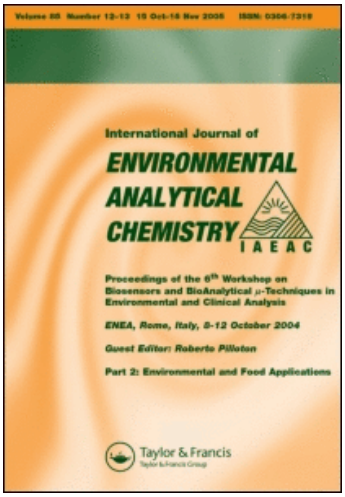

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MICROWAVE DIGESTION OF FISH TISSUES AND DETERMINATION OF Cu, Se AND Hg BY ATOMIC ABSORPTION SPECTROMETRY

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A microwave digestion procedure has been developed for the accurate determination of Cu, Se and Hg in fish tissues (muscle and liver). Digestion parameters such as the time and power of the microwave system, the composition of the digestion solution and the adjustment of oxidation state of Se and Hg prior to analysis have been studied. For Se and Hg, a comparison of different analytical techniques was done. The efficiency of both the digestion and the analytical procedures was tested by using certified reference materials (CRM). The determination of Cu, Se and Hg in fish tissues from Ramsey Lake (Sudbury, Canada) indicated that Cu and Se were more efficiently accumulated in liver whereas Hg had more affinity for muscle tissues. The differences in tissue concentrations of the three elements could be partially explained by fish feeding habits.

Keywords: Microwave digestion; fish tissues; Cu; Se; Hg; AAS

INTRODUCTION

Trace elements (TE) can enter the aquatic environment through natural processes and anthropogenic activities. Contamination of aquatic systems by TE has been a well recognized problem for decades. However, bioaccumulation of TE in aquatic life, especially in fish, remains of special interest because of the direct toxic effects to aquatic animals and the potential detrimental influence on people's health [1].

Copper, particularly under the ionic forms Cu^{2+}, $Cu_2OH_2^{2+}$ and $CuOH^+$, is one of the most toxic heavy metals to fish[2]. Selenium is a unique element, since it is a micronutrient for plants at natural levels but becomes toxic to aquatic life at

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levels sometimes only 10 times higher[3]. Furthermore, Se is suspected to have an antagonistic effect on the toxicity and bioavailability of some heavy metals (particularly Hg)[4,5]. Mercury, due to its high volatility and its potential to be accumulated by fish especially under the methylated forms, can pose a serious risk to human being[6, 7].

Microwave treatments have been developed for TE determination in a wide variety of environmental samples[8, 9]; special attention must be given to volatile elements such as Hg and Se and to the contamination problems often associated with TE determination. Once digested, TE present in biological specimens can be determined by several instrumental techniques[10,11] among which atomic absorption spectrometry (AAS) remains commonly used in most laboratories[12].

In this study, the technique and procedure were developed for an efficient microwave digestion of fish tissues (muscle and liver). The accuracy and precision of Cu, Se and Hg analyses following this procedure were verified by measuring certified reference materials (CRM) using various atomic absorption spectrometry techniques.

METHODOLOGY

Sampling and sample pre-treatment

Fish samples including yellow perch *Perca flavescens*, northern pike *Esox lucius*, and brown bullhead *Ameiurus nebulosus* were collected from Ramsey Lake located in Sudbury, Canada. Livers and boneless, skinless fillets of dorsal muscle tissue were collected and stored in pre-acid-cleaned plastic bags at −100°C. All the tools and equipment used in this study including bags or containers were washed or acid-treated, i.e. soaked in 10% HNO_3 for at least 24 hours, rinsed with double demineralized (DD) water, dried in an oven at low temperatures (30–40°C), then kept in clean environment to avoid surface contamination. Bench tops and working areas were covered with Teflon-coated film for the processing of fish samples. Muscle and liver samples were freeze-dried to specifically minimize the loss of volatile elements. It has been shown that this treatment does not affect the Hg content of biological and environmental samples[13–15]. After drying, the fish samples were homogenized in pre-cleaned mortars, ground into very fine powders, then transferred into pre-cleaned brown plastic containers. All were kept in the freezer before subsequent analysis.

Digestion procedure

All acids used in the digestion and pre-treatment procedures were trace-metal grade (Fisher Scientific); the other chemical reagents were analytical-reagent grade. Dried fish tissues along with the certified reference materials (CRM) DORM-2 and TORT-2 from NRC Canada were digested using a kitchen microwave oven. A series of tests was done to find an appropriate reagent mixture and procedure to digest efficiently fish samples and CRM for Hg determination. The experimental data are given inTables I and II (see also Results & Discussion). For the determination of Hg, a precisely weighed 0.1 g of dry ground fish sample was put in a pre-cleaned PTFE Teflon container (30 mL). Then, 2.0 mL of concentrated nitric acid and 2.0 mL of hydrochloric acid were added to the Teflon containers which were capped and well-sealed. The selected digestion procedure was the following: after a cool overnight digestion, the sample was microwaved once at low power (20% or 200W) for 1 min followed by six times at high power (100% or 1000W) for 30 s. An ice-bath was used to cool the containers between each digestion interval. After digestion, the sample was allowed to cool at room temperature, the solution was transferred into a 25.00 mL volumetric flask and brought to mark by adding DD water. For the determination of Cu and Se, the procedure was the same except that only concentrated HNO_3 (2.0 mL) was used as the digestion reagent.

TABLE I Comparison of reagent combinations in the microwave digestion of fish tissues for Hg determination

Digestion mixtures Reagents (mL)	*Value Obtained* [a] *(μg/g)*	*Recovery* [b] *(%)*
HNO_3 (2.0)	2.78 ± 0.25	60
HNO_3 (1.0) + HCl (2.0)	4.41 ± 0.26	95
HCl (2.0) + H_2O_2 (1.0)	4.27 ± 0.13	92
HNO_3 (1.0) + HCl (1.0)	4.40 ± 0.18	95
HCl (1.0) + H_2O_2 (0.5) + HNO_3 (0.5)	4.18 ± 0.07	90
HCl (1.0) + H_2O_2 (0.5) + HNO_3 (1.0)	4.42 ± 0.23	95
HCl (3.0) + HNO_3 (1.0) *(aqua regia)*	4.41 ± 0.28	95
HCl (2.0) + HNO_3 (2.0)[c]	4.62 ± 0.16	99.6

[a] The CRM DORM-2 (certified value of 4.64 ± 0.26 ug/g) was analyzed by CVAAS in this comparison.
[b] For triplicate samples.
[c] Selected combination of reagents.

TABLE II Effect of Power and Time of the Microwave Digestion on Hg recovery

Digestion Program[a]	*Recovery (%)*[b]
1 min at L; 2 times 30 s at M	73
1 min at L; 10 times 30 s at M	76
1 min at L; 2 times 30 s at M; 2 times 30 s at H	88
5 min at L; 2 times 30 s at M; 2 times 30 s at H	93
5 min at L; 4 times 30 s at M; 2 times 30 s at H	101
10 min at L; 2 times 30 s at M; 2 times 30 s at H	97
1 min at L; 4 times 30 s at H	88
1 min at L; 6 times 30 s at H[c]	101

[a] The power levels of the microwave are as follows: L for low or 200W; M for medium or 500W and H for high or 1000W.
[b] The Hg analyses (n = 3) of the CRM DORM-2 were done on the Perkin-Elmer MHS-10 system.
[c] Selected procedure.

Analyses

Copper and selenium in fish tissues were determined by a Perkin-Elmer 5000 atomic absorption spectrometer equipped with a Zeeman graphite furnace module and an HGA-500 graphite furnace controller. Pyro-coated graphite tubes without platform were used in the furnace and argon was the circulating gas. The instrumental parameters for Cu and Se determinations are given in Table III. For comparison, Se was also analyzed by Hydride Generation Atomic Absorption Spectrometry (HGAAS) combined to a system of cooling traps to remove water and preconcentrate the Se hydride before analysis[16,17]. Before being analyzed by HGAAS, Se should be converted into Se(IV). The digested solutions were transferred to a 25.00 mL volumetric flask, filled with 4.0M HCl to the mark, then transferred to a 50 mL reflux flask, and heated at 70–80 °C for 15 minutes. The reflux flask was connected to a condenser cooled by tap water to minimize the loss of volatile Se species during the heating process. In this study, a 1.2M HBr was also tested as reducing solution. For the analysis, 40mL of 4.0M HCl and 1.0–2.0mL of the previously-reduced sample solution were added into the stripper[16], 1.0mL of 4%(w/v) sodium borohydride was added to the mixture over a period of 2 minutes by using a syringe. After 6 minutes, the liquid nitrogen trap was removed and the hydrogen selenide inside the tube would be evaporated immediately and carried into the detection system by helium gas. The H_2Se peak was then detected and measured by AAS.

TABLE III Instrumental parameters of the Zeeman GFAAS for Cu and Se analysis

Parameter	*Cu*	*Se*[a]
Lamp type	HCL	EDL
Wavelength (nm) and Slit	324.7 0.7	196.4 0.7
Sample Size (uL)	10–20	20–30
Drying Temp (°C)	110	110
Time (s)[b]	50	60
Ashing Temp (°C)	800	800
Time (s)[b]	30	30
Atomizing Temp (°C)[c]	2500	2500
Time (s)[d]	8	10

[a] For Se, a 1000 mg/L Ni solution was used as matrix modifier.
[b] This time includes 5 s of ramping time.
[c] The gas-stop mode was activated at this step.
[d] This time includes 1 s of ramping time.

For the determination of Hg in CRM, a Perkin-Elmer MHS-10 Cold Vapour Generation Atomic Absorption Spectrometry (CVAAS) system was used with $SnCl_2$ as reducing agent. The digested solutions were also analyzed directly on a Perkin-Elmer Sciex Elan 5000 Inductively Coupled Plasma Mass Spectrometry (ICP-MS) equipped with a Gilson 212B autosampler and a CETAC U-5000AT ultrasonic nebulizer. The experimental parameters for ICP-MS are given in Table IV.

TABLE IV Instrumental parameters of the ICP-MS for Hg determination

Parameter	*Selection*	
ICP Gas Flow Rate (L/min)	Plasma	14.60
	Auxiliary	0.90
	Nebulizer	1.10
Mass Spectrometer	Sample Cone Orifice (mm)	1.14
	Skimmer (mm)	0.89
	Mass Spectrum	^{202}Hg

RESULTS AND DISCUSSION

Microwave digestion for Hg determination

A special attention was given to Hg since it was the most difficult element to recover. The pre-treatment step requires that all forms of mercury in fish be converted into Hg(II) and in soluble forms. Only nitric acid was used first since it was proven efficient for Cu and Se, but a low recovery of mercury in CRM samples was obtained probably due to the incomplete oxidation and solubilization of Hg compounds present in fish tissues. Since organomercuric compounds were found to be completely degraded by UV irradiation[18], this procedure was tested in the present study. However, no significant improvement was observed. In order to improve the digestion efficiency, mixed volumes of HNO_3 and HCl as well as H_2O_2[19] were tested in this study. The best recoveries were obtained with a mixture of 2.0 mL of both HCl and HNO_3 as confirmed by the analysis of Hg in CRM using CVAAS with the MHS-10 system (Table I).

Time and power are crucial parameters to adjust in microwave procedure for insuring complete digestion and avoiding losses of volatile elements. Among the different tested procedures, the best selection for saving time and for a good recovery of the studied elements was found to be 1 min at low power (25% or 225 W) followed by 6 times 30 s at high power (100% or 1000W). The results are presented inTable II only for Hg but recoveries of Cu and Se in CRM were also very good (95 – 102%) with this procedure.

Analysis of Cu and Se by GFAAS

Copper and selenium were determined by GFAAS using the conditions given in Table III. For Se analysis, a volume of 1000 mg/L nickel nitrate (stock solution for AAS) equal to the sample volume was employed as the matrix modifier. The purpose of Ni addition is to stabilize the selenide by forming NiSe until it is atomized. In our study, the standard calibration curve and the method of standard addition were tested and gave very close results. Therefore only standard calibration curve method was used when analyzing fish samples since it is less time consuming. To test the accuracy of the analytical technique, certified reference materials DORM-2 and TORT-2 were digested and analyzed. The results obtained by Zeeman-GFAAS are presented in Table V. For both CRM, the results obtained were very close to the certified values. The relative standard deviations (RSD) for replicates of the two CRM were less than 6%.

TABLE V Concentrations of Cu and Se in CRM measured by GFAAS

	Cu		*Se*	
CRM	DORM-2	TORT-2	DORM-2	TORT-2
Cert. value (μg/g)	2.34 ± 0.16	106 ± 6	1.40 ± 0.09	5.63 ± 0.67
Value obtained (μg/g)	2.22 ± 0.05	103 ± 5	1.37 ± 0.06	5.66 ± 0.33
Nb. of replicates	4	7	6	13
Recovery (%)	95	97	98	101

Analysis of Se and Hg by HGAAS

HGAAS is a very sensitive technique for the determination of Se and Hg often present at low concentrations in the environment[20]. In the case of Se, it can be used with or without preconcentration in cooling traps[16,17].

Before being analyzed by the hydride generation, all Se species should be converted into Se(IV). It was done by refluxing the digested sample in a 4M HCl solution[17]. Some authors recommended HBr as the reducing reagent to minimize the potential interference of chlorine generated by the HCl reduction as well as interferences due to HNO_3[21]. The HBr technique was also tested in this work. Digested samples were submitted to a reduction with 1.2M HBr at 70–80 °C for 15 minutes. The results for CRM samples measured by HGAAS with cooling traps are given in Table VI. All the Se results for CRM were acceptable, good recoveries (93–99%) and small RSD (3–5%) were obtained. The method involving GFAAS was selected for the analysis of fish samples because it is less time consuming than HGAAS.

TABLE VI Concentrations of Se in CRM measured by HGAAS after reduction by HCl and HBr

	4.0M HCl		*1.2M HBr*	
CRM	DORM-2	TORT-2	DORM-2	TORT-2
Cert. value (μg/g)	1.40 ± 0.09	5.63 ± 0.67	1.40 ± 0.09	5.63 ± 0.67
Value obtained (μg/g)	1.38 ± 0.05	5.25 ± 0.19	1.39 ± 0.07	5.31 ± 0.16
Nb. of replicates	5	5	4	4
Recovery (%)	99	93	99	94

For the determination of Hg in CRM by CVAAS, good recoveries and small RSD were obtained (Table VII). However, this technique could not be used for

our samples because the sensitivity was not high enough to detect the very low Hg concentrations in our fish tissues.

TABLE VII Concentrations of Hg in CRM measured by CVAAS

CRM	*DORM-2*	*TORT-2*
Cert. value (μg/g)	4.64 ± 0.26	0.27 ± 0.06
Value obtained (μg/g)	4.62 ± 0.16	0.24 ± 0.01
Nb. of replicates	24	13
Recovery (%)	99.6	88.9

Analysis of Hg by ICP-MS

ICP-MS is now a well-established technique widely used in environmental and biological materials analysis. Many unique properties make it a very powerful technique in elemental analysis. These properties include: high sensitivity; broad linear dynamic range of at least six orders of magnitude, excellent precision (0.1–1.0%), ease and speed, multi-element determination ability and high sample throughput. However, the conventional standard calibration solution was not applicable in this study because the matrix of the standard solution was very different from that of fish samples. Important analytical errors could be introduced by using the standard calibration method not only due to the matrix effect but also to the high viscosity of sample solutions. This difference in viscosity can cause significant divergence in the aspiration of samples when compared to that of standard solutions.

Therefore, to test the accuracy of ICP-MS determination, two sets of spiked standard solutions were made by spiking CRM samples (DORM-2) with known amounts of a Hg standard solution. They were subjected to the same pretreatment and determination procedures as for fish and were used for calibration. In addition, several CRM samples were determined as unknowns and the results were compared with the certified values. The results for solutions of CRM samples spiked by a Hg standard and CRM results analyzed by ICP-MS are presented in Table VIII. Good recoveries were obtained for CRM either spiked or analyzed as unknown samples.

Concentrations of Cu, Se and Hg in fish samples: Environmental implications

The mean concentrations of Cu, Se and Hg measured in fish samples from Ramsey Lake are given in Table IX. Levels of Cu and Se in liver tissues were higher than those in muscle for the three studied fish species. The concentration ratio of liver over muscle was much higher for Cu than for Se and could reach values as high as 50 in bullhead. It has been found that liver is one of the main organs for bioacccumulation of some TE [2]. At the opposite, Hg levels were found higher in fish muscle than in liver tissues. Unlike Cu and Se, Hg was reported to accumulate mainly in muscle tissues rather than in other organs or tissues[2]. This may result from the lipophilic character of methyl Hg which is the main chemical form of Hg in fish.

TABLE VIII Results of Hg in spiked CRM solutions and in measured samples determined by ICP-MS

CRM	*Certified value*[a] *(μg/L)*	*Spike (μg/L)*	*Value obtained*[b] *(μg/L)*	*Recovery*[c] *(%)*
TORT-2	0.53	0.00	0.53 ± 0.03	100
		1.00	1.47 ± 0.06	94
		2.00	2.44 ± 0.11	96
		3.00	3.46 ± 0.17	98
Measured (μg/g)	0.27 ± 0.03[d]		0.26 ± 0.06	95
DORM-2	4.73	0.00	4.53 ± 0.22	96
		2.00	6.55 ± 0.28	91
		4.00	8.17 ± 0.31	86
		6.00	10.00 ± 0.40	88
Measured (μg/g)	4.64 ± 0.26[e]		4.41 ± .0.30	95

[a] Expressed as the value in solution.
[b] For n = 3.
[c] Calculated as: (Value Obtained – Certified Value) / Spike × 100.
[d] Seven replicates.
[e] Six replicates.

Although the three studied species did not show any significant difference in their muscle Cu concentrations, bullhead samples had higher average Cu levels in liver tissues. One possible explanation can come from the feeding habits of this species. Indeed, brown bullhead feeds mainly on organisms living at the bottom of the lake or in the sediments while the two other species feed mainly in the water body[22]. The sediments of Ramsey Lake have been severely contaminated due to intensive mining and smelting activities in Sudbury. As often happens for most TE, the concentrations in sediments are usually much higher than in lakewater.

TABLE IX Mean concentrations and range[a] of Cu, Se and Hg (μg/g dr. wt) measured in fish samples from Ramsey Lake

	Cu		*Se*		*Hg*	
Fish Species	*Muscle*	*Liver*	*Muscle*	*Liver*	*Muscle*	*Liver*
Yellow Perch	2.22 ± 0.77	74.20 ± 9.80	7.67 ± 0.47	14.28 ± 0.66	0.193 ± 0.116	0.127 ± 0.045
	1.04 – 3.45	14.78 – 305.0	5.18 – 10.64	5.45 – 29.21	0.055 – 0.394	0.060 – 0.200
	(n = 31)	(n = 26)	(n = 31)	(n = 26)	(n = 12)	(n = 10)
Northern Pike	1.96 ± 0.59	63.06 ± 6.38	6.44 ± 0.67	9.22 ± 0.29	0.284 ± 0.133	0.077 ± 0.18
	0.73 – 4.83	39.50 – 92.53	5.80 – 7.52	6.87 – 11.12	0.100 – 0.531	0.047 – 0.108
	(n = 13)	(n = 15)	(n = 13)	(n = 15)	(n = 10)	(n = 10)
Brown Bullhead	2.75 ± 0.63	131.2 ± 4.6	2.25 ± 0.71	10.50 ± 0.92	0.135 ± 0.083	0.055 ± 0.033
	1.71 – 4.14	76.60 – 188.79	1.27 – 3.24	8.41 – 14.65	0.035 – 0.338	0.027 – 0.145
	(n = 10)	(n = 10)	(n = 10)	(n = 10)	(n = 10)	(n = 11)

[a] Represent the mean and range values in fish samples of various lengths and the standard deviation for n measured samples.

Average concentrations of Se in bullhead muscle were lower than in perch and pike but the concentrations in liver did not show a clear difference from the two other species. Recent studies show that the main pathway of Se transfer to fish is via Se-contaminated food, while uptake of Se from solution is a less important pathway due to the very low concentration in natural waters[23]. The Se and Hg concentrations in Ramsey lakewater were below our detection limits (0.5 μg/L and 0.1 μg/L for Se and Hg respectively, representing 3 SD of the blank). Average Hg concentrations in pike muscle were higher than in the other two species. Since pike is the potential predator on perch, and sometimes on brown bullhead as well, it may explain the highest Hg level in its muscle. Although bullhead is a sediment feeder, it did not appear to accumulate Hg more than the other two species. It may suggest that bioaccumulation of Hg occur more from the water or food than from the sediments where it could be less bioavailable. However, it is important to mention that the bioacccumulation of TE in aquatic organisms can be influenced by many biotic and abiotic factors and cannot be necessarily explained by considering a single parameter.

Finally, in this study, we noticed that neither mean nor individual concentrations of Cu, Se and Hg were higher than suggested toxic threshold values.

CONCLUSION

Microwave conditions and reagent mixtures were systematically studied to obtain accurate concentrations of Cu, Se and Hg in fish tissues. A HCl-HNO_3 (1:1) mixture gave the best recovery of the three elements. CRM were used to control the quality of the digestion and analytical procedures. Cu and Se could be precisely determined by GFAAS; Se could also be measured successfully using hydride generation with cooling traps but this method is more time consuming than GFAAS. For Hg, CVAAS was satisfactory for CRM but not sensitive enough for the low concentrations found in fish samples from Ramsey Lake. ICP-MS was used to measure Hg in muscle and liver tissues of three fish species. The analyses showed that liver could accumulate Cu and Se more efficiently than muscle while muscle had greater affinity than liver for Hg. Differences in TE concentrations among the studied fish species seem related to their feeding habits. However, more investigation will be necessary to confirm our observations.

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