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Micro-columns packed with *Chlorella vulgaris* immobilised on silica gel for mercury speciation

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Abstract

A method has been developed for mercury speciation in water by using columns packed with *Chlorella vulgaris* immobilised on silica gel. The method involves the retention of CH_3Hg^+ and Hg^{2+} in micro-columns prepared by packing immobilised algae in polypropylene tubes, followed by selective and sequential elution with 0.03 and 1.5 M HCl for CH_3Hg^+ and Hg^{2+}, respectively. The adsorption capacity of the micro-algae for Hg^{2+} and CH_3Hg^+ has been evaluated using free and immobilised *C. vulgaris*. The efficiency uptake for both species at pH 3 was higher than 97%. Studies were carried out on the effect of retention and elution conditions for both species. Furthermore, the stability of mercury species retained on algae-silica gel micro-columns and lifetime of the columns were also investigated. Hg^{2+} showed a higher stability than CH_3Hg^+ at 0 °C (21 and 3 days, respectively) and a better lifetime than for the organic species.

The developed method was applied to the analysis of spiked tap, sea and wastewater samples. Recovery studies on tap and filtered seawater provided results between 96 ± 3 and 106 ± 2 for Hg^{2+} and from 98 ± 5 to 107 ± 5 for CH_3Hg^+, for samples spiked with single species. For samples spiked with both CH_3Hg^+ and Hg^{2+}, the average recoveries varied from 96 ± 5 to 99 ± 3 and from 103 ± 6 to 115 ± 5 for Hg^{2+} and CH_3Hg^+, respectively. However, the percentages of retention and elution on wastewater and unfiltered seawater were only adequate for the inorganic species.

Keywords: Hg; Speciation; *Chlorella vulgaris*; Silica

1. Introduction

Mercury is not an essential element for plant or animal life and it is a potential environmental toxic because of its tendency to form covalent bonds with organic molecules and the high stability of the Hg–C bond. CH_3Hg^+ and Hg^{2+} are the most significant species of mercury in aquatic medium and CH_3Hg^+ can be naturally produced from Hg^{2+} by sulphate reducing-agent bacteria. Moreover, CH_3Hg^+ is more harmful than Hg^{2+} due to its ready diffusion through biological membranes. These facts have impelled the development of methods for mercury speciation in water. To determine the different mercury species (methyl mercury, phenyl mercury, ethyl mercury, etc.) hyphenated techniques has been widely used. These systems are based on the use of a powerful separation technique (gas chromatography (GC), high performance liquid chromatography (HPLC), capillary electrophoresis (CE)) coupled to a sensitive atomic detector [1]. However, taking into account that 95% of the organic mercury in the environment is present as CH_3Hg^+, it is important to develop easy analytical procedures to selectively determine CH_3Hg^+ and Hg^{2+}. In this way, a simple alternative is the use of biological substrates such as algae, bacteria, yeast and fungi.

The wide spectrum of potential interactions between metal ions and biological substrates make them selective adsorbents for metal species. So, bio-sorption has been largely employed for trace metal accumulation and evaluated as a potential speciation and preconcentration method [2]. However, only a few microorganisms have been used for metal speciation: yeast as *Saccharomyces cerevisiae* (Hg [3–5], Cr [6,7], As [8], Sb and Se [9]); bacteria as *Pseudomonas putida* (Se [10], Se-cystamine [11], Se-methionine and Se-urea [12], Hg(II) and Hg(I) [13,14]), *Escherichia coli* (Se [10], Hg(II) and Hg(I) [13,14]), *Spirulina platensis* (Se [15], Cr and Sb [16]), and fungus as *P. purpurogenum* (As(III), Hg(II), Cd(II) and Pb(II) [17]) and *P. chrysosporium* (Hg(II) [18,19]).

Unfortunately, microorganisms are formed by small particles with poor mechanical strength and rigidity. Therefore, the use of

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native biomass is not practical being necessary to carry out the microorganism immobilisation in or on a solid support. But, the immobilisation procedure could affect the adsorption capacity of microorganisms. So, it is necessary to evaluate the immobilised biomass properties [20].

On the other hand, the speciation in water samples still presents several difficulties related to losses or species transformations during sampling, handling and storage stages. Thus, alternative sampling and handling procedures are interesting for speciation analysis [21]. In this way, sampling methods based on "in situ" species separation using solid adsorbents packed in micro-columns have been developed (Amberlite [22], polymers [23], diatomeous earth [24], C18 [25], sulphidryl cotton fibre [26], silica gel [27] or alumina [28]). Moreover, "in situ" analyte elution after sampling for unstable species can be realized [29].

The aim of the paper is to develop a method for Hg^{2+} and CH_3Hg^+ speciation by using columns packed with a biological substrate (*Chlorella vulgaris*) immobilised on a solid support (silica gel). The algae was chosen due to it can be grown in large quantities with relative ease and it presents a simple handling. Silica gel was chosen because of its large specific surface ($675\ m^2\ g^{-1}$) and homogeneous porous surface. Several analytical applications (sampling of different water and the storage of adsorbed species) were also evaluated.

2. Experimental

2.1. Reagents

A $1000\ mg\ L^{-1}$ Hg^{2+} stock standard solution (Panreac) was used. A $1000\ mg\ L^{-1}$ CH_3Hg^+ stock standard solution was prepared by dissolving the suitable amount of CH_3HgCl (Aldrich) in Milli-Q water. Working standard solutions were prepared daily and a higher concentration for CH_3Hg^+ ($100\ \mu g\ L^{-1}$) than for Hg^{2+} ($20\ \mu g\ L^{-1}$) was used in the different experiments. This choice was realised due to the cold vapour formation is lesser effective from CH_3Hg^+ than from Hg^{2+}. Furthermore, the CH_3Hg^+ solutions were stored away from light to prevent decomposition.

A 0.2% (w/v) sodium tetrahydroborate(III) solution was daily prepared by dissolving $NaBH_4$ powder (Merck) in Milli-Q water, stabilising in 0.05% (w/v) NaOH (Panreac) and filtering. Hydrochloric acid solution (3%) was prepared by appropriate dilution of 37% (v/v) hydrochloric acid (max. 0.0000005% of Hg, Panreac).

Silica gel Merck grade, 35–70 meshes, particle size 40 Å (Aldrich) was employed.

All chemical reagents were of analytical reagent grade and deionised water was obtained using a Milli-Q System (Millipore).

2.2. Culture conditions

C. vulgaris biomass was supplied and characterised by Microbiology Department from the University of A Coruña. The biomass cultures were grown in an aquarium in glass bottles of 1 L, with airflow of $10\ L\ h^{-1}$, at a constant temperature of 22 °C and the cultures were illuminated for 12 h every day.

The algae were harvested in a medium with a mixture of micro and macronutrient solutions in the ratio of 3 mL (micro) per litre (macro). The macronutrient solution composition ($mg\ L^{-1}$) was: $NaNO_3$ 1000; K_2HPO_4 75; KH_2PO_4 175; $MgSO_4{\cdot}7H_2O$ 750; NaCl 250; $CaCl_2$ 196. Micronutrient solution composition ($mg\ L^{-1}$) was: H_3BO_3 186; $MnCl_2$ 415; $ZnCl_2$ 3.2; $CuCl_2$ 11.4×10^{-3}; $CoCl_2$ 1.3; $FeCl_3$ 159.9; $Na_2MoO_4{\cdot}2H_2O$ 7.2; $Na_2EDTA{\cdot}2H_2O$ 300. The nutrient solutions were added to the cultures fortnightly.

Cell density is an important parameter, which affects the percentage retention. Ageing and growth of algae have been regulated by the feeding frequency and culture conditions. The optimum value for mercury species retention was in the range from 5th to 10th growth day (10 mL of culture are equivalent to 1 mg of dry weight). The media and all materials used were sterilised by autoclaving at 120 °C.

2.3. Instrumentation

A Perkin-Elmer Model AAnalyst 800 Atomic Absorption Spectrometer coupled to a flow injection system (FIAS 400) equipped with two multichannel-peristaltic pumps, a five port-valve with a PTFE-reaction coil (500 μL) and a mixture manifold coupled to a membrane gas–liquid separator was employed for mercury species determination. An electrode less lamp operating at 170 mA was used and a spectral bandwidth of 0.7 nm was selected to isolate the 253.7 nm mercury line.

Mercury cold vapour was generated in hydrochloric acid medium (3% v/v) using 0.2% (w/v) $NaBH_4$ as reducing agent and it was carried by an argon stream ($50\ mL\ min^{-1}$) to the atomisation cell. The analytical measurement was based on peak height and three replicate determinations were realised each time.

An Eppendorf centrifuge Model 5804 and a solid phase extraction system (Supelco) coupled to a Millipore vacuum pump was employed.

2.4. Analytical procedure

2.4.1. Immobilisation procedure

The algae cells were immobilised using a method developed by Mahan and Holcombe [30]. Silica gel (0.2 g) was washed twice with 10 mL of 0.2 N HCl for 10 min with continuous stirring. The acid solution was discarded and the silica was washed using 10 mL of deionised water to eliminate the acid medium. A 20 mL of *C. vulgaris* (2 mg of dry weight) were centrifuged at $2000 \times g$ for 5 min. The supernatant was discarded and the wet algae biomass was homogeneously mixed with the silica gel. The resulting paste was heated in an oven at 70 °C for 4 h. Afterwards the mixture was wetted with 2 mL of deionised water and mixed until homogeneous. Then it was heated at 70 °C until it was completely dry. The silica-algae briquette obtained was cooled and gently broken to retrieve the original particles size.

To check the effect of the support (silica gel) on mercury species retention, a treated silica gel without biomass (acid-washed silica gel dried at 70 °C using the same procedure mentioned above) was prepared.

2.4.2. Batch procedure

A 20 mL of *C. vulgaris* were centrifuged at 2000 × *g* for 5 min, the supernatant was discarded and the centrifuged alga biomass was collected. The standard solutions of mercury species (10 mL) were added to the collected biomass. They were in contact at the optimum pH for 5 min with continuous stirring. The supernatant was separated from the algae by filtration (filter paper Whatman 41) and the species in it were measured using flow injection analysis-cold vapour atomic absorption spectrometry (FIA-CVAAS). The assay and analytical measurements were realised by triplicate.

To study the effect of the *C. vulgaris* immobilisation and the treated silica gel on the retention of mercury species the same procedure mentioned above was employed.

2.4.3. Column procedure

The adequate adsorbent amount (0.5 g of treated silica gel or *C. vulgaris* immobilised on silica gel) was packed in a polypropylene micro-column (6.5 cm × 0.9 cm i.d.) with a porous polyethylene frit. Afterwards, the columns were coupled to a solid phase extraction system with an air vacuum pump.

Before running the samples, the columns were washed with 10 mL of deionised water in order to eliminate non-immobilised biomass particles, which could interfere in the measurement. Then, 10 mL of working solution containing Hg^{2+} (20 μg L^{-1}) and/or CH_3Hg^+ (100 μg L^{-1}) were passed through the column at a flow rate of 2 mL min^{-1}. The mercury concentration in the eluate was measured by FIA-CVAAS to calculate the retention.

The selective elution of the retained CH_3Hg^+ and Hg^{2+} was carried out sequentially with 10 mL of 0.03 and 1.5 M HCl, respectively. The mercury species concentrations were determined by FIA-CVAAS.

The assay and analytical measurements were realised by triplicate.

3. Results and discussion

3.1. Study of CH_3Hg^+ and Hg^{2+} retention on a batch system

The batch procedure described above was used to establish the optimal conditions of accumulation by the algae (free and immobilised) and the effect of the support (treated silica gel) on CH_3Hg^+ and Hg^{2+} retention.

3.1.1. Effect of pH on species retention

Retention of ionic species is highly dependent on pH, due to the surface charge of the adsorbent (silica gel or biomass) and the ionisation degree of the analyte are conditioned by the solution pH. The influence of pH on mercury species retention was assessed in the range 2–12 for each case. In Fig. 1, the retention of CH_3Hg^+ and Hg^{2+} by silica gel and *C. vulgaris* is shown. As it can be seen, the species retention on silica gel was dominated by the protonation of silanol superficial groups (pK_a of silanol groups is between 4 and 5 [31]). So, the adsorption of mercury species decreased for $pH < pK_a$ due to silanol groups are protonated, and the retention was negligible and 18 ± 2% for CH_3Hg^+ and Hg^{2+}, respectively at pH 3.

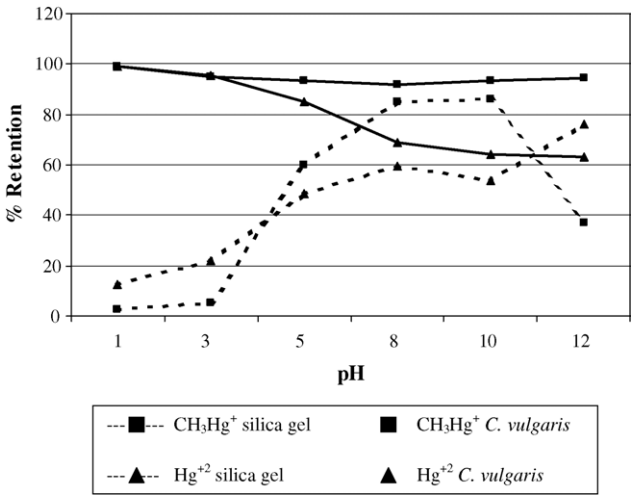

Fig. 1. Effect of pH on Hg^{2+} and CH_3Hg^+ retention by *C. vulgaris* or silica gel.

On the other hand, it has been found that the retention of CH_3Hg^+ on *C. vulgaris* was quantitative at the pH-range studied; whereas the retention of Hg^{2+} decreased at basic pH and reached values higher than 93% at pH ≤ 3. This behaviour could be explained because of the organic moiety of CH_3Hg^+ allows bioaccumulation by passive diffusion into the cell independently of the pH. However, in the case of Hg^{2+} there is the possibility of different bioaccumulation mechanisms (i.e. passive diffusion, complexation or adsorption to the cell wall).

It was observed that with pH 3 there were not significant adsorption of mercury species by silica gel and quantitative retention of CH_3Hg^+ and Hg^{2+} by *C. vulgaris* was achieved. In this way, the algae immobilised on silica gel may be used for the retention of mercury species, being the biomass responsible for the uptake.

3.1.2. Effect of C. vulgaris immobilisation on species retention

Algae immobilisation on silica gel is based on an adsorption process, which requires a dry step of the biomass-support mixture. The temperature can produce variations on the algae structure changing the retention of mercury species. Thus, the effect of drying temperature on the retention of CH_3Hg^+ and Hg^{2+} by *C. vulgaris* was investigated.

For this study, the batch procedure using biomass dried at different temperatures (25–110 °C) and pH 3 was performed. As it can be seen in Fig. 2, the retention for both species decreased with drying temperature especially between 70 and 110 °C (from 96 ± 3% to 81 ± 2% for Hg^{2+} and from 85 ± 3% to 74 ± 3% for CH_3Hg^+). This fact is probably due to biomass was charring

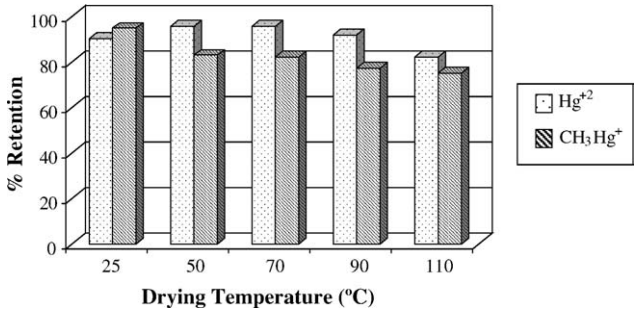

Fig. 2. Effect of the immobilisation temperature on Hg^{2+} and CH_3Hg^+ retention.

and a shrinking of cell membrane was produced. So, 70 °C was chosen as the optimum drying temperature for the algae immobilisation.

Before packing the immobilised algae on columns, a study of the species retention by *C. vulgaris* immobilised on silica gel was realised. For this assay, 0.5 g of immobilised biomass and pH 3 were employed. The results had shown a quantitative retention of CH_3Hg^+ ($99 \pm 3\%$) and Hg^{2+} ($101 \pm 1\%$). The same study using 0.5 g of support (acid-washed silica gel dried at 70 °C) was carried out and negligible CH_3Hg^+ ($0 \pm 5\%$) and $8 \pm 9\%$ Hg^{2+} were retained.

3.2. Speciation study using a column system

Uptake and elution processes are governed by different factors (pH, flow rate, sample volume, analyte concentration, elution volume and sample matrix). To check the effect of the experimental conditions, separate solutions containing CH_3Hg^+ and Hg^{2+} were passed through the columns and the column procedure described above was carried out. Once the retention and elution procedure was optimised, mixtures of CH_3Hg^+ and Hg^{2+} were used.

3.2.1. Effect of flow rate on species retention

It is important to choose a flow rate that ensures an adequate mercury species uptake. So, the influence of flow rate on species retention was studied between 2 and 10 mL min^{-1}. Although flow rate did not affect the species retention, it was observed that standard deviation (S.D.) values increases at flow rate higher than 2 mL min^{-1} (from $98 \pm 1\%$ to $94 \pm 6\%$ for Hg^{2+} and from $99 \pm 1\%$ to $96 \pm 12\%$ for CH_3Hg^+ with 2 and 10 mL min^{-1}, respectively). Thus, sample flow rate of 2 mL min^{-1} was chosen as appropriate in the retention processes.

To check the effect of silica gel on mercury species retention, columns filled only with silica gel were also tested. Analysis of the eluates showed that Hg^{2+} and CH_3Hg^+ remained in the solution.

3.2.2. Selective species elution

Since the selective retention of both mercury species was not possible, the separation of Hg^{2+} and CH_3Hg^+ through selective elution was investigated. Several mercury complex agents (e.g. Cl^-, NO_3^-, CN^-, EDTA, $S_2O_3^{2-}$, cysteine, thiourea, etc.) can be used for species elution. However, some of them are adsorbed on silica gel surface, which get damage the immobilised adsorbent and produces non-reproducible values when recycling column was used. Due to the high affinity of hydrochloric acid by mercury species and its low interaction with silica, it was chosen as eluent.

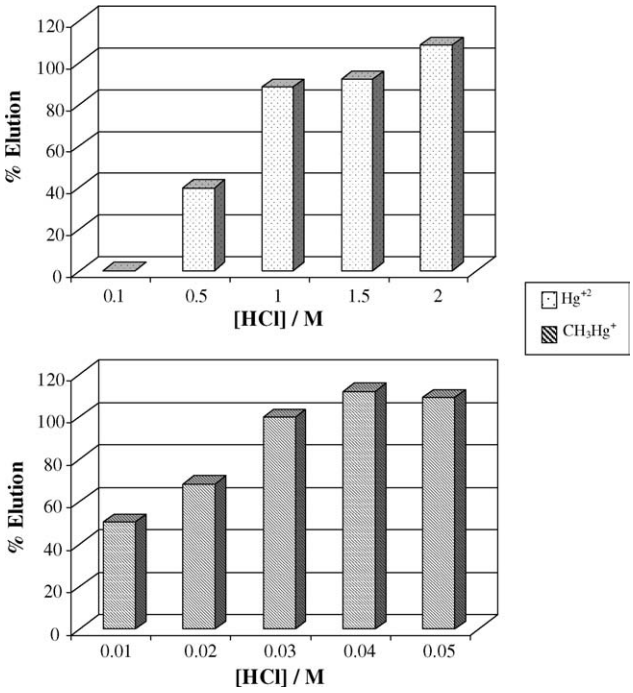

Fig. 3. Influence of the HCl concentration on Hg^{2+} and CH_3Hg^+ recovery.

To check the influence of the HCl concentration on Hg^{2+} and CH_3Hg^+ elution, different concentrations of the HCl solutions (10 mL) were tested for each case. For this study, solutions of 10 mL containing either Hg^{2+} or CH_3Hg^+ were passed through the columns at the optima conditions and the elution was carried out. As it can be seen in Fig. 3, the use of 0.03 M HCl allowed the selective elution of CH_3Hg^+ in the presence of Hg^{2+}. The quantitative elution of Hg^{2+} was only possible with HCl concentrations higher than 1.5 M. The recovery (% elution) was calculated for Hg^{2+} or CH_3Hg^+ retained. A 2 M HCl solution was tested as a blank for the mercury determination by FIA-CVAAS. The signal obtained was negligible.

The next step was to determine the minimum volume of eluents needed to release Hg^{2+} and CH_3Hg^+ quantitatively from the columns. Different volumes of 0.03 M HCl for CH_3Hg^+ elution and 1.5 M HCl for Hg^{2+} elution were evaluated. The results are shown in Fig. 4. The retained organic species was quantitatively eluted with 10 mL of acid and it was observed that the percentage of elution decreased with volumes lower than 10 mL. The elution of Hg^{2+} was quantitative with 4 mL of 1.5 M HCl (recovery of $90 \pm 3\%$).

In order to check the suitability of the method, 10 mL of working solutions containing mixtures of CH_3Hg^+ and Hg^{2+} were passed through the columns at the optima conditions. Results had shown that both species were retained up to the same extent than the separated ones ($95 \pm 3\%$ and $96 \pm 2\%$ for CH_3Hg^+ and Hg^{2+}, respectively). Afterwards, the sequential elution of

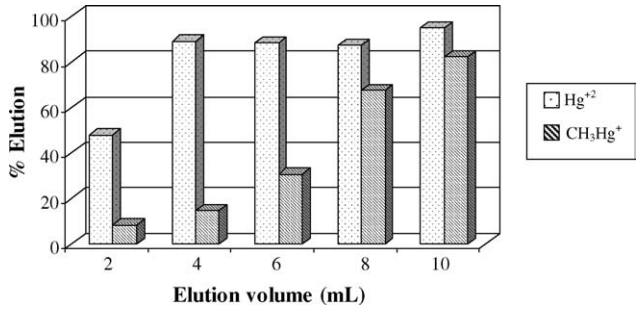

Fig. 4. Effect of the elution volume on Hg^{2+} and CH_3Hg^+ recovery.

CH_3Hg^+ and Hg^{2+} was carried out by passing through the column 10 mL of 0.03 M HCl and 4 mL of 1.5 M HCl. The elution yields were 95 ± 8% for CH_3Hg^+ and 91 ± 5% for Hg^{2+}.

3.2.3. Effect of sample volume on species retention: preconcentration factor

There is a maximum volume (breakthrough volume), which can be used in solid phase extraction. This parameter depends on the affinity of the adsorbent by the analyte.

To study the influence of sample volume on species retention, a constant total amount of analyte (2000 ng Hg^{2+} or 5000 ng CH_3Hg^+) in different volumes (10–500 mL) was passed through the columns at the optima pH and flow rate. Fig. 5 shows that for higher volumes than 10 mL a sharply decrease on CH_3Hg^+ retention is observed (97 ± 3% for 10 mL and 74 ± 4% for 25 mL). This fact is probably due to a washing effect for large sample volume was produced. However, the amount of Hg^{2+} retained in the columns remains constant up to a volume of 300 mL.

Considering the minimum elution volumes (see previous section), the preconcentration factor was 75 for Hg^{2+} and preconcentration of CH_3Hg^+ was not achieved.

3.2.4. Evaluation of the adsorption capacity of immobilised algae

The rate of Hg^{2+} and CH_3Hg^+ uptake by immobilised *C. vulgaris* is an equilibrium process, which becomes gradually saturated, and it is highly dependent on surface-active sites. The retention capacity of the adsorbent was investigated by using 10 mL of solutions at different Hg^{2+} or CH_3Hg^+ concentrations. As it can be seen (Table 1) a 10-fold increase of Hg^{2+} concentration did not produce a significant variation on the retention, and for higher concentrations than 200 μg L^{-1} a sharp decrease was observed. The retention of CH_3Hg^+ remains quantitative up to 300 μg L^{-1}. However, it was observed that the total amount of retained analyte appeared to be increased with the concentration of mercury species. The saturation on the retention of Hg^{2+} was reached at 6.6 μg Hg (g adsorbent)$^{-1}$.

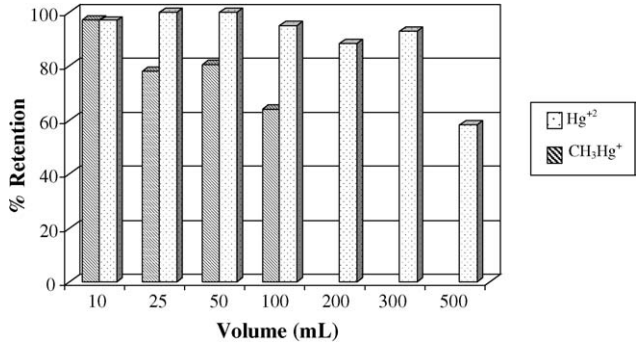

Fig. 5. Influence of sample volume on uptake of Hg^{2+} and CH_3Hg^+ by *C. vulgaris* immobilised on silica gel.

Table 1
Retention capacity of immobilised *C. vulgaris*

	% Adsorption	(μg Hg retained/g adsorbent)
$[Hg^{2+}]_0$ (μg L^{-1})		
20	97 ± 2	0.39 ± 0.01
50	97 ± 1	0.97 ± 0.01
100	97 ± 3	1.94 ± 0.06
200	91 ± 2	3.64 ± 0.08
500	66 ± 4	6.60 ± 0.40
1000	33 ± 3	6.60 ± 0.60
$[CH_3Hg^+]_0$ (μg L^{-1})		
100	98 ± 1	1.82 ± 0.02
200	95 ± 1	3.51 ± 0.07
300	90 ± 3	5.00 ± 0.11
400	88 ± 2	6.51 ± 0.22
1000	75 ± 3	13.90 ± 0.56
2500	73 ± 4	34.10 ± 1.88

3.2.5. Study of column reutilization

Lifetime of the columns was investigated. Three different columns were used for each mercury species and the retention–elution processes were consecutively carried out. Between the working cycles a column-washing step with 10 mL deionised water at 2 mL min^{-1} was performed.

For inorganic mercury better results than for CH_3Hg^+ were observed. The retention and elution of Hg^{2+} was quantitative up to five working cycles (retention of 98 ± 2% and 98 ± 3% and elution of 97 ± 4% and 101 ± 10% for the first and the fifth cycle, respectively). Afterwards, the columns could not be used due to the biomass was dragged from the support.

However, the retention and elution of CH_3Hg^+ was only quantitative up to two working cycles and a strong variation for the third cycle was observed (retention of 97 ± 3% and 65 ± 6% and elution of 99 ± 6% and 148 ± 14% for the first and the third cycle, respectively).

3.2.6. Influence of other ions on mercury species retention

The effect of other metal ions on the retention and elution of the mercury species was investigated. For this purpose, the following ions: Al^{3+}, Ca^{2+}, Cd^{2+}, Co^{2+}, Cu^{2+}, Fe^{3+}, Mg^{2+}, Mn^{2+}, Ni^{2+}, Pb^{2+} and Zn^{2+} as NO_3^-, K^+ and Na^+ as Cl^-, Se^{6+} as SeO_3^{2-}, V^{5+} as VO_3^- and Cr^{6+} as $Cr_2O_7^{2-}$ were added individually to the working solution at 1000 or 100 μg L^{-1} (for Hg^{2+} and CH_3Hg^+ studies, respectively) and the procedure was carried out. The results obtained were compared using a *t*-test (95% confidence level) to those found when the procedure was realised without ions in the solution.

A significant decrease on the elution recovery of Hg^{2+} was observed with the presence of V^{5+}, and the others

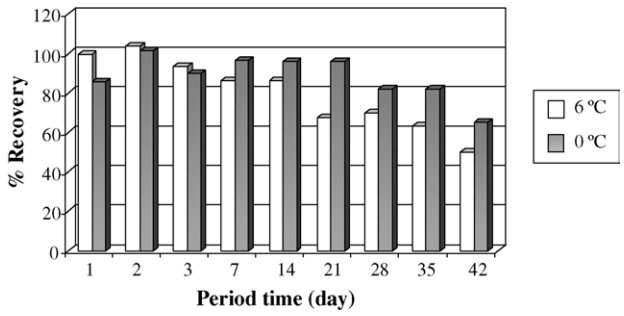

Fig. 6. Stability of Hg^{2+} on the column as function of time and temperature.

aforementioned ions did not produced any variation on the retention and elution yield at 95% confidence level. However, the retention of the organic species was significantly different when Al^{3+}, Zn^{2+} and $SeO_3{}^{2-}$ were added. Furthermore, it was observed that the elution of CH_3Hg^+ was interfered by Cd^{2+}, Mn^{2+} and Zn^{2+}. The variation on the retention and/or elution of CH_3Hg^+ could be explained by competition for the superficial active sites of the adsorbent.

On the other hand, the influence of the mentioned ions on the mercury species determination by FIA-CVAAS was also investigated. For this purpose, the standards solutions of calibrate were spiked with the ions at 1000 or 100 μg L^{-1} for Hg^{2+} and CH_3Hg^+ studies, respectively. The results had shown a slight and reproducible decrease on CH_3Hg^+ signal (approximately 30%) when Al^{3+} and $SeO_3{}^{2-}$ were added.

3.3. Applications

3.3.1. Study of species stability

After loading the species on the column it may be advantageous store them for extend periods of time. Therefore, the stability of the mercury species loaded on the columns was investigated under different storage conditions. Three columns were employed for each time, temperature and mercury species studied. Different columns were loaded with Hg^{2+} or CH_3Hg^+ solutions and their elution was carried out 1–3, 7, 14, 21, 28, 35 and 42 days after storage in different temperature conditions. The results are given in Fig. 6 for Hg^{2+} and Fig. 7 for CH_3Hg^+. As it can be seen, Hg^{2+} had a high stability at 0 °C being the recovery quantitative after 21 days. However, the recovery was only quantitative up to the third day at 6 °C, and it was decreasing gradually for higher storage periods of time than 3 days. The

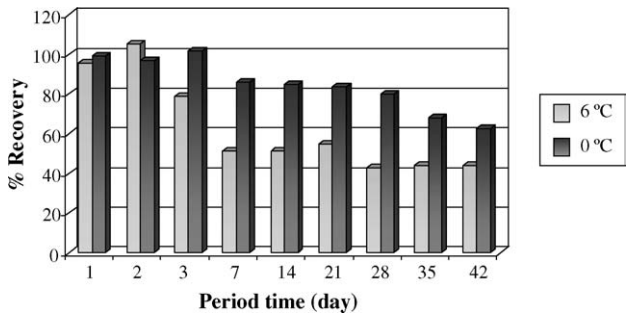

Fig. 7. Stability of CH_3Hg^+ on the column as function of time and temperature.

stability of methyl mercury was considerably lower than for the inorganic ones. Thus, the recovery was quantitative after 2 and 3 days of storage at 6 and 0 °C, respectively.

The main processes responsible of mercury species losses are decomposition, volatilisation and adsorption on the wall of the columns. To quantify the later process, the columns used for the study of 42 days after storage were washed with 10 mL of 7% HNO_3 to desorbs species from the wall of the columns. Then, Hg^{2+} or CH_3Hg^+ was measured in the washing solutions. The results showed that the adsorption of inorganic species to the wall of the column was not produced, while 30 ± 7% and 19 ± 3% of total CH_3Hg^+ at 6 and 0 °C, respectively was adsorbed to the wall.

3.3.2. Application of speciation method to real water samples

It is very well known that the use of substrates for metallic species retention is highly dependent on sample matrix. In order to ascertain the analytical possibilities of the proposed method for real samples unpolluted water samples (tap water, seawater and industrial discharge water) were spiked with Hg^{2+} and/or CH_3Hg^+ and analysed following the proposed method.

To collect the water samples bottles of 1 L (previously washed for 24 h with 10% HNO_3 and thoroughly rinsed three times with deionised water) were used. The container was rinsed with the water sample and the sampling was performed. Preserving agent was not added due to samples were processed within 1 h. The mercury speciation method was carried out using filtered and unfiltered water samples (five replicates in every cases). To filter the samples Millipore 45 μm filters were used. Furthermore, filtered water sample blanks at pH 3 were analysed and signal from mercury species was not detected.

The retention and elution yields were quantitatively determined by using calibrate prepared with the sample matrix in each case. Observed percentage of retention and elution are given in Table 2. The retention of Hg^{2+} did not significantly change with the sample matrix, while the retention of CH_3Hg^+ was strongly affected by the complexity of the matrix (industrial wastewater and unfiltered seawater). This could be explained by the presence of organic matter, which can form stable complexes with CH_3Hg^+.

On the other hand, the recovery obtained in the elution process depends on the complexity of the matrix. So, CH_3Hg^+ elution yields for wastewater were higher than 100% (it could be due to complexation with matrix components, which can vary cold vapour formation) and the elution of Hg^{2+} was slightly decreased in this kind of samples. So, the proposed method only allowed mercury speciation for tap water and filtered seawater.

3.3.3. Analytical characteristics of the method

The analytical characteristics of the developed method for CH_3Hg^+ and Hg^{2+} speciation in aqueous solutions were evaluated. The analytical parameters have been calculated in deionised water, tap water and filtered seawater due to the retention and elution of mercury species in these matrixes were not affected by strong interferences.

Table 2
Influence of sample matrix in uptake and elution yields

Sample	Added ($\mu g\,L^{-1}$)		Uptake (%)		Elution (%)	
	Hg^{2+}	CH_3Hg^+	Hg^{2+}	CH_3Hg^+	Hg^{2+}	CH_3Hg^+
Deionised water	20	0	98 ± 2		97 ± 4	
	0	100		97 ± 3		99 ± 6
	20	100	96 ± 2	95 ± 3	91 ± 5	95 ± 8
Tap water	20	0	99 ± 2		106 ± 2	
	0	100		90 ± 2		107 ± 5
	20	100	97 ± 2	90 ± 2	99 ± 3	115 ± 5
Filtered seawater	20	0	99 ± 2		96 ± 3	
	0	100		84 ± 6		98 ± 5
	20	100	99 ± 2	84 ± 6	96 ± 5	103 ± 6
Unfiltered seawater	20	0	99 ± 2		90 ± 2	
	0	100		50 ± 4		103 ± 2
	20	100	99 ± 2	42 ± 10	108 ± 8	131 ± 20
Filtered wastewater	20	0	96 ± 3		83 ± 4	
	0	100		66 ± 9		108 ± 4
	20	100	96 ± 3	66 ± 9	73 ± 4	119 ± 6
Unfiltered wastewater	20	0	87 ± 3		76 ± 13	
	0	100		78 ± 6		211 ± 15
	20	100	87 ± 3	78 ± 6	82 ± 10	168 ± 11

The limits of detection were calculated using procedural blanks prepared by performing the retention–elution processes with each water sample. The limits of detection achieved (estimated by 3S.D./*b*, where S.D. is the standard deviation of 10 measurements of the blank and *b* is the slope of the calibration line) were 0.5, 0.5 and 1 $\mu g\,L^{-1}$ for Hg^{2+} and 2.0, 2.0 and 4.0 $\mu g\,L^{-1}$ for CH_3Hg^+ in deionised, tap and filtered seawater, respectively.

In order to evaluate the recovery of the mercury species and the precision of the method, different water samples (deionised water, tap water and filtered seawater) were employed. For each case, five columns were loaded with 10 mL of water sample spiked with 100 and/or 20 $\mu g\,L^{-1}$ of CH_3Hg^+ and Hg^{2+}, respectively. Afterwards, the elution procedure was carried out. The recoveries and standard deviations (S.D.) for both species were adequate (see Table 2).

4. Conclusions

This study shows that *C. vulgaris* immobilised on silica gel allows the retention of CH_3Hg^+ and Hg^{2+} from water samples and their sequential elution and determination. So, the method described provides a simple and inexpensive way for mercury speciation. A drawback of the procedure is that it requires large elution volumes to quantitatively remove the previously retained CH_3Hg^+. Furthermore, the maximum volume that can be run through the column without any decrease in recovery of CH_3Hg^+ was 10 mL. So, the organic species cannot be concentrated. Pérez-Corona et al. had used another substrate (*S. cerevisiae*) free [3] and immobilised on silica gel for Hg speciation [4,5] and the preconcentration of both mercury species was obtained. The proposed method presents the advantage that Hg^{2+} is more stable since it remains unaltered for 21 days at 0 °C, while with the method using *S. cerevisiae* the maximum period of time of 1 week at −20, 4 and 20 °C was achieved. This difference may be due to the inorganic mercury is bound to the support in the latter method and it could be transformed by the substrate during storage. However, *S. cerevisiae* is a medium more adequate than *C. vulgaris* to stabilize CH_3Hg^+ when long periods of storage are required before analysis. These results are of great interest since the use of these micro-columns allows the storage of mercury species until analysis, avoiding the problem associated with maintaining species integrity in aqueous solution. This fact makes *C. vulgaris* immobilised on silica gel as a promising alternative to conventional water sampling for mercury species analysis. Furthermore, the half-life of the column (five and two working cycles for Hg^{2+} and CH_3Hg^+, respectively) is poor, but this adsorbent presents the advantage of a very low cost.

On the other hand, the inconvenience of the method for real sample is that the complexity of the water matrix affects the retention of CH_3Hg^+ and the elution of both species. So, an important problem has emerged for wastewater and unfiltered seawater, which requires further careful study about the effect of the dissolved organic matter on the retention and elution processes. So, the developed method can be applied to mercury speciation of tap and filtered seawater, and to the analysis of inorganic mercury for unfiltered seawater and filtered wastewater.

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