Liquid chromatography coupled to molecular fluorescence with post-column UV sensitization for thimerosal and derivative compounds monitoring in environmental samples

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ABSTRACT

A high performance liquid chromatography coupled (HPLC) with molecular fluorescence (MF) spectrometry method for determination of thimerosal (sodium ethylmercurythiosalicylate, $C_9H_9HgNaO_2S$), and derivatives is proposed. A sensitization of molecular fluorescence was provoked by UV irradiation of analytes in a home-made photoreactor that served as interface between the LC column and MF spectrometer. This method is applied to determination of thimerosal (THM), ethylmercury (EtHg) and thiosalicylic acid(TSA) in samples of pharmaceutical industry effluents, and waters of La Carolina and Jáchal rivers situated in the center-west side of San Luis city and in the east of San Juan city (Middle West, Argentine) where the effluents are dumped. The limit of detection, calculated on basis of 3σ criterion, was of 1.8; 5 and 0.05 μmol L^{-1} for THM, EtHg and for TSA, respectively.

Keywords: Thimerosal; Pharmaceutic industry effluents; Contaminated river waters; HPLC-UV-postcolumn photochemical sensitization; Molecular fluorescence.

1. Introduction

Researchers first detected drugs in groundwater and surface water in the 1990s, and there are reports indicating the presence of pharmaceutical compounds and their active metabolites in fresh water environment in ng L^{-1} to µg L^{-1} range, occurring with increasing frequency[1]. Pharmaceuticals enter the aquatic environment through municipal effluents, being detoxification processes in wastewater treatment plants not enough to manage them. The constant discharge, then goes directly to the aquatic environment and gives them a state of pseudo-persistence. Moreover, they may cause diverse effects on aquatic biota[2]. Nevertheless, research is limited to a low number of drugs compared to the actual current compounds discharged (dumped). 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[3, 4]. 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. [5]. 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 [6].

Thimerosal (or Thiomersal) is a trade name for an organomercurial compound (sodium ethyl-mercury thiosalicylate, $C_9H_9HgNaO_2S$) that is 49.55% Hg by weight. Thimerosal (THM) has been used as a preservative in cosmetics, pharmaceutical preparations, and biological products such as eye shadows, make-up removers, mascaras, and soap-free cleansers; ear, eye and nose drops and ointments, antiseptic sprays, etc [7, 8]. The most controversial use of this conserving has been as additive in children vaccines. Experimental studies that addressed early life exposure to THM-containing vaccines indicate

that in exposed fetuses, neonates and infants, mercury remains in their blood at sufficient concentration and for enough time to reach the brain and to exert a neurologic impact and affect neurodevelopment. However, the mechanism of toxicity has not been well understood and studies with EtHg and THM are scarce in vivo and in vitro [9, 10]. THM is reported to decompose by oxidation to 2,2´-dithiosalicylic acid and to thiosalicylic acid, EtHg and elemental mercury[11, 12]. 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 [13, 14].

The detection of THM at trace level in environmental samples is of crucial importance. In the past, various analytical techniques including high-performance liquid chromatography (HPLC) with electrochemical detection, enzymatic amperometry, colorimetry, atomic absorption spectrometry (AAS) and atomic fluorescence spectrometry (AFS) have been developed to detect thimerosal and derivatives [15-22]. As an alternative, mass spectrometry detectors are preferable in terms of sensitivity, accuracy, generated waste and others requirements of routine analysis, however, the equipment and running costs are prohibitive to many laboratories [11, 23]. HPLC with UV detection, on the contrary is a 'low cost' substitute, but the affordable detection limits are not compatible with environmental levels of THM and its derivatives. Alternatively, absorption spectra can be sensitized photochemically through UV irradiation of samples [24]. The alternative of using molecular fluorescence (MF) as detection is an attractive approach to the above mentioned, but the low fluorescence of THM and its derivatives requires adding a fluorophore, or other approaches for reconfiguration of molecules aiming to generate new electronic configurations with more π-electrons [25]. Nevertheless, to the best of our knowledge, there are no reports describing

the configuration of HPLC with post column sensitization for MF detection of THM and derivatives determination.

In a previous 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 was described using AFS with UV photo reduction of mercurial species [26]. Despite this method was attractive for environmental studies, the cold vapor technique is reagent consuming i.e.; high amounts of argon gas, mercurial standards, acids and sodium tetrahydroborate were wasted. This situation complicates the analysis of high number of samples.

The aim of this work was to develop a new approach for THM and the derivatives ethyl-Hg, thiosalicylic acid and di-thiosalicylic acid separation by polarity-gradient reversed-phase HPLC with post-column photoreaction and molecular fluorescence detection. The method was developed studying first the photochemical reaction in a continuous flow system, and after that, spectroscopic conditions were assessed. After that, chromatographic conditions for total separation of the four species were stablished. The method was successfully applied for the analysis of environmental samples.

2. Materials and methods

2.1 Instrumentation

A Shimadzu spectrofluorometer model RF-5301PC (Kyoto, Japan), equipped with a Xenon discharge lamp was used for the fluorescent measurements and a 12-µL volume flow-cell unit for the continuous measurements were used to record transient signals. The THM fluorescence measurements were carried out operating the spectrofluorometer in the time-course mode (λ_{ex} = 315 nm through a slit of 10 nm, and λ_{em} = 442 nm with a slit 15 nm).

Separations were performed with a Series 200, Perkin-Elmer (Thornhill, Canada) binary pump. The column used was Zorbax SB-Aq C18-RP (1.6 x 150 mm, 5 µm) with a column guard (1.6 x 5mm, 5µm) Agilent Technologies.

The UV photoreactor was made as follows: a Hg vapor lamp (15W G15T8 UV-C LONG LIFE high pressure Hg, PHILIPS) that ignited with a suitable starter and chock surrounded by a 2.5-m PTFE tubing (internal diameter = 0.75 mm and outer diameter = 1.0 mm), and covered by an aluminum foil.

2.2 Reagents and stock solutions

All operations for the HPLC-UV-MF method were performed on a clean bench. A1000 mg L^{-1} standard solution of ethylmercury chloride (CH_3HgCl) in water were obtained from Fluka (Germany). A ≥ 97% (HPLC) standard of thimerosal ($C_9H_9HgNaO_2S$) in water was obtained from Sigma-Aldrich (Germany). Thiosalicylic acid (98%) was obtained from Sigma-Aldrich (Germany). Analytical calibration standards were prepared daily for the HPLC-UV-MF method by step-wise dilutions of the stock solution in the mobile phase (A: 0.1% HCOOH (v v^{-1}) and B:methanol, 50:50). Additional chemicals for the speciation studies were: HPLC grade methanol (99.9% v/v) and formic acid from Sigma-Aldrich (USA).

2.3 Samples and sample preparations

Two effluent samples were collected immediately at the end of the ending pipe of the effluent system of a pharmaceutical industry that produces cosmetics and other beauty products in San Luis province, (Argentine Middle East). Additionally, two 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). 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. HPLC-UV-MF measurements

A system using HPLC-UV-MF was used for THM and derivatives determination in effluent and water samples. The effluent from the LC column was directly connected to the PTFE tubing of the UV-photoreactor (section 2.3) with PEEK tubing (1.59 mm o.d.) and a low dead volume PEEK connector. Samples were loaded with a syringe into a 100 µL sample loop of the load/injection valve of the HPLC. All separations were performed at room temperature under gradient conditions (Table 1). The flow rate was 0.8 mL min^{-1}.Data evaluation was performed using MF Shimadzu Software supplied with the instrument, and quantification was based on peak area by external calibration. The optimum experimental conditions for MF, UV-photochemical reaction and HPLC separation are given in Table 1.

3. Results and discussion

3.1 Spectroscopic conditions

As it was discussed, THM lacks fluorescent activity, or it is negligible. On the other hand, from the main degradation products, EtHg and thiosalicylic acid, only the last one is fluorescent, with absorption and emission wavelengths at 315 and 442 nm, respectively. The effect of direct UV irradiation on the THM fluorescent spectrum was evaluated. To this aim, pure solutions (100 µL) were passed through LC (pumps only)-UV-MF system, in isocratic mode (MeOH:H_2O - 70:30) without the column. The spectra were recorded after stopping the pumping system when the signal reached a maximum. The procedure was repeated with and

without light irradiation, as it is shown in figure 2. THM spectrum after irradiation depicted a maximum fluorescence intensity at 390 nm when 256 nm was used as excitation. As can be observed in Figure 2, the emission spectra of THM photoproduct (B) accomplished a noticeably enhancement of fluorescence intensity compared to THM solution without UV irradiation (A). In order to preserve adequate sensitivity for THM but also for the main degradation products (EtHg and TSA), the spectroscopic conditions were fixed at 315 and 442 nm for excitation and emission wavelengths, respectively.

Considering the small volume (12 µL) of the LC-flow cell of the spectrofluorometer, there is a noticeably loss of sensitivity in comparison with a 1-cm conventional quartz cell. A reasonable compensation to this drawback was applied by widening monochromators slits width. Thus, the signal-to-background ratio (SBR) was evaluated using slits width of 5/5 to 15/15 nm, and the best SBR was achieved with slits at 10/15.

3.2 THM and derivatives UV-photoreaction

In our previous work [26] we decompose THM and its derivatives to generate free Hg atoms that were then measured by atomic fluorescence spectroscopy. The evidences demonstrated that exposition to UV radiation within a flowing scheme could provide THM with enough energy to be decomposed in presence a suitable electron donor reagent. In our case, we used formic acid, based on a work of Yepsen *et al.* [24] that performed a catalytic photodegradation of THM by UVA in presence of TiO_2 nanoparticles with HCOOH as 'sacrifice' reagent.

Thimerosal is an aromatic molecule with very low native fluorescence, and this fact can be attributed at least partially to the intramolecular heavy-atom effect of the mercury atom bound to the aromatic ring through its thiol. Thus, the fact that strongly fluorescent

photoproducts are formed upon UV irradiation can be explained in part by the occurrence of a Hg photoreduction and photohydrolysis of the side chain that might lead also to the formation of oxygenated derivatives. Even though the chemical forms are not indeed identified, the photoproducts consist probably in substituted salicylic acids, and besides, sensitization was experimentally corroborated (Figure 3).

The optimization of this sensitization process aimed to interface the HPLC separation with the fluorometric quantitation, but maintaining the column efficiency in terms of chromatographic resolution and time of analysis. Considering this last, the photoreaction should take place in a hydro-alcoholic media such as the optimized mobile phase for THM and derivatives separation (i.e., gradients between 50:50 and 70:30 MeOH:H_2O composition). Consequently, the effect of the irradiation time was studied observing the effect of the mobile phase flow rate, and the photoreactor coil length (Figure 3). Since the mobile phase suffers changes during chromatographic separation, optimization was carried out under extreme conditions (i.e. MeOH:H_2O 70:30). As can be seen, outstanding enhancement of signal was observed at high irradiation periods that occurred with longer coils and lower flowing rates. The selected conditions based on sensitivity reached were 250 cm coil length and 0.75 mL min^{-1}. It was further observed that faster separation with adequate resolution and sensitivity could be obtained if the chromatographic separation was done even at higher flow rates; i.e. 0.8 mL min^{-1}.

3.3 HPLC-UV-MF determination

The method described by Tleugabulova *et al.* [27] was applied in this research to separate thimerosal (THM), thiosalicilic acid (TSA) and other degradation products such as EtHg and di-thiosalicylic acid with some modifications. In this research, a C18 column (1.6 mm × 150

mm × 5 µm) was used. Mobile phase consisted in A: 0.1% HCOOH (v v^{-1}) and B:methanol according to our previous research [26].

As observed in Figure 4a, introducing a mobile phase of 65% B in isocratic mode conditions reported by Tleugabulova *et al.*[27], did not resolve TSA and THM to the base line (Chromatographic resolution = 1.73). New conditions were evaluated for the column used in this work (figure 4b). The flow rate was increased from 0.6 to 0.8 mL min^{-1} to avoid excessive axial dispersion in the photoreactor coil.

The new LC separation program (Table 1) consisted of four steps:1) 6´, 50% B (isocratic); 2) 2´, 70% B (gradient); 3) 4´, 70% B (isocratic) and 4) 2', 50% B (gradient). Results can be observed in Figure 4b. In step 1), B percentage was at 50% to favor interaction of analytes with stationary phase (C18). Then in step 2 a lower polarity of mobile phase was introduced by increasing A percentage to 70%, for a faster elution of THM (Chromatographic resolution = 5.84). This allowed base line separation of TSA and THM with convenient retention times, i.e. 6.76 minutes for TSA, 10.12 minutes for THM. It is worth mentioning that EtHg eluted without overlapping at 4.0 min, and at 12.4 min, a wide peak that was attributed here to di-thiosalicylic acid, was also observed.

3.4 Analytical performance and validation

The limit of detections (LOD), calculated based on the 3σ criterion, and the precisions, calculated as the relative standard deviations (RSD) for five replicate determinations, can be found in Table 2. Linearity was attained from levels close to the detection limits throughout three orders of magnitude. Since there is not water standard reference material available with certified content of thimerosal, EtHg and thiosalicylic acid species, a series of recovery

studies were carried out to check the accuracy (Table 3). Accuracy was verified through spike-recovery tests as follows: each sample was divided into 9 aliquots to which, increasing amounts of a standard was added at two levels. (i.e., three aliquots were not added, three aliquots were added with $4.0x10^{-5}$, $4.1x10^{-7}$ and $1.5x10^{-5}$ mol L^{-1}, and the last three ones were added with $1.2x10^{-4}$, $8.1x10^{-7}$, $3.1x10^{-5}$ mol L^{-1} of Ethyl Hg, Thiosalycilc Acid and Thimerosal, respectively). Recoveries were evaluated according to Equation (1) and compared with t-test showing no statistically significant differences (p=0.05, n=3).

3.5 Application to real samples

Figure 5 shows the experimental chromatogram obtained after the injection of an effluent sample collected in San Luis province, Argentina. The results obtained for EtHg, TSA and THM are presented in table 4. The uncertainty of each determination was calculated as the confidence interval (Eq. 2) corresponding to a 95% of confidence limit with 2 degrees of freedom. The standard deviation (S) was propagated (Eq. 3) for EtHg, TSA and THM considering the contributions of sampling (s_s^2), volume measurement for dilutions (s_V^2), data acquisition (s_m^2) and estimated recovery (s_R^2). The sampling variance was negligible for homogeneous effluents, and the recovery variance, which was estimated at the two spike levels, was the average variance because the obtained values at the two levels (for the three analytes) were very close each other (Table 3).

4. Conclusions

A novel chromatographic method with molecular fluorescent detection for thimerosal and derivatives determination was developed. The photochemical modification of analytes after chromatographic separation was the key to the lack of fluorescent emission of THM, and it was accomplished with a very simple device made at the laboratory with a bactericide UV lamp and PTFE tubing. This procedure is fast and simple, becoming adequate for screening

studies and routine analysis. This is the first time that thimerosal, EtHg and TSA were separated and determined via chromatography and UV-MF detection. The method was successfully applied to the determination thimerosal in pharmaceutic industry effluents and river waters.

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Figure Captions

Figure 1 – Scheme of the experimental configuration for Thimerosal and derivatives determination by HPLC-UV-MF.

Figure 2 – Fluorescent emission spectra for thimerosal (A) and its photoproduct (B) generated by UVC irradiation in a continuous flow scheme. Experimental conditions: excitation wavelength, 256nm; slit widths, (A):10/10 and (B):5/5.

Figure 3 – Influence of UV radiation exposition upon THM fluorescent intensity in terms of photoreaction coil length and mobile phase flowing rate ($mLmin^{-1}$).

Figure 4 – Optimization of chromatographic conditions. a) Isocratic separation with 65:35 MeOH:H2O mobile phase, and b) gradient chromatographic mode.

Figure 5 – Analysis of a plastic industry effluent by HPLC-UV-MF for THM and derivatives determination. A: Unspiked sample; B: Spiked sample with 15, 0.4 and 15 $\mu molL^{-1}$ of EtHg, TSA and THM, respectively. Maximized chart: EtHg peak at 4.00 min in the unspiked sample.

FIGURES

Figure 1.

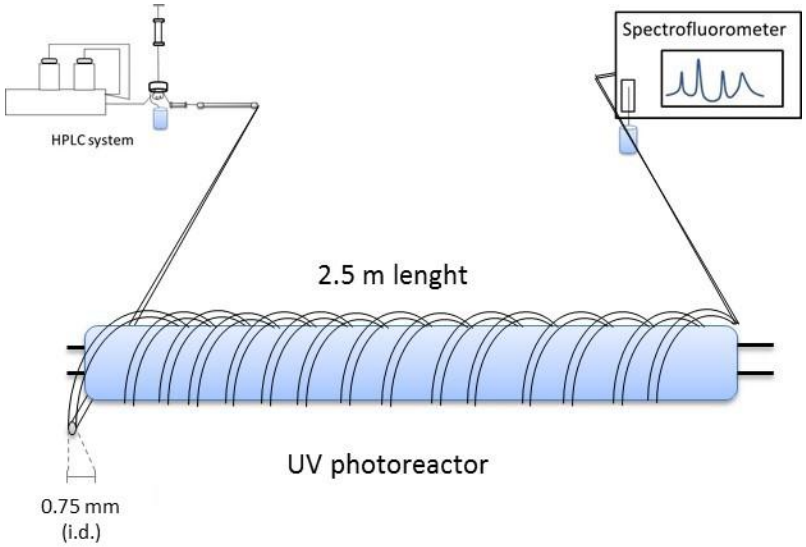

Figure 2.

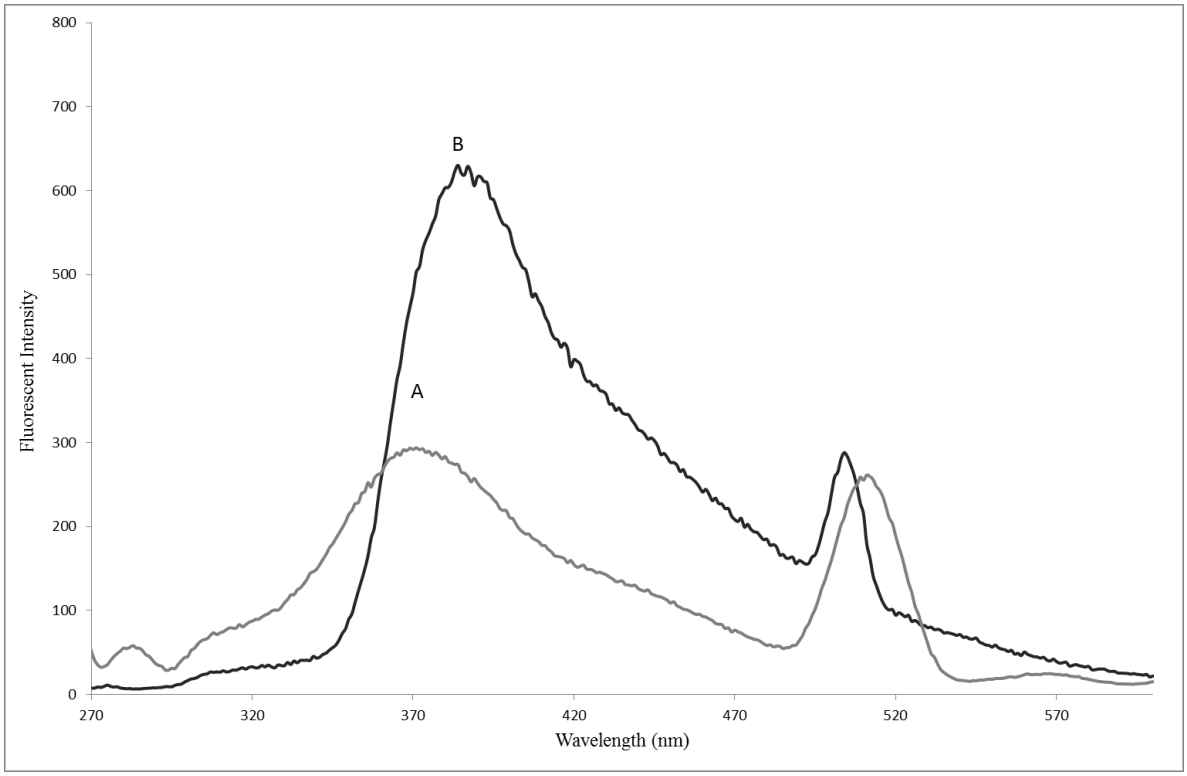

Figure 3.

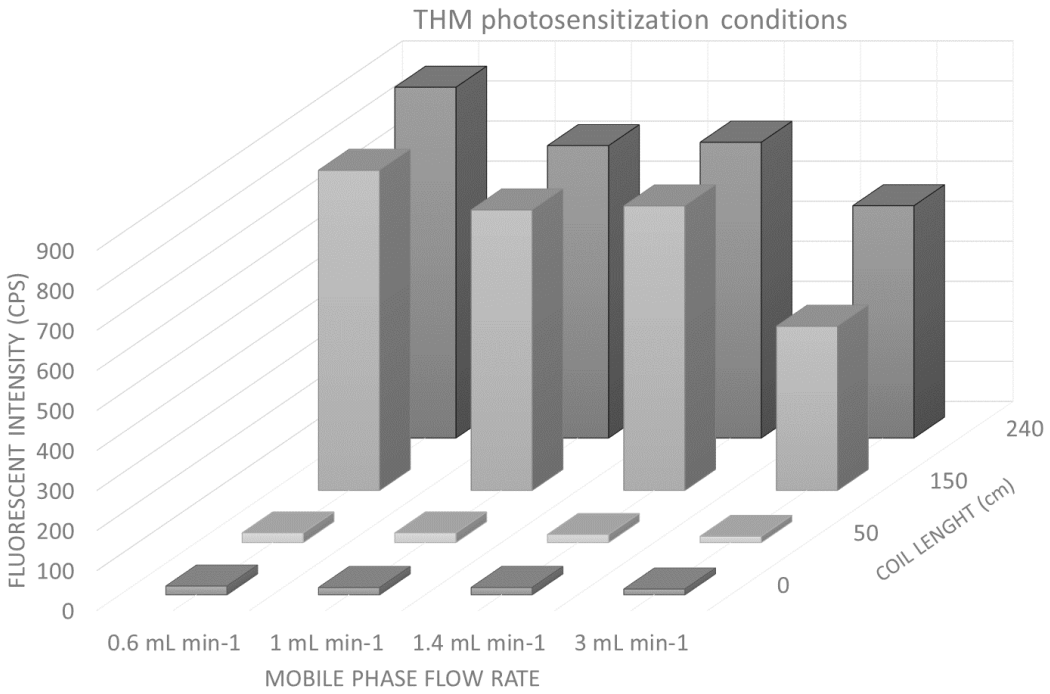

Figure 4

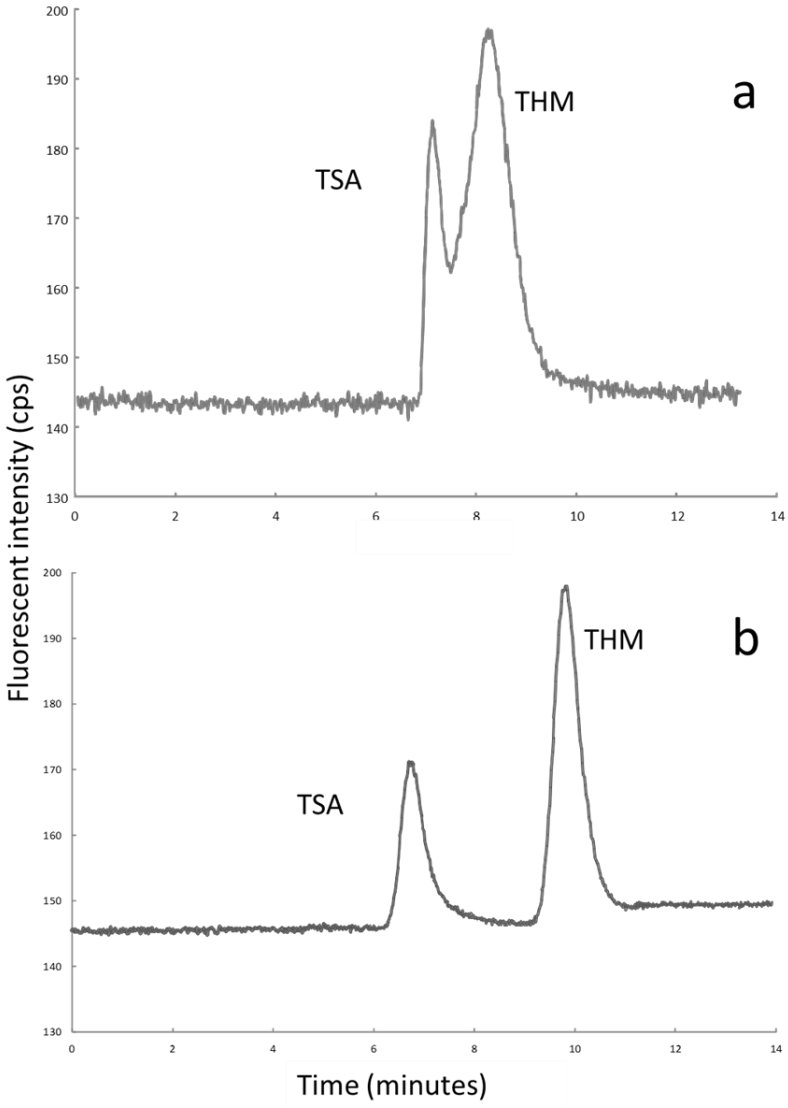

Figure 5

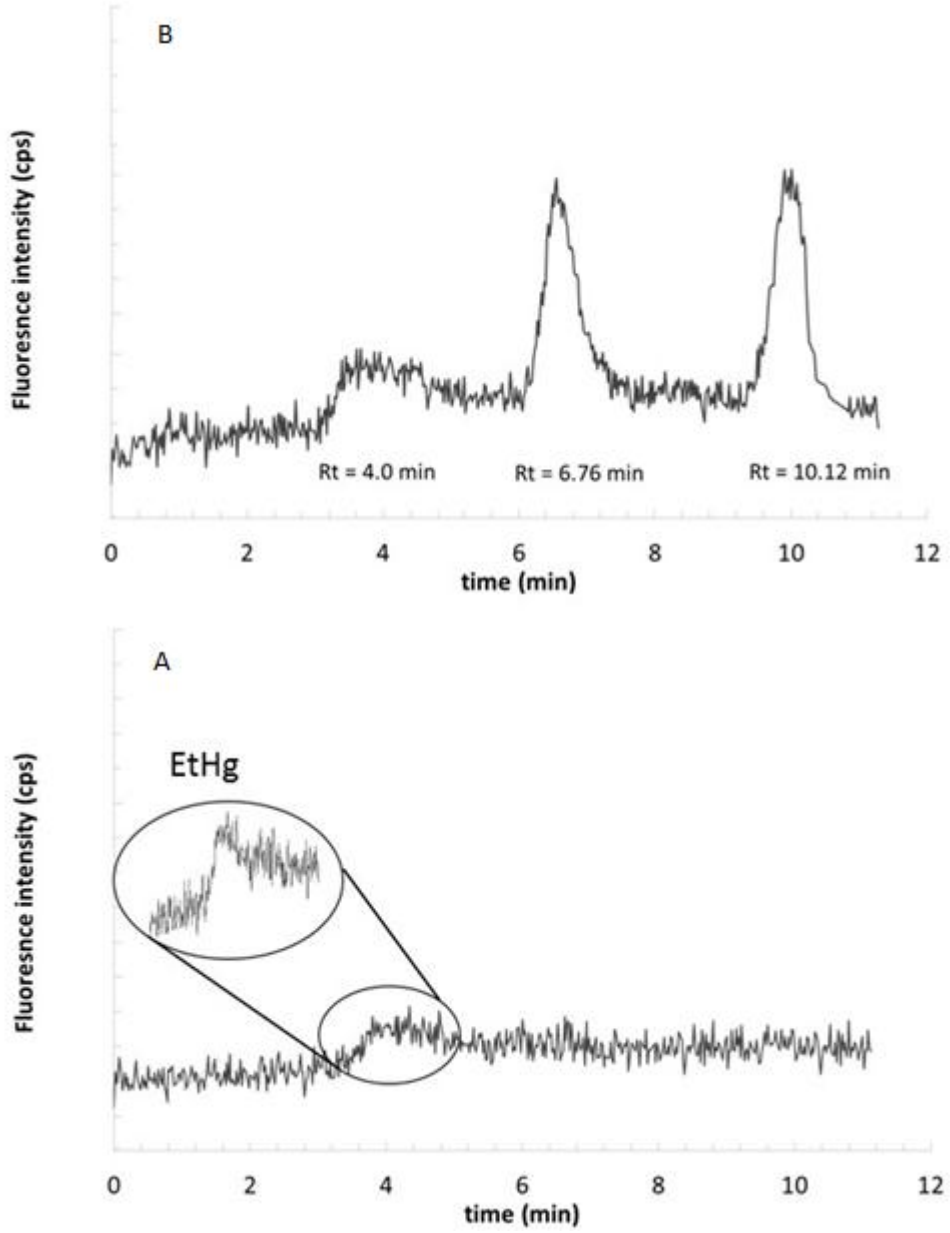

Table 1 – Instrumental operating conditions for THM and derivatives determination by HPLC-UV-MF

HPLC			
Operation mode	Time (mode)	% A	% B
	0-6 min (isocratic)	50	50
	6-8 min (gradient)	30	70
	8-12 min (isocratic).	30	70
	12-14 min (gradient)	50	50
Mobile phase	A: 0.1% formic acid		
	B: CH_3OH		
Maximum pressure	6000 psi		
Flow rate	0.8 mL min^{-1}		
Column / column guard	C18 (1.6 x 150 mm x 5 µm) / C18 (1.6 x 5mm x 5µm)		
Injector loop	100 µL		

Table 2 – Analytical figures of merit for the HPLC-UV-MF method

	Calibration curve[a]	R^2	Limit of detection (μmol L^{-1})	Percent relative standard deviation (RSD)
Thimerosal	FI = 9.6 x 10^6 C	0.9961	1.8	1.8
Thiosalicylic acid	FI = 2.0 x 10^7 C	0.9996	0.05	2.2
Ethyl-Hg	FI = 4.0 x 10^6 C	0.9970	5	1.4
di-thiosalicylic acid	Not calibrated	---	---	---

[a]FI: fluorescent intensity; C: concentration [mol L^{-1}]

Table 3- Recovery study and method validation

Analyte	**Base**	**1st spike (added)**	**1st spike (found)**	**% Recovery for the 1st spike**	**Variance of the recovery**	**2nd spike (added)**	**2nd spike (found)**	**% Re the 1**
	1.4x10^{-5}		5.5 x10^{-5}	101			1.4 x10^{-4}	108
EtHg	1.4 x10^{-5}	4.0 x10^{-5}	5.4 x10^{-5}	100	4.1	1.2 x10^{-4}	1.4 x10^{-4}	107
	1.5 x10^{-5}		5.6 x10^{-5}	104			1.5 x10^{-4}	110
	---		4.2 x10^{-7}	104			8.8 x10^{-7}	109
TSA	---	4.0 x10^{-7}	3.8 x10^{-7}	95	20.5	8.1 x10^{-7}	8.7 x10^{-7}	108
	---		4.0 x10^{-7}	98			8.3 x10^{-7}	103
	---		1.6 x10^{-5}	102			3.0 x10^{-5}	94
THM	---	1.5 x10^{-5}	1.6 x10^{-5}	101	0.4	3.1 x10^{-5}	2.8 x10^{-5}	93
	---		1.6 x10^{-5}	103			2.9 x10^{-5}	94

Concentrations in mol L^{-1}

Table 4- Determination of thimerosal, ethylmercury and thiosalicylic acid in pharmaceutical industry and river waters by HPLC-UV-MF

Sample	**THM** **(µmol L^{-1})**	**EtHg** **(µmol L^{-1})**	**TSA** **(µmol L^{-1})**
Plastic industry effluent	< LOD	2.4±0.2	< LOD
Pharmaceutic industry effluent	2.5±1.0[a]	36.1±1.0[b]	0.207±0.080[c]
La Carolina river	< LOD	1.4±0.1	< LOD

< LOD: Below limit of detection. [a]$Confidence\ interval\ of\ the\ mean = t(0.05,2)xS/\sqrt{n}$ With t= tabulated value for t of the t-test – 95% confidence level and two degrees of freedom. n= number of observations (3).

EQUATIONS

Equation 1

$$\%\text{Recovery} = 100\text{x}\left(\frac{\text{Found} - \text{Base}}{\text{Added}}\right)$$

Equation 2

$$confidence\ limit = \frac{t(0.05,2)xS}{\sqrt[2]{n}}$$

Equation 3

$$S = \sqrt[2]{s_s^2 + s_m^2 + s_w^2 + s_R^2}$$