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Determination of Total Mercury in Muscle Tissues of Marine Fish Species by Ultrasonic Assisted Extraction Followed by Cold Vapor Atomic Absorption Spectrometry

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Abstract

A simple and rapid ultrasonic assisted extraction procedure (UEP) was developed for the determination of total mercury (Hg) in muscle tissues of marine fish species. For this purpose four fish species were collected from fish markets of Karachi, Pakistan. Total Hg concentration was determined by cold vapor atomic absorption spectrometry (CV-AAS), following UEP. Certified reference material DORM-2 (dogfish muscle) was used to validate the results. No significant difference was observed between the experimental results and the certified values of CRM (paired t-test). The limit of detection (LOD) and limit of quantitation (LOQ) of Hg were 0.133 and 0.445 µg/kg respectively. The Hg concentration in muscle tissues were obtained in the range of 0.721 – 1.41 mg/kg on dry weight. The contribution of the daily intake of Hg, based on the consumption of 250 g fresh fish muscles per day was found in the range of 0.615 – 1.22 µg/kg body weight/ day, which is greater than WHO permissible limit.

Keywords: Mercury, Marine fish species, Ultrasonic assisted extraction, Cold vapor atomic absorption spectrometry.

Introduction

Over the last few decades, there has been growing interest in determining heavy metals in the marine environment and attention was drawn to the measurement of contamination levels in public food supplies, particularly fish [1–2]. Heavy metals like mercury (Hg) are environmental contaminant of special concern due to a wide distribution in the environment and likely adverse effects for human health [3-4]. In recent decades, the high concentration of Hg in the environment is due to the use of hard coal, brown coal, and also due to un controlled waste combustion [5]. Globally, the major sources of Hg in coastal systems are atmosphere deposition and anthropogenic origin [6]. The increasing level of Hg found in the food chain caused serious health impact. Minamata disease, which appeared among inhabitants of Minamata Bay, Japan, was caused by the consumption of fish and shellfish containing high concentrations of MeHg. In Iraq, 459 people died due to consumption of Hg-contaminated bread. Hg pollution has also been reported in Quebec, Canada, the Amazon in Brazil, and in many other areas of the world [7]. Mercury has become the subject of most concern due to its biomagnification potential and toxic effects to aquatic organisms and human health. The sources of Hg pollution came from industrial effluents and sewage sludges relating to chloro-alkali industry, the manufacturing of electrical equipment and paint. Water chemistry (e.g. pH, temperature and turbidity), the chemical form of Hg in the environment and food chain structure will affect Hg accumulation in fish [8]. Fish has been the main supply of cheap and healthy protein to a large percentage of the world's population. In most

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Asian countries, especially those in Southeast asia, fish is the main source of protein in the diet. Besides good health benefits of fish, there were many reports on contamination of fish by chemicals in the environment [9]. Hg is accumulated through the food chain, especially in an aquatic medium. Fish have higher Hg contents than other foods, but it is difficult to give an average content, because that depends on the fish species, its age, size and conditions of the water in which it lives [10].

In analytical chemistry, ultrasonic radiation has been used infrequently, although it could be a powerful tool for accelerating various steps in the analytical process [11]. Ultrasound radiation is of great help in the pretreatment of samples. The effects of extremely high temperature and pressure were at the interface of the sonicated solution and the solid matrix, along with the oxidative power of strong acids, results in high extractive power [12]. The use of an ultrasonic device is also a good alternative to minimize the disadvantages of conventional extraction procedures in terms of number of analytical steps, time, extraction efficiency and reagent consumption by facilitating and accelerating pre-treatment process of various biological and environmental samples. The efficiency of both microwave and ultrasound-assisted extraction methods for the sample preparation has been evaluated for various biological and environmental matrices [13].

Nowadays, the most widespread analytical technique for Hg determination is cold vapor atomic absorption spectrometry (CVAAS). One of the early example of this technique was described by Hatch and Ott in 1968 [14]. The CVAAS was adopted as a standard method for analysis of Hg in foodstuffs. This technique is based on the chemical reduction of mercury, usually by Sn^{2+} or BH^{4-} ions to elemental Hg which is swept from the solution by a carrier gas to a quartz cell placed in the optical path of an atomic absorption spectrophotometer where the absorption of Hg is measured [15].

Pakistan has a coastline of around 960 km, of which 745 lies in Balochistan and the remaining about 30 km adjoining Karachi. Karachi is located on the northern border of the Arabian sea. Karachi discharges around 292 million gallons per day of untreated industrial and municipal waste into the sea through its two main drains known as the Lyari and Malir Rivers. The bulk of it passes through the Lyari into the sea, western part of Karachi harbor [16]. Extremely high levels of toxic heavy metals such as Hg have been documented, especially in the coastal waters and sea near Karachi [17].

The aim of the present work was the extraction of total Hg by ultrasonic assisted acid extraction procedure from muscle tissues of marine fish species collected from Karachi, Pakistan. All fish species are edible and local people as well as other peoples of country frequently use in their diet. The extraction of total Hg from muscle tissues used as bio- indicators for marine fishes of Karachi coastal of Arabian sea to know whether consumption of these fishes threatens human health. The extracted Hg was determined by cold vapor atomic absorption spectrometry. The proposed method was validated by certified reference material DORM-2. Results were also compared from microwave assisted acid digestion in closed vessels. The estimated daily intake (EDI) of Hg by adults consuming understudied fish species were also calculated for possible human health risks.

Experimental

Instrumentation

The ultrasonic-assisted acid extractions were carried out with an ultrasonic bath Sonicor, Model No. SC-121TH, Sonicor Instrument Corporation (Copiague, NY, USA) with technical specifications; programmable for temperature ranging from 0 to 90 ◦C, timer 0–30 min, 220V, 50-60 Hz, intensification frequency 35 kHz for the ultrasound energy and a total volume was used to induce the acid extraction process. Microwave-assisted digestion was carried out in a milestone microwave system (Bergamo, Italy), using a five-step program: 2 min at 250W, 2 min at 0W, 6 min at 250W, 5 min at 400W, and 5 min at 650W, followed by 5 min of ventilation. Hg was determined in both digests obtained by both procedures using a model Analyst 700 atomic absorption spectrometer (Perkin-Elmer, Norwalk, CT, USA). Hg was determined by cold vapor

technique using MHS-15 chemical vapor generation system (Perkin-Elmer), coupled to the AA spectrometer. A Hg hollow cathode lamp (Perkin-Elmer) operated at 6mA was used. Measurements were carried out at the wavelength of 253.7 nm. Argon 99.9 % was used as the carrier gas. The calibration curves (1-5 μg/L) for Hg were established with solutions prepared from a 1000 g/L certified stock solution.

Digestion methods

Ultrasound-assisted extraction procedure (UEP)

Three replicate samples of dried muscle tissues of each fish species and six replicate samples of CRM DORM-2 (100 mg) were taken by accurately weighing separately in 25 ml flasks. 2 ml of a mixture of concentrated (65 %) HNO_3 and 30 % H_2O_2 in (2+1) ratio was added to each flask, the flasks were placed inside the ultrasonic water bath and subjected to ultrasonic energy at 35 kHz for different time intervals at a fixed temperature of 80 °C. After sonication for different time intervals, the resulted extracted solution were centrifuged at 3000 rpm for 5 min, and supernatant liquids were collected in a polyethylene flask for the determinations of Hg by CVAAS. Blanks were also prepared in the similar procedure. At the end of the experiment, the solution in the flasks were diluted with ultra pure water to 10 ml and placed into polyethylene bottle as stock solution at -4 °C. To evaluate the efficiency of the process, the results obtained with the UEP were compared with those obtained from MAD, using a domestic microwave oven on same CRM and real samples

Microwave acid digestion method (MAD)

A microwave assisted digestion procedure was carried out to obtain total Hg for comparison purpose. Six replicates of DORM-2 and duplicate samples (dry weight) of muscles tissues of each studied fish samples were directly weighed into PTFE tubes. Added to each tube 2 mL of a freshly prepared mixture of concentrated HNO_3 and H_2O_2 (2:1, v/v) and kept for 10 minutes at room temperature, then closed the tubes and placed in covered PTFE container. It was then heated following a one-stage digestion programmed at 80% of total power (900 W), 1-2 min for complete dissolution. The digestion tubes were cooled, and the resulting solution was diluted up to 10 mL with 2 M HCl and centrifuged, the supernatants were stored in Teflon flasks as master sample solutions.

Mercury determination was carried out by cold vapor atomic absorption spectrometry (CVAAS), using sodium tetrahydroborate as reducing agent and hydrochloric acid as carrier solution. About 500 μL of the each stock sample solution of CRM, and real samples were transferred to the PTFE flasks of the MHS-15 system and 10 mL of a 0.15 mol/L HCl solution were added, along with 40μL of the antifoam agent. The system was sealed and 3% (m/v) $NaBH_4$ was added for 5 sec to the PTFE flask. A stream of high purity argon gas at a flow rate of 200 mL/min carried the Hg vapor generated in the system to the quartz cell, and the absorbances of the generated Hg atoms were measured. Calibration was performed using aqueous standards (1 -5 ppb) and subjected to CV AAS procedure described above.

Reagent

The ultrapure water obtained from ELGA lab water system (Bucks, UK) was used throughout. Concentrated HNO_3 and H_2O_2 were analytical reagent grade from Merck (Germany) and were checked for possible trace contamination. Standard solutions were prepared by appropriate dilution of the certified stock solution of mercury (1000 g/L), obtained from Fluka Kamica (Bushs, Switzerland). The method was validated by certified reference material, DORM-2 (Dogfish Muscle) from the National Research Council of Canada (Ottawa, Ontario Canada). Solution of sodium tetrahydroborate was prepared by dissolving $NaBH_4$ powder (Acros Organics New Jersey, USA) in 0.05 M KOH. All glassware and polyethylene bottles were thoroughly washed and then soaked overnight in 5 M HNO_3, thoroughly rinsed with distilled and deionized water before use.

Sample collection

Four marine fishes were collected from the fish markets of Karachi. The fishes were caught from Karachi coast of the Arabian sea. Samples were delivered in an ice box filled with ice brought

to the laboratory for further treatment. In first step, muscles were removed from the bone. The muscles tissues were freeze-dried for 20 h at a chamber pressure of 0.225 torr. The lyophilized samples were crushed and homogenized to a fine powder in an agate ball mill. The resulting powder was stored in polyethylene bottles at -20 °C till further preparation and analysis.

Moisture content

Moisture content was determined by drying samples to a constant weight in freeze-drying system (Labconco, USA) and was calculated as percent of water loss.

Validation of results

The method was assured by the analysis of triplicate samples, reagent blank and standard reference material. The detection and quantitation limits were calculated by $\mathrm{LOD} = 3 \times s/m$ and $\mathrm{LOQ} = 10 \times s/m$, respectively, where s is the standard deviation of 10 measurements of a reagent blank and m is the slope of the calibration graph corresponding to Hg and good precision could be seen for the calibration. The calculated LOD was 0.133µg/kg and LOQ was 0.445µg/kg. The precision of the methods, expressed as the relative standard deviation (RSD) of 8 independent analyses of the same sample, provided values less than 10% for the determination of total Hg. The accuracy of analytical method was determined with certified reference material DORM-2 (dogfish muscles) (Table 1). The method was also validated by the muscle tissues of fish *Mushka* in triplicate at four concentration levels 1.0, 2.0, 4.0 µg/ml and was analyzed by CVAAS (Table 2).

Table 1. **Validation of the proposed method for determination of THg against CRM (DORM-2) (µg/g).**

Methods	Certified values (µg/g)	Found values $\bar{x} \pm ts/\sqrt{n}$	% recovery	[b]Paired t test $t_{Experimental}$
Ultrasound acid extraction	4.64±0.26	4.635±0.21	99.9	0.146
Microwave acid digestion	4.64±0.26	4.629±0.23	99.7	

[b]Paired t test between Certified /literature values and found values, Degree of freedom (n-1) = 5, $t_{Critical}$ at 95% confidence limit = 2.57

$\%\mathrm{Recovery} = [\mathrm{Certified\ value}]/[\mathrm{Found\ values}] \times 100$

Table 2. **Recovery of total Mercury (THg) spiked in muscle tissues of Mushka (µg/Kg).**

Amount added	Amount found	% recovery
0	1.47	-
1.0	0.99	99.0
2.0	2.03	101
4.0	4.11	103

Results and Discussion

In the framework of a broad survey of Hg contamination, four marine fish species were purchased from fish markets of Karachi, Pakistan which are commonly consumed by local and people residing in other areas of Pakistan. These fish species were used as a bioassay indicator for the pollution condition of Karachi coastal area, which is polluted due to untreated waste and sewage of industries as well as oil spills from ships and fishing trawlers transiting the port [18].

Total Hg was determined by the cold vapour atomic absorption spectrometry (CVAAS), following UEP. The duration of ultrasound exposure was studied in the range of 2-20 min. It can be seen in (Fig. 1), that the maximum recovery of Hg was achieved after 10 min at temperature 80 ºC of ultrasonic bath, as compared to those obtained by microwave assisted digestion. The short heating time may possibly minimize the losses of Hg due to its inherent volatility. The method was validated by the analysis of triplicate samples, reagent blank, certified reference material DORM-2 (dogfish muscles) and standard addition method. Application of paired t-test also showed that there is no difference between two methods. The paired t-test was calculated for n-1 = 5 degrees of freedom, t_{exp} (0.146) was less than the t_{crit} (2.57) at 95% confidence interval (P = 0.05). Average recovery of Hg spikes from fish muscles matrix was found in the range of 99.0–103 %.

The percentage recoveries of total Hg were calculated by the equation:

%Recovery = measured value / Certified values x 100

The calibration graph for Hg was linear with correlation coefficients of 0.9994, at level near the detection limits up to 10 µg/L.

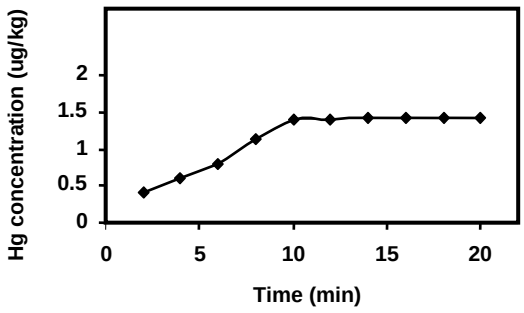

Figure 1. **Caption. Effect of sonication time for optimum recovery of THg.**

Application

In the present study the Hg concentrations in muscle tissues of commonly consumed four marine fish species was determined. The developed ultrasonic assisted acid extraction method was applied for the analysis of muscle tissues of fish species. The moisture contents in fish muscle tissues were 78.0 ± 2.5%. A 100 mg of dried fish muscles were subjected to extraction for total Hg at optimized conditions of different variables (Fig. 1). The mean Hg concentration expressed as x± s, where x is the mean and (S) is the standard deviation for n = 10 measurements is given in (Table 3).

Table 3. **Estimation of total mercury in muscle tissues of sea fish species by ultrasonic acid extraction (UAE) and MAD on dry weight basis. (n=100).**

Fish (Scientific name with common name)	Total Hg (µg/kg) UAE ($\bar{X} \pm S$)
Scombermorus commersoni (Surmai)	0.861±0.58
Arius spp. (Khagga)	1.09±0.94
Otolithes ruber (Mushka)	1.47±0.38
Pampus argenteus (Poplet)	0.739±0.27

The broad distributions of Hg concentration in muscles tissues of four fish species were found in the range of, 0.739 –1.47 mg/kg, and great variations in accumulation of Hg among muscles tissues of understudied fishes were observed. The highest level of Hg was observed in muscles tissues of *Mushka* (1.47 mg/kg); while low concentration was found in muscles tissues of *poplet* (0.739 mg/kg). Trace amounts of Hg are soluble in bodies of water or settles to the bottom, where bacteria can cause chemical changes that transform mercury to methyl mercury, a more toxic form. Fish absorb methyl mercury from water as it passes through their gills and eat smaller aquatic organisms. Larger and older fish absorb more Hg as they eat other fish. In this way, the amount of Hg builds up as it passes through the food chain. Fish eliminate Hg slowly, and so it builds up in fish in much greater concentrations than in the surrounding water [20].

Consumption of fish contaminated with Hg may be a risk to human health. Estimated daily intake of Hg based on the consumption of 250 g fresh fish muscles per day ranges from 0.615–1.22 µg Kg^{-1} body weight day^{-1} (Table 4), which is greater than the FAO/WHO tolerable daily intake of 0.22 µg/person/day of Hg [21].

Table 4. **Daily intake of total mercury by consumption of 250 g of fish muscles/kg body weight/day.**

Fish (Scientific name with common name)	µg of Hg /kg body weight/day
Scombermorus commersoni (Surmai)	0.717
Arius spp. (Khagga)	0.908
Otolithes ruber (Mushka)	1.22
Pampus argenteus (Poplet)	0.615

Conclusion

The ultrasonic assisted acid extraction has been demonstrated to be an efficient methodology for determination of total Hg by cold vapor generation atomic absorption spectrometry. Cold vapor AAS has been shown to be a highly efficient procedure due to its simplicity, low cost and sensitivity. The accuracy of method was checked with certified reference material and by spiking recovery test. The good analytical features of the method allow for its application for routine

analysis of large number of samples and a variety of foodstuff since there are no risks for interferences or matrix effects. Information on the levels of Hg in the marine fish species should be properly maintained, which help for the effective monitoring of both environmental quality and the health of organisms inhabiting in marine ecosystem. The collected data indicate that marine fishes are responsible for a significant source of Hg to human population. The concentrations of Hg found in this study lead us to conclude that the public's exposure to Hg from marine fishes demands reconsideration by regulatory agencies.

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