1,2} = A(\lambda_2) - A(\lambda_1)$ as the analytical signal (Fig. 1(a)) from equations (1) and (2), and considering that $b_0 = A_0 = 0$, and that for every i, $b_i' = A_i'$ because the reagent shows the same absorbance values at λ_1 and λ_2, yields:

$$\Delta A_{1,2} = A(\lambda_2) - A(\lambda_1)$$
$$= [M(\lambda_2) - M(\lambda_1)] \times C_i. \quad (3)$$

Plotting $\Delta A_{1,2}$ *vs* C_i leads to a straight line with zero intercept and a slope of $M(\lambda_2) - M(\lambda_1)$ value. This expression shows that the absorbance increments depend exclusively on the analyte concentration. The excess of free reagent does not affect the straight line because for each point its contribution is cancelled, because their absorbance values are the same at the two previously selected wavelengths, although its absorbance values were different in

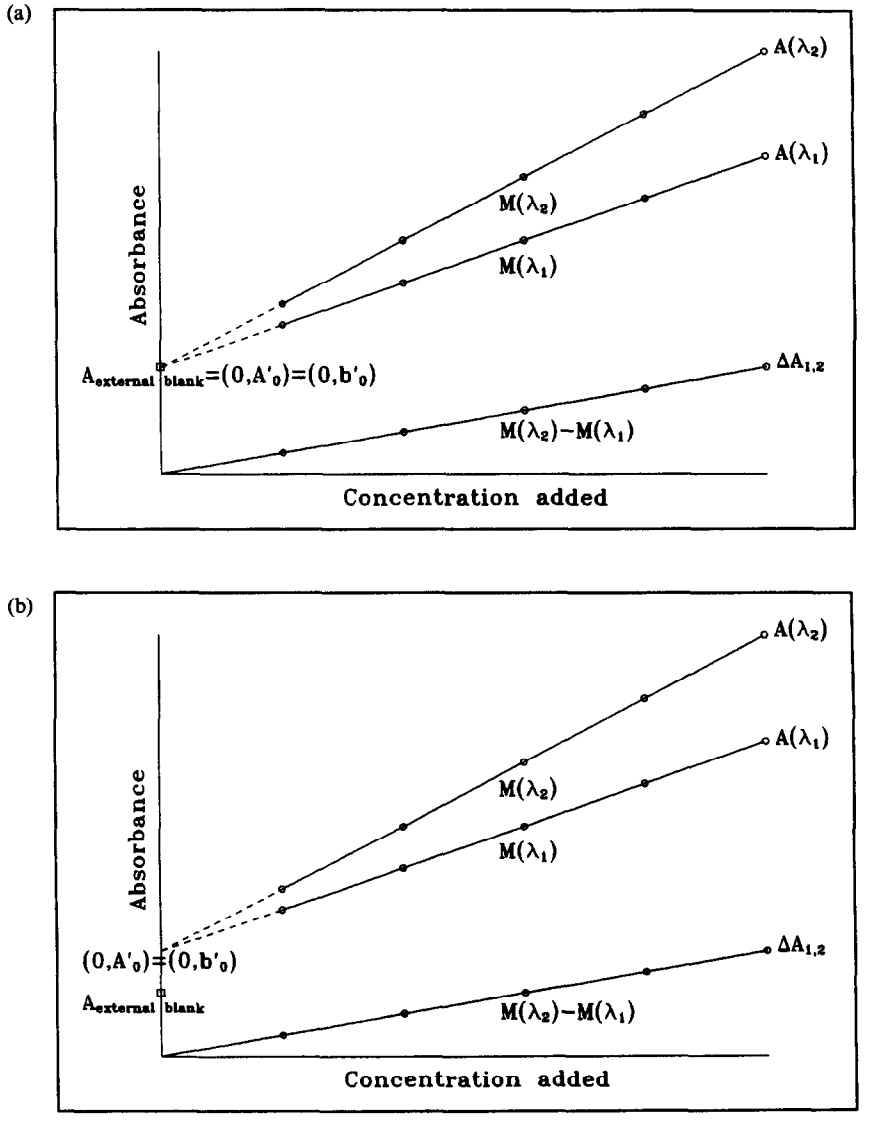


Fig. 1 (a and b)

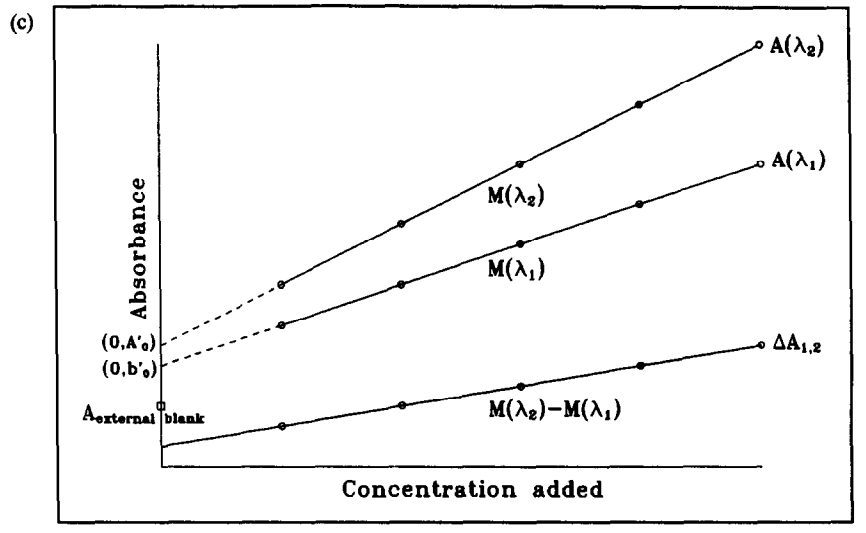


Fig. 1(c)

Fig. 1. The different situations that the application of the HPSAM leads to: (A) The intercepts for the calibration lines at λ_1 and λ_2 (internal blank) and the value for the external blank are coincident. (B) The intercepts for the calibration lines at λ_1 and λ_2 (internal blank) are coincident between them, but not with the value for the external blank. (C) The intercepts for the calibration lines at λ_1 and λ_2 (internal blank) are not coincident between them, neither with the value for the external blank.

each solution. The intercepts of the lines $A(\lambda_1)$ and $A(\lambda_2)$ must be equal by the basis of the HPSAM, and equal to the absorbance value for the external blank, if it is equivalent to the internal one.

This is the most general situation. It is plotted in Fig. 1(a), and corresponds with the examples studied in Ref. 5.

But the application of the HPSAM to the analytical procedure studied shows that the described situations in Figs 1(b) and (c) are also possible. These situations are applicable to other analytical procedures, and so, the following considerations and methodology will also be applicable. Each one corresponds with a different behaviour between the external and the internal blank. There are examples of this in the Results and Discussion section, where the procedure to evaluate and characterize the existing errors is described, and a guide for optimizing the measuring procedure is presented.

EXPERIMENTAL

Material and methods

Reagents. Phosphate buffer (pH = 7.5) was prepared by dissolving 3.00 g of sodium monohydrogenphosphate dodecahydrate (Probus) and 0.25 g of sodium dihydrogenphosphate dihydrate (Probus) in 100 ml of distilled water. 1,2-Naphthoquinone-4-sulphonate stock solution was prepared by dissolving 0.50 g of the sodium salt (Sigma) in 100 ml of distilled water. This solution was prepared fresh for each experiment and it was stored in dark. Methamphetamine hydrochloride (Sigma) and deoxiephedrine hydrochloride (Sigma) stock solutions were prepared by dissolving 100 mg in distilled water up to 100 ml. d-l-Amphetamine sulphate (Sigma) stock solutions were prepared by dissolving 100 mg in distilled water up to 100 ml. These stock solutions were then diluted further to yield appropriate working solutions for the preparation of the calibration standards. *n*-Hexane 95% HPLC grade (Scharlau), chloroform LC grade (ethanol as preservative) (Scharlau), ethyl acetate LC grade (Scharlau), concentrated ammonium (Probus) and hydrochloric acid (Probus) were also used.

Apparatus. pH measurements were made in a Crison micropH 2000 pH-meter.

The organic solvent (*n*-hexane) was evaporated to dryness using a rotary evaporator R110 Büchi.

The mixture samples were heated in a calefactory RM 20 Lauda, vortex-mixed in a Vibromatic-384 and centrifuged in a Medifuge Heraeus Sepatech.

All spectrophotometric measurements were made on a Hewlett-Packard HP 8452 A diode array spectrophotometer furnished with quartz cuvettes of 1 cm pathlength, and interfaced to

an HP Vectra ES/12 computer and an HP Think Jet printer. A home-made computer program was used to obtain the calibration lines at all of the measured wavelengths from the spectral data.

Procedures

Standard solutions

Different volumes of the amine stock solution (d-l-amphetamine sulphate, deoxyephedrine or methamphetamine hydrochloride) were added to 1 ml of phosphate buffer (pH = 7.5), 1 ml of 0.5% (w/v) NQS and distilled water up to a volume of 3 ml. The mixture was heated at 70°C for 30 min. After cooling, the mixture was shaken with the same volume of organic solvent for 2 min and then it was centrifuged for 5 min. The absorbance between 350 and 500 nm, in 2-nm steps, of the organic phase was registered. Absorbances were measured against a solvent blank at 25°C, measuring it first and subtracting its spectrum from the spectra of the samples.

Urine sample

Samples of urine (5 or 10 ml, previously spiked or not with methamphetamine stock solution) were made alkaline with 0.1 or 0.2 ml of concentrated ammonium solution, and the free bases were extracted twice with the same volume of *n*-hexane. A small amount (50–100 μl) of

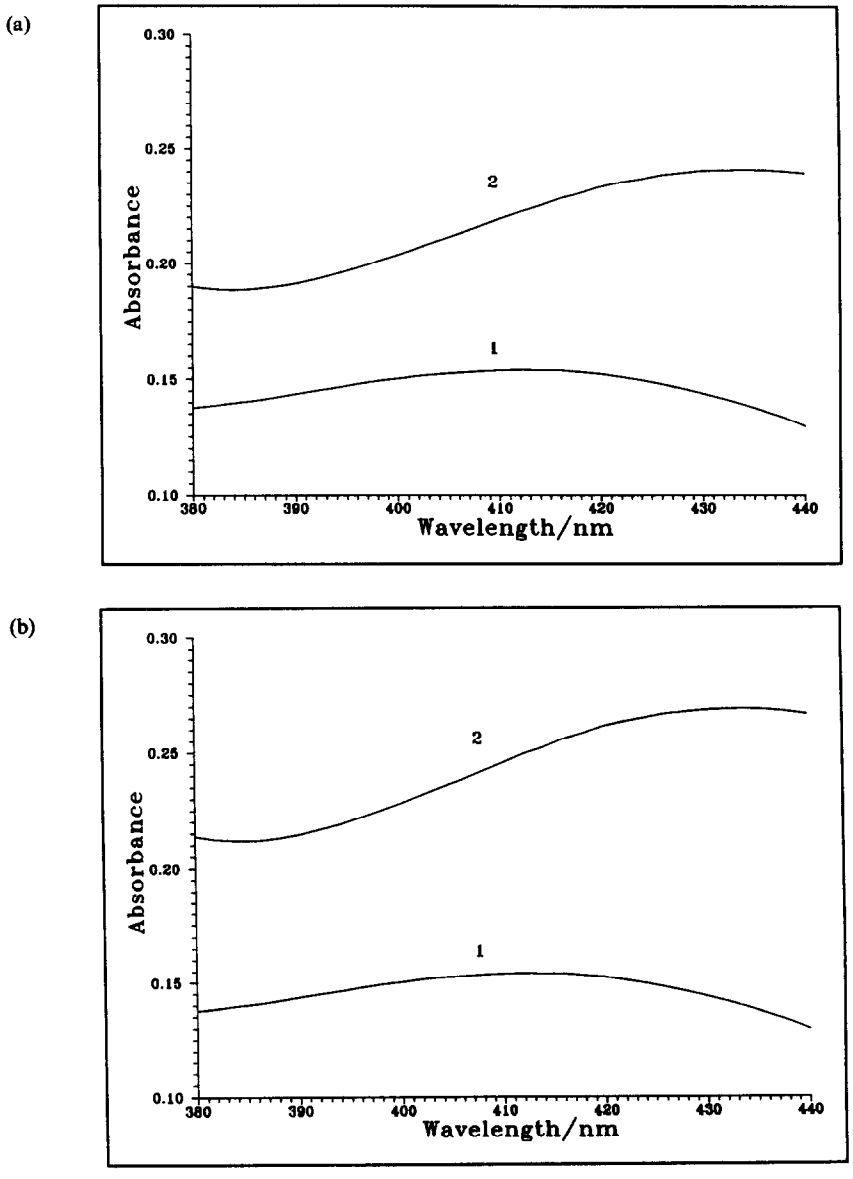


Fig. 2 (a and b)

(c)

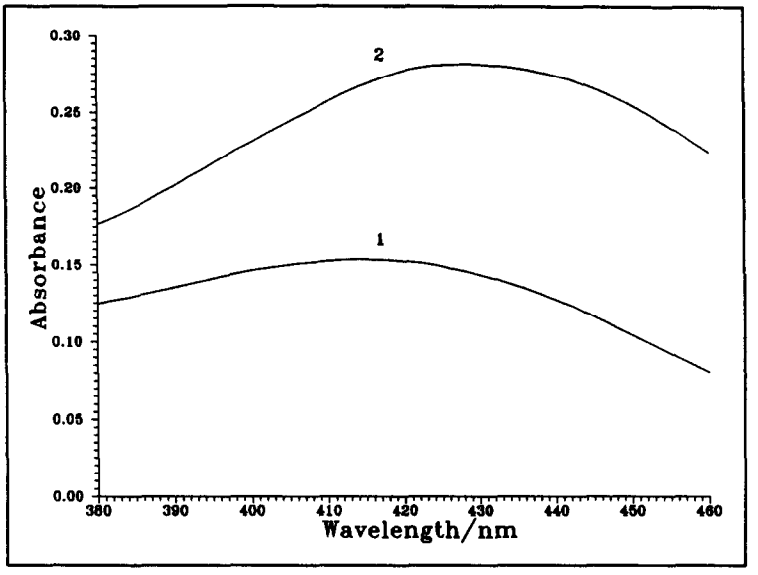
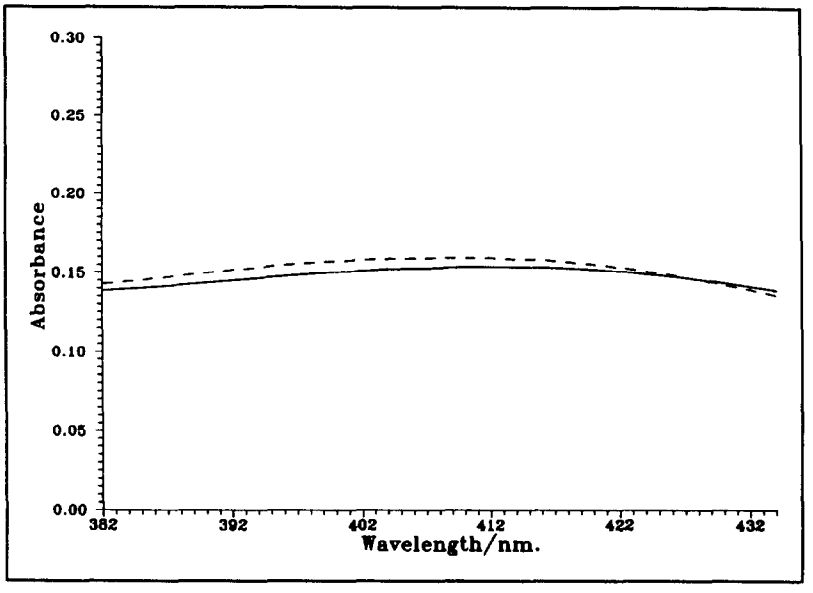


Fig. 2(c)

Fig. 2. (A) Spectra of the reagent blank (1) and reagent blank plus 9.6 ppm of deoxiephedrine against solvent blank (2). (B) Spectra of the reagent blank (1) and reagent blank plus 8.66 ppm of metamphetamine against solvent blank (2). (C) Spectra of the reagent blank (1) and reagent blank plus 8.66 ppm of d-l-amphetamine against solvent blank (2).

(a)



(b)

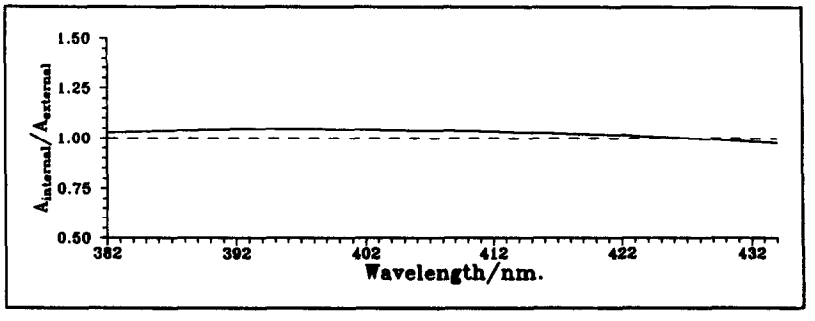


Fig. 3. (A) Representation of the internal (– – –) and external (——) blanks for the calibration of deoxiephedrine using NQS as reagent and $CHCl_3$ as extractant. (B) Quotient internal blank/external blank.

Table 1. Results obtained for the calibration lines in the determination of deoxiephedrine in presence of NQS reagent. $a \pm s_a$ represent the value for the intercept and its variance[7] in absorbance units; $b \pm s_b$ represent the value for the slope and its variance,[7] in /ppm; r is the correlation coefficient; $t_{calc} = b/s_b$ is the statistical used for test linearity[6] and n represents the number of points used to construct the calibration line

Wavelength measured	434	382	430	390	426	396
$a \pm s_a$	0.136 ± 0.005	0.143 ± 0.004	0.143 ± 0.005	0.150 ± 0.005	0.149 ± 0.005	0.155 ± 0.005
$(b \pm s_b) \times 10^3$	11.22 ± 0.11	4.97 ± 0.10	10.46 ± 0.11	4.47 ± 0.10	9.63 ± 0.12	4.67 ± 0.11
r	0.99990	0.99963	0.99988	0.99949	0.99985	0.99947
t_{calc}	101.754	51.986	91.887	44.240	81.091	43.470
$A_{external\ blank}$	0.1387	0.1385	0.1436	0.1436	0.1478	0.1479

Wavelength measured	464 (reagent blank)	ΔA(434–382)	ΔA(430–390)	ΔA(426–396)
$a \pm s_a$	-0.015 ± 0.003	-0.0070 ± 0.0009	-0.0074 ± 0.0008	-0.0063 ± 0.0007
$(b \pm s_b) \times 10^3$	14.67 ± 0.07	6.248 ± 0.019	5.990 ± 0.019	4.962 ± 0.017
r	0.99998	0.99999	0.99999	0.99999
t_{calc}	219.734	314.472	314.608	295.022
n	4	4	4	4
Detection limit/ppm	1.32	0.92	0.92	0.98

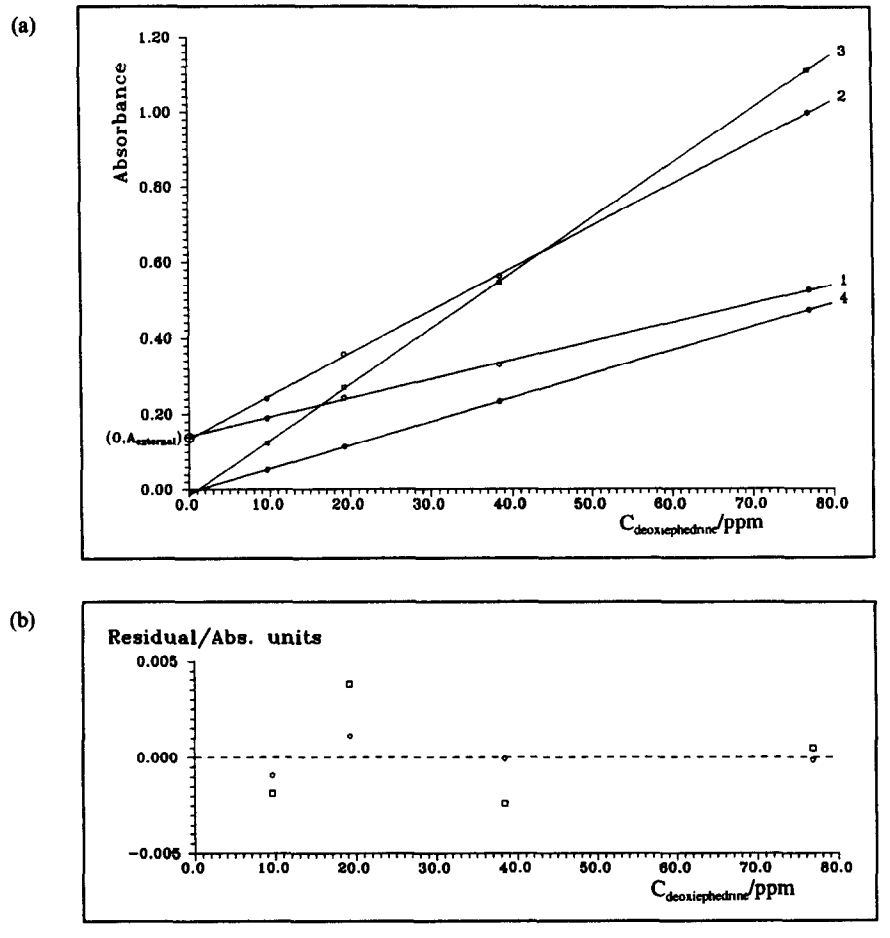


Fig. 4. (A) Calibration lines for the determination of deoxiephedrine. (1) Using absorbance at 382 nm against solvent blank as analytical signal. (2) Using absorbance at 434 nm against solvent blank as analytical signal. (3) Using absorbance at 464 nm, against reagent blank as analytical signal. (4) Using ΔA (434–382) as analytical signal. The equations are shown in Table 1. (B) Plot of residuals (absolute values) *vs* $C_{deoxiephedrine}$; ○ using ΔA(434–382) as analytical signal; □ using absorbance at 464 nm against reagent blank as analytical signal.

Table 2. Results obtained for the calibration lines in the determination of metamphetamine in the presence of NQS reagent. The symbols are as in Table 1

Wavelength measured	430	390	426	396	424	398
$a \pm s_a$	0.174 ± 0.006	0.175 ± 0.003	0.180 ± 0.006	0.180 ± 0.003	0.182 ± 0.005	0.182 ± 0.003
$(b \pm s_b) \times 10^3$	10.03 ± 0.14	4.09 ± 0.07	9.21 ± 0.13	4.29 ± 0.08	8.79 ± 0.13	4.45 ± 0.08
r	0.99973	0.99958	0.99970	0.99952	0.99968	0.99951
t_{calc}	74.188	59.541	70.354	56.011	68.683	55.285
$A_{external\ blank}$	0.1436	0.1436	0.1478	0.1479	0.1494	0.1493

Wavelength measured	464(reagent blank)	ΔA(430–390)	ΔA(426–396)	ΔA(424–398)
$a \pm s_a$	0.014 ± 0.007	-0.001 ± 0.004	-0.0008 ± 0.0024	-0.0005 ± 0.0021
$(b \pm s_b) \times 10^3$	14.26 ± 0.16	5.94 ± 0.07	4.92 ± 0.06	4.35 ± 0.05
r	0.99982	0.99979	0.99981	0.99981
t_{calc}	90.686	83.688	88.318	87.885
n	5	5	5	5
Detection limit/ppm	2.25	2.44	2.31	2.32

concentrated hydrochloric acid:ethanol (1:6) was added to the combined n-hexane extracts to convert the free amines into the hydrochlorides. After this, the solvent was evaporated to dryness. The residue was reconstituted in 1 ml of distilled water, 1 ml of phosphate buffer (pH = 7.5) and 1 ml of 0.5% NQS. These samples were processed according to the procedure

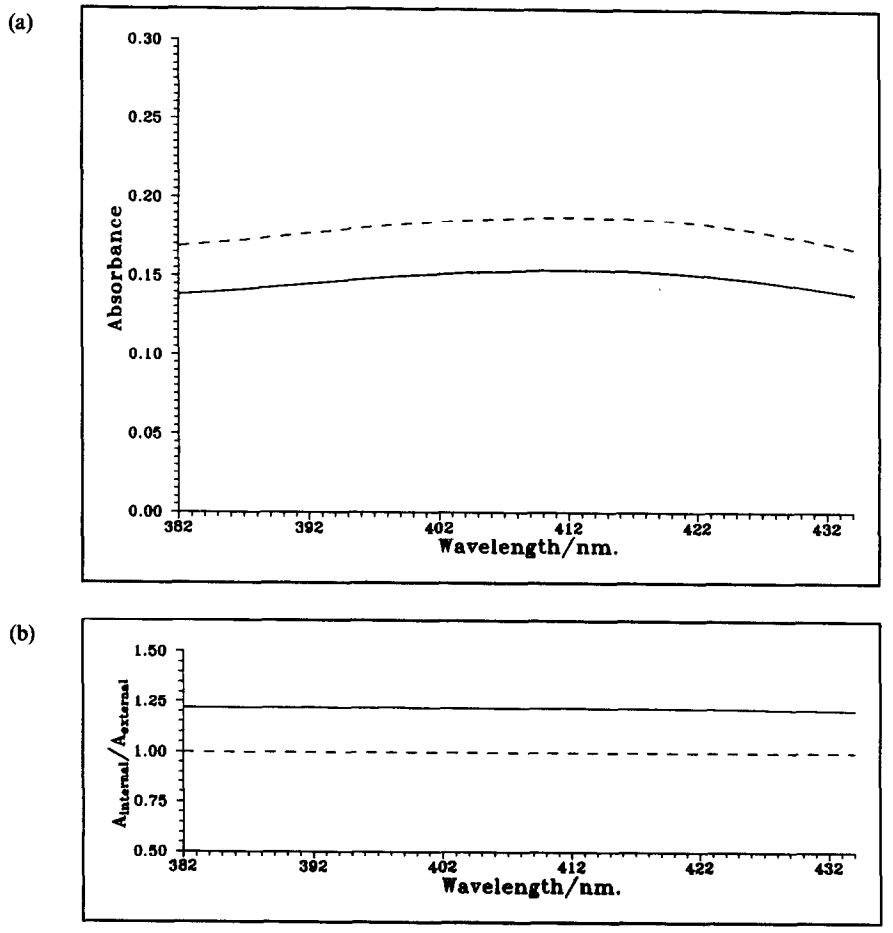


Fig. 5. (A) Representation of the internal (– – –) and external (——) blanks for the calibration of metamphetamine using NQS as reagent and $CHCl_3$ as extractant. (B) Quotient internal blank/external blank.

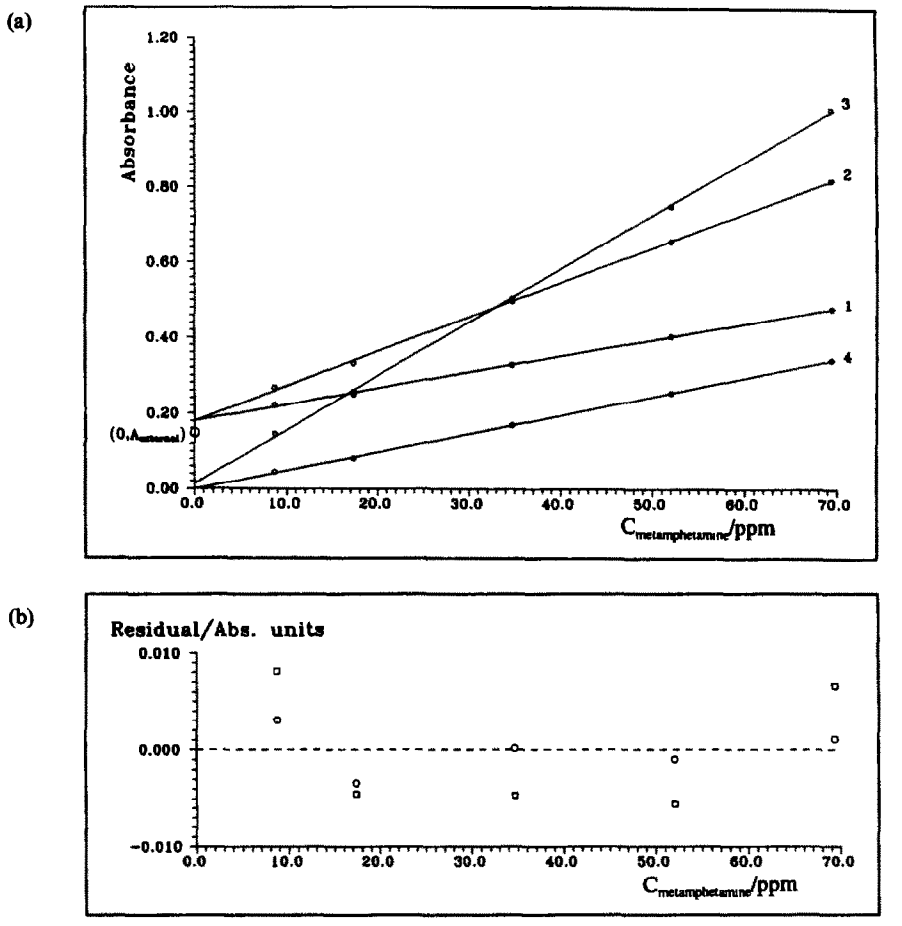


Fig. 6. (A) Calibration lines for the determination of metamphetamine. (1) Using absorbance at 396 nm against solvent blank as analytical signal. (2) Using absorbance at 426 nm against solvent blank as analytical signal. (3) Using absorbance at 464 nm against reagent blank as analytical signal. (4) Using ΔA(426–396) as analytical signal. Equations shown in Table 2. (B) Plot of residuals (absolute values) *vs* $C_{metamphetamine}$; ○ using ΔA(426–396) as analytical signal; □ using absorbance at 464 nm against reagent blank as analytical signal.

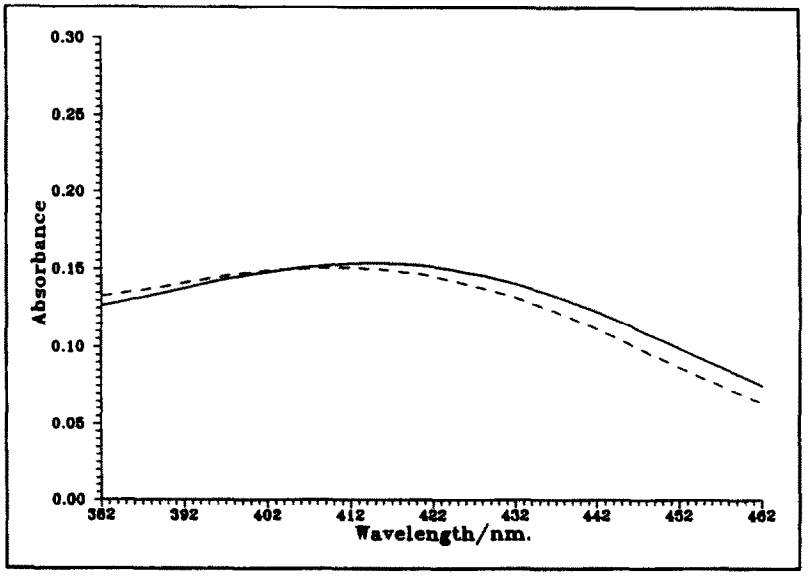


Fig. 7. Representation of the internal (– – –) and external (——) blanks for the calibration of d-l-amphetamine using NQS as reagent and $CHCl_3$ as extractant.

Table 3. Results obtained for the calibration lines in the determination of d-l-amphetamine in presence of NQS reagent. The symbols are as in Table 1

Wavelength measured	440	382	432	394	424	404
$a \pm s_a$	0.116 ± 0.003	0.133 ± 0.006	0.132 ± 0.004	0.143 ± 0.005	0.143 ± 0.004	0.150 ± 0.005
$(b \pm s_b) \times 10^3$	18.67 ± 0.11	5.99 ± 0.20	17.72 ± 0.12	8.69 ± 0.17	16.49 ± 0.13	11.26 ± 0.15
r	0.99997	0.99887	0.99995	0.99963	0.99994	0.99981
t_{calc}	176.594	29.761	144.414	51.623	125.176	73.160
$A_{external\ blank}$	0.1436	0.1436	0.1478	0.1479	0.1494	0.1493

Wavelength measured	450(reagent blank)	ΔA(440–382)	ΔA(432–394)	ΔA(424–404)
$a \pm s_a$	-0.0122 ± 0.0025	-0.016 ± 0.006	-0.011 ± 0.004	-0.0066 ± 0.0022
$(b \pm s_b) \times 10^3$	19.14 ± 0.08	12.68 ± 0.20	9.03 ± 0.13	5.23 ± 0.07
r	0.99998	0.99976	0.99981	0.99983
t_{calc}	240.709	64.528	71.717	76.509
n	4	4	4	4
Detection limit/ppm	0.89	3.24	2.93	2.75

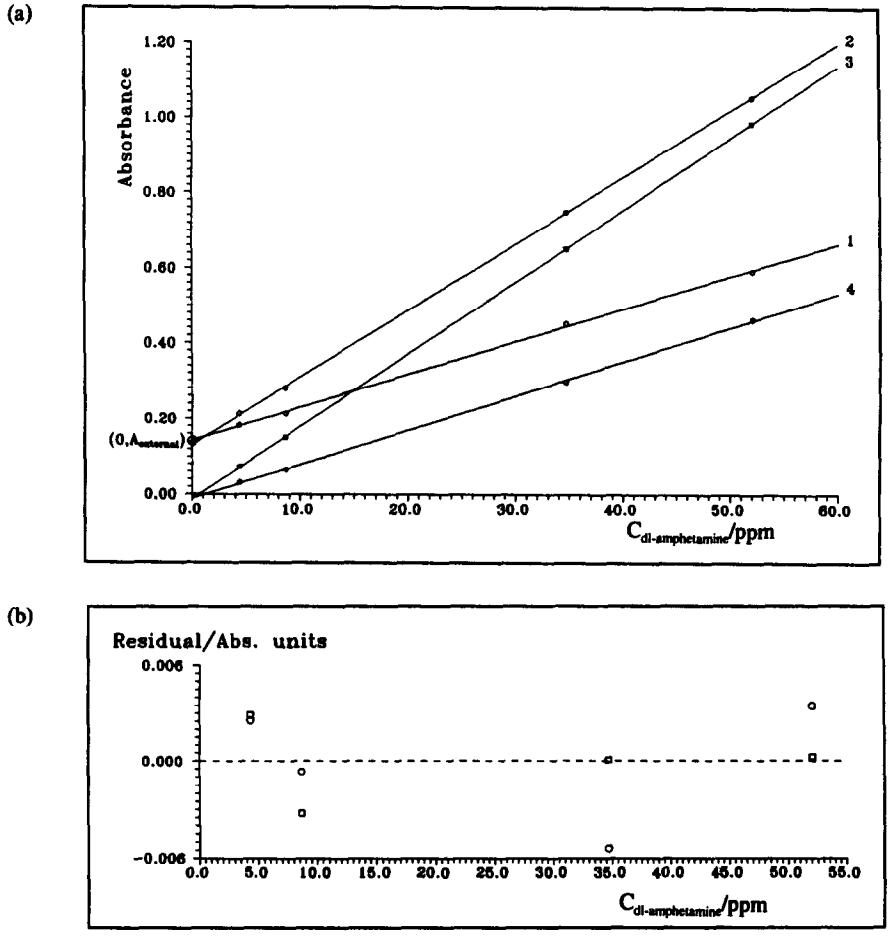


Fig. 8. (A) Calibration lines for the determination of d-l-amphetamine. (1) Using absorbance at 394 nm against solvent blank as analytical signal. (2) Using absorbance at 432 nm against solvent blank as analytical signal. (3) Using absorbance at 450 nm against reagent blank as analytical signal. (4) Using ΔA(432–394) as analytical signal. The equations are shown in Table 3. (B) Plot of residuals (absolute values) *vs* $C_{d\text{-}l\text{-}amphetamine}$; ○ using ΔA(432–394) as analytical signal; □ using absorbance at 450 nm against reagent blank as analytical signal.

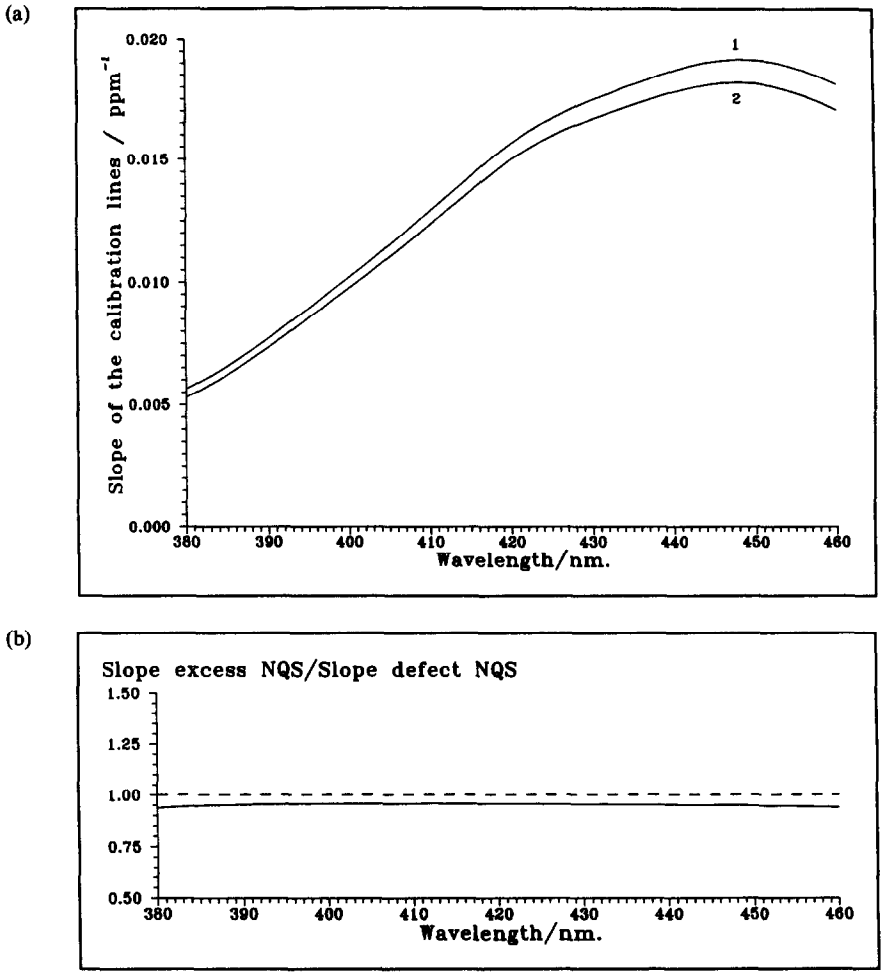


Fig. 9. Equivalence of the slopes of the calibration lines for the complex d-l-amphetamine-$(NQS)_2$ using defect (1) and excess (2) of reagent.

described for the standard solutions, but the reaction product were extracted with *n*-hexane/ethyl acetate (1:1).

RESULTS AND DISCUSSION

The situation when internal and external blank coincide completely is shown in Fig. 1(a). In this situation, the use of ΔA as analytical signal leads to better results in spectrophotometric determinations that the ones obtained by using the traditional method with absorbance values against reagent blank, as it was demonstrated in Ref. 5, due to HPSAM application allows us to annul the possible diminution of reagent concentration when analyte concentration enhances. Deoxiephedrine calibration lines, which were obtained as it has been described in the experimental section, follow this behaviour. In Fig. 2(a) is represented the spectra of the reagent blank alone and reagent blank plus 9.6 ppm of deoxiephedrine. As can be seen in Fig. 3, the internal and the external blanks are coincident, with values which are not more different than 5%. So that, by selecting two pairs of wavelength where external blanks show the same absorbance, we can obtain two straight lines which intersect at the so-called H-Point of coordinates (O, $A_{\text{external blank}}$) (Fig. 4(a)). The use of ΔA as analytical signal leads to straight lines with zero intercept, and where the blank contribution has been cancelled. In Table 1 are summarized the results obtained for three pairs of selected wavelength, where the absorbance of external blanks show the same value. In all of the cases, we can see that the results obtained are according with the mentioned theoretical basis.

By using ΔA as analytical signal, the linearity improves respected than that obtained by using $A - A_b^{ex} = f(C)$, where A_b^{ex} is the absorbance value measured for the external blank, as the

greater t_{calc} values show.[6] By using ΔA signals, calibration lines with zero intercepts are obtained, because at each point the reagent absorbance is cancelled, even when its contribution to the absorbance value differs from one solution to another, In this case, the absorbance increments depend only on the analyte concentration. The residual plots *vs* analyte concentration added (Fig. 4(b)) are also shown. As it can be seen, the proposed method describes better the experimental points, and lower detection limits are reached.[7]

Figure 1(b) shows the HPSAM plots when the intercepts for the calibration lines at λ_1 and λ_2 (internal blank) are coincident between them, but not with the value for the external blank. This result could be because the preparation of blank reagent was not correct or because it was made an irreproducibility addition of it to the analyte standards (for example when immobilized reagents are used). In this case it is important to take into account another factor, such as an inefficient extraction of the reagent in presence of analyte. In this kind of situation, the use of ΔA as analytical signal can cancel this systematic error, which should not be eliminated by applying the usual method using absorbance values against reagent blank added in the initial concentration.

Metamphetamine determination, whose spectra are shown in Fig. 2(b), is an example of this, as can be seen in Fig. 4. In this instance,

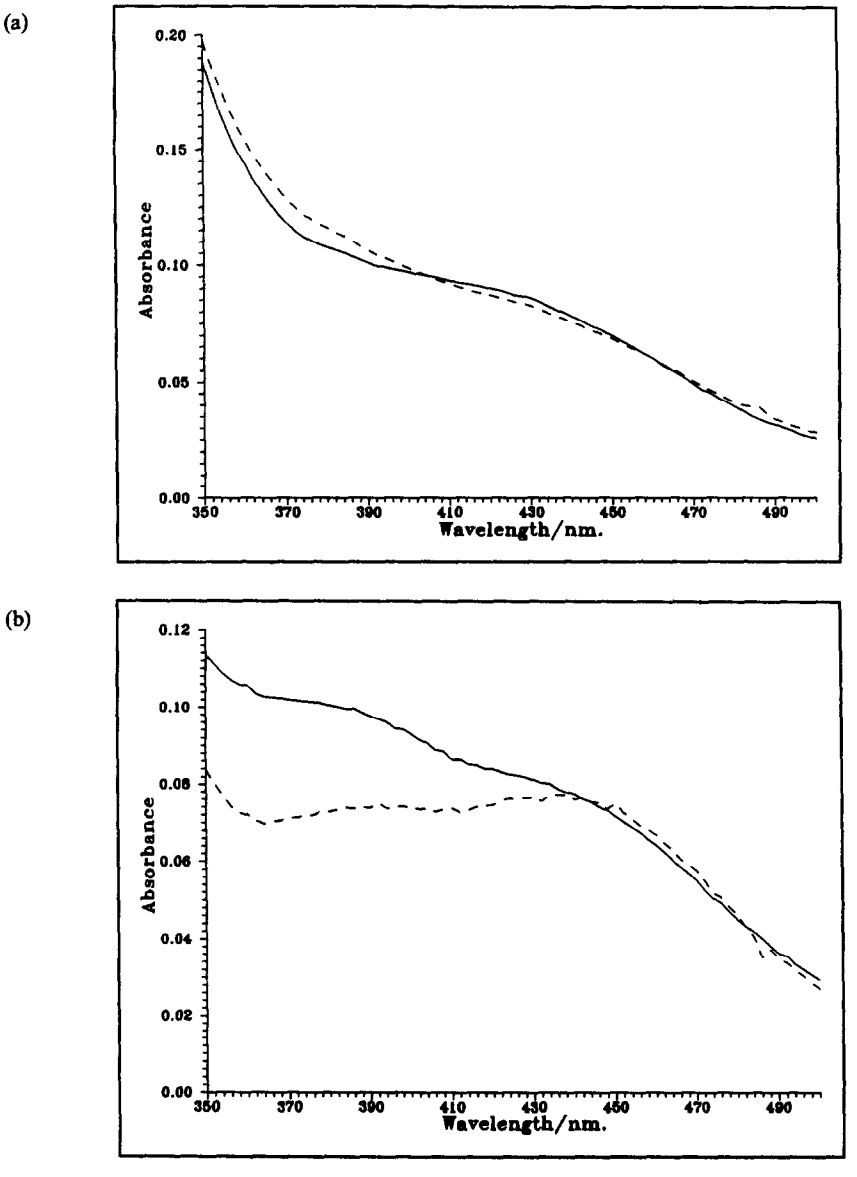


Fig. 10 (a and b)

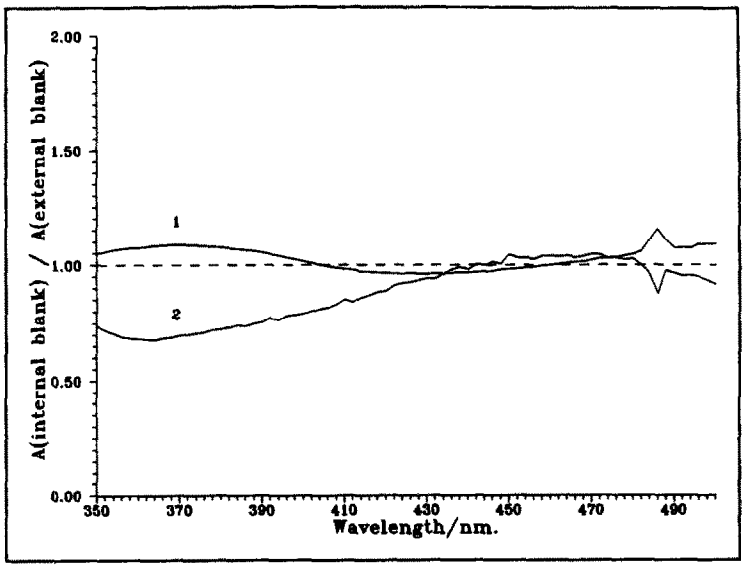


Fig. 10(c)

Fig. 10. (A) and (B) Representation of the internal (- - -) and external (——) blanks for the two calibrates of amphetamine in urine samples assayed. (C) Quotient internal blank/external blank for the two replicates assayed.

although the internal and external blanks have different absolute values, the ratio between them is constant in the wavelength interval studied, due to the spectral behaviour being the same for both blanks, but not their magnitude.

The results obtained for three pairs of wavelength where the external blank has the same absorbance are shown in Table 2. The absolute values obtained for external blank are not coincident with that obtained for internal blank, although they are constants at the same wavelengths. These results agree with that obtained in Fig. 5.

The graphical representation for one pair of wavelengths is shown in Fig. 6(a). The application of HPSAM allows us to cancel the blank bias error, although its analytical signal does not correspond with the one that should be obtained for the initial concentration. All of this leads to calibration lines with zero intercepts, as in the prior case. The intercept values obtained are near from zero than those obtained by the usual method. All of this demonstrates that if the spectral behaviour of the blank is constant, but not the added concentration in the different solutions, the use of the HPSAM is able to eliminate the blank bias error present, as the residual plots *vs* analyte concentration added (Fig. 5(b)) shows.

In Fig. 1(c) are shown situations where the spectra of internal and external blank reagent do not coincide, as it can be demonstrated by applying the HPSAM. In this case, different replicates of chloroformic solutions of NQS treated as the analyte solutions, show spectral differences in the wavelength interval down to 410–420 nm. The use of calibration lines obtained against organic solvent allow to obtain the minimum possible error when the measurements were made at the wavelength interval where there are not differences between internal and external blank. However, the HPSAM method is not possible to apply due to the absorbance of internal blank is not the same at the two pairs of selected wavelengths in the external blank.

Reagent blank alone and reagent blank plus 8.66 ppm of d-l-amphetamine spectra are showed in Fig. 2(c). In Fig. 7 is shown the spectra obtained for the external blank and the intercepts of the calibration plots for the determination of d-l-amphetamine as analyte. It can be observed that they are not coincident. In the Table 3 are shown the results for three pairs of wavelengths where the external blank shows the same absorbance, but not the internal blank, as it can be observed when the different intercepts of the calibration plots at different wavelengths are compared. In Fig. 8(a) is represented the application of the HPSAM at the wavelength pair of 432,394 nm. The obtained line using ΔA as analytical signal does not intercept at the zero value, because in this case $b'_0 \neq A'_0$. In addition, the t_{calc} statistic is lower than the one for the line

that uses absorbance values as analytical signal, because the HPSAM fundamentals are not fulfilled, and the absorbance increment is not only due of the analyte concentration. The plot of residuals *vs* concentration of analyte added (Fig. 8(b)) for this situation shows that the proposed method describes worse the experimental data. When this situation is present, the external blank does not allow us to choose the pairs of wavelengths to apply the HPSAM. The treatment of the data by the usual methodology, or by using the HPSAM, leads to measurements affected by error, if a procedure to eliminate it is not described. The usual procedure could be applied if it can be demonstrated that at least at one wavelength the external and the internal blank are coincident, and, in addition, the concentration of reagent can be considered constant for all the dynamic range of concentration of analyte. The last condition is not fulfilled if there is not present a great excess of reagent, or in the cases of extractive-colorimetric procedures, if the organic phase was not saturated with the reagent, independently of the present concentration of analyte. If all of these conditions are not fulfilled, the slope of the calibration plot is changed respected the real value. The calibration line is then distorted, and the results obtained when it is used leads to values affected by systematic error, as it was demonstrated in Ref. 5.

In the studied determination, it can be demonstrated that the NQS concentration extracted is constant and independent of the amine concentration present. This is true because the slope of the calibration lines are the same in excess and in defect of NQS. In this late case, the reagent directs the reaction. In Fig. 8 has been plotted the slope values for the calibration plots for the complex amphetamine–$(NQS)_2$, in excess and in defect of NQS. Values are equals for more than 95% (Fig. 8(b)). It shows that the free reagent concentration are the same in all of the cases, because its saturation in the organic phase makes its concentration constant in all of the solutions prepared for application of the method.

From Fig. 7, and taking into account the fundamentals now exposed, it can be deduced that in the 430–460 nm interval, the external and the internal blanks show a similar behaviour. It indicates choosing a wavelength in this interval where the error in the determination of an analyte is minimum.

Another similar case is observed when the blanks corresponding to amphetamine calibrations lines in urine samples are studied. In this case, the sample matrix produces different blank measurements for different urine samples, as it can be observed in Figs 10(a) and (b). By the same considerations before shown, for the correct determination of the analyte we must find a measurement wavelength interval where the spectral behaviour of the external and internal blanks are the most similar possible. In Fig. 10(c) is shown the quotient external/internal blank for the two replicates studied. In the wavelength zone of 440–480 nm, are the bigger coincidences between them, so this zone must be chosen for the analyte determination. This zone coincides with the experimental cited in the bibliography for the amphetamine determination in urine samples when NQS is used as derivatizing reagent.[8]

CONCLUSIONS

In experimental procedures where a reagent blank is used, its analytical signal can be different in presence of analyte in the different solutions prepared for the calibration plots from the signal presented when it is alone in the usually named "reagent blank". For these cases it is necessary to define two kinds of blank: the external blank (in reagent alone solutions) and the internal blank (in reagent plus analyte solutions). The comparison between both spectral behaviours gives us evidence and characterizes the error, if any exists, that the presence of the reagent blank introduces in the measuring procedure.

The application of the HPSAM, or the use of the maximum coincidence zone between external and internal blank, when HPSAM is not applicable by the spectral behaviours of the external and the internal blank, obtains the best results for the analyte quantifying, because it allows elimination of the systematic error due to the use of absorbance values against the reagent blank in the initial added concentration, and the processing through the analytical method.

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