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Determination of thimerosal in pharmaceutical industry effluents and river waters by HPLC coupled to atomic fluorescence spectrometry through post-column UV-assisted vapor generation

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ABSTRACT

A high performance liquid chromatography coupled with atomic fluorescence spectrometry method for the determination of thimerosal (sodium ethylmercury thiosalicylate, $C_9H_9HgNaO_2S$), ethylmercury, and inorganic mercury is proposed. Mercury vapor is generated by the post-column reduction of mercury species in formic acid media using UV-radiation. Thimerosal is quantitatively converted to Hg(II) followed by the reduction of Hg(II) to Hg(0). This method is applied to the determination of thimerosal (THM), ethylmercury (EtHg) and inorganic Hg in samples of a pharmaceutical industry effluent, and in waters of the San Luis River situated in the west side of San Luis city (Middle West, Argentine) where the effluents are dumped. The limit of detections, calculated on the basis of the 3σ criterion, where 0.09, 0.09 and 0.07 μg L^{-1} for THM, EtHg(II) and for Hg(II), respectively. Linearity was attained from levels close to the detection limit up to at least 100 μg L^{-1}.

1. Introduction

A large variety of organic compounds are used by society in vast quantities for different purposes including human and animal healthcare, production and preservation of food and drugs as well as industrial manufacturing processes [1,2]. Among these substances, numerous harmful chemicals can be found such as pharmaceuticals, personal care products, endocrine disruptor chemicals, polycyclic aromatic hydrocarbons, polychlorinated biphenyls, pesticides, etc. [3]. Pharmaceuticals are a class of emerging environmental contaminants that enter municipal sewage and sewage treatment plants mainly as effluents of pharmaceutic industries; but also they reach municipal effluents due to incomplete metabolism after administration. If pharmaceuticals are not eliminated during sewage treatment, they may enter the aquatic environment and eventually reach drinking water [4].

Thimerosal (sodium ethylmercurythiosalicyclate or thiomersal – THM), which contains 49.6% ethylmercury (EtHg), has been employed as a vaccine preservative since the 1930s. Additionally, THM has been widely used as antimicrobial agent in a variety of products including topical anti-septic solutions, cosmetics, cleaning solutions for contact lenses and other injectable biological products [5,6]. The discussion on the safety of THM-containing vaccines started in 1999. The recent popular media attention given to pediatric Hg exposure reflects growing concerns by public, health officials, and policymakers about the detrimental effects of Hg on health and development of exposed children. Especially organic mercury species like di- or monoalkylated Hg show very high neurotoxicity due not only to the total concentration, but to the amount of different Hg species, that needs to be investigated in order to evaluate the risk to human health [7,8].

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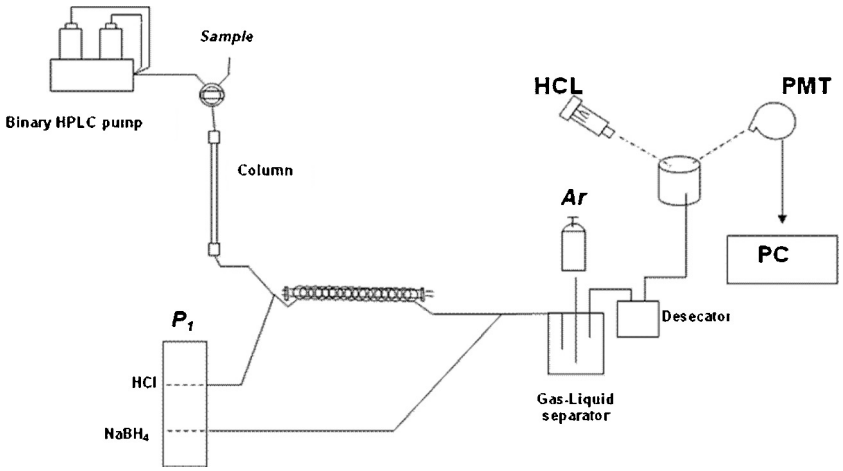


Fig. 1. Schematic diagram of the instrumental setup. P_1, pump 1; HCL, hollow cathode lamp; PMT, photomultiplier tube.

THM is reported to decompose by oxidation to 2,2′-dithiosalicylic acid and to thiosalicylic acid, EtHg and elemental mercury [9]. Although all forms of mercury are poisonous, alkylmercury compounds (di- or monoalkylated Hg) are of special concern because of their easy penetration through biological membranes, efficient bio-accumulation, high volatility and long-term elimination from tissues [10,11].

Quantification of mercury species normally requires the use of hyphenated techniques, involving a more complex instrumentation (usually a chromatography-based technique coupled with atomic spectrometry) in comparison with that needed for single element measurement [12–15]. The coupling of UV radiation, cold vapor generation and atomic fluorescence spectrometry (UV-CV-AFS) allowed organic and inorganic mercury fractions determination [16]. Even more, four Hg species, mercury (Hg^{2+}), methylmercury (MeHg), dimethylmercury (Me_2Hg), and phenylmercury (PhHg) were determined employing the same coupling [6]. THM has been determined by microwave-assisted photochemical online oxidative decomposition and CV-AFS [17], and by photochemical vapor generation coupled to inductively coupled plasma optical emission spectrometry (ICP OES) [18]. Despite the fact that THM has been separated from decomposition compounds like thiosalicylic acid, salicylic acid, and dithiosalicylic acid [19]; and from ethylmercury [20], no attempts have been made to separate inorganic Hg, EtHg and THM. These hyphenated methods are attractive for mercury speciation due to their excellent detection limits and selectivity, and to use in routine analysis [21,22].

Cold vapor atomic fluorescence spectrometry (CV-AFS) is a well-known and widely used technique for mercury determination at ultra-trace levels. Generation of a cold vapor from organo mercury species requires a step to achieve their conversion to Hg(II). The discrimination between inorganic mercury and total mercury was based on the differential behavior of mercury species with several reducing agent [23,24]. This conversion, usually performed in "on line" mode, has been aided by different chemical or photochemical reactions [15,25,26]. Although the chemical oxidation can be achieved at room temperature, the reaction time for an efficient conversion can be long. The use of UV irradiation is a valid alternative to facilitate the decomposition of mercury species [27–29].

In this study, a chromatographic method for the determination of thimerosal and its degradation products, EtHg(II) and Hg(II), in pharmaceutical industry effluents and river waters is presented. Determination is based on the chromatographic separation (reversed phase-high performance liquid chromatography; RP-HPLC) followed by CV-AFS. UV-photochemical induced oxidation aided with formic acid was accomplished to convert organic Hg to inorganic species before vapor generation with sodium tetrahydroborate (THB). The optimized method was applied with success to the analysis of pharmaceutical industry effluents and river waters for the determination of THM, EtHg(II) and Hg(II). Validation took place through spike-recovery tests. Total Hg content was also determined in Certified Reference Material (QC METAL LL3 mercury in water, with a mercury content of $6.48 \pm 0.51\ \mu g\ L^{-1}$).

2. Materials and methods

2.1. Instrumentation

Mercury fluorescence measurements were carried out with an AFS, AI 3300, Aurora Instruments (Vancouver, BC, Canada). The apparatus was equipped with a two-channel peristaltic pump for the continuous fluorescence measurements. Hg hollow cathode lamp from Aurora Instruments (Vancouver, BC, Canada) was employed as radiation source. Samples and reagents involved in cold vapor generation (CV) were delivered by a Minipulse 3 peristaltic pump Gilson (Villiers-Le-Bell, France). Separations were performed with a Series 200, Perkin-Elmer (Thornhill, Canada) binary pump. The column used was Zorbax SB-Aq C18-RP (1.6 mm × 150 mm, 5 μm) with a column guard (1.6 mm × 5 mm, 5 μm) Agilent Technologies.

2.2. Reagents and stock solutions

For solutions and samples preparation, a laminar-flow hood capable of producing class 100 ambient was used. Materials were cleaned by soaking in 10% v/v HNO_3 for 24 h, rinsing three times with ultrapure water and dried in a class 100 laminar flow hood before use. All operations for the HPLC-UV-CV-AFS method were performed on a clean bench. A $10\ mg\ L^{-1}$ standard solution of inorganic mercury was obtained from Perkin-Elmer (PerkinElmer, Norwalk, CT). A $1000\ mg\ L^{-1}$ standard solution of ethylmercury chloride (CH_3HgCl) in water was obtained from Fluka (Germany). A ≥97% (HPLC) standard of thimerosal ($C_9H_9HgNaO_2S$) in water was obtained from Sigma–Aldrich (Germany). Analytical calibration standards of mercury species were prepared daily over the range of 0.0–80.0 $\mu g\ L^{-1}$ for the HPLC-UV-CV-AFS method by stepwise dilutions of the stock solution in the mobile phase. Additional chemicals for the speciation studies were HPLC grade methanol (99.9% v/v) and β-mercaptoethanol (Sigma–Aldrich, USA). Formic acid was purchased from Sigma–Aldrich (USA).

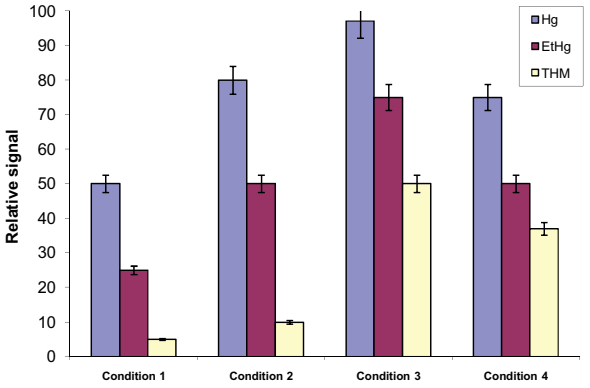


Fig. 2. Evaluation of UV-VG process for THM and EtHg(II), compared to Hg(II) response. Four conditions were studied: Condition 1: 0.1% (v/v) formic acid, 0.3% (m/v) $NaBH_4$ and 10% (v/v) HCl at 1 mL min^{-1}; Condition 2: 0.1% (v/v) formic acid, 0.5% (m/v) $NaBH_4$ and 10% (v/v) HCl at 1 mL min^{-1}; Condition 3: 0.1% (v/v) formic acid, 0.5% (m/v) $NaBH_4$ and 10% (v/v) HCl at 2 mL min^{-1}; Condition 4: 0.1% (v/v) formic acid, 0.5% (m/v) $NaBH_4$ and 40% (v/v) HCl at 1 mL min^{-1}.

2.3. Samples and sample preparations

Three effluent samples were collected immediately at the end of the ending pipe of the effluent system of a pharmaceutic industry that produces cosmetics and other beauty products in San Luis province (Argentine Middle East). Additionally, three surface water samples (0.1–0.3 m) were collected downstream the river where the effluents are deposited. The samples were collected in polyethylene bottles (100 mL) and immediately acidified with few drops of hydrochloric acid (to reach pH below 4.0). Samples were transported to the laboratory within 2 h after collection and stored at 4 °C until determination. All instruments and equipment used in the sample collection were acid-washed and rinsed several times with de-ionized water.

2.4. Measurements of total mercury

After sampling and filtering, samples were diluted 1:10 with mobile phase (0.5% of formic acid diluted in ultrapure water) and analyzed by UV-photoreduction-CV-AFS as previously described [16], with a single modification. Briefly, 100 μL of sample added with 0.1% β-mercaptoethanol (blanks and standards) were injected onto a formic solution stream at 1 mL min^{-1} and mixed before the gas–liquid separator with 0.5% (m/v) THB solution (in 0.5% NaOH). Detection was accomplished by AFS.

2.5. Measurements of THM, EtHg(II) and Hg(II)

A system using HPLC-UV-CV-AFS, described in Fig. 1, was used for mercury speciation in effluent and water samples. The effluent from the LC column was directly connected to the PTFE tubing of the UV-photoreactor (described in previous works [6]) with PEEK tubing (1.59 mm o.d.) and a low dead volume PEEK connector. The reducing solution ($NaBH_4$) was added in confluence between UV-photoreactor and the gas–liquid separator (GLS) to form the vapor species of mercury. Samples were loaded with a syringe into a 100 μL sample loop. All separations were performed at room temperature under gradient conditions (Table 1). The flow rate was 1.0 mL min^{-1}. Data evaluation was performed using Aurora Instruments software (Version 3.1.; AI 3300 AFS) supplied with the instrument, and quantification was based on peak area by external calibration. The optimum experimental conditions for AFS, UV-cold vapor and HPLC are given in Table 1.

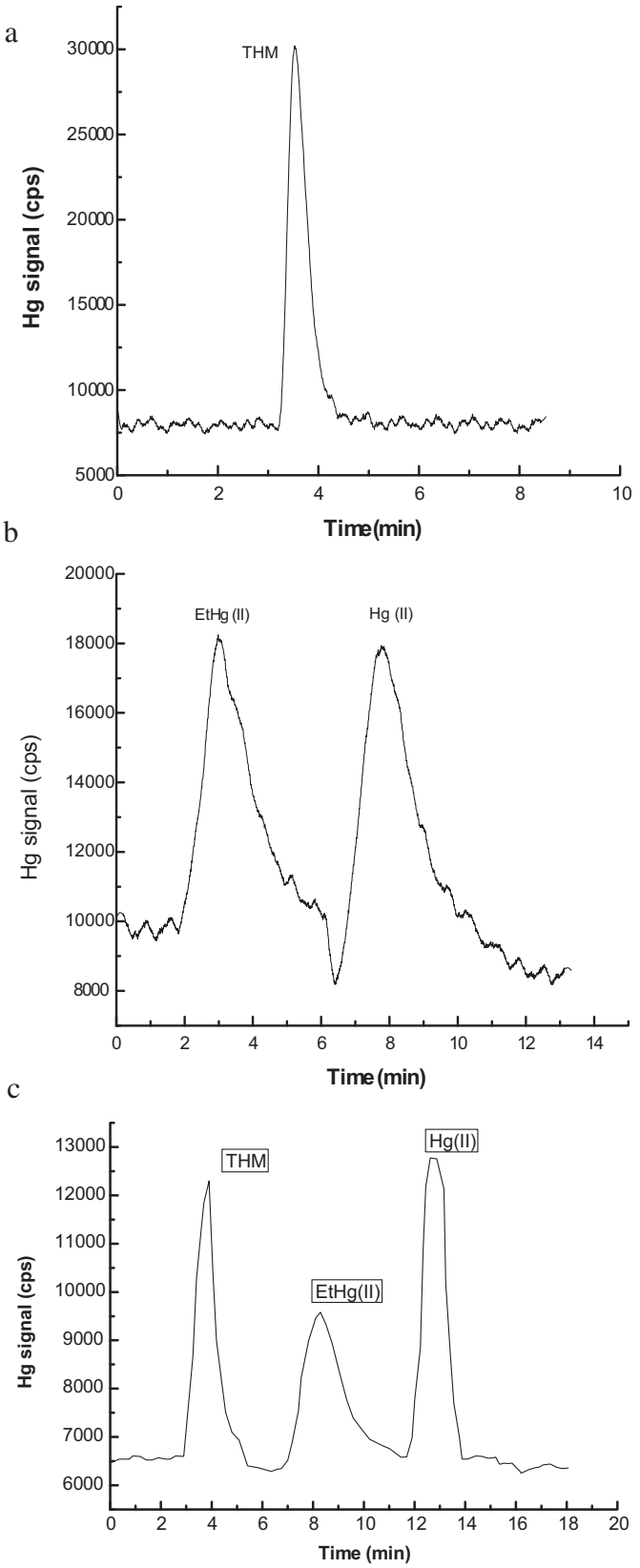


Fig. 3. Chromatogram obtained in a C18 column with a mobile phase of 0.5% formic acid (a); with a mobile phase of 0.5% formic acid and 0.1% β-ME (b); and with the gradient program described in Table 1 (c).

3. Results and discussion

3.1. Optimization of thimerosal and ethylmercury cold vapor generation

Mercury cold vapor formation from THM and EtHg in the mobile phase was evaluated in a univariate approach. It has been stated that THM decomposition can be achieved employing a 40 cm UV reaction coil at a sample flow rate of 2.6 mL min^{-1} [14]. In this work a sample flow rate of 3 mL min^{-1} was employed, and then a 1-m reaction coil was introduced to assure a quantitative decomposition of THM.

Four conditions were evaluated for cold vapor generation and they are depicted in Fig. 2. The efficiency of the oxidative decomposition of TMH was calculated on the basis of the peak area with respect to the peak area of an equimolar concentration of Hg(II) analyzed in the same operating conditions (75.7% of Hg signal for EtHg(II) and 49.1% for TMH). Initial condition (condition 1) was proposed considering that mobile phase from chromatographic separation contains 0.1% (v/v) formic acid. It has been stated that the signal of Hg concentration of 10 μg L^{-1} increases significantly with formic acid in UV reactor [6]. Then the mobile phase contributes to TMH and EtHg oxidation, representing an advantage of the system.

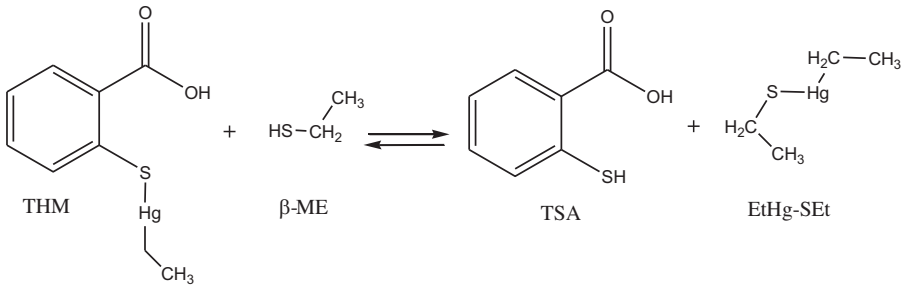
As observed in Fig. 2, condition 1 did not achieve an equimolar response of TMH and EtHg compared to Hg. Condition 2 introduced a higher $NaBH_4$ concentration (0.5%, v/v). Under this condition, a higher Hg signal was obtained, but an equimolar response of TMH and EtHg was not achieved. In condition 3, the acid flow rate was increased to 2 mL min^{-1} achieving an equimolar response of TMH and EtHg. In condition 4 the acid concentration was increased to 40% (v/v), however the background signal increased, decreasing Hg signal. For this reason condition 3 was chosen as optimal for future analysis.

Table 1
Instrumental operating conditions for THM, EtHg(II) and Hg(II) determination by HPLC-UV-CV-AFS.

HPLC	
Operation mode	Gradient (hold 100% A, 5 min; change directly to 100% B, hold 10 min. Recondition: hold 100% A, 5 min)
Mobile phase	A: 0.1% formic acid B: 0.1% formic acid + 0.1% β-mercaptoethanol
Maximum pressure	6000 psi
Flow rate	1.0 mL min^{-1}
Column/column guard	C18 (1.6 mm × 150 mm × 5 μm)/C18 (1.6 mm × 5 mm × 5 μm)
Injector loop	100 μL
UV-CV-AFS	
UV-reaction coil	PTFE, 1-m length, 0.75 mm i.d.
Reductant	$NaBH_4$ 0.5% in 0.5% NaOH
Reductant flow rate	1.0 mL min^{-1}
Acid media	Hydrochloric acid (20% v v^{-1})
Acid flow rate	1.0 mL min^{-1}
Gas liquid separator	Acrylic, 6 mL internal volume
Radiation source (operating current)	High intensity Hg-hollow cathode lamp (30 mA)

3.2. HPLC-UV-AFS determination

Based on the conditions proposed by Barbosa Jr. et al. [5] for the determination of ethyl-, methyl, and inorganic Hg by HPLC-CV-ICPMS, it was assayed the separation of THM, EtHg and Hg. In brief, they proposed the chromatographic separation of mercury species on a C8 column with a mobile phase containing 0.05% v/v β-mercaptoethanol, 0.4% m/v L-cysteine, 0.06 mol L^{-1} ammonium acetate and 5% v/v methanol. However, when using β-mercaptoethanol (β-ME) (or L-cysteine) THM decomposition in ethylmercury thioethanoate (EtHg-SEt) and thiosalicylic acid (TSA) is produced (Eq. (1)).



(1)

Under the above mentioned conditions, the obtained chromatogram using a C18 column, showed only two resolved peaks for a mixture of THM, EtHg(II) and Hg(II) (20 μg L^{-1} with 100 μL of sample injected). The first identified peak at 7.2 min retention time was assigned to EtHg and THM – both as EtHg-SEt; and the other one corresponded to Hg(II) – as $(EtS)_2Hg$ at 10.4 min retention time.

Further studies showed that THM eluted quickly (Fig. 3a) without decomposition using formic acid 0.5% mobile phase, whilst both Hg(II) and EtHg(II) did not elute even at elution times as long as 30 min. Hg(II) and EtHg(II) species eluted well, however, in a mobile phase composed by 0.5% of formic acid and 0.1% of β-ME (Fig. 3b).

The optimized conditions were thus joined in a unique gradient program (Table 1) in which THM eluted in 0.5% formic acid after 3.9 min, and after switching to a mobile phase with 0.5% formic acid and 0.1% β-ME (5 min elapsed time), EtHg(II) and Hg eluted at 8.3 and 12.5 min respectively (Fig. 3c).

3.3. Analytical figures of merit

The limit of detections (DL), calculated on the basis of the 3σ criterion, and the precisions, calculated as the relative standard deviations (RSD) for five replicate determinations, can be found in Table 2 where are also compared with other reported method. Linearity was attained from levels close to the detection limit up to at least 100 μg L^{-1}.

3.4. Application to real samples and recovery studies

Since there is not standard reference material available with certified content of thimerosal and EtHg species, a series of recovery studies were carried out to check the accuracy. The solutions obtained before filtration were spiked with different mercury concentrations according to the mercury levels found after the total amount of Hg found by UV-CV-AFS. The samples were spiked with 5.0, 10.0, and 20.0 μg L^{-1} of Hg^{2+}, EtHg(II), and THM. Mercury species concentrations in each one of the studied samples are listed in Table 3. Mercury levels of each species found in the different samples and materials were in good agreement with total

Table 2
Compared analytical figures of merit for THM and related compounds determination.

	LC-UV-CV-AFS method for THM, EtHg(II) and Hg(II) (this work)	Non-chromatographic UV method AFS for Hg(II), MeHg(II), $Me_2Hg(II)$, and PhHg(II) [6]	FI-microwave-photochemical oxidation and CV-AFS for THM [17]	Photochemical vapor generation coupled to ICP OES for THM and Hg(II) [18]	Reversed-phase HPLC for thio-salicylic acid, THM and dithio-salicylic acid [19]
LOD	0.09 $\mu g L^{-1}$; 0.09 $\mu g L^{-1}$; 0.07 $\mu g L^{-1}$	0.001, 0.04, 0.068, and 0.099 $\mu g L^{-1}$	0.003 $\mu g L^{-1}$	0.6 $\mu g L^{-1}$; 0.3 $\mu g L^{-1}$	5 $\mu g mL^{-1}$
LOQ	0.29 $\mu g L^{-1}$; 0.28 $\mu g L^{-1}$; 0.25 $\mu g L^{-1}$	N.I.	0.009 $\mu g L^{-1}$	N.I.	N.I.
Precision	3.5% (as percent relative standard deviation; 4.0 $\mu g L^{-1}$)	1.5, 3.1, 4.7 and 5.8%	5%; 0.9% and 2% for 0.02; 0.2 and 2 $\mu g L^{-1}$ THM concentrations respectively	2.9% (repeatability) and 4.4% (reproducibility)	1–2.5% (intraday) 1–4% (inter day)
Linearity	0.29–100 $\mu g L^{-1}$; 0.28–100 $\mu g L^{-1}$; 0.25–100 $\mu g L^{-1}$	Linearity was attained from levels close to the detection limit up to at least 100 $\mu g L^{-1}$	0.01–2 $\mu g L^{-1}$	0.5–10 $\mu g L^{-1}$	20–80 $\mu g mL^{-1}$
Retention time (for HPLC)	3.9; 8.3 and 12.5 min	Non-chromatographic	Non-chromatographic	Non-chromatographic	6.1; 9.3 and 11.6 min

MeHg(II), methyl mercury; $Me_2Hg(II)$, di-methyl mercury; PhHg(II), phenyl mercury.
FI, flow injection.
N.I., not informed.

Table 3
THM, EtHg(II) and Hg(II) determination in pharmaceutical industry effluents, river water and in a CRM with certified total Hg concentration.

Sample[f]	THM ($\mu g L^{-1}$)[a,e]	EtHg(II) ($\mu g L^{-1}$)[a,e]	Hg(II) ($\mu g L^{-1}$)[a,e]	Total Hg ($\mu g L^{-1}$)[b,e]
A	N.D.[c]	10.0 ± 0.5	6.2 ± 0.1	17.0 ± 0.6
1	5.0 ± 0.1	14.8 ± 1.1	11.2 ± 0.1	–
2	10.0 ± 0.8	22.1 ± 0.3	16.8 ± 0.3	
3	19.8 ± 0.5	30.1 ± 0.5	27.1 ± 0.5	
B	N.D.[c]	7.7 ± 0.5	5.1 ± 0.5	12.1 ± 0.5
1	4.7 ± 0.4	12.0 ± 0.3	10.0 ± 0.2	–
2	9.8 ± 0.8	18.1 ± 0.5	15.0 ± 1.1	–
3	17.5 ± 0.5	28.1 ± 1.0	25.1 ± 0.3	
QC LL3 Mercury in water[d]	N.D.[c]	N.D.[c]	6.3 ± 0.8	6.5 ± 0.6

[a] Determined by HPLC-UV-CV-AFS.
[b] Determined by UV-CV-AFS.
[c] Not detected.
[d] Certified value 6.48 ± 0.51 $\mu g L^{-1}$ of Hg (total).
[e] Confidence limits as: $t_{(2;0.05)}\, s/\sqrt{n}$ ($n = 3$).
[f] A, river water where effluents are dumped. B, pharmaceutical industry effluent. 1, 2 and 3 are spiked samples with 5.0, 10.0 and 20.0 $\mu g L^{-1}$ of the respective compound expressed as Hg concentration.

mercury levels. In addition, total mercury amounts in each sample were determined by UV-CV-AFS, following the conditions described previously [6]. A CRM (QC LL3 mercury in water) was also analyzed.

Despite, no significant THM levels were found in the studied samples; the developed method was able to monitor the actual situation in that sampling point, and showed that a large number of samples could be analyzed to undertake a comprehensive study of contamination of surface water due to pharmaceutical industrial activity using Hg-containing components.

4. Conclusions

A novel chromatographic methodology for mercury species determination was developed. The proposed method achieved the determination of three mercury species. This procedure is fast and simple becoming adequate for screening procedures and routine analysis. This is the first time that thimerosal, EtHg(II) and Hg(II) species are determined via a chromatography and UV-CV-AFS.

The introduced method only required simple and low cost instrumentation, compared with some hyphenated techniques (e.g. HPLC-ICP-MS). AFS demonstrated to be a sensitive technique for the determination of Hg at low concentration levels. The method was successfully applied to the determination of Hg(II), EtHg(II), and thimerosal in pharmaceutic industrial effluents and river waters.

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