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Mercury speciation analysis in terrestrial animal tissues

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ABSTRACT

No previous analytical procedures are available and validated for mercury speciation analysis in terrestrial animal tissues. This analysis is a difficult task both because the expected concentrations are low, since important accumulation process are not likely to occur, and also because there are not commercially available certified reference material. Thus, an analytical methodology has been developed and validated for mercury speciation for the specific case of terrestrial animal tissues. The proposed method is based on the quantitative extraction of the species by closed-vessel microwave assisted heating with an alkaline reagent, followed by ethylation. The ethylated derivatives were then submitted to head-space solid phase microextraction with a 100 μm polidimethylsiloxane-coated fiber, and desorbed onto a gas chromatograph coupled to atomic fluorescence detection via pyrolysis unit (HS-SPME-GC-pyro-AFS). Procedural detection limits were 31.8 ng g^{-1} and 52.5 ng g^{-1} for CH_3Hg^+ and Hg^{2+}, respectively, for liver and 35.3 ng g^{-1} and 58.1 ng g^{-1} for CH_3Hg^+ and Hg^{2+}, respectively, for kidney. These limits of detection are 5.5 and 6 times better than the obtained without solid phase microextraction for CH_3Hg^+ and Hg^{2+}, respectively. The methodology was found linear up to 120 μg L^{-1} and reproducible from one day to the following. It was validated with certified reference materials NCS ZC 71001 (beef liver) and BCR No 186 (pig kidney) for total mercury, calculated as the sum of species, and with spiked red deer liver and kidney for speciation. Finally, it was applied to the analysis of samples of red deer liver, red deer kidney and wild boar kidney coming from the Almadén's mercury mining area (Ciudad Real, Spain), the longest and largest producer of mercury in the world until its closure in 2002.

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1. Introduction

Mercury is a non-essential, toxic, and naturally occurring element that is widespread in the environment. Mercury occurs in different chemical and physical forms [1,2]. The most important forms are elemental (Hg^0), inorganic (Hg^{2+}) and methylmercury (CH_3Hg^+), which differ with respect to kinetics and toxicology [3–7]. CH_3Hg^+ is known as a very important neurotoxicant that bioaccumulates in the aquatic food webs, being the main source of human exposure the fish and/or seafood consumption. According to European Union's legislation, the maximum levels set for total mercury in fishery products are 0.5 mg kg^{-1} [8], with the exception of certain listed fish species for which 1 mg kg^{-1} applies. However, due to the special behavior of CH_3Hg^+, the FAO/WHO Joint Expert Committee on Food Additives (JECFA) set a Provisional Tolerable Weekly Intake (PTWI) for methylmercury [9] of 1.6 μg kg^{-1} body weight in 2003, instead of doing so for total mercury.

Mercury's chemistry and biochemistry in aquatic environments has been largely studied but the role of mercury in terrestrial animals has been neglected, even though the majority of total Hg (approximately 60%) is estimated to be deposited in terrestrial environments, which becomes an important focus of transfer and bioaccumulation in local food webs [10]. As a result, data on levels of mercury species in terrestrial animals are scarce. It is also remarkable that levels of Hg in meat of terrestrial animals are still under no regulation, as opposed to other heavy metals such as Pb and Cd [11]. Concerning to analytical methodology, no analytical procedures are available and validated for terrestrial animal tissues. Up to now, the validation of the different methodologies for mercury speciation has been carried out with certified reference materials of fish tissue even though the samples to be analyzed were of completely different nature [12]. In this way special attention should be paid at the fact that mercury accumulation and transfer of the species in terrestrial food chains in terrestrial animals is very different to marine food chains [13].

The speciation of mercury has been based on coupling chromatographic separation to mercury specific detection. Although

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liquid chromatographic techniques have been used for these analyses [14–17], gas chromatography is the most frequently employed technique due to its excellent separation efficiency and the availability of coupling to a number of highly sensitive and specific detectors, such as mass spectrometry (GC–MS) [18–23], atomic fluorescence spectrometry via pyrolysis (GC-pyro-AFS) [1,2], cold vapor atomic fluorescence spectrometry (GC-CV-AFS) [25,26], microwave-induced plasma atomic emission spectrometry (GC-MIP-AFS) [27,28] or inductively coupled plasma mass spectrometry (GC-ICP-MS) [24,29,30]. GC-pyro-AFS is preferred by many groups due to its low cost and robustness, but also because it is simple, fast, sensitive and selective. In fact, in previous papers, we described a procedure for the speciation of mercury in fish reference materials [31] and sediments [32] by using GC-pyro-AFS. In spite of the developments in instrumentation, the most important drawbacks are still in the sample preparation. The analysis of mercury species by gas chromatography requires a previous and efficient derivatization process to form volatile derivatives. There are different options but the most frequently used is ethylation by $NaBEt_4$ reagent [33,34]. The extraction and separation of mercury compounds in solid samples, such as sediments or biotissues, is also one of the key steps. At present, the most popular extraction techniques are steam distillation [35], supercritical fluid extraction [36] and acid or basic liquid extraction either at room temperature [37] or using different heating sources such as sonication [38] or microwaves [39]. The use of microwave-assisted extraction technique has been confirmed as one of the best methods for mineralization and selective leaching of analyte compounds [31].

In the particular case of terrestrial animals, levels of mercury species are expected to be lower than in fish and fishery products, i.e. tens of ng g^{-1}, because important accumulation process are not likely to occur. Therefore low detection limits are required and, consequently, a pre-concentration step has to be included in the analytical methodology. In this way, solid phase extraction [40–42] and, recently, even cloud point extraction [43] has been assayed, but the most used technique for this purpose has been solid-phase microextraction (SPME) [44]. This technique simplifies sample preparation while retaining the merits of GC, is solvent free, and offers low costs and feasibility of automatization.

The aim of this work is to develop and validate a methodology for mercury speciation fulfilling requirements, particularly limit of detection, so as to be applied to the analysis of tissues of terrestrial animals. For this purpose, GC-pyro-AFS will be used after head space solid-phase microextraction (HS-SPME), which replaces the liquid–liquid extraction with hexane that we used up to present. The mercury species extraction and derivatization conditions will be optimized. Moreover, the validation will be carried out with reference materials and/or fortified samples, both coming from terrestrial animal tissues. The methodology will be applied to the speciation of red deer and wild boar samples from the area of the recently closed Almadén Hg mining district (Ciudad Real province, Southern Spain).

2. Experimental

2.1. Standards, solutions and reagents

Stock standard solutions of 1000 μg mL^{-1} of Hg^{2+} and CH_3Hg^+ were prepared by dissolving mercury (II) chloride (Panreac) in 5% HNO_3 (Merck) and methylmercury chloride (Strem Chemicals) in methanol, respectively. All stock solutions were stored in amber glass bottles in a cold room at 4 °C. Working standards were prepared daily by proper dilution with ultrapure water.

For the sample extraction, methanolic tetramethylammonium hydroxide (25%, w:w) was obtained from Sigma-Aldrich (Steinheim, Germany). Sodium tetraethylborate 98% was purchased from Strem Chemicals (Bischeim, France). All chemicals were analytical grade.

Ultrapure water (18.2 MΩ × cm) was obtained from an Elga Purelab Ultra Analytic water purification system.

Helium C-50 was used as carrier gas and argon C-50 was used as make-up and sheath gas at the transfer line and the AFS detector, respectively (Carburos Metálicos, Spain).

2.2. Instrumentation

A gas chromatograph (Shimadzu GC-2010, Shimadzu Corporation, Kyoto, Japan) was coupled to an AFS detector (Millenium Merlin, P. S. Analytical, United Kingdom) via pyrolysis unit. The instrumental configuration is described elsewhere [31]. The GC was provided with a non-polar capillary column (DB-5, 30 m × 0.25 mm × 0.25 μm, Teknokroma, Barcelona, Spain) and with an inlet liner with an internal diameter of 0.75 mm. Helium was used as carrier gas. The chromatographic conditions are summarized in Table 1. Finally, data were acquired using the Speciation Application Millenium Systems Software (P. S. Analytical, United Kingdom) and processed by Microcal Origin 5.0 (Microcal Software, Inc., Northampton, MA, USA).

A manual SPME device, equipped with a Supelco 57300-U fused silica fiber coated with a 100 μm film of polydimethylsiloxane (PDMS) (Supelco, Bellefonte, USA), was used for the sampling of ethylated species from the head space above the aqueous solutions.

A laboratory microwave system (Ethos Plus, Milestone, Monroe, CT, USA), equipped with temperature and pressure feedback control was used in this study. This device is accurate in sensing temperature within ±2.0 °C of set temperature, and automatically adjusts the microwave field output power. It is prepared for extracting ten samples simultaneously. The high pressure closed digestion vessels used for extraction are made of high purity TFM (a thermally resistant form of Teflon) and have a capacity of 100 mL.

2.3. Sample preparation

Mercury extraction for speciation analysis in terrestrial animals was carried out with 0.2 g of sample and 2.0 or 5.0 mL of tetramethylammonium hydroxide (TMAH) solution for liver and kidney, respectively. The final volume was adjusted to 10 mL with ultrapure water for microwave requirements. A clear solution was obtained after microwave irradiation. Then the vessels were cooled down to room temperature, made-up to a known volume and stored in the cold room until analyzed. Blanks were prepared along with the samples in each batch.

Table 1
Operating conditions for GC-pyro-AFS system.

Gas chromatograph	
Column	DB-5, 30 m × 0.25 mm × 0.25 μm
Temperature program	40 °C (3 min), 40 °C min^{-1}, 200 °C (1 min)
Carrier gas	He at 3 mL min^{-1}
Pyrolyser	
Pyrolysis temperature	800 °C
Atomic fluorescence detector	
Make-up gas	Ar at 150 mL min^{-1}
Sheath gas	Ar at 300 mL min^{-1}
AFS gain	1000
Filter factor	16

Volumes of 2 mL of the alkaline extracts were used for derivatization in 10 mL capped amber vials. The pH was adjusted to 3.9 using 5 mL of 0.1 M acetic acid-sodium acetate buffer. Then, 500 μL or 1.3 mL of sodium tetraethylborate at 3% (w:v) for liver and kidney, respectively, was added for derivatization and later, the vial was capped and shaken for 5 min. An aliquot of 1 mL of this solution was put in a 10 mL vial and encapsulated. The SPME needle was inserted through the septum and HS sampling was performed for 10 min. The fiber was desorbed onto the GC injection port at a depth of 2.5 cm. A 0.5 min desorption time at 200 °C ensured maximum desorption of the analytes from the fiber.

2.4. Certified reference materials and samples

The certified reference materials used were NCS-ZC 71001 (beef liver) from China National Analysis Center for Iron and Steel (Beijing, China) and BCR no. 186 (pig kidney) from the Institute for Reference Materials and Measurement (IRMM, Geel, Belgium). Both are certified for total mercury and they were used as provided.

Red deer (*Cervus elaphus*) and wild boar (*Sus scrofa*) were sampled from regular shooting allocations during 2005–2006 hunting season in Ciudad Real province (Spain). Sampling took place after "monterías" (large driven hunts), at the end of which the animals are butchered on site. Red deer liver and kidney and wild boar kidney were taken out from the body, transferred to a −80 °C freezer within 2 h. Later on, they were freeze-dried and stored at −20 °C until analysis.

3. Results and discussion

3.1. Optimization of extraction and derivatization

Preliminary experiments were carried out to select conditions for the extraction from solid matrix and derivatization. The initial point was a previously reported methodology for mercury speciation in fish tissues [31]. The procedure is based on the quantitative closed-vessel microwave-assisted extraction with an alkaline extractant (TMAH, tetramethylammonium hydroxide) and the derivatization by ethylation with $NaBEt_4$. This procedure was applied to two terrestrial animal tissues certified reference materials, NCS ZC 71001 of beef liver and BCR 186 of pig kidney, which are certified only for total mercury. For beef liver, the results showed that the found value of total mercury (0.167 ± 0.013 μg g^{-1}, $n=3$), calculated as sum of species, was comparable to the certified value (0.18 μg g^{-1}). In this case, mercury species distribution found was 42.9% for CH_3Hg^+ and 56.1% Hg^{2+}. On the other hand, the found values for pig kidney were poor in terms of accuracy and reproducibility. As a consequence, the sample preparation for liver was decided to be the same as for fish tissues, whereas specific extraction and derivatization for kidney tissues had to be carried out.

The optimization process was carried out using 0.2 g of kidney tissue spiked at 2.5 μg g^{-1} (expressed as Hg) of both species.

3.1.1. Optimization of TMAH volume

Kidney samples spiked as explained above were extracted with 2.0, 3.0 and 5.0 mL of the TMAH solution and followed the sample preparation as described previously for fish tissues [31]. The results showed that recoveries for CH_3Hg^+ were over Hg^{2+}, but in all cases from 50 up to 85%, so therefore they could not be considered as quantitative. A volume of 5.0 mL of TMAH solution was selected for further studies because it provided the most similar recoveries for both species (69.5% for CH_3Hg^+ and 60.5% for Hg^{2+}).

3.1.2. Optimization of $NaBEt_4$ concentration

This step was carried out in order to find out whether the concentration of the derivatizing agent, $NaBEt_4$, had an influence on the efficiency of the sample preparation. Thus, spiked kidney samples were extracted with 50 mL of TMAH and derivatized with different concentrations of $NaBEt_4$ from 0.1 up to 1.2% (w:v) in the vial. The results showed that the recoveries increased with the concentration of $NaBEt_4$ (Fig. 1). Apparently, an unidentified constituent(s) of the sample consume(s) the bulk of the derivatization reagent before it reacts with the target species and that is why an increase in the concentration of ethylation reagent was necessary. Quantitative recoveries were obtained for 0.8% (w:v) and over, so this concentration was selected as optimum (Fig. 1).

3.2. Optimization of HS-SPME conditions

Head space mode was chosen because our analytes have been previously derivatized to volatile species. In this mode, no optimization of pH or NaCl added is required, as opposed to what happens in the immersion mode, and exposure of SPME fiber to the sample matrix is minimized, thereby enhancing its lifetime. All the parameters were optimized using 1 mL of a solution containing the ethylated species at 25 μg L^{-1} expressed as Hg, placed in a 10 mL amber glass vial, which was allocated in a water bath at 35 °C. The initial conditions were an absorption time of 15 min, a desorption time of 1 min at 250 °C, and a fiber depth of 3 cm. Optimization was carried out varying the different factors one by one whilst keeping the rest unchanged. All experiments were carried out by triplicate, and the average of the areas obtained was used in order to monitor each analyte.

3.2.1. Absorption time

At this point it is convenient to clarify that the selected PDMS fiber works by absorption, which means that analytes can be placed in the surface and also in the interior of the fiber. Other fibers work by adsorption, which means that analytes are retained only on the surface of the fiber. Thus, for this study, the absorption time was varied from 5 to 40 min. The maximum peak area for Hg^{2+} and CH_3Hg^+ was found for an absorption time of 10 min. Therefore, an absorption time of 10 min was selected as optimum.

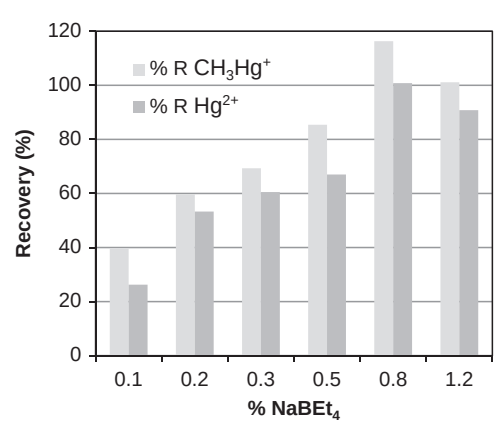

Fig. 1. Influence of the $NaBEt_4$ concentration on the mercury species recovery in kidney.

3.2.2. Desorption time

The influence of desorption time on peak area was studied from 0.5 to 10 min and it is plotted as Fig. 2. In this case, the maximum peak area for Hg^{2+} and CH_3Hg^+ was found for 0.5 min, but sharply decreased for a time of 1 min. Then, a moderate decrease was observed as time increased. This could be because, at the initial moments, the fiber is saturated with analytes and the injection port is free from them. As a consequence, there is a strong desorption from the fiber onto the liner. Then, as time becomes longer, there seems to be a re-equilibration between both the fiber and the gaseous phase in the liner and, as a consequence, an increasing fraction of analytes come back to the fiber. Once the equilibrium is reached, the concentrations of the analytes in the liner remain stable, and that is why the areas do not change significantly.

Owing to the fact that our interest is to find the conditions to obtain the best signal to noise ratio for our analytes, in other words, the maximum peak area, a desorption time of 0.5 min was selected as optimum.

3.2.3. Desorption temperature

The desorption temperature was varied from 200 to 270 °C in steps of 10 °C. The peak areas of the analytes were similar up to 210 °C. However, for temperatures over 230 °C, the area of CH_3Hg^+ decreased as the area of Hg^{2+} increased, which could indicate a degradation of the former into the latter. As a consequence, a temperature of 200 °C was selected as optimum for desorption.

3.2.4. Depth of the fiber in the injector

The optimization of the position of the fiber and the use of liners of narrow internal diameter, ca. 0.75 mm, are common strategies to get a fast desorption, minimize the dispersion and increase the sensitivity. Thus, the depth of the fiber in the injector was varied from 2.5 upto 4.5 cm in steps of 0.5 cm. As shown in Fig. 3, the areas of the analytes decreased as the depth increased. The best result in terms of signal to noise ratio was obtained for a depth of 2.5 cm, so this was considered as optimum.

The optimized conditions are summarised in Table 2, and a chromatogram of a standard of 25 μg L^{-1} of the analytes (expressed as Hg), obtained under these conditions, is shown in Fig. 4. Analytical blanks were prepared and injected after a standard in such conditions without finding any peak from the analytes, which means no carryover existed from one injection to the following.

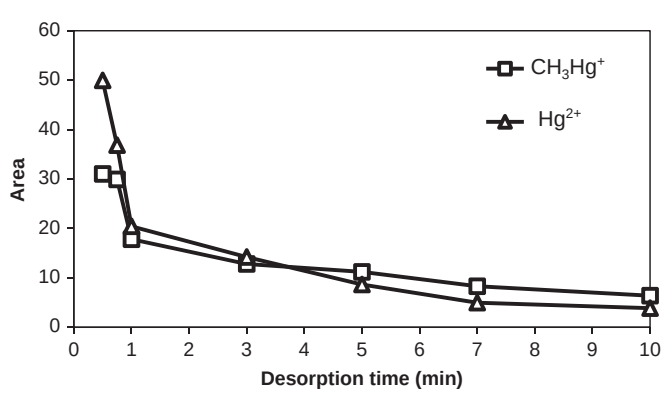

Fig. 2. Influence of the desorption time on the peak area.

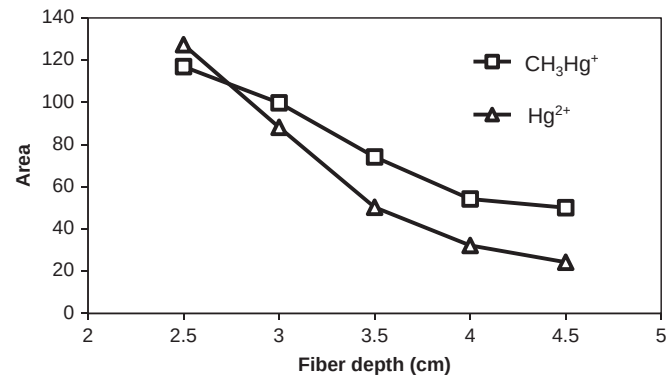

Fig. 3. Influence of the fiber depth on the peak area.

Table 2
Optimized HS-SPME sampling conditions with a PMDS fiber of 100 μm.

Absorption		Desorption	
Absorption time	10 min	**Injection mode**	Splitless
Absorption temperature	35 °C	**Desorption time**	0.5 min
Vial volume	10 mL	**Desorption temperature**	200 °C
Sample volume	1 mL	**Depth of the fiber**	2.5 cm

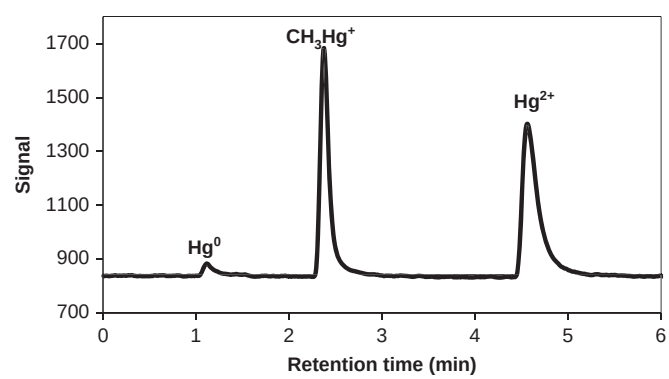

Fig. 4. Chromatogram of a standard of 25 μg L^{-1} of the analytes (expressed as Hg) under the optimised conditions of HS-SPME-GC-pyro-AFS.

3.3. Validation of the methodology

3.3.1. Analytical figures of merit

The instrumental limits of detection (LOD) and quantification (LOQ) were estimated according to the signal to noise ratio of the chromatogram, as described elsewhere [31], and summarized in Table 3. These LODs are 5.5 and 6 times lower for CH_3Hg^+ and Hg^{2+}, respectively, than the typical LOD obtained without HS-SPME in the same chromatographic conditions. According to the sample preparation procedures, which are specific for each tissue, these LODs would allow the detection of concentrations of 31.8 ng g^{-1} and 52.5 ng g^{-1} for CH_3Hg^+ and Hg^{2+}, respectively, for liver and 35.3 ng g^{-1} and 58.1 ng g^{-1} for CH_3Hg^+ and Hg^{2+}, respectively, for kidney.

The linearity was checked in a range from 2 upto 120 μg L^{-1} of the species. The equations and regression coefficients are summarized in Table 3. In all cases, the calibration curves showed good linear relation between area and concentration. Moreover, intercepts were found not different from zero, according to Student's test "t" ($p=0.05$), proving that no blank offset was obtained.

The reproducibility over time was evaluated by running 6 replicates of a standard of 25 μg L^{-1} (as Hg) of the analytes

along one day, and 4 replicates of the same standard along the following day. The standard deviations of the areas were compared between both days using the Snedecor test "*F*" for two tales ($p=0.05$). The results showed no significant differences in the precisions obtained for each individual day.

3.3.2. Accuracy

The accuracy of the methodology was evaluated by the analysis of two terrestrial animal tissues certified reference materials, NCS ZC 71001 (beef liver) and BCR 186 (pig kidney). Unfortunately, only total mercury is certified in these materials, so we used the sum of the concentrations of both CH_3Hg^+ and Hg^{2+}, obtained in the speciation analysis as total content of mercury, in order to compare with the certified concentration. The concentrations obtained for the reference materials are given in Table 4, showing satisfactory agreement with the certified values, with recoveries of 96% and 109.6% of total mercury.

Owing to the fact that no reference materials of terrestrial animal tissues are certified for mercury species, TMAH extracts from red deer liver and kidney were spiked so that the concentration of the derivatized species before HS-SPME sampling was 25 μg L^{-1}, expressed as Hg. An analytical blank was also prepared accordingly. The recoveries obtained are shown in Table 4, and were considered satisfactory.

3.4. Applications

The present methodology was applied to the analysis of two samples of red deer liver, two samples of red deer kidney, and two samples of wild boar kidney coming from Almadén's mining district area. These samples were initially analysed by GC-pyro-AFS without HS-SPME preconcentration, without detecting CH_3Hg^+ in any of them. Samples were then analyzed by triplicate using the presented methodology with the HS-SPME step. The results, summarized in Table 5, indicate that HS-SPME allows the detection of species that could not be detected without. As examples, two chromatograms corresponding to sample RDL#1 with and without HS-SPME are shown in Fig. 5.

Table 3
Calibration curves, determination coefficients (r^2) and instrumental limits of detection and quantification.

	CH_3Hg^+	Hg^{2+}
Calibration curve	$A=28\ (\pm 14)+3.14\ (\pm 0.26)\ c$	$A=10.6\ (\pm 7.5)\pm 4.18\ (\pm 0.14)\ c$
r^2	0.9742	0.9942
LOD (μg L^{-1})	0.17	0.28
LOQ (μg L^{-1})	0.58	0.92

A: peak area (no units). c: concentration (μg L^{-1} as Hg).

Because of the limited number of samples, no significant conclusions on the biological significance of the mercury speciation can be obtained. However, as an initial approach, it can be observed that, in terms of chemical pattern, CH_3Hg^+ is the dominant species in red deer liver samples, whereas Hg^{2+} is the dominant species in both red deer and wild boar kidney. Further experiments will be carried out with a more representative number of samples to obtain significant conclusions for mercury species mechanism of accumulation and transfer in terrestrial animals.

4. Conclusion

This paper constitutes a step forward in the field of mercury speciation in terrestrial animal tissues, where literature, namely reporting levels, is scarce up to present. From the analytical point of view, the main contribution is to include HS-SPME in the analytical methodology, which has successfully replaced liquid–liquid extraction with hexane and has improved LODs up to

Table 5
Analysis of samples of red deer liver (RDL), red deer kidney (RDK) and wild boar kidney (WBK) for mercury species (expressed in ng g^{-1} as Hg) by HS-SPME-GC-pyro-AFS.

Sample name	CH_3Hg^+	Hg^{2+}	% CH_3Hg^+
RDL#1	329.4 ± 3.8	117.14 ± 0.62	73.8
RDL#2	425 ± 21	95.9 ± 2.7	81.6
RDK#1	< LOD	735 ± 75	–
RDK#2	78.09 ± 0.60	675 ± 70	10.4
WBK#1	< LOD	698.3 ± 2.0	–
WBK#2	< LOD	266 ± 67	–

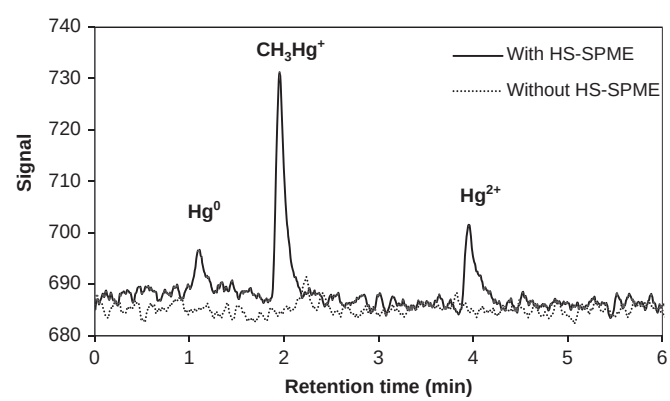

Fig. 5. Chromatograms corresponding to the sample RDL#1 (reed deer liver) with and without HS-SPME.

Table 4
Analysis of certified reference materials for mercury species (expressed in μg g^{-1} as Hg) and recoveries (%) obtained in spiked blank, red deer liver and red deer kidney.

	Found			Certified
	CH_3Hg^+	Hg^{2+}	Total Hg*	Total Hg
CRMs				
NCS ZC 71001	0.076 ± 0.012	0.097 ± 0.048	0.173 ± 0.035 ($n=3$)	0.18
BCR no 186	< LOD	2.160 ± 0.057	2.160 ± 0.057 ($n=4$)	1.97 ± 0.04
Spiked samples				
Blank	98.0 ± 7.4	110.2 ± 6.2		
Red deer liver	78.0 ± 5.2	82.1 ± 6.1		
Red deer kidney	116.3 ± 7.7	100.8 ± 7.5		

* Calculated as sum of species.

5.5 and 6 times for CH_3Hg^+ and Hg^{2+}. Moreover, this is the first work using terrestrial animal tissues for validation in the analyses of samples of terrestrial animals, which improves the quality of the results obtained.

Finally, HS-SPME has proved to be a technique of choice for the speciation of mercury in samples of terrestrial animal tissues whose levels were not detectable without it.

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