Challenge
Mercury species extraction is crucial for speciation, but requires preserving species integrity in a compatible medium for separation

Solution
New method for mercury species extraction from fish and seafood using thiol-containing reagent [1] and microwave-assisted extraction to avoid inter-conversions

Intended audience
For ICP-MS users in food, environmental testing, and academia: Analyze mercury species in tuna fish to examine contaminations in natural ecosystems

Speciation of Mercury in Fish and Seafood using HPLC-ICP-MS

Introduction

Mercury is one of the most studied environmental pollutants due to its high toxicity and mobility in the environment. Considering the ability to travel over long distances in the atmosphere as gaseous elemental species, mercury is regarded as a 'global pollutant' [2]. The high toxicity of mercury is given, *inter alia*, by its methylated form, methylmercury ($MeHg^+$), which is widely recognized as a neurotoxin affecting humans [2].

The main pathway of human exposure to mercury is the food chain. The great ability to bioaccumulate in the aquatic food chain leads to considerably elevated levels of $MeHg^+$ in aquatic organisms of higher trophic levels of the food chain despite nearly immeasurable quantities of Hg^{2+} in the water. Many foodstuffs, particularly fish, contain most of the Hg as $MeHg^+$. The provisionally tolerable human consumption of $MeHg^+$ is limited to 1.6 µg/kg body mass, per week [3]. However, taking into account that a mass fraction of Hg of 1.0 µg/g is permitted for certain fish species, a consumer could easily exceed such a recommendation [4].

Nowadays the reliable analysis of mercury species has been mainly achieved by hyphenated techniques, including gas chromatography (GC) or high-performance liquid chromatography (HPLC) coupled with a mercury-selective detection technique, such as atomic fluorescence spectrometry (AFS), atomic emission spectrometry (AES), atomic absorption spectrometry (AAS) or inductively coupled plasma-mass spectrometry (ICP-MS) [5]. Among the methods mentioned above, the coupling of HPLC to ICP-MS appears to be one of the most considered methods for mercury speciation analysis because of its ease of sample preparation, simplicity of the interface.

A successful extraction procedure for speciation analysis requires high extraction efficiency, and more importantly, all original species must be kept intact prior to analysis. To lower the possibility of species transformation and quantitatively extract inorganic and methylmercury from fish and seafood samples, a mild microwave-assisted extraction (MAE) method using an extractant solution containing 1% L-cysteine and 0.1% thiourea was developed in this work. After extraction, total mercury and mercury species were determined by ICP-MS and HPLC–ICP-MS, respectively. The developed method was validated by analyzing threefish and seafood certified reference materials: IAEA 436a (Tuna Fish), DORM-2 (Dogfish Muscle), and TORT-1 (Lobster Hepatopancreas).

Materials and Methods

Instrumentation

Microwave-assisted extraction

A microwave oven system (speedwave XPERT, Analytik Jena) with a rotor for 12 PTFE digestion vessels (DAP60X) was used for sample preparation and extraction.

Inductively coupled plasma-mass spectrometry

A PlasmaQuant MS Q ICP-MS was used to determine total Hg concentration. Nickel sample and skimmer cones and a Peltier-cooled Scott type spray chamber were used.
The ICP-MS system was equipped with a CETAC ASX-560 autosampler (Teledyne CETAC Technologies) and an ESI oneFast injection valve (Elemental Scientific Inc.) for automation of the analysis.

HPLC-ICP-MS

A PQ LC HPLC System, non-metal, PEEK (Analytik Jena) equipped with a Phenomex C18 separation column was used to determine the Hg species in fish and seafood. The outlet of the separation column was directly connected to a Seaspray® nebulizer, and the PlasmaQuant MS Q was used as an element specific detector. The ICP-MS and HPLC operating conditions are given in Table 1. Hg was detected at m/z 202. The ICP-MS and HPLC systems were checked daily, and the data was processed using the Clarity workstation.

Table 1: Optimized HPLC-ICP-MS operating parameters

Parameter (ICP-MS)	Specification	Parameter (HPLC)	Specification
Plasma gas flow	9.0 L/min	Reverse phase C-18	Phenomex, Synergi (4 µm, 150 mm × 4.6 mm)
Auxiliary gas flow	1.50 L/min	Mobile phase	0.2% L-cysteine + 0.1% thiourea, pH 2.3
Sheath gas flow	0.00 L/min	Injection volume	100 µL
Nebulizer gas flow	1.05 L/min (Seaspray®)	Flow rate	1.4 mL/min
Sampling depth	5.0 mm	Gradient	Isocratic
Plasma RF power	1.40 kW	Elution times	Hg^{2+} – 1.44 min MeHg – 2.30 min EtHg – 4.91 min
Pump rate	100 rpm – blue/blue PVC pump tubing	Total run time	10 min
Stabilization delay	0 s	Column temperature	40 °C
iCRC gas setting	H_2 20 mL/min	Evaluation mode (Clarity)	Peak Area - ^{202}Hg
Dwell time	^{202}Hg – 500000 µs		

Samples and reagents

All solutions were prepared with deionized water (Milli Q Plus system; Millipore, Bedford, MA) with an 18.2 MΩ/cm resistivity. For the speciation studies, standard solutions (1,000 mg/l Hg) of Hg compounds were prepared as follows: Mercury (II) chloride ($HgCl_2$, ACS grade, Merck Sigma-Aldrich, Germany) in 0.1% (v/v) HCl; Methylmercury(II) chloride (CH_3HgCl, purity ≥ 95%, Sigma Aldrich Fine Chemicals Biosciences, Germany) and Ethylmercury chloride (CH_3CH_2HgCl, purity ≥ 95%, Fisher Scientific, Portugal) were dissolved in water and 20% (v/v) methanol (CH_3OH, purity ≥ 99.8% for HPLC, VWR International, LLC, Germany). All the stock standard solutions were protected from light in pre-cleaned Pyrex® glass vials and stored at 4 °C. The mercury working standards were prepared daily by proper dilution with mobile phase. Mobile phase and extraction solution were prepared from L-Cysteine, free base ($C_3H_7NO_2S$ purity ≥ 98.5%, Merck Sigma-Aldrich, Germany) and thiourea (CH_4N_2S ACS grade, Merck Sigma-Aldrich, Germany). The mobile phase solution was adjusted to pH 2.3 with 1 mol/L hydrochloric acid (HCl, Suprapur 30%, Merck Sigma-Aldrich, Germany) and 1 mol/L ammonium hydroxide ((NH_4OH) 20%, OPTIMA ultra-pure grade, Fisher Scientific, Portugal).

For total mercury measurements, calibration was performed with a mercury single element standard (1.000 ± 3 mg/ml in 2% (v/v) HNO_3, VWR International, LLC, Germany). Gold single element standard (1.000 ± 2 mg/ml in 2% (v/v) HNO_3, VWR International, LLC, Germany) was used to stabilize mercury in all the solutions.

Three certified reference materials for individual Hg species, IAEA 436a (Tuna Fish) from the International Atomic Energy Agency, DORM-2 (Dogfish Muscle), and TORT-1 (Lobster Hepatopancreas) from the National Research Council Canada were used to validate the results.

Determination of total Hg

To evaluate the efficiency of the applied extraction procedure, total mercury concentrations in extracts of IAEA 436a, DORM-2 and TORT-1 were determined by ICP-MS after mild MAE. Three independent weights of each material (0.2 g) were weighed into a dry and clean PTFE vessel, followed by the addition of 20 mL of 1% L-Cysteine + 0.1% thiourea + 50 µg/L of Au. The vessels were closed, placed into the rotor, and tightened. The loaded rotor was then placed into the microwave oven. The microwave oven program for Hg species extraction from fish and seafood samples was comprised of two steps as follows: a microwave power of 100 W with a first ramp for 3 min up to 65 °C followed by 15-min hold; a second ramp for 3 min up to 80 °C followed by 15-min hold. After cooling for 30 min, the vessels were opened carefully. Each extraction was centrifuged for 5 min at 3,500 rpm. The supernatant was then filtered through a Millipore membrane (0.45 µm), transferred to 15-ml Pyrex® glass vials, and stored at 4 °C for analysis.

Total mercury concentrations in the extracted reference materials were determined by ICP-MS. A calibration was performed by using concentration levels between 0.1 and 5 µg/L in1% (v/v) HNO_3 + 500 µg/L of Au to ensure the stability of Hg in the standards and to avoid memory effects from the mercury load in the system.

Determination of Hg species in certified reference materials of fish and seafood

The CRM extracts were further diluted 2, 5 and 10 foldswith the mobile phase and directly injected into the HPLC within 24 h after extraction for Hg species quantification. Spikes recoveries (5 µg/L) of individual Hg species were also performed in different dilutions to evaluate accuracy of the proposed method.

Results and Discussion

Calibration curve, LODs and MDL

Before the analysis of the CRM extracts, the HPLC was calibrated with a mix of the three Hg species ranging from 0.05 to 25 µg/L. Calibration curves are shown in Figure 1, which reveals the linearity ($r^2 > 0.9997$) for all the three species monitored.

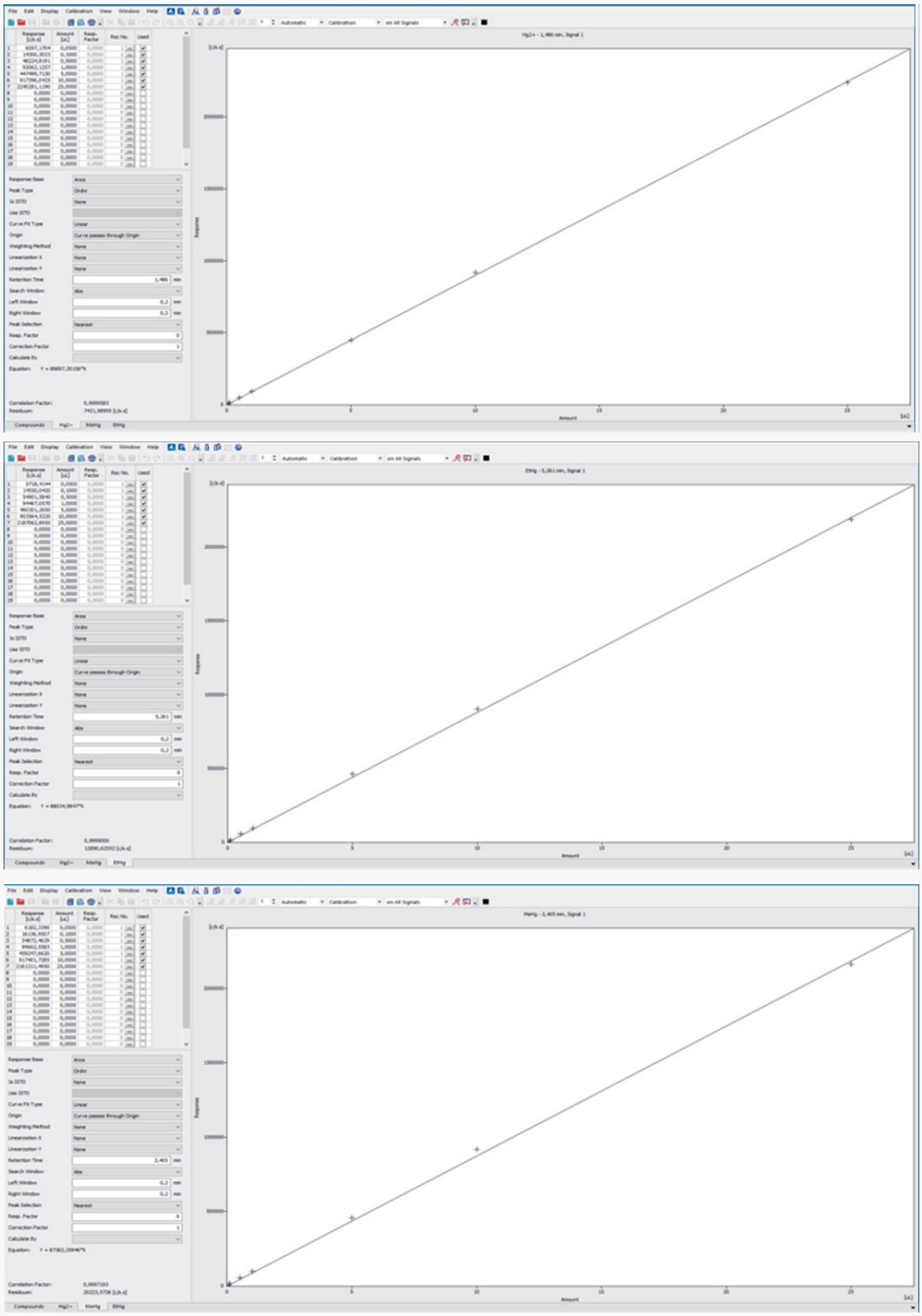

Figure 1: Calibration curves for the determination of the three Hg species in fish

The limits of detection (LOD) for the Hg species were calculated as three times the chromatographic peak-to-peak signal-to-noise ratio (S/N) of the lowest calibration standard in comparison to blank noise level, as follows:

Table 2: Details of 0.05 standard for LOD and MDL estimation

Analyte	0.05 µg/L standard	Noise	S/N Ratio	LOD (ng/L)	MDL (µg/kg)
Hg^{2+}	6097.17	184	33	1.1	0.55
MeHg	6182.24	167	37	1.3	0.64
EtHg	5718.41	142	40	1.4	0.69

Total Hg and Hg species concentrations

Table 3 shows the Hg total concentrations obtained in the extracts (n=3) by ICP-MS, which are in agreement with the certified values, since the recoveries are within 96-113%, RSD < 4.5% and Z-score within [-2;2]. Table 4 shows the concentration of Hg species obtained in the three CRMs. According to the results, quantitative recoveries from 95% to 122% were achieved. This demonstrates the accuracy of the results, which are in agreement with the certified values for the two Hg species in all the tested materials. In addition, spikes recoveries were within 82%-102% with %RSD below 13.8%, indicating the extraction procedure was effective for all the species, including EtHg, without any inter conversion between them or any matrix effects. Figure 2 shows an exchange or DOR-2 with TORT-1 chromatogram profile for inorganic Hg, MeHg, and EtHg diluted 2-fold and spiked with 5 µg/L of each species.

Table 3: Concentration of total mercury in IAEA 436a (Tuna Fish), DORM-2 (Dogfish Muscle) and TORT-1 (Lobster Hepatopancreas) determined by ICP-MS after microwave extraction

CRM ID	Certified values (mg/kg)	Concentration (mg/kg)	RSD, % (n=3)	Recovery (%)	Z-score
IAEA 436a	4.26 ± 0.36	4.49 ± 0.20	4.5	105	0.63
DORM-2	4.64 ± 0.26	4.46 ± 0.03	0.7	96	-0.69
TORT-1	0.33 ± 0.06	0.374 ± 0.005	1.2	113	0.74

Table 4: Concentration of mercury species, precision (n=3) and spike recoveries (5 µg/l) in IAEA 436a (Tuna Fish), DORM-2 (Dogfish Muscle) and TORT-1 (Lobster Hepatopancreas) determined by HPLC-ICP-MS after microwave extraction. nd: not detected

CRM ID	Certified values (mg/kg)	Concentration (mg/kg)	RSD, % (n=3)	Recovery (%)	Spike recovery (%)
	Hg^{2+} (mg/kg)				
IAEA 436a	0.64 ± 0.11	0.61 ± 0.08	13.8	95	99
DORM-2	0.17 ± 0.06	0.21 ± 0.02	10.4	122	93
TORT-1	0.202 ± 0.06	0.23 ± 0.01	4.3	114	102
	MeHg (mg/kg)				
IAEA 436a	3.62 ± 0.47	3.56 ± 0.21	5.8	98	100
DORM-2	4.47 ± 0.32	4.31 ± 0.05	1.7	96	91
TORT-1	0.128 ± 0.014	0.128 ± 0.01	2.8	100	95
	EtHg (mg/kg)				
IAEA 436a	not certified	nd	-	-	82
DORM-2		nd	-	-	86
TORT-1		nd	-	-	94

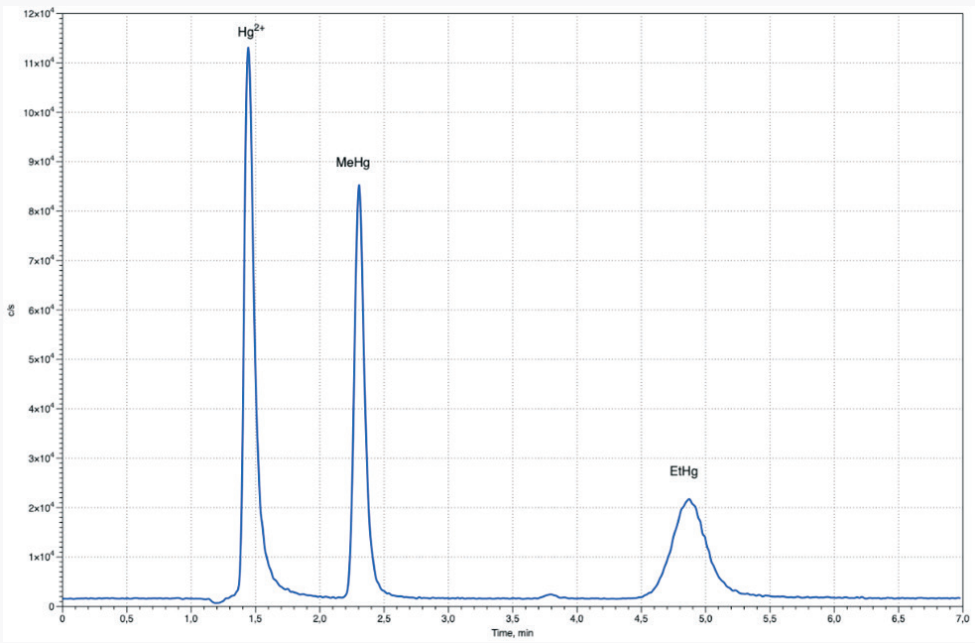

Figure 2: Chromatogram profile of TORT-1 diluted 2-fold and spiked with 5 µg/L of each species

Quality control (QC)

During the analysis sequence, a check standard (QC - 1 µg/L) was measured periodically. The precision was evaluated in terms of %RSD, where values less than 6.5% at 1 µg/L level were obtained, demonstrating the stability of the HPLC-ICP-MS system (Table 5).

Table 5: QC check at 1 µg/L during analysis sequence

Runs	Hg^{2+}	MeHg	EtHg
1	0.920	1.003	0.973
2	0.979	0.979	1.014
3	1.019	1.019	1.030
Mean	0.950	1.016	1.012
SD	0.06	0.01	0.04
%RSD	6.5	1.3	3.6

Conclusion

A HPLC method for the determination of inorganic (Hg^{2+}), methylmercury (MeHg), and ethylmercury (EtHg) with ICP-MS detection has been developed. Under optimized conditions, separation of all these three Hg species could be achieved on a Phenomex C-18 column within 7 min of run. Furthermore, a simple, rapid, effective, and reliable sample preparation procedure, without loss of species or interconversion and loss, has been successfully applied to the quantitative extraction of inorganic and methylmercury from reference materials. This allowed for further determination through the use of HPLC-ICP-MS, with LODs in the range of low ng/L.

Figure 3: PlasmaQuant MS Q

Recommended device configuration

Table 6: Overview of devices, accessories, and consumables

Article	Article number	Description
Microwave-assisted extraction		
speedwave XPERT - Microwave Pressure Digestion System	819-5005000-2	speedwave XPERT is a universally applicable microwave digestion system for the preparation of organic and inorganic sample materials that impresses with its reliability, safety, and economy
Inductively Coupled Plasma Mass Spectrometry		
PlasmaQuant MS Q	818-08011-2	The PlasmaQuant MS Q is a high-performance ICP-MS, capable of measuring over 75 elements in a single measurement, from ultra-trace to major levels
(ESI) oneFAST Complete (or eqivalent)	810-88100-0	The oneFAST sample introduction accessory increases sample throughput and lowers operating costs by reducing sample uptake and washout times
Teledyne-Cetac ASX 560 Autosampler	810-88015-0	The Teledyne CETAC Technologies ASX-560, next generation autosampler with integrated rinse function is sleek and durable by design
HPLC		
PQ LC - HPLC System, non metal, PEEK	810-88409-0	The PQ LC system is a versatile and customizable solution, with a Quaternary Gradient Pump, optional vacuum degasser, and micro- or analytical Pump head

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Acknowledgements

This work was supported by the Fundacão para a Ciência e Tecnologia (FCT) (funded by national funds through the FCT/MCTES (PIDDAC)) to CIQUP, Faculty of Science, University of Porto (Project UIDB/00081/2020), IMS-Institute of Molecular Sciences (LA/P/0056/2020)).

Headquarters
Analytik Jena GmbH+Co. KG
Konrad-Zuse-Strasse 1
07745 Jena · Germany

Phone +49 3641 77 70
Fax +49 3641 77 9279

info@analytik-jena.com
www.analytik-jena.com

Version 1.0 · Author: RuSa/RoGa
en · 05/2023