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Generalised *H*-point standard addition method for the isolation of the analyte signal from the sample signal when coelution of unknown compounds occurs in liquid chromatography

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Abstract

The generalised *H*-point standard addition method (GHPSAM) is proposed for isolating the analytical signal of an analyte from the signal of an unknown sample. Samples containing two and three coeluting compounds have been analysed. The accuracy of the predictions depends on the shape of the analyte and interferent spectra but not on the degree of chromatographic overlap. This methodology involves the location of linear intervals for the unknown interference spectrum from the spectrum of the sample. Once the linear interval has been found the selection of three wavelengths within the interval will allow the cancellation of the signal of the unknown interferent. The method has been applied to the determination of diuretics, amphetamines and phenols in water.

Keywords: Generalised *H*-point standard addition method; Detection, LC; Diuretics; Phenols; Amphetamines

1. Introduction

The physical separation of the components of a mixture provided by chromatographic techniques allows the analyst the simultaneous determination of several analytes in complex matrices. Use of high-efficiency columns has become commonplace due to their commercial availablity. In spite of the use of these columns when analysing real mixtures of great complexity, some unresolved peaks may appear. Several methods have been developed for the detection of overlapped peaks [1–4] since the occurrence of overlapped peaks may be much more prevalent than expected [5]. When overlapping peaks are detected the mobile phase and the column characteristics must be adjusted to improve resolution. However, when a large number of compounds are present in the sample, it may be impossible to resolve all the peaks under the same chromatographic conditions. On the other hand, the optimising process is a tedious task that increases the analysis time.

In the literature there are a number of reports where numerical methods are used to resolve overlapping peaks. Among these methods are self-modelling curve resolution (SMCR), generalised rank

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annihilation method (GRAM) and deconvolution. These methods try to calculate the individual chromatograph of each compound from the total one.

SMCR [6] has been applied to two of the major combinations of spectrometry and chromatography: gas chromatography with mass spectrometry (GS–MS) [7] and liquid chromatography with diode array UV–Vis detection (LC–DAD) [8]. Several self-modelling approaches [9] have been developed since the pioneering work [6]. Depending on the approach selected, the resolution of two [10], three [11], four [12] or an unlimited number [13] of unresolved components in the peak, is possible. It has to be stated that the performance of some of these methods depends on the presence of pure variables (variables of which intensities are due to only one component in the mixture).

GRAM [14] is a calibration and curve resolution method for multicomponent bilinear data arrays. Bilinear data arrays are defined as two-dimensional data arrays where the contribution from each chemical component to the data can be expressed as the outer product of two vectors [15]. This method allows the quantitative analysis of an analyte in the presence of several unknown interfering components. The quality of the results obtained are a function of the noise level, of the number of overlapped components, of the similarity of the concentration ratios, of the similarity of spectra and of the degree of chromatographic overlap.

At present, methods for deconvolving overlapped peaks can be categorised into three kinds: geometrical, algebraic and pattern recognition methods. The peaks are represented by mathematical models with some undetermined parameters. The process consists on the optimisation of these undetermined parameters to approximate the chromatogram curve. Several mathematical models have been developed trying to fit the experimental peaks [16]. This situation is due to the fact that although the most simple chromatographic model predicts Gaussian elution profiles, in practice the shapes of the chromatographic peak change considerably. Each available peak-shaped function can fit a limited number of real peaks. An attempt to develop flexible models can be found in Refs. [16,17].

Partial least-squares (PLS) and principal components regression (PCR) have been also proposed [18,19] for the determination of the overlapping species. Only the spectra at the top of the peaks are required. It is not necessary to know the spectra of all the compounds in the sample, but their concentrations must change from sample to sample to allow the method to model the changes in the analytical signal.

The *H*-point standard addition method (HPSAM) has been described for the resolution of two coeluting species in chromatography when both compounds are known [20], or they can be identified. This paper deals with the problem of solving the analyte in presence of unknown interferences in chromatography. For this purpose we will employ the generalised HPSAM (GHPSAM) [21,22], developed by our investigation group for the spectrophotometric field. The method isolates the signal of the analyte from the sample signal avoiding the signal of the unknown global interference.

2. GHPSAM fundamentals. Theoretical background

Let us suppose a mixture consisting of an analyte (X) and an unknown interference (Y). The method is based on the selection of three wavelengths (λ_1, λ_2 and λ_3) within an interval of wavelengths which linearly relate the absorbance of the interferent with the wavelength.

From Fig. 1 it can be seen that the following equation must be satisfied if the interferent presents a linear behaviour

$$\frac{A_{Y2} - A_{Y1}}{\lambda_2 - \lambda_1} - \frac{A_{Y3} - A_{Y2}}{\lambda_3 - \lambda_2} \tag{1}$$

Two parameters, p and q, can be defined as:

$$p = \frac{\lambda_2 - \lambda_1}{\lambda_3 - \lambda_1};\ q = \frac{\lambda_3 - \lambda_2}{\lambda_3 - \lambda_1} \tag{2}$$

Eq. (1) can be rewritten into:

$$q(A_{Y2} - A_{Y1}) = p(A_{Y3} - A_{Y2}) \tag{3}$$

this means that if three wavelengths where the interferent absorbance can be considered as linear can be found, the interferent contribution to the

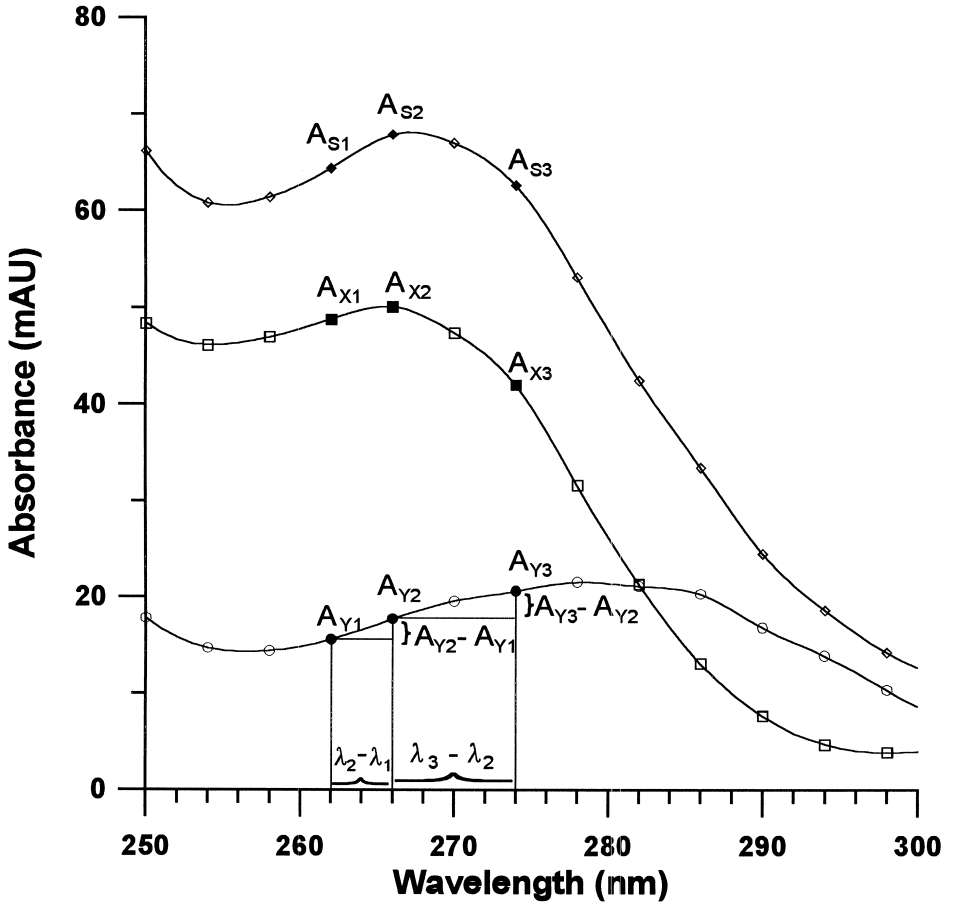

Fig. 1. Spectral features for the X analyte (bumetanide), the Y interferent (ethacrynic acid) and the S sample in the wavelength interval selected as linear for the interferent.

signal of the sample can be cancelled if the analytical signal used is, $q\Delta A_{S(2,1)} - p\Delta A_{S(3,2)}$ (where $\Delta A_{S(2,1)}$ and $\Delta A_{S(3,2)}$ are the measured absorbance increments of the sample at these lambdas), for estimating the analyte concentration.

The representation of $q\Delta A_{S(2,1)}$ and $p\Delta A_{S(3,2)}$ vs. the added concentration of analyte gives a plot with two straight lines which intersect in a point, called a *H*-point (Fig. 2). The coordinates of the *H*-point are $(-C_H, \Delta A_H)$, with C_H the concentration of the analyte in the sample and $\Delta A_H = q(A_{Y2} - A_{Y1}) = p(A_{Y3} - A_{Y2})$. The concentration of the unbiased analyte concentration in the sample ($C_X^0 = C_H$) is given by:

$$-C_H = \frac{q\Delta A_{S(2,1)} - p\Delta A_{S(3,2)}}{p(\epsilon_{X3} - \epsilon_{X2}) - q(\epsilon_{X2} - \epsilon_{X1})} = \frac{A_{X2}^0 - qA_{X1}^0 - pA_{X3}^0}{q\epsilon_{X1} + p\epsilon_{X3} - \epsilon_{X2}} \quad (4)$$

where A_{X1}^0, A_{X2}^0 and A_{X3}^0 are the absorbances of the analyte in the sample at the wavelengths 1, 2 and 3, respectively. ϵ_{X1}, ϵ_{X2} and ϵ_{X3} are the molar absorption coefficients of the analyte at the wavelengths 1, 2 and 3, respectively [20]. The abscissa of the *H*-point includes the $q\Delta A_{S(2,1)} - p\Delta A_{S(3,2)}$ increment and then cancels the interferent contribution to the signal. This increment value is only related with

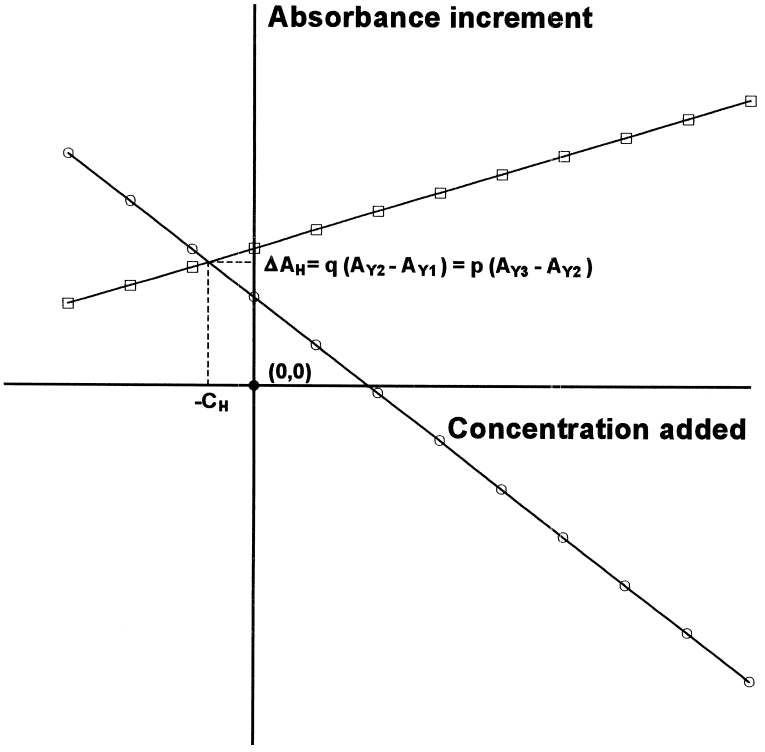

Fig. 2. Graphic representation of the lines $q\Delta A_{S(2,1)}$ (□) and $p\Delta A_{S(3,2)}$ (○) for the different amounts of added analyte.

analyte parameters as is demonstrated in the following.

Let us multiply p and q parameters by the absorbance increments of the sample $\Delta A_{S(2,1)}$ and $\Delta A_{S(3,2)}$:

$$q\Delta A_{S(2,1)} = q(A_{X2} - A_{X1}) + q(A_{Y2} - A_{Y1}) \quad (5)$$

$$p\Delta A_{S(3,2)} = p(A_{X3} - A_{X2}) + p(A_{Y3} - A_{Y2}) \quad (6)$$

If the method of standard additions (MOSA) is followed as a calibration model, Eqs. (5) and (6) can be expressed as:

$$q\Delta A_{S(2,1)} = q(A^0_{X2} - A^0_{X1}) + q(A_{Y2} - A_{Y1}) + q(\epsilon_{X2} - \epsilon_{X1})C^i_X \quad (7)$$

$$p\Delta A_{S(3,2)} = p(A^0_{X3} - A^0_{X2}) + p(A_{Y3} - A_{Y2}) + q(\epsilon_{X3} - \epsilon_{X2})C^i_X \quad (8)$$

where C^i_X denotes the different solutions from 0 to n additions of known amounts of the analyte (X) added to the sample.

At the intersection point (H-point) in the representation of $q\Delta A_{S(2,1)}$ and $p\Delta A_{S(3,2)}$ vs. added concentration of analyte (see Fig. 2), $q\Delta A_{S(2,1)}$ equals $p\Delta A_{S(3,2)}$, so Eqs. (7) and (8) can be rearranged yielding Eq. (4).

Similar equations can be deduced in an analogous way if the area under the chromatographic peak is preferred over the peak height. Although the method was first developed for the MOSA calibration model, if it is known that no matrix effect is affecting the measurement, no standard addition is required since the molar absorption coefficients of the pure analyte can be used.

2.1. Location of linear intervals for the interferent

Two methods for the location of linear intervals for the spectrum of unknown interferences have been proposed.

The first one [20] makes use of second derivatives of the spectra of the sample and the second derivative of the molar absorption coefficient of the analyte, obtained from the standard addition carried out in the sample or from the analyte spectrum when the standard addition is not carried out.

If the interferent (Y) has a linear behaviour in an interval of wavelengths $[\lambda_1, \lambda_n]$ it will follow that:

$$A_Y = a + b\lambda \tag{9}$$

the first and second derivative will be, respectively:

$$A'_Y = b \tag{10}$$

$$A''_Y = 0 \tag{11}$$

where b is null if the spectral behaviour can be considered constant in the selected range.

For the sample, S, the second derivative in this interval of wavelengths will yield:

$$A''_S = C^0_X \epsilon''_X \tag{12}$$

that can be easily rewritten as:

$$C^0_X = \frac{A''_S}{\epsilon''_X} \tag{13}$$

The location of the linear interval is based on the calculation of the quotient A''_S/ϵ''_X. The interval where this value could be considered constant is selected. The possible divisions by zero, ϵ'' becomes zero for the inflexion points of the analyte spectrum, must be considered in order to eliminate them because of the quotient value goes to infinity. The C^0_X value obtained from the A''_S/ϵ''_X plot must be improved as is demonstrated in Ref. [20]. A reason for that can be that the S/R ratio is not advantageous for second derivative methods.

The second approach [21], recently developed, to locate the linear interval is based on the first and second derivatives of the spectra of the samples (A'_S and A''_S) and a reference analyte solution ($A^{R'}_X$ and $A^{R''}_X$), of concentration C^R_X.

Considering that the interferent (Y) presents a linear behaviour in an interval of wavelengths $[\lambda_1, \lambda_n]$ it can be written:

$$A'_S - A^{R'}_X = \frac{C^0_X - C^R_X}{C^R_X} A^{R'}_X + b \tag{14}$$

$$A''_S - A^{R''}_X = \frac{C^0_X - C^R_X}{C^R_X} A^{R''}_X \tag{15}$$

Therefore, by plotting the values of the difference between the first derivative of the sample and the first derivative of the reference solution, $(A'_S - A^{R'}_X)$, vs. the first derivative of the reference solution $(A^{R'}_X)$, at each wavelength, a straight line with intercept b and slope $(C^0_X - C^R_X)/C^R_X$ must be obtained for the interval where the spectral behaviour of the unknown interferent can be considered linear. The representation of $(A''_S - A^{R''}_X)$ vs. $A^{R''}_X$ is used to confirm whether the interferent presents a linear behaviour, since in such a case a straight line with zero intercept and $(C^0_X - C^R_X)/C^R_X$ slope must be obtained.

The estimated spectral linearity hypothesis for the interference is corroborated when the GHPSAM method is processed. In this case, the analyte concentration is estimated more accurately [20,21].

3. Experimental

3.1. Apparatus

A Hewlett-Packard Model 1040A liquid chromatograph, equipped with a diode array detector linked to a data system (Hewlett-Packard Chemstation) was used for data acquisition and storage. The system was coupled to a quaternary pump (Hewlett-Packard, 1050 Series) with a 25-μl sample loop injector for diuretics and amphetamines and a 100-μl sample loop injector for phenols.

The detector (Hewlett-Packard, 1100 series) was set to collect a spectrum every 640 ms (over the range 200–400 nm and every 4 nm). All the assays were done at ambient temperature.

For the analysis of diuretics (mixtures of acetazolamide–amiloride and ethacrynic acid–bumetanide) the range of measured wavelengths was set to 222–302 nm since the analysis of these drugs in biological fluids are, generally, carried out in this zone. The mixtures of phenols and amphetamines were measured in the ranges 222–398 and 272–600 nm, respectively. All the spectra were obtained at the maximum of the peaks of the chromatograms.

3.2. Reagents

All reagents were of analytical grade. Methanol (Scharlau, Barcelona, Spain) and acetonitrile (J.T. Baker, Deventer, The Netherlands) were of HPLC grade.

The following reagents were used: amiloride hydrochloride (ICI-Pharma, Pontevedra, Spain), acetazolamide (Cynamid Ibérica, Spain), amphetamine sulphate (Sigma, St. Louis, MO, USA), methamphetamine hydrochloride (Sigma), β-phenylethylamine hydrochloride (Sigma), 1,2-naphtoquinone-4-sulphonic acid sodium salt (Sigma), bumetanide (Boehringer Ingelheim), ethacrynic acid (Sigma), phenol, *o*-cresol and *m*-cresol (Merck, Darmstadt, Germany) and 2-chlorophenol (Aldrich, Steinheim, Germany).

Sodium hydroxide and sodium chloride (Panreac, Barcelona, Spain), phosphoric acid (Probus, Badalona, Spain), sodium dihydrogenphosphate monohydrate (Merck), amonium hydroxide (Probus) hydrochloric acid (Probus), acetate trihydrate (Probus), acetic acid (Probus) sodium hydrogencarbonate (Probus) and propylamine hydrochloride (Fluka, Buchs, Switzerland) were also used. Water was distilled, deionized and filtered in 0.45-μm nylon membranes (Teknokroma, Barcelona, Spain).

3.3. Columns and mobile phases

The column for the determination of diuretics was a HP-Hypersyl ODS-C_{18}, 5 μm, 250 mm×4 mm I.D. A phosphate or acetate buffer (pH 3 and pH 4, respectively)–acetonitrile gradient with an increasing acetonitrile content from 15% at the start to 80% at 8 min was used. The flow-rate was set at 1 ml/min.

A LiChrospher Si-60, 5 μm, 125 mm×4 mm I.D. (Merck) column was used for separation of amphetamines. The mobile phase was ethanol–chloroform–ethyl acetate–*n*-hexane (1:22:32:45, v/v). The flow-rate was set at 2 ml/min.

A LiChrospher 100 RP_{18}, 5 μm, 125 mm×4 mm I.D. (Merck) column was used for separation of phenols. A 0.1 *M* phosphate buffer (pH 7)–acetonitrile mixture was used as mobile-phase, at a flow-rate of 0.75 ml/min. The acetonitrile content was increased from 20% at zero time to 30% at 10 min, and to 50% at 15 min. After 15 min, the acetonitrile content was kept constant.

All solvents were filtered with nylon membranes, 0.45 μm, (Teknokroma) and degassed with helium before use.

3.4. Preparation of solutions

Stock standard solutions of diuretics (100 μg/ml) were prepared in methanol. Working solutions were prepared by dilution of the stock solutions with distilled deionized water.

Phosphate buffer (0.05 *M*) for bumetanide–ethacrynic acid mixtures was prepared daily by dissolving sodium dihydrogenphosphate monohydrate in water, the solution being 0.03 *M* in propylamine hydrochloride. The pH was adjusted to 3 by adding the minimum amount of concentrated phosphoric acid. Acetate buffer for amiloride–acetazolamide mixtures was prepared from sodium acetate trihydrate and acetic acid in the same way and at the same concentrations as the phosphate buffer. The pH was adjusted to 4.

The standard solutions of amines were prepared by dissolving 100 mg of the pure compounds in 100 ml of water. Working solutions were prepared by dilution of the stock solutions with distilled deionized water. The 1,2-naphtoquinone-4-sulphonic acid (NQS) stock solution (0.5%, w/v) was prepared freshly for each experiment. The bicarbonate solution was prepared by dissolving 8 g of sodium hydrogencarbonate in 100 ml of distilled water. All solutions were stored in the dark at 2°C.

Stock standard solutions of phenols (1000 μg/ml) were prepared in water. Working solutions of the phenols were prepared by dilution of the stock solutions with a NaCl solution (at a concentration of 35 g/l) acidified to pH 3 with phosphoric acid.

The 0.1 *M* phosphate buffer was prepared daily by dissolving sodium dihydrogenphosphate monohydrate in water. Next, the pH was adjusted to 7 with 10% (w/v) NaOH. All solutions were stored in the dark at 2°C.

3.5. Derivatization of amphetamines

Derivatization with NQS was performed as follows. Different volumes of the stock solution of the

amines were added to 0.5 ml of bicarbonate solution (8%), 0.5 ml of NQS and distilled water up to 1.5 ml. The mixture was heated at 70°C for 20 min. After cooling, the mixture was shaken with the same volume of chloroform for 2 min and was then centrifuged for 5 min at 1500 *g*. The aqueous phase was discarded, and sulphate anhydrous sodium was added to the organic solution to remove the water. The chloroform layer was filtered through 0.45-μm (13 mm diameter) Nylon filters from Teknokroma.

4. Results and discussion

4.1. Selection of linear intervals

As explained in the theoretical section the GHPSAM is based on the selection of three wavelengths which linearly relate the signal of the unknown interferent (absorbance or area) with the wavelength. Two methods have been proposed to carry out the selection. The first one [20] is preferred due to the simplicity of the calculations. The horizontal intervals for the A''_S/ϵ''_X plots are well described when the analytical signal for the analyte is higher than the interference one for the sample assayed. If the analyte signal is equal or lower than that of the interferent, the second methodology [21] to select the linear wavelength intervals leads to more accurate results. For more details see Refs. [20,21].

For the determination of acetazolamide, the quotient A''_S/ϵ'' (with A''_S the second derivative of the samples spectra and ϵ'' the second derivative of the molar absorption coefficients of the acetazolamide, obtained from a spiked sample with known amounts of the analyte) was plotted vs. the wavelength. As can be seen in Fig. 3 the value of A''_S/ϵ'' can be considered constant in the range 258–274 nm, so the behaviour of the unknown interferent (amiloride) is expected to be linear in this interval. The selection of three wavelengths within this interval will yield the analyte concentration free of bias error. In Fig. 3 it can be also seen that the values of the quotient A''_S/ϵ'' give an approximate idea of the concentration of the analyte in the samples.

Another set of mixtures was prepared for the analysis of amiloride in the presence of acetazolamide, as unknown interference. The linear interval for the interferent was also found by means of the quotient A''_S/ϵ'', using for these mixtures the molar absorption coefficient of amiloride, calculated from a sample spiked with known amounts of it. A wavelength range of 282–298 nm was found as a linear interval for the interferent.

A sample containing amphetamine, methamphetamine and β-phenylethylamine was also analysed. The peaks corresponding to amphetamine and methamphetamine were partially overlapped. Both analytes were treated to be solved in the mixture, considering amphetamine or methamphetaine as analyte. The A''_S/ϵ'' plots were used to locate the linear interval of the unknown interferent in each determination. The ϵ'' values for the amphetamine (ϵ''_A) were calculated from the spectrum at the top of the peak after the injection of a pure standard of amphetamine of known concentration. The same procedure was followed to calculate the ϵ'' values (ϵ''_M) for the methamphetamine.

A wavelength range of 272–324 nm was found as a linear interval for the determination of amphetamine. For the determination of methamphetamine in the same sample, the range 272–304 nm can be considered as linear. In view of the plot (Fig. 4) A''_S/ϵ''_M vs. wavelength it is better to divide it into two sections. The quotient value at 292 nm does not follow the trend of the former points, so the interval 272–304 nm was divided into 272–292 and 292–304 nm.

When analysing a mixture of phenols, three partially overlapped peaks were obtained (Fig. 5). The coeluting compounds were *o*-cresol, *m*-cresol and 2-chlorophenol. Every compound was treated to be calculated in the presence of the other two. This means that for the determination of *o*-cresol (i.e.) it must be found an interval of wavelengths where the *m*-cresol and 2-chlorophenol, being the global unknown interference, present a linear behaviour. Here is not an easy task due to the similarity among the spectra. When following the previous method (plot of A''_S/ϵ'' values) for the location of linear intervals no-one could be found for any determination. In view of the high degree of spectral overlap and the difficulty of finding linear intervals the spectra of the analyte and the sample was calculated at every nm (it was measured every 4 nm) using a cubic spline

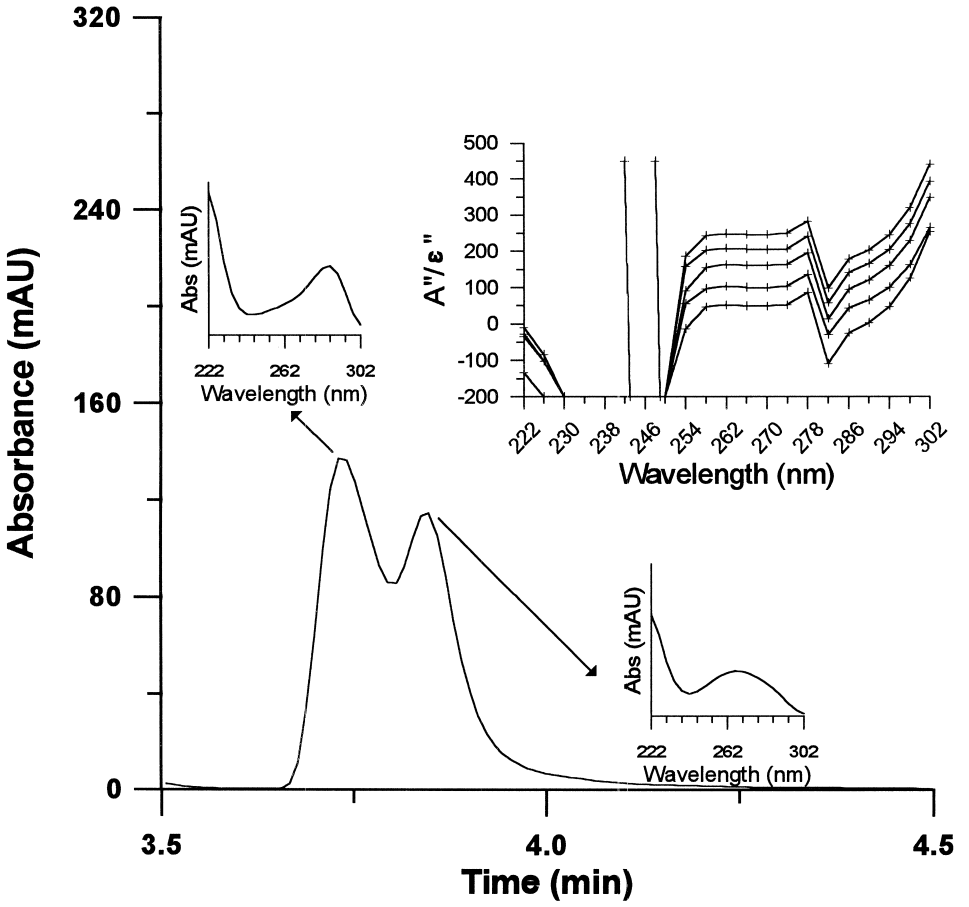

Fig. 3. Chromatogram at 275 nm of a mixture of 150 ppm of amiloride and 50 ppm acetazolamide, respectively. Insets: Spectra obtained at the top of the peaks and location of the linear interval for the interferent in the determination of acetazolamide (the samples contained 50, 100, 150, 200 and 250 ppm of acetazolamide and 100 ppm of amiloride).

interpolation formula to dispose of a great number of variables.

The second method proposed for the location of linear intervals in the unknown interferent spectrum is based on the use of first- and second-order derivatives of the sample and analyte spectra. A linear regression between $(A'_S - A'_X)$ vs. A'_X and $(A''_S - A''_X)$ vs. A''_X can be established in the range where the interferent presents a linear behaviour. The values of the second derivatives are used to decide whether an interval is linear or not (the same slope must be obtained for both regressions).

No linear intervals were found for the determination of *o*-cresol and *p*-cresol. The analysis of the graph obtained when plotting $(A'_S - A'_X)$ vs. A'_X, for the determination of 2-chlorophenol, gives as possible linear intervals: 244–255, 254–264 and 266–284 nm. The comparison of the regression values for the first and second derivatives suggested that the real one was 266–271 nm. The equations of the regressions $(A'_S - A'_X)$ vs. A'_X, and $(A''_S - A''_X)$ vs. A''_X for this interval were: $(A'_S - A'_X) = 0.012 + 0.4554A'_X$, $r^2 = 0.9996$ and $(A''_S - A''_X) = -0.0008 + 0.46A''_X$, $r^2 = 0.992$. It can be seen that no significant differences between both slopes exists and that the intercept value for the second derivative regression is nearly zero.

Bumetanide was determined in samples that also contained ethacrynic acid as unknown interference. Fig. 6 shows the plot of A''_S/ϵ'' (ϵ'' calculated from a

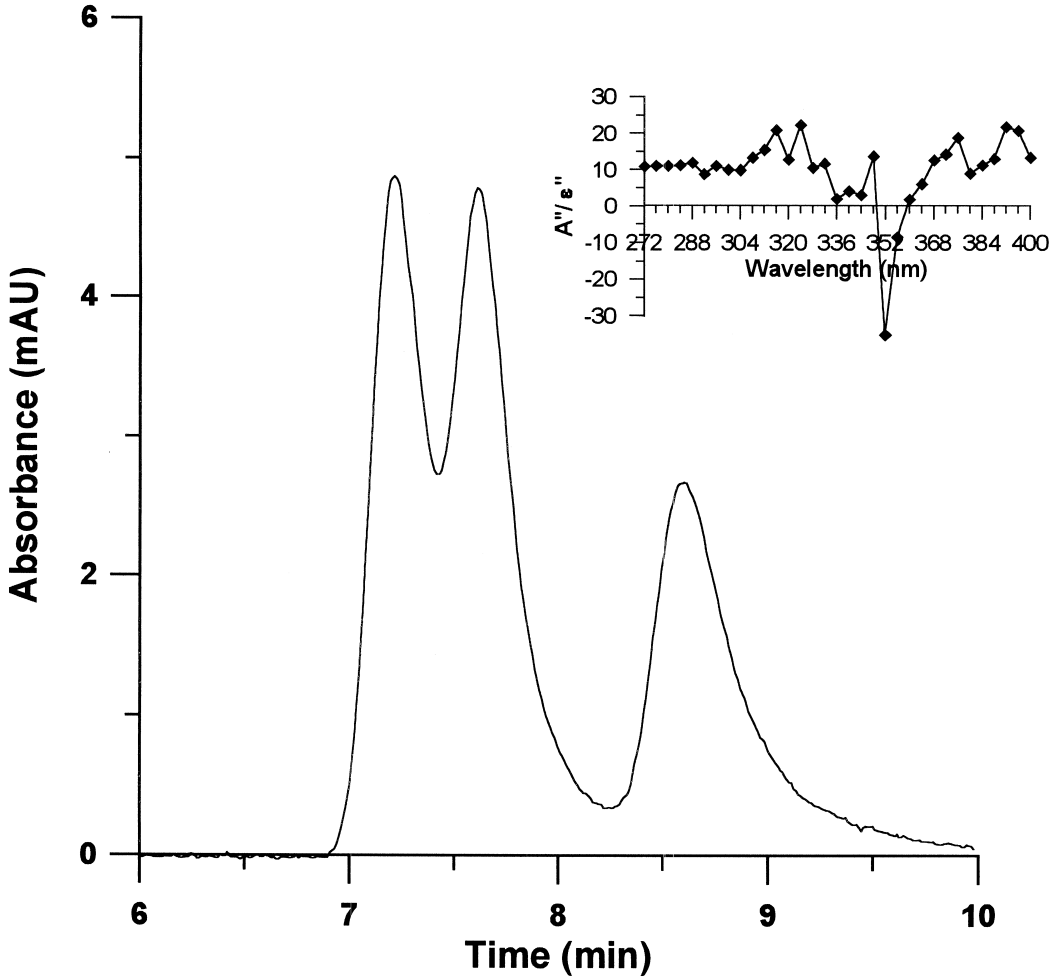

Fig. 4. Chromatogram at 275 nm of a mixture of 10 ppm of amphetamine, 10 ppm of methamphetamine and 10 ppm of β-phenylethylamine (in order of elution). Inset: location of the linear interval for the interferent in the determination of methamphetamine.

sample spiked with known amounts of bumetanide). The existence of four linear intervals in the spectrum of the interferent can be seen. They were: 222–234, 242–258, 262–278 and 278–302 nm.

4.2. Determination of the concentration of the analytes

The GHPSAM corroborates and optimises the chosen wavelength intervals and permits to obtain better results for the analyte concentration than those obtained using the whole selected interval.

4.2.1. Partially overlapped peaks

The injection of the mixtures acetazolamide–amiloride (Fig. 3), the mixture of amphetamines (Fig. 4) and the mixture of phenols (Fig. 5) led to the obtaining of partially overlapped peaks.

For the analysis of acetazolamide and amiloride one of the samples to be determined was spiked with known amounts of the analyte. This spiked set was used to predict the concentration of the sample. Once it was known, the calibration curve calculated for this set was used to predict the concentration of the other samples, that were measured without any addition of analyte.

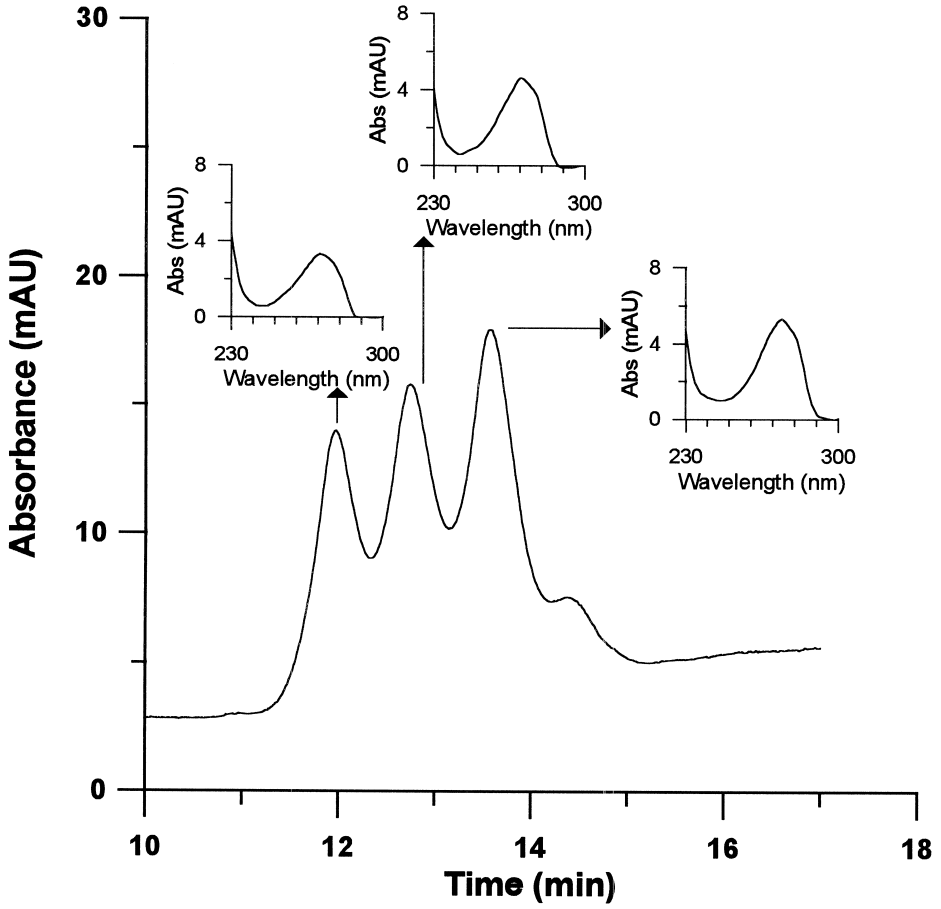

Fig. 5. Chromatogram at 220 nm of a mixture of *m*-cresol, *o*-cresol and 2-chlorophenol (in order of elution) at the concentrations: 3.6, 4.8 and 6 ppm, respectively. Inset the recorded spectra at the top of each peak.

For the prediction of amiloride and acetazolamide in the samples three wavelengths were selected within the previously calculated linear intervals. All the positions for the three wavelengths were assayed (10 possible combinations for both determinations). The absorbance increments ($A_{S2}-qA_{S1}-pA_{S3}$) that presented a slope ≥ 0.1 and a correlation coefficient, $r \geq 0.99$ when regressed versus the added concentration of analyte were selected. The mean prediction of the concentration of acetazolamide and amiloride in the spiked samples was 53 ± 2 ppm ($n=7$) and 49.8 ± 0.6 ppm ($n=7$), the real content in both cases being 50 ppm. These samples also contained 50 ppm of amiloride and 100 ppm of acetazolamide, respectively. The mean of the predictions for the remainder samples can be found in the Table 1.

Amphetamine was successfully predicted in the mixture that also contained methamphetamine. All the absorbance increments (three wavelengths chosen from 14 possible positions, yields 364 combinations) were assayed. From all these increments those with a regression coefficient ≥ 0.99 were selected. In this case as no standard addition was made the slope parameter of the regression was not available for the selection of analytical signals. Twenty absorbance increments were selected, they were those that provided the highest signals for an amphetamine standard. The concentration of amphetamine in the sample was calculated by comparison of the analytical signals of the sample with the analytical signal of the standard. The mean prediction and standard deviation for these 20 increments was

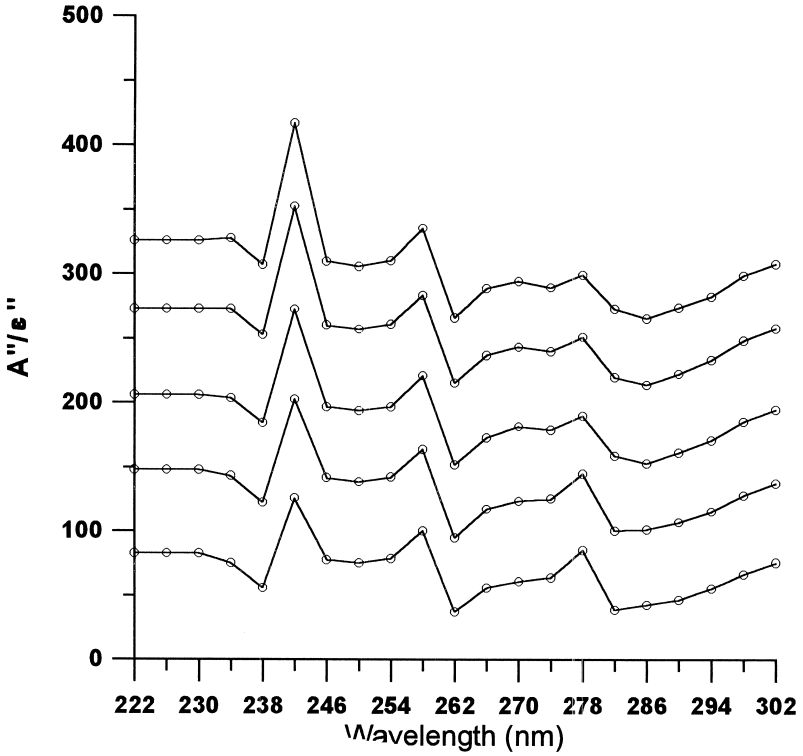

Fig. 6. Location of the linear interval for the interferent in the determination of bumetanide in presence of ethacrynic acid. The samples contained 50, 100, 150, 200 and 250 ppm of bumetanide and 100 ppm of ethacrynic acid.

10.2±0.4 ppm ($n=20$), being the real content of the sample 10 ppm of amphetamine and 10 ppm of methamphetamine.

Two linear intervals can be considered for the determination of methamphetamine, the first one at 272–292 nm and the second one 292–304 nm. The A''_S/ϵ''_M values, where they can be considered constant, (see Fig. 4) suggest that the concentration of methamphetamine in the sample is around 10 ppm. The first linear interval is not a useful one since the results provided by the absorbance increments in this range are not consistent. The interval 292–304 gives four absorbance increments predicting 10 ppm of methamphetamine in the mixture (the same mixture

Table 1
Calculated values for the analyte concentration (mean±standard deviation) in the mixtures of acetazolamide and amiloride[a]

True acetazolamide (ppm)	True amiloride (ppm)	Measured acetazolamide (mean±SD) (ppm)	Error	Measured amiloride (mean±SD) (ppm)	Error
50	100	46.5±0.2	−7.0	–	–
100	100	97±1	−3.0	–	–
150	100	152±1	1.3	–	–
200	100	204±1	2.0	–	–
50	100	–	–	95.4±0.4	−4.6
50	150	–	–	145.2±0.5	−3.2
50	200	–	–	198.9±0.2	0.6

[a] Every result is the mean of seven predictions. Error=(mean measured value−true value)×100/true value.

than analysed previously). In order to assure the result, the absorbances of the sample and a methamphetamine standard spectra at every nanometer within the interval were calculated. For doing this, a cubic spline interpolation formula was used. With these new number of variables 286 combinations are possible. The best 20 were selected (with the same criterion than for amphetamine) giving a mean prediction of 9.9 ± 0.6 ppm ($n = 20$).

As it was said previously, when analysing the mixture of phenols only a linear interval for the determination of 2-chlorophenol could be found. In this case it was measured one mixture of the three compounds and one standard of every one. The concentration of 2-chlorophenol in the sample was obtained by comparison of the signal (at the top of the third peak) with the signal of the standard of known concentration. The concentration of 2-chlorophenol found in the mixture was 6.1 ± 0.7 ppm ($n = 4$), with the real one being 6 ppm (*o*-cresol and *m*-cresol were 4.8 and 3.6 ppm, respectively). The absorbance increments values were very low, since all the spectra (analyte and interferents) were so similar, this has repercussion on the standard deviation of the prediction, which is slightly high.

4.2.2. Total overlapped peaks

Under the selected experimental conditions for the analysis of bumetanide in presence of ethacrynic acid only one peak was obtained. Working under the assumption that the composition of the sample is unknown the purity of the obtained peak must be assured. The absorbance ratio factor was calculated and normalised to 1 for each sample. A deviation from 1 indicates an impure peak. The absorbance ratio plot showed that the peak obtained was composed by, at least, two different compounds. Fig. 7 shows the absorbance ratios at four different wavelengths, as it can be seen in the plots obtained suffer

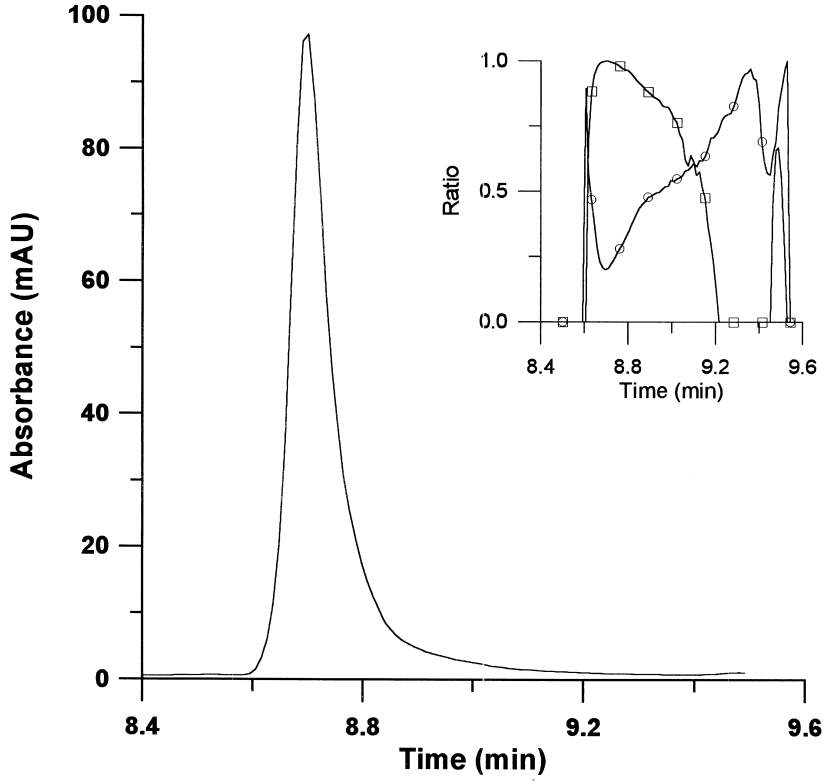

Fig. 7. Chromatogram at 275 nm of a mixture of ethacrynic acid and bumetanide. Absorbance ratio at 254/266 nm (○) and at 286/266 nm (□) for a solution of composition: 50 ppm of bumetanide and 50 ppm of ethacrynic acid.

Table 2
Calculated values for bumetanide (mean±standard deviation) in the mixtures of bumetanide and ethacrynic acid (every value is the mean of three predictions)

True bumetanide[a] (ppm)	True ethacrynic acid (ppm)	Measured bumetanide (mean±SD) (ppm)	Error
50	50	49.6±0.5	−1.3
100	50	104±1	4.0
150	50	143.5±0.4	4.3
200	50	202.7±0.7	1.4
50	50	43.0±0.7	−14.0
100	50	104.4±0.3	4.4
150	50	160.2±0.3	6.8
200	50	188.4±0.7	−5.8
250	50	243±1	−2.8
50	100	51±1	2.0
100	100	104.1±0.9	4.1
150	100	148.4±0.8	−1.1
200	100	213±2	−11.5
250	100	235±1	−6.0

[a] Independent samples. Error=(mean measured value−true value)×100/true value.

from severe deviations from the theoretical square wave function.

The experimental work for the determination of bumetanide was done in an analogous way than that for the determinations of acetazolamide and amiloride. One sample was spiked with known amounts of bumetanide, and once the concentration of this was known the spiked set was used as calibration set. The selected interval for carrying out the calculations was 262 to 278 nm since the other intervals found did not give consistent results. Ten combinations of three wavelengths within this interval are possible. All the combinations were calculated. Three absorbance increments were found with a slope ≥ 0.1 and a correlation coefficient ≥ 0.99, when regressed against the added concentration of analyte. The prediction of bumetanide in the spiked sample was 53.8 ppm ($s=1.7$ ppm). The prediction of the concentrations of bumetanide in the other samples can be found in Table 2.

5. Conclusions

The usefulness of the GHPSAM has been proved for the resolution of overlapped chromatographic peaks when the interference is unknown. In contrast with other proposed methods for chromatography the results obtained do not depend on the degree of overlap of the peaks but on the degree of spectral overlap between the analyte and the interferents. Two ways to locate the linear spectral interval have been shown.

The advantage of the method is that only a spectrum of the sample and one of the analyte is required. The successful determination of the analyte will depend on the characteristics of the interference but no a priori data of the later is required.

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