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H-Point standard additions method for resolution of overlapping chromatographic peaks with diode array detection by using area measurements Determination of phenol and cresols in waters[1]

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Abstract

The H-point standard additions method (HPSAM) is proposed to resolve overlapped peaks in liquid chromatography. The method uses the areas obtained at two previously selected wavelengths as analytical signals, and good results are obtained for highly overlapped peaks with highly overlapped spectra. The principal benefits of the method are the ease with which the required wavelengths can be selected, its insensitivity to changes in the retention time of the peak from one injection to other, and the possibility of using it in highly or only partially overlapped peaks. We have applied it to phenol and cresol determinations and the highly overlapped peak of *m*- and *p*-cresol is resolved by this method.

Keywords: Peak overlap; Standard additions method; H-Point standard additions method; Water analysis; Phenol; Cresols

1. Introduction

Quantitative analysis of several overlapping chromatographic peaks is still a difficult problem. Two options are available for achieving quantitative information in such a situation: the resolution of the peaks can be improved by choosing an alternative column packing or mobile phase, or by performing a mathematical resolution of the component of interest.

One of several mathematical approaches proposed is the transformation of the elution profile to its first, second or higher derivative [1–3]. Another simple method is the deconvolution by peak supression utilizing difference chromatograms [4–6]. D'Allura and Juvet proposed [7] a least-squares procedure for liquid chromatography (LC). A series of non-linear simultaneous equations are solved for the concentration of each component in the mixture. Many published recently rely on factor analysis [8–10]. The generalized rank annihilation method is a calibration and curve resolution method for multi-component bilinear data arrays [11,12]. Another aproach uses Kalman filtering [13–15].

Despite the great achievements in this field, the acquisition of reliable results is difficult when severely overlapping chromatographic peaks occur. This problem needs to be solved to obtain information about the components of the mixture.

We have suggested a modification of the equilib-

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rium standard additions method, called H-point standard additions method (HPSAM), where the error resulting from the presence of a direct interferent in the determination of an analyte is transformed into a systematic constant error that can be evaluated and permits the determination of the analyte concentration free from bias error, also in the presence of a direct interferent and/or total Youden blank, TYB [16]. The basis of the method was established in [17,18], and it has been applied to the determination of two species with extensively or even coincident overlapping spectra [19]. It was also developed for coupled chromatography and UV-Vis spectrophotometry [20], and it allows the determination of an analyte, X, in the presence of a direct interferent, Y, which coelutes with it. The method used as analytical signals the peak height (absorbance values) at two selected wavelengths λ_j and λ_k at which the species Y should have the same absorbance. Because an additional variable, time, is involved, it was selected at the retention time of the analyte to provide the better sensitivity.

In this work, we propose the use of the HPSAM with peak areas (absorbance values), selecting as analytical signals to record the chromatograms at two wavelengths where the interferent shows the same absorbance. We apply it to phenol and/or cresol determination. Phenol and its derivatives are among the most toxic and widely spread pollutants in industrial effluents and natural waters. It is often extremely important to identify individual pollutants and to determine individual concentrations, as the permissible concentration limits for various phenols and their isomers differ significantly. The separation of phenol and *m*-, *o*- and *p*-cresol by normal or reversed-phase liquid cromatography shows overlapped peaks for two of the three isomers [21–24]. The HPSAM can improve these determinations.

2. Theoretical background

Let us suppose a chromatographic separation of species X and Y, which coelute, and in addition show overlapped spectra (Fig. 1). In order to apply the proposed method, we have to select two wavelengths where the species which we will suppose as the interferent (Y) shows the same absorbance (A):

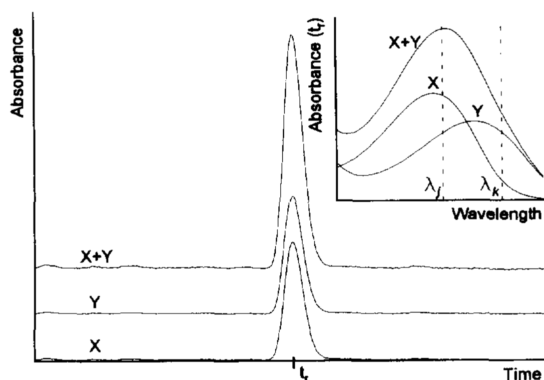

Fig. 1. Chromatographic separation of two species X and Y which coelute, and their absorption spectra at their retention time. Two wavelengths, λ_j and λ_k, where the interferent Y shows the same absorbance, have been selected.

$$A_{Y,j} = A_{Y,k} \tag{1}$$

If we register the chromatograms for species X and Y at these wavelengths, we will obtain the results shown in Fig. 2. The chromatograms for species Y are coincident, because of the particular selection of wavelengths. However, species X does not show the same absorbance at these wavelengths, and so, its chromatograms are not coincident.

In order to use the method, we prepared different solutions which contain the sample and the sample spiked with known amounts of analyte X, in accordance with the well-known method of standard additions.

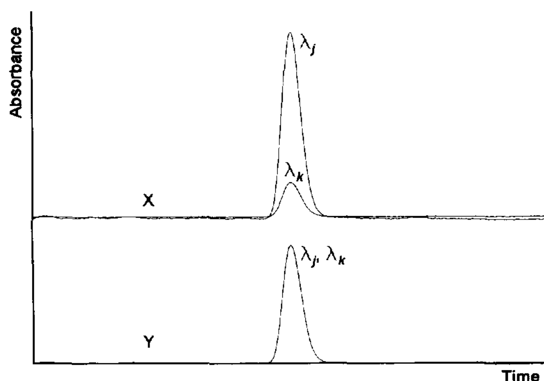

Fig. 2. Chromatograms obtained for the species X and Y at the two previously selected wavelengths λ_j and λ_k, where the species Y shows the same absorbance.

The lines obtained by representing measured area peak for these solutions at the previously selected wavelengths versus analyte added concentration fit the following equations (Fig. 3):

$$a(\lambda_j) = a^0_{X,j} + a_{Y,j} + M_j\, C^i_X \quad (2)$$

for λ_j, and

$$a(\lambda_k) = a^0_{X,k} + a_{Y,k} + M_k\, C^i_X \quad (3)$$

for λ_k, where $a^0_{X,j}$ and $a^0_{X,k}$ are the peak areas due to species X in the solution which only contains sample at λ_j and λ_k; $a_{Y,j}$ and $a_{Y,k}$ are the peak area due to species Y in the solution which only contains sample at λ_j and λ_k (and they will be the same for all the solutions prepared); M_j and M_k are the slopes of the calibration lines at λ_j and λ_k and C^i is the analyte added concentration for i=0 to n additions. When i=0, we have the solution which only contains sample.

H-Point, with coordinates ($-C_H$, a_H), is common for both lines, so:

$$a^0_{X,j} + a_{Y,j} + M_j(-C_H) = a^0_{X,k} + a_{Y,k} + M_k(-C_H) \quad (4)$$

from where

$$-C_H = \frac{(a^0_{X,k} - a^0_{X,j}) + (a_{Y,k} - a_{Y,j})}{M_j - M_k} \quad (5)$$

Because λ_j and λ_k have been selected to give the

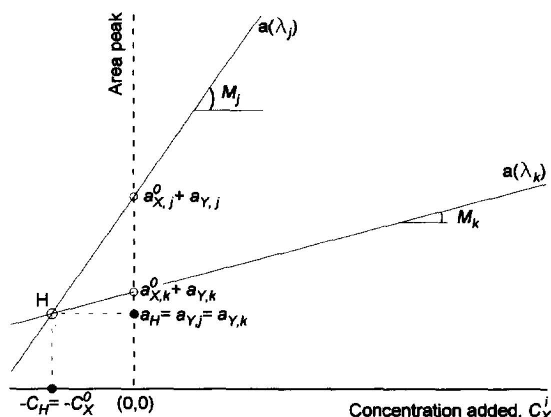

Fig. 3. H-Point standard additions lines obtained by representing the area peak vs the concentration of analyte added.

same analytical signal for species Y (1), Eq. 5 transforms to:

$$-C_H = \frac{a^0_{X,k} - a^0_{X,j}}{M_j - M_k} \quad (6)$$

which is equivalent to the unbiased analyte concentration in the sample, C^0_X:

$$-C_H = \frac{a^0_{X,k} - a^0_{X,j}}{M_j - M_k} = \frac{-a^0_{X,k}}{M_k} = \frac{-a^0_{X,j}}{M_j} = -C^0_X \quad (7)$$

If we substitute the value of C_H obtained in Eq. 6 in Equations 2 or 3, we obtain:

$$a_H = a_{Y,j} = a_{Y,k} \quad (8)$$

So, from the abcissa of the H-point and a calibration graph of species Y at the selected wavelengths, we can obtain its concentration in the sample.

If matrix effects are known not to be present, as usually occurs in liquid chromatography, the methodology can be simplified by using as M_j and M_k values the ones obtained from calibration lines of pure species X. The equations of interest now will be:

$$-C_H = \frac{a^0_{S,k} - a^0_{S,j}}{M_j - M_k} \quad (9)$$

where $a^0_{S,j}$ and $a^0_{S,k}$ are the peak areas of the sample at the selected wavelengths λ_j and λ_k and M_j and M_k are the slopes of calibration lines at λ_j and λ_k for pure solutions of X. The concentration for species Y is obtained from:

$$a_H = a^0_{S,j} - C_H\, M_j = a^0_{S,k} - C_H\, M_k \quad (10)$$

This possibility permits experimental simplification of the method.

3. Experimental

3.1. Apparatus

A Hewlett-Packard Model 1040A liquid chromatograph (Palo Alto, CA, USA), equipped with a

Hewlett-Packard 1040M Series II 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 and an automatic injector (Hewlett-Packard, 1050 Series).

The column was a HP-Lichrospher 100 RP-18 (5 μm) (250×4 mm I.D.) (Merck, Darmstadt, Germany). The detector was set to record the chromatogram at six different analytical signals. All assays were performed at room temperature.

3.2. Reagents

All the reagents were of analytical-reagent grade, and acetonitrile was LC grade (Scharlau, Barcelona, Spain). Water was distilled, deionized and filtered through 0.45-μm nylon membranes (Teknokroma, Barcelona, Spain). Standard solutions of phenols (1000 ppm) were prepared by dissolving phenol, *o*-cresol, *m*-cresol and *p*-cresol in water (all from Merck). They were stored in borosilicate-glass vessels in the refrigerator.

3.3. Mobile phases

A water–acetonitrile mixture (64:36, v/v) was used as mobile phase. All the solutions were prepared daily, filtered through a 0.45-μm nylon membrane and degassed with helium before use. The flow-rate was set at 1 ml/min and 50 μl of each sample were injected.

3.4. Solid-phase extraction

Bond Elut C_{18} (500 mg, Analytichem International, Harbor City, CA, USA), Bond Elut CH (500 mg, Analytichem) and Isolute C_{18} EC (500 mg, International Sorbent Technology, Hengoed, UK) extraction columns were used. Before sampling, each column was conditioned with 5 ml of methanol and then with 15 ml of water, pH 3.0 (acidified with H_3PO_4). Water samples were adjusted to pH 3.0 with phosphoric acid, and 35 g/l of NaCl was added in order to improve the recoveries. Different volume samples were then filtered through the sorbent bed under reduced pressure by using the Vac Master-10 sample processing station (International Sorbent Technology) at a flow-rate of about 5 ml/min, and dried with air under the same conditions for 5 min. Phenols were eluted from column with 1.5 ml of a mixture water–acetonitrile (1:1, v/v). Methanol must not be used, because it gives broad peaks in the chromatogram, and more than 2 ml of mobile phase is required to elute all the phenols from the column.

3.5. Real samples

Natural waters were sampled near Valencia (from Albufera lake, located in an industrial area and from the Valencia harbour). 100 ml of the samples were adjusted to pH 3.0 with phosphoric acid and filtered through a 0.45-μm nylon membrane. A 3.5-g amount of NaCl was added to the samples from Albufera lake, before the solid-phase extraction, whereas sea samples were directly extracted, using the procedure described above.

4. Results and discussion

As the content of phenol derivatives in effluents is often rather low, preconcentration by absorption is recommended. Because a comparison of published results shows differences in the recoveries of phenols [25], three kinds of extraction columns were tested. Cyclohexyl columns gave good results for cresols at low volumes, but for phenol it gave poor recoveries, and from the two C_{18} columns, the Bond Elut was selected, because of its better results. This difference can be explained because the C_{18} EC has the silane groups end-capped, and the polar secondary interactions associated with surface silanol groups are reduced. The recoveries obtained for these columns and for the four phenols versus the sample volume passed through are shown in Fig. 4. A sample volume of 100 ml was selected, because it gives a recovery of almost 100% for cresols. For the more polar phenol, only a recovery of ca. 30% was achieved. However, the final signal was higher in these conditions if a sample size of 25 ml was selected, because the major sample volume passed. This procedure gives a concentration factor of about 20 for phenol and 67 for cresols.

Under the selected experimental conditions, and according to the previously described separations,

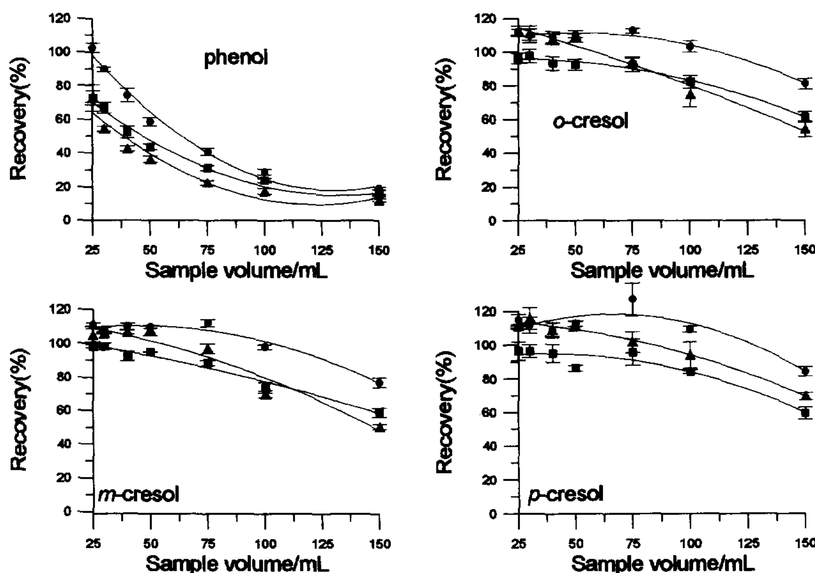

Fig. 4. Recoveries for phenol and cresols in the solid-phase extraction columns. (●) Bond Elut C_{18}, (■) Isolute C_{18} EC, (▲) Bond Elut CH. n=2–5, depending on the analyte and the volume.

the phenolic mixture is resolved in three peaks (Fig. 5): the first peak corresponding to phenol, the second to *m*-/*p*-cresol and the third to *o*-cresol. The chromatogram shows baseline resolution between the last two peaks. Fig. 6 shows that the spectra for *m*-cresol and *p*-cresol are overlapped. So, the conditions for applying the HPSAM to resolve this peak are of use now.

First of all, we have to find two analytical signals where one of the species shows the same value. Because of the position of the first maximum, it was selected as interferent *p*-cresol, because it was easier find these signals for it. A chromatographic signal from the diode-array is made up of four parameters: a wavelength and a bandwidth for detection and a wavelength and a bandwidth for the reference signal. The wavelength value gives the central wavelength of the signal and defines the center diode. The bandwidth value defines the range of diodes either side of this center, those diodes whose responses are to be averaged to give the signal [26]. So, an appropiate combination of these factors can be selected to find the required signals. In our case, the selected signals were 215(8)/224(6), 210(6)/226(4) and 271(4)/281(4), with 450(80) as the reference signal (the first number gives the central diode, and the number in brackets the bandwidth). The chromatograms obtained are shown in Fig. 5. Because of the particular wavelength selection, the chromatograms for *p*-cresol are completely coincident, but not those for *m*-cresol. Thus, HPSAM can be used to resolve the mixture. Because phenol and *o*-cresol absorb at the same wavelengths, it was not necessary select any special signal to determine them, and those previously selected were of use.

The calibration lines obtained for *p*-cresol at the selected wavelengths in three different situations are shown in Table 1. All the statistical tests showed that the selected signals are coincident [27]. This is in agreement with the chromatograms shown in Fig. 5.

Five synthetic samples were then prepared to test the method. The composition of the samples and the results obtained from the proposed method by using Eqs. 7 and 8 are shown in Table 2. Excellent results were obtained, even when the concentration of one of the unresolved compounds was ten times higher than the other (samples 3 and 5). No significant differences were observed from the results obtained for the sustances used as analyte and interferent (*m*- and *p*-cresol). Less precise results were obtained for phenol in low concentrations, in accordance with its lower recovery.

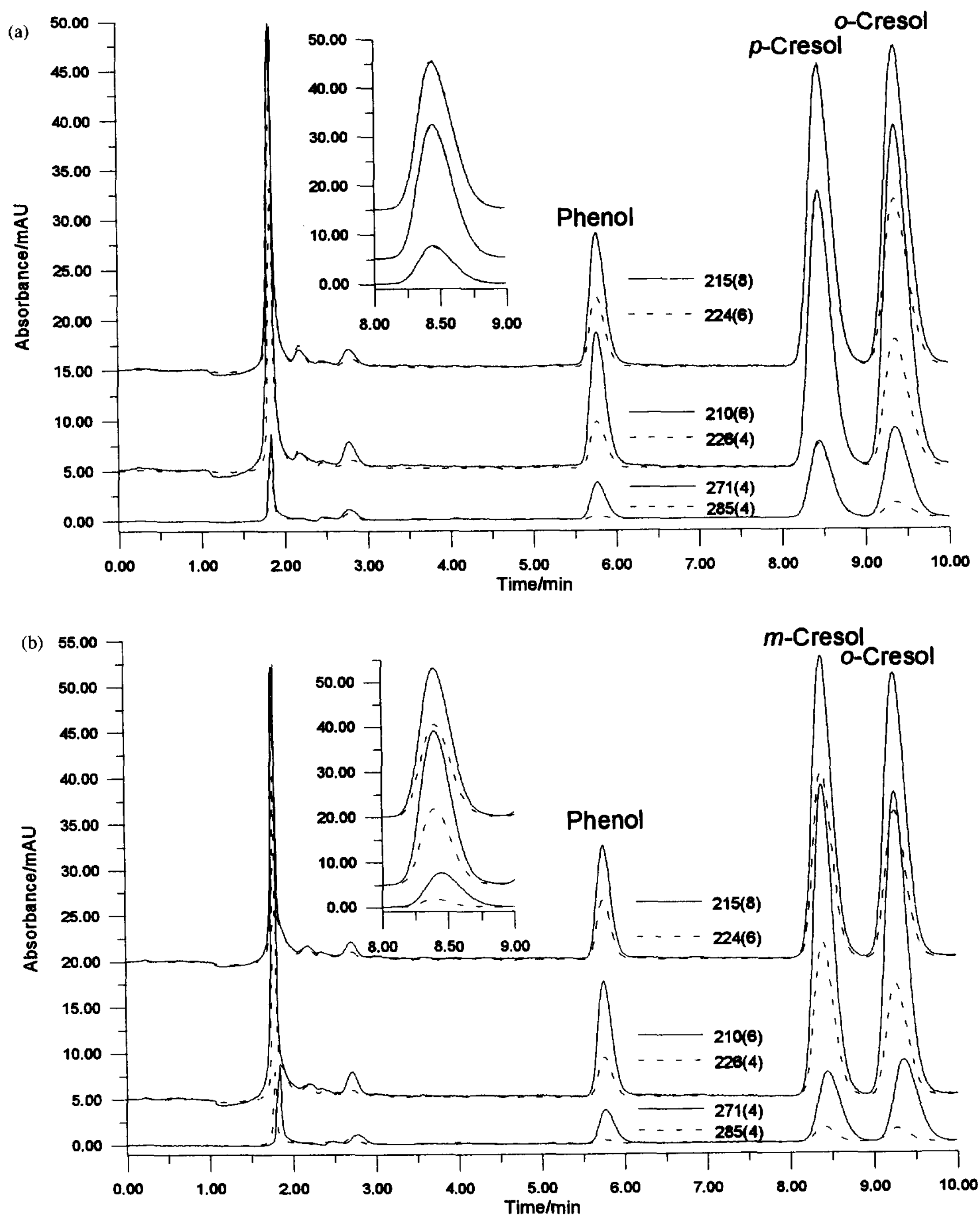

Fig. 5. Chromatographic separation of phenol, o-, m- and p-cresol at the selected wavelengths with the HPSAM. For experimental conditions see text.

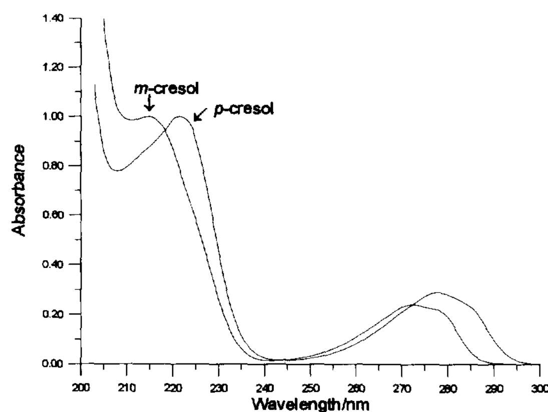

Fig. 6. Absorption spectra for *m*- and *p*-cresol in the mobile phase.

Table 2 also contains the results from the direct calibration of samples, by using Eqns 9 and 10, with sample area values and slopes of calibration lines. Similar results are obtained, because the reproducibility in the sample treatment and in the detector response.

The detection limits for the method are 1 ppb for phenol and <1 ppb for cresols. As stated previously, 20 ppb of each phenol can be quantified without error.

In an application of the method, two real samples were analysed: one from the Albufera lake (near of Valencia), and the other from the harbour of Valencia. Both extracts were very contaminated, but fortunately the bulk of the contaminants appear at the

Table 1

Calibration lines obtained for *p*-cresol ($n=5$) for: (a) 6.11–30.57 ppb; (b) 23.38–477.62 ppb; (c) 23.38–477.62 ppb, in the presence of 95.62 ppb of *m*-cresol

	Wavelength (bandwidth) (nm)					
	215(8)	224(6)	210(6)	226(4)	271(4)	285(4)
(a) Parameter						
$a \pm s_a$	0.2±5.9	−2.6±5.4	0.6±4.6	−1.9±5.7	0.8±1.9	−0.6±1.7
$(b \pm s_b)$ (ppb)	5.73±0.29	5.95±0.27	5.22±0.22	5.24±0.28	1.43±0.09	1.44±0.08
r	0.996185	0.996996	0.997228	0.995676	0.993861	0.995208
s_{yx}	5.61	5.41	4.56	5.46	1.78	1.58
F test						
(homogeneity of variances)	1.18		0.63		1.27	
F test(coincidence)	0.40		0.44		1.29	
(b)						
$a \pm s_a$	−13.8±19.9	−11.6±18.6	−12.9±16.9	−11.3±15.6	−2.9±4.8	−4.7±4.4
$(b \pm s_b)$ (ppb)	5.86±0.08	5.90±0.08	5.32±0.07	5.26±0.07	1.47±0.02	1.45±0.02
r	0.999710	0.999748	0.999746	0.999767	0.999736	0.999767
s_{yx}	30.58	28.7	26.0	24.6	7.33	6.78
F test						
(homogeneity of variances)	1.13		1.12		1.17	
F test(coincidence)	0.38		0.63		2.46	
(c)						
$a \pm s_a$	642.7±30.7	405.8±22.8	663.9±29.6	335.0±24.6	154.5±7.3	37.8±5.7
$(b \pm s_b)$ (ppb)	5.28±0.12	5.34±0.11	4.81±0.12	4.77±0.10	1.33±0.03	1.32±0.02
r	0.999147	0.999318	0.999049	0.999330	0.999232	0.999525
s_{yx}	47.33	42.8	45.5	37.9	11.3	8.8
F test						
(homogeneity of variances)	1.22		1.44		1.64	
F test(parallelism)	0.12		0.06		0.05	

Variances are homogeneous if $F_{tab} > F_{calc}$ [$F_{0.95}(3;3)=9.28$]. Lines are coincident if $F_{tab} > F_{calc}$ [$F_{0.95}(1;6)=5.99$]. Lines are parallels if $F_{tab} > F_{calc}$ [$F_{0.95}(1;6)=5.99$]. S_a=standard deviation of the intercept; S_b=standard deviation of the slope; s_{yx}=standard deviation of the fit.

Table 2
Results obtained for five different synthetic samples by using the standard additions method and calibration slopes

Sample (ppb)		Phenol (ppb)	*o*-Cresol (ppb)	*m*-Cresol (ppb)	*p*-Cresol (ppb)
1	Amount added	38.1	38.1	19.1	19.1
	Amount found (SA)[a]	44.3±1.6	40.3±0.8	21.3±2.1	19.5±1.7
	Amount found (CS)[b]	43.2±1.5	41.8±0.6	21.6±1.8	20.7±0.7
2	Amount added	119.0	119.2	23.9	95.5
	Amount found (SA)	126.5±1.5	126.0±1.7	24.6±2.8	96.1±2.0
	Amount found (CS)	134.8±2.1	118.3±1.1	23.9±2.8	94.7±1.8
3	Amount added	209.5	209.7	19.1	191.0
	Amount found (SA)	197.7±3.0	212.2±2.4	21.6±3.5	186.8±2.8
	Amount found (CS)	191.1±2.3	207.2±2.6	20.1±2.6	181.8±2.1
4	Amount added	95.2	95.3	76.5	19.1
	Amount found (SA)	96.0±2.3	94.8±2.1	75.6±1.8	19.2±0.4
	Amount found (CS)	87.5±1.1	92.2±0.9	74.0±1.1	18.5±0.6
5	Amount added	209.5	209.7	191.2	19.1
	Amount found (SA)	210.0±1.4	212.9±1.5	193.5±1.2	19.9±0.8
	Amount found (CS)	208.0±1.2	220.3±1.0	200.7±0.8	20.0±0.7

[a]SA=standard additions method (Eqs. 7 and 8).
[b]CS=calibration slopes (Eqs. 9 and 10).

beginning of the chromatogram, and one little peak (unknown compound) at the left of phenol in the Albufera sample is well resolved. The results for Albufera water show that it contains 16±4 ppb of phenol, and cresol values were lower than 1 ppb (Fig. 7). These results are in agreement with those reported in previous work [28], where the concentration of total phenols in Albufera lake water was 18 ppb. In the harbour sample, phenol concentration was 14±3 ppb, and it contains cresols at about the 1 ppb level, but their concentration is under the quantification limit for the method (Fig. 8). Applying the HPSAM to the spiked samples, the results obtained are shown in Table 3.

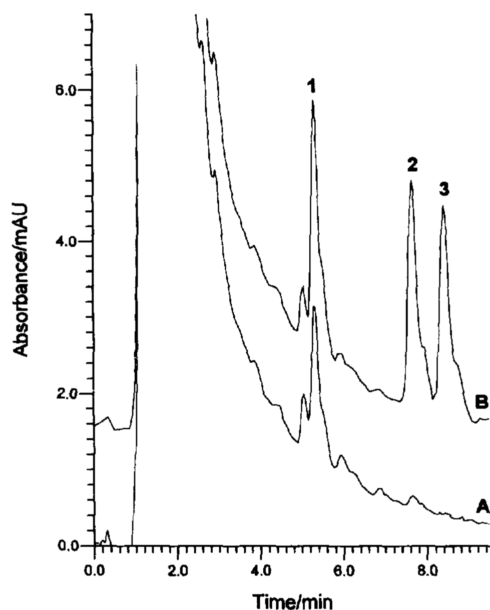

Fig. 7. Chromatograms obtained for (A) the Albufera water sample and (B) the Albufera water sample spiked with 7.9 ppb of phenol (peak 1), *m*-cresol (peak 2) and *o*-cresol (peak 3).

5. Conclusions

A new method is proposed to resolve highly overlapped peaks in liquid chromatography. The method uses the areas obtained at two previously selected wavelengths as analytical signals, and good results are obtained for highly overlapped peaks with highly overlapped spectra. The principal benefits of the method are the ease with which the required wavelengths can be selected, its insensitivity to changes in the retention time of the peak from one injection to other, and the possibility of using it in highly or only partially overlapped peaks.

Table 3
Results obtained for two real samples

Sample[a]		Phenol (ppb)	*m*-Cresol (ppb)	*p*-Cresol (ppb)	*o*-Cresol (ppb)
Albufera	Amount added	–	–	–	–
	Amount found	16.6±1.3	–	–	–
	Amount added	7.9	7.9	–	7.9
	Amount found	26.0±0.8	6.3±0.8	ND[a]	8.4±0.9
	Amount added	15.7	15.7	–	15.7
	Amount found	33.9±1.6	17.4±1.9	ND	15.2±0.7
Harbour	Amount added	–	–	–	–
	Amount found	16.1±1.6	–	–	–
	Amount added	7.9	7.9	–	7.9
	Amount found	26.6±1.5	8.2±0.3	ND	6.9±1.4
	Amount added	15.7	15.7	–	15.7
	Amount found	34.4±1.6	16.3±1.9	ND	15.6±1.0

[a]ND: not detected.

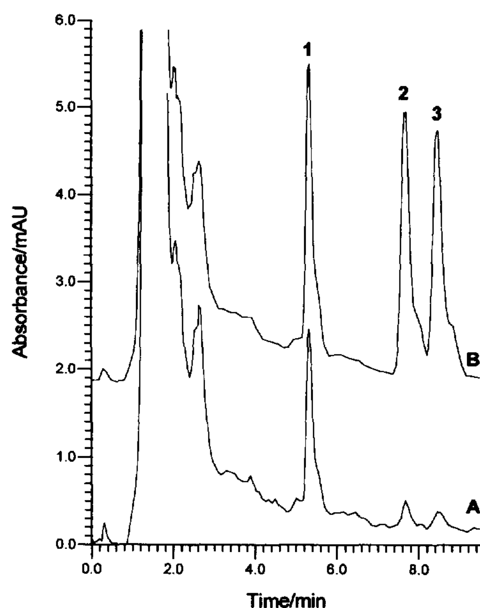

Fig. 8. Chromatograms obtained for the (A) Valencia harbour water sample, and (B) the Valencia harbour water sample spiked with 7.9 ppb of phenol (peak 1), *m*-cresol (peak 2) and *o*-cresol (peak 3).

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