CHEMICAL REMOVAL OF HUMIC SUBSTANCES INTERFERING WITH THE ON-LINE SOLID-PHASE EXTRACTION - LIQUID CHROMATOGRAPHIC DETERMINATION OF POLAR POLLUTANTS

ABSTRACT

Different methods for removing interference by humic substances in the analysis of polar pollutants have been compared in the analysis of environmental water by solid-phase extraction (SPE) with a chemically modified polymeric resin coupled on-line to liquid chromatography with UV detection.

The methods were based on the use of chemical reagents. The best method was found to be addition of sodium sulphite to humic-containing water before SPE. The appropriate amount of sulphite depends on the amount of humic substances dissolved in the sample - for analysis of 50 ml tap and Ebro river water, respectively, 250 µl and 500 µl 10% Na_2SO_3 solution had to be added. In both cases, the recovery values after chemical treatment were similar to those when a Milli-Q quality water standard was analysed.

Keywords: Column liquid chromatography*;* On-line preconcentration; Humic substances; Polar pollutants in water.

INTRODUCTION

Methods in which solid-phase extraction is hyphenated with high-performance liquid chromatography (HPLC), with UV and diode-array (DAD) detection, are those most frequently used for determining polar pollutants in environmental water. On classical SPE sorbents (bonded silicas and styrene-divinylbenzene copolymers) these compounds have low breakthrough volumes, but new chemically modified polymeric resins with different functional moieties (e.g. acetyl, hydroxymethyl, benzoyl and *o*-carboxybenzoyl) [1-4] and highly crosslinked polymers [5,6], which have higher sorption power than the other sorbents, mainly for polar analytes, have recently been developed.

When on-line SPE-HPLC is used to analyse surface water samples containing high levels of humic substances, a band at the beginning of the chromatogram hampers the determination of the early-eluting peaks from the most polar analytes [2-7]. This matrix interference increases when a SPE sorbent with high sorptive power is used (e.g. a chemically modified polymeric resin or a highly crosslinked polymeric sorbent) [2-6].

Humic substances, including humic and fulvic acids, are acidic, yellow-to-black polyelectrolytes which usually account for most of the dissolved organic matter [8]. They are polymers with an average molecular mass of 1000-10000 containing heterogenic structures such as methoxy aromatic compounds, phenols and aryl ketones [9]. Normal concentrations of humic acids in ground, surface and estuarine water are extremely variable and range between 0 and 10 mg l^{-1} [10] and the humic acid concentration might be different in samples taken at the same site with only a few days difference between sampling times [11]. These substances are UV-absorptive because of their high percentage of aromaticity [8]. This is the origin of the initial broad band during analysis of real water samples with UV detection.

To avoid or reduce humic interference, other detection systems which are more selective than DAD and UV can be used; these include mass spectrometry (MS) [9,10,12], fluorescence detection [2,9] or electrochemical detection [13,14]. Although these systems enable the determination of some analytes without this matrix interference, sometimes, depending on the amount of humic substances dissolved in the sample, these detection systems are not sufficiently selective and humic interference still occurs. UV detection is, in addition, the system most frequently used in laboratories and so other methods are required to eliminate the band corresponding to humic and fulvic acids.

Some analysts have solved matrix effects in humic-containing water samples by using a precolumn (alkyl-diol-silica) based on the non-adsorptive size- exclusion of macromolecules and simultaneous partitioning of the analytes, but the retention of this precolumn is not sufficient for the most polar analytes and the humic hump is not wholly eliminated [10]. Clean-up at high pH (pH 11) followed by heart-cutting of the precolumn eluent has also been used. The explanation given is that humic substances are highly polar molecules which are easily eluted from the precolumn without incorporation of the analytes of interest [15].

Other workers have reduced this band by chemical treatment with $KMnO_4$. This treatment consists in oxidation of humic acids before the extraction process [11,16].

In previous work [4,17,18] we have used Na_2SO_3 to eliminate free chlorine in tap water, because it reacts with phenols to produce chlorophenols, and observed that the initial peak corresponding to fulvic and humic acids decreased in size. For this reason, we tried to reduce the initial hump by use of different chemical reagents such as sodium thiosulphate and oxalic acid with reducing properties similar to those of sodium sulphite.

This paper focuses on the use of different chemical reagents to reduce the initial hump which can prevent the analysis of polar organic pollutants in humic-containing

water samples. Analysis of the organic pollutants was performed by use of an SPE sorbent with high sorptive power (a chemically modified polymeric resin) coupled on-line to HPLC with UV detection.

EXPERIMENTAL

Equipment

Experiments were performed using two Shimadzu (Tokyo, Japan) LC-10AD pumps with a Shimadzu SPD-10A UV spectrophotometric detector. The mobile phase was degassed with a Shimadzu DGU-4A degasser. The temperature of the column was controlled by means of a Shimadzu CTO-10A oven. Chromatographic data were collected and recorded by use of an HP-3365 Series II Chemstation. The compounds were separated on a 25x0.46 cm I.D. Kromasil 100 C_{18} column, particle size 5 μm, supplied by Teknokroma (Barcelona, Spain).

To check the response of the instrument standard solutions were injected through a Rheodyne (Cotati, CA, USA) valve with a 20 μl loop; an automatic Must column-switching device (Spark Holland, Emmen, The Netherlands) was used for on-line SPE. The on-line trace enrichment process was performed with 10x3 mm I.D. steel precolumns laboratory-packed with a chemically modified polymeric sorbent, with an *o*-carboxybenzoyl moiety, which had been synthesized in our laboratory [4]. A Waters (Milford, MA, USA) M45 pump was used to deliver the sample and to condition the precolumn.

Reagents and Standards

The compounds studied were a mixture of polar pesticides and phenolic compounds. The phenolic compounds were resorcinol, phenol (Ph), 4-nitrophenol (4-NP) and 2-chlorophenol (2-CP); these were purchased from Aldrich-Chemie (Steinheim, Germany). The pesticides were oxamyl and methomyl (carbamates) and bentazone (diazine) from Riedel-de Haën (Seelze, Germany). Stock standard solutions (2000 mg l^{-1}) of each compound were prepared in methanol. Standard solutions were prepared weekly by diluting the stock standard solutions with Milli-Q water (Millipore, Bedford, MA, USA), and more dilute working solutions were prepared daily by diluting with Milli-Q, tap or river water. All solutions were stored at 4ºC. HPLC-quality acetonitrile (Fisher Scientific, Leicestershire, UK) and Milli-Q quality water, adjusted to pH 3 with sulphuric acid (Probus, Badalona, Spain), were used to prepare the mobile phase.

Hydrochloric acid (Probus, Badalona, Spain) was added to adjust the pH of the sample to 2.5 before SPE to prevent the analytes from occurring in the ionic form. Tap and river water samples were filtered through a 0.45 µm nylon membrane (Whatman, Maidstone, UK) before SPE to eliminate particulate matter. Different volumes of sodium sulphite (Na_2SO_3) (Panreac, Barcelona, Spain), sodium thiosulphate ($Na_2S_2O_3$) (Probus), oxalic acid ($H_2C_2O_4$) (Probus) and potassium permanganate ($KMnO_4$) (Probus) solutions were added to real samples to reduce the peak that appears at the beginning of the chromatogram because of the presence of humic and fulvic acids in these samples. Humic acid of M_r ~600-1000 from Fluka (Buchs, Switzerland) and of M_r ~5000-10000 from Aldrich-Chemie were used to determine the relationship between the concentration of humic acid in the sample and the amount of Na_2SO_3 added.

Chromatographic Conditions

Gradient elution was performed with Milli-Q quality water at pH 3 (solvent A) and acetonitrile as organic modifier (solvent B). The flow rate was 1 ml min^{-1} and the temperature of the column oven was set at 65ºC. The gradient profile was 15% B initially, 45% B after 20 min, 100% B at 22 min, isocratic for 2 min, and back to the initial conditions in 2 min. Post-run time was 10 min.

Detection was performed at 280 nm for phenolic compounds and at 240 nm for pesticides. The wavelength program used enabled detection of each compound at its wavelength maximum absorbance.

On-line Trace Enrichment Procedure

The on-line solid-phase extraction process was performed using a chemically modified polymeric resin with an *o*-carboxybenzoyl moiety, prepared from a polystyrene-divinylbenzene resin; the synthesis has been described elsewhere [4]. The program used for conditioning the precolumn and for sample preconcentration with the Must automatic column-switching device is shown in Table 1.

Table 1
Sample preconcentration program for on-line SPE

Step	Time (min)	Flow rate (ml min^{-1})	Event	Solution
1	0	2	Washing tubes	Acetonitrile
2	5	2	Conditioning precolumn	Acetonitrile
3	6	2	Washing tubes	Milli-Q water at pH 2.5
4	11	2	Activating precolumn	Milli-Q water at pH 2.5
5	12	2	Washing tubes	Sample

6	17	4	Sample preconcentration	Sample
7	*		Analyte desorption	Acetonitrile of mobile phase

* Depends on sample volume

Analytes were desorbed in the backflush mode, using only the organic solvent (solvent B, acetonitrile) of the mobile phase. This effectively suppressed peak broadening as a result of the different properties of the analytical column and the precolumn sorbent [19,20]. Solvents A and B were mixed before they reached the analytical column. When this equipment design was used for SPE of environmental water a slight increase in the humic hump was observed.

RESULTS AND DISCUSSION

Chromatographic Separation

Before the on-line solid-phase extraction study, the gradient elution and the wavelength program were optimized for separation of the seven compounds studied in a short time (<20 min). The optimum conditions have already been described in the section on chromatographic conditions.

Direct injection of 20 µl samples resulted in good linearity for all compounds at 0.025-20 mg l^{-1}; regression coefficients (r^2) were >0.9998. Detection limits were calculated by the statistical program ULC (Univariate Linear Calibration) with k=6 [21] and the values found were between 5 µg l^{-1} for oxamyl and 12 µg l^{-1} for bentazone. For this reason, samples must be preconcentrated to enable detection of the low levels allowed in environmental waters.

Breakthrough Volumes

The SPE sorbent, a chemically modified polymeric resin with an *o*-carboxybenzoyl moiety, was chosen because it resulted in higher breakthrough volumes for polar analytes than did other commercial sorbents [4,18]. An experimental method for determining both breakthrough volumes and recoveries has been described elsewhere [7,22-24]; it is easily performed with this on-line arrangement. One advantage of this method is that these values can be obtained from few on-line preconcentrations for all analytes and under experimental conditions that correspond to those used for real analysis (trace level and several analytes together) [23].

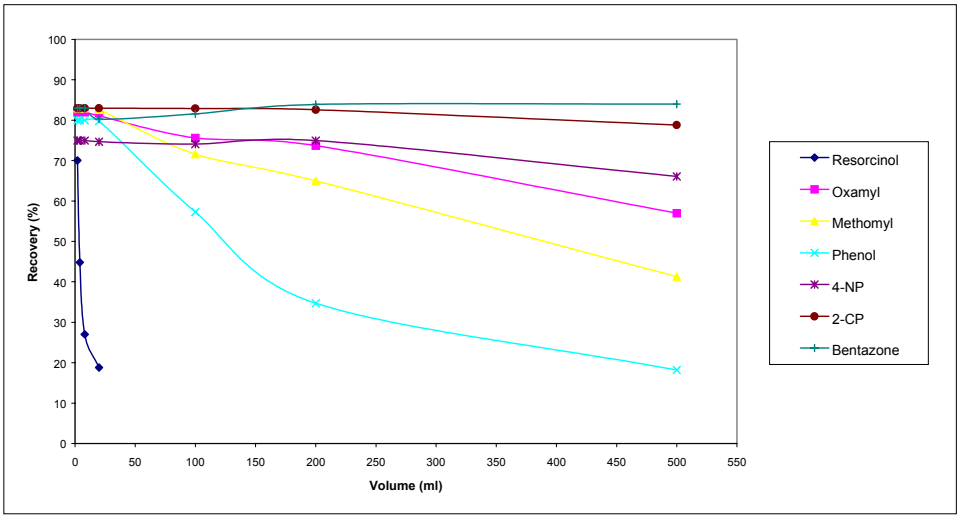

Fig. 1. Experimental variation of the recovery obtained by on-line preconcentration of different volumes of Milli-Q water samples containing a constant amount (0.1 µg) of the analytes.

Different sample volumes (2, 4, 8, 20, 50, 100, 200 and 500 ml) of Milli-Q quality water were spiked with different concentrations of the analytes so that the amounts of the analytes in the samples were constant (0.1 µg injected). These

were then percolated through the precolumn. The recoveries were calculated by dividing the peak area obtained for a given sample volume by the peak area obtained by direct loop injection of the same amount of compound; breakthrough occurred when the recovery began to decrease. The results of measuring breakthrough volumes are shown in Fig. 1. It can be seen that except for phenol and resorcinol recoveries sample for all analytes were >70% after preconcentration of a 200 ml sample. Recovery values were 57 and 76% for phenol after preconcentration of 100 and 50 ml, respectively, whereas 45% of resorcinol was recovered from a 4 ml sample and none was recovered when 50 ml was preconcentrated. From the results obtained 50 ml was selected for further analysis, except for the determination of resorcinol, the breakthrough volume of which was 2 ml.

Breakthrough was only studied using Milli-Q water because it has been reported that breakthrough volumes for different types of water do not vary substantially [7,23].

Reduction in Interference by Humic Substances

A method must be found which considerably reduces the characteristic frontal humic hump and which enables polar analytes to be determined more accurately. For this reason, various chemical reagents with different reductive properties, 10% (*w/v*) sodium sulphite, sodium thiosulphate or oxalic acid solutions, were added to tap water samples (pH 2.5) to remove this humic hump. Different volumes of these solutions (150, 250 or 500 µl for every 50 ml of sample) were added to the sample before extraction. The best results were obtained using Na_2SO_3 – addition of 250 µl Na_2SO_3 to 50 ml tap water was sufficient to eliminate the initial broad band whereas with the other reagents there was no significant reduction in the matrix interference peak. Fig. 2 shows

the chromatograms obtained after preconcentration of 50 ml tap water spiked with 2 μg l^{-1} of each target analyte and addition of 250 μl of these reagents. It should be pointed out that resorcinol was not retained in the precolumn with this sample volume (50 ml).

In contradiction of our initial supposition, these results suggest that the humic band is not reduced by the reducing power of the chemical reagents, because sulphite is a less powerful reducing agent than the other two reagents used. One possible explanation of the reduction in size of the frontal humic hump on addition of sodium sulphite is that some of the ketone and aldehyde carbonyl groups present in the humic substances [25,26] undergo addition of bisulphite at the pH of the sample (2.5) [27]. This reaction might transform the humic substances into more highly polar compounds which are not sufficiently retained by the precolumn sorbent in the sample preconcentration step, resulting in cleaner chromatograms. Further studies are required to improve our knowledge of this reaction.

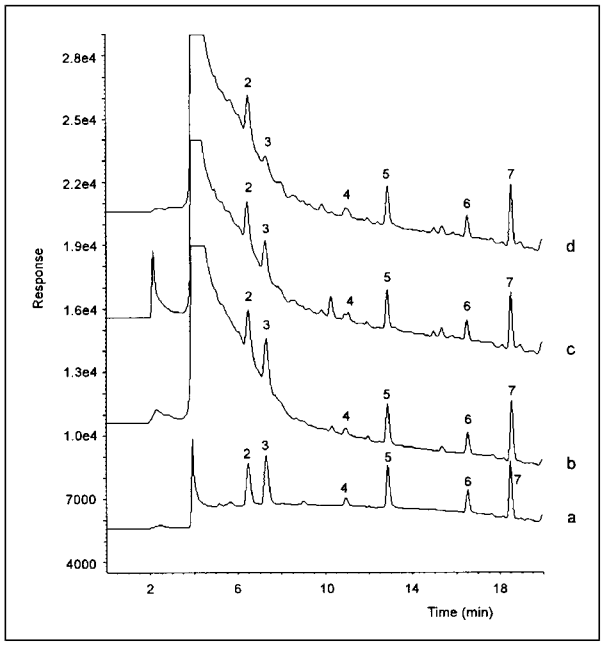

Fig. 2. Chromatograms obtained after on-line trace enrichment of 50 ml tap water spiked with 2 μg l^{-1} of each analyte after adding 250 μL of (a) sodium sulphite, (b) sodium thiosulphate, (c) oxalic acid and (d) no reagent solution. Peaks: 2, oxamyl; 3, methomyl; 4, phenol; 5, 4-NP; 6, 2-CP; 7, bentazone.

Another method of reducing this band, which has been used by Bonifazi et al. [11,16], is to oxidize the humic substances with potassium permanganate. The sample was acidified to pH 1 with HCl, and then 0.2M $KMnO_4$ solution was added dropwise until the sample turned violet. The solution was stirred for 30 min, then H_2O_2 (30%) was added until the sample became colourless, and finally, to prevent the hydrolysis of the sorbent, the sample pH was adjusted to 2.5 by addition of 6N sodium hydroxide. After this treatment the band decreased only slightly, so the method was less effective than addition of Na_2SO_3.

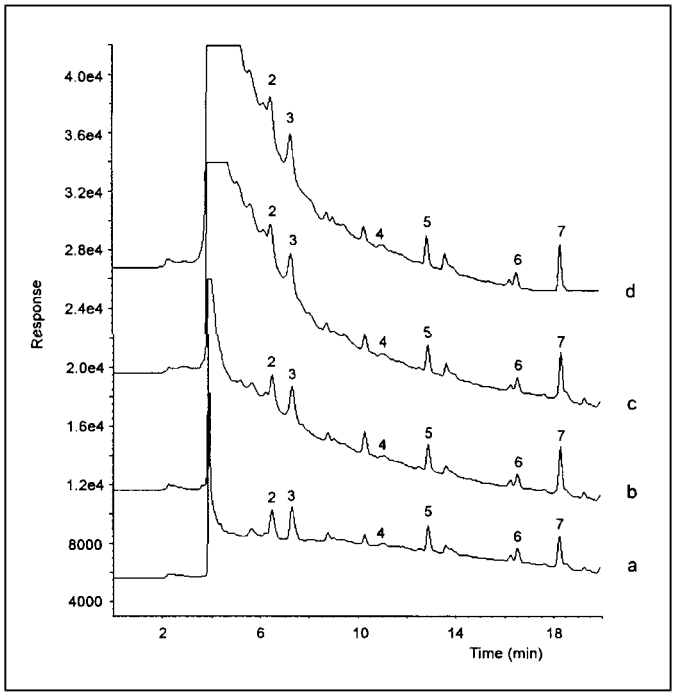

Fig. 3. Chromatograms obtained after on-line trace enrichment of 50 ml Ebro river water spiked with 2 μg l^{-1} of each analyte with or without addition of different volumes of 10% Na_2SO_3 solution: (a)

500 µl, (b) 250 µl, (c) 150 µl, (d) without addition of Na_2SO_3 solution. Peak designation as for Fig. 2.

Addition of Na_2SO_3 was also studied in the preconcentration of 50 ml of Ebro river water. For this sample matrix, 500 µl of 10% Na_2SO_3 solution had to be added to reduce the frontal humic hump sufficiently – after addition of 250 µl the initial hump still prevented analysis of the most polar compounds. Fig. 3 shows the chromatograms obtained after preconcentration of 50 ml of Ebro river water spiked with 2 µg l^{-1} and addition of 150, 250 and 500 µl Na_2SO_3 solution.

Table 2
Recovery values obtained in the on-line preconcentration of a 50 ml sample spiked with 2 µg l^{-1} of each analyte.

Compound	Milli-Q water	Tap water[a]	Ebro river water[b]
Resorcinol	6	-----	-----
Oxamyl	88	85	87
Methomyl	89	78	77
Phenol	77	58	47
4-NP	82	81	75
2-CP	81	84	78
Bentazone	86	76	66

For all results % RSD (*n*=6) values were <9, except for resorcinol (13).
[a]With addition of 250 µl 10% Na_2SO_3 solution.
[b]With addition of 500 µl 10% Na_2SO_3 solution.

The recoveries obtained by preconcentrating 50 ml tap and river water with the addition of sulphite were similar to those obtained with Milli-Q quality water, except for phenol and bentazone, the recoveries of which were lower when the amount of sulphite added was increased. These recoveries are shown in Table 2.

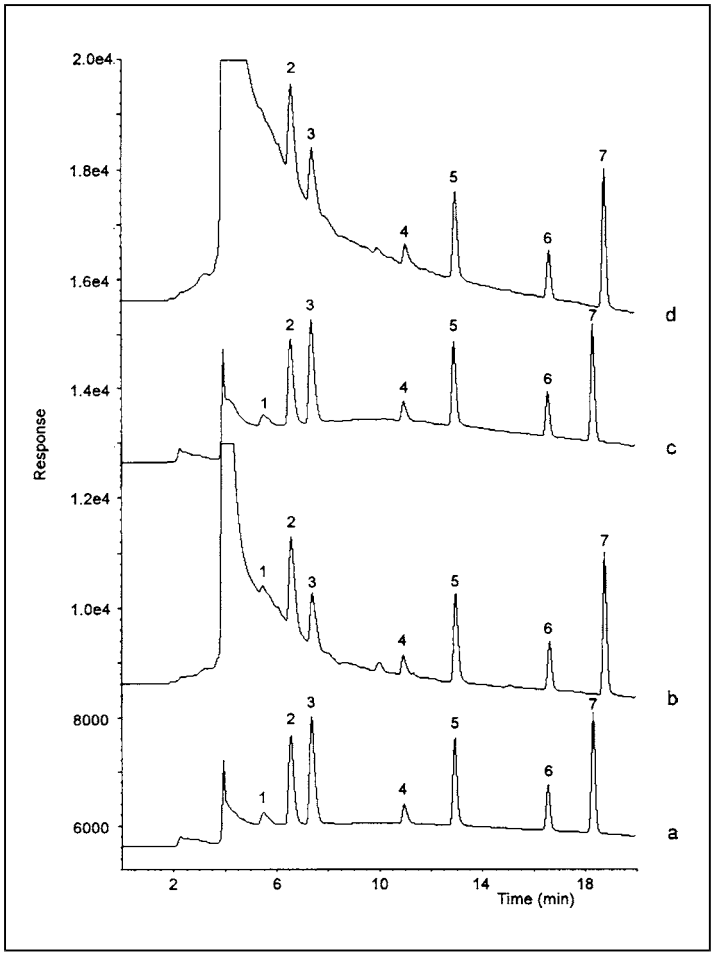

Fig. 4. Chromatograms obtained after on-line trace enrichment of 8 ml tap water spiked with 12.5 µg l^{-1} of each analyte with or without addition of 250 µl sulphite solution: (a) with sulphite, (b) without sulphite, (c) with sulphite and addition of 10 mg l^{-1} reference humic acids, and (d) without sulphite and addition of 10 mg l^{-1} reference humic acids. Peak designation: 1, resorcinol; others as for Fig. 2.

For resorcinol, the peak of which is completely obscured by matrix peak interferences when 8 ml tap water is preconcentrated, it was found that after addition of 250 µl 10% Na_2SO_3 solution the peak could be detected with the same

recovery (29%) as when Milli-Q quality water is used. When tap water sample was spiked with 10 mg l^{-1} reference humic acid solution it was observed that the treatment with 250 µl sulphite was still effective and there was no significant decrease in the recovery of resorcinol. The chromatograms obtained in this study are compared in Fig. 4 and demonstrate that the treatment enables the detection, without interferences, of very polar compounds usually covered by the hump of humic substance.

Relationship Between the Concentration of Humic Substances and the Amount of Na_2SO_3 Added

This study was performed to determine the amount of 10% Na_2SO_3 solution appropriate for a specific concentration of humic substances in the sample. Two different reference humic acids, M_r ~600-1000 and M_r ~5000-10000, were dissolved in Milli-Q quality water at 500 mg l^{-1} (250 mg l^{-1} of each) and the solution was diluted to 5 and 10 mg l^{-1}. These simulated surface water samples were spiked with 2 µg l^{-1} of the analytes and 50 ml was preconcentrated on the chemically modified resin. The matrix interference in the resulting chromatograms was similar to that from real water samples. Different volumes of 10% Na_2SO_3 solution (150, 250 and 500 µl) were added to reduce the humic acid band. Addition of 150 µl sulphite solution was sufficient for water samples containing 5 mg l^{-1} of humic acids; 250 µl was needed for water samples containing 10 mg l^{-1} of humic acids.

As has been mentioned above, 250 µl sulphite solution was sufficient to reduce the initial humic hump in the analysis of 50 ml of tap water. These results agree with those obtained for the simulated surface waters (250 µl for 10 mg l^{-1} humic acids, the maximum concentration found in real water). When 50 ml Ebro river water was analyzed, however, 500 µl of this solution was necessary. This might

be because the dissolved organic matter is made up of more than just humic and fulvic acids [25,26].

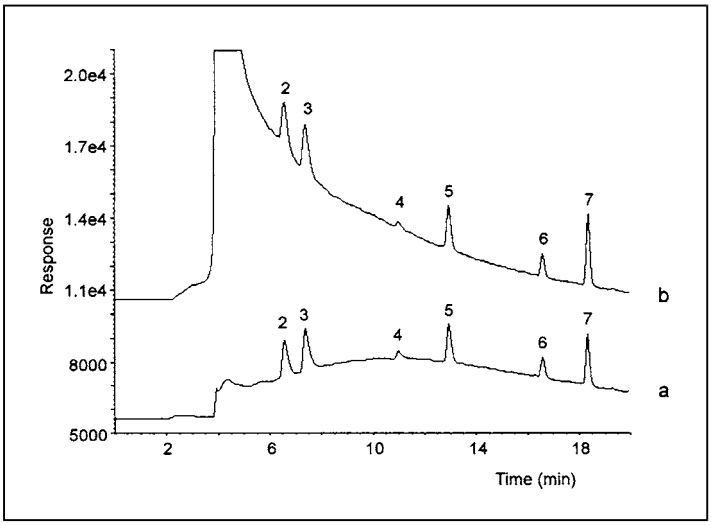

Fig. 5. Chromatograms obtained after on-line trace enrichment of 50 ml Milli-Q water spiked with 5 mg l^{-1} humic acid solution and 2 µg l^{-1} each analyte: (a) with addition of 150 µl sulphite solution and (b) without sulphite addition. Peak designation as for Fig. 2.

It seems that addition of sulphite reduces the matrix interference which appears at the beginning of the chromatogram but does not reduce interference from humic acids which elute later, because a small hump still appears in the middle of the chromatogram. This phenomenon is shown in Fig. 5, and might be because the last-eluted humic acids have fewer carbonyl groups than the humic acids which form the frontal hump. It should be pointed out that, fortunately, the hump of humic substances in the middle of the chromatogram did not appear after analysis of any of the real water samples, whereas the frontal hump appeared in all the samples.

CONCLUSIONS

This study has demonstrated that addition of sodium sulphite is the best method for removing the humic substance band which can make the early-eluted polar analytes difficult to determine. This method could be used for determination, by HPLC with low selectivity UV detection, of polar pesticides and phenolic compounds preconcentrated on a sorbent of high sorption power (chemically modified polymeric resin).

The appropriate volume of sulphite solution depends on the concentration of humic substances dissolved in the sample. When preconcentrating 50 ml tap water, it was necessary to add 250 µl sulphite solution; when the same volume of Ebro river water was analyzed, however, 500 µl of sulphite solution had to be added.

The recovery values obtained after chemical treatment of environmental water samples with sulphite were similar to those obtained when a Milli-Q water standard was analyzed, except for phenol and bentazone, the recoveries of which were lower when the amount of sulphite added was increased.

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