Pergamon

Talanta, Vol. 41, No. 2, pp. 195–200, 1994

0039-9140/94 $6.00 + 0.00

COMPARISON OF FOUR MICROWAVE DIGESTION METHODS FOR THE DETERMINATION OF SELENIUM IN FISH TISSUE BY USING HYDRIDE GENERATION ATOMIC ABSORPTION SPECTROMETRY

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(*Received* 22 *June* 1993. *Revised* 17 *August* 1993. *Accepted* 18 *August* 1993)

Summary—Four microwave digestion methods of fish tissue for selenium determination by hydride generation atomic absorption spectrometry were compared, in which potassium hexacyanoferrate(III) was chosen as a masking agent for eliminating matrix interferences. The results showed that the methods employing HNO_3/H_2O_2, $HNO_3/K_2S_2O_8/H_2O_2$ and $HNO_3/H_3PO_4/H_2O_2$ digestion media were unreliable. However, the decomposition using the digestion media of $HNO_3/H_2SO_4/H_2O_2$ enabled adequate digestion of fish tissue and retention of selenium in a state amenable for determination. Therefore, the digestion procedures with $HNO_3/H_2SO_4/H_2O_2$ media are proposed for the determination of selenium in fish tissue by hydride generation atomic absorption spectrometry. The recoveries of the spiked samples investigated ranged from 90 to 102%. The result obtained from analyzing the NIES CRM No. 6 mussel was in good agreement with the reference value (reference value: 1.5 μg/g; found: 1.45 ± 0.05 μg/g). The limit of detection for selenium was 0.03 μg/g dry mass for a 100 mg sample. The contents of selenium in local fish species investigated ranged from 0.49 to 2.90 μg/g, and the relative standard deviation for the determination of selenium was less than 8%.

Selenium, which is an essential micronutrient for most aquatic organisms, has recently been receiving considerable attention in studies on fish nutrition.[1–3] Signs of nutritional deficiencies have been experimentally induced in fish that were fed on diets containing low levels of selenium (<0.1 μg/g of dry feed).[1] However, at concentrations above normal physiological requirements, selenium can be toxic to aquatic organisms. It is claimed that dietary selenium levels in excess of 3 μg/g of dry feed may ultimately be toxic to fish if maintained over long periods of time.[1] Although the exact mechanism is poorly understood, substitution of selenium for sulfur in amino acids may result in the production of unstable enzymes and proteins that interfere with normal metabolic pathways.[4] Since the difference between the essential and the toxic levels in fish is rather narrow, precise knowledge of the selenium concentrations in fish tissue is important.

A number of methods are available for the determination of selenium in biological samples. These methods include fluorimetry, various electroanalytical methods, chromatographic techniques, neutron activation analysis, and various atomic absorption (or emission) spectrometric procedures.[5] Of these, hydride generation atomic absorption spectrometry (HG–AAS) is a well-established technique. It has been widely applied to the determination of selenium in a variety of materials because of its high sensitivity and simplicity.[6–8] As in many other analytical techniques, the measurement of selenium in biological samples by HG–AAS requires the organic matrix be destroyed and the residue taken up in solution, and organoselenium compounds must be completely decomposed in order for the resulting selenium species to be reduced and thus form hydrides. It is quite apparent that with many analyses, sample preparation is the rate determining step for analysis throughput. Fortunately, the microwave digestion approach offers a reasonably economical solution to this bottleneck.[9] Recently, several rapid methods for selenium determination based on the decomposition of biological samples by digestion medium/media such as HNO_3 only,[10] HNO_3/H_2O_2,[11] $HNO_3/$

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$HClO_4$,[12-13] and $HNO_3/H_2SO_4/H_2O_2$[14-15] with microwave heating and subsequent determination by HG–AAS and other instrumental methods have been described.[10-15] However, few authors present a comparison of results from different digestion medium/media. In addition, the HG–AAS technique is prone to interferences of transition metal such as copper, nickel, *etc.*[16-18] these interference elements are often encountered in a sample matrix of aquatic animals. Therefore, it would be desirable to find a means of eliminating matrix interferences during the sample preparation.

The aim of the present study is to compare four microwave digestion methods of fish tissue for selenium determination by HG–AAS. The digestion methods investigated were those employing HNO_3/H_2O_2, $HNO_3/H_2SO_4/H_2O_2$, $HNO_3/H_3PO_4/H_2O_2$, and $HNO_3/K_2S_2O_8/H_2O_2$ digestion media. It has been reported that potassium hexacyanoferrate(III) masks copper, nickel and other interferences in the determination of selenium by HG–AAS.[19] This method has been successfully applied to the determination of selenium in foods.[20] For this reason, the suitability of potassium hexacyanoferrate(III) as a masking agent for eliminating matrix interferences in fish tissue was also investigated in the present study.

EXPERIMENTAL

Apparatus

An atomic absorption spectrometer (Perkin–Elmer model 280) equipped with a selenium hollow cathode lamp, connected to a hydride vapour generation accessory (Perkin–Elmer MHS-10 system), was used for the determination of selenium. The signals (peak–height mode) were recorded on a Shimadzu U-135 recorder.

An unmodified Delonghi MW-155 domestic microwave oven (made in Italy) with 15–100% full power (600W) capability in 15–25% increments was used for digestion. Sample digestion was carried out in a closed polytetrafluoroethylene (PTFE) bomb.

Reagents

All chemicals used were of analytical reagent grade from Merck, unless otherwise indicated. All-glass apparatus was used to produce doubly distilled water, which was used throughout the study.

Standard solutions. Stock standard selenite solution containing 1000 $\mu g/cm^3$ Se for AAS was purchased from BDH; stock standard selenate solution containing 1000 $\mu g/cm^3$ Se was prepared by dissolving 0.2393 g sodium selenate (Fluka) in 100 cm^3 of 1 mol/dm^3 HCl; standard organo-selenium solution was prepared by dissolving 0.0124 g seleno-DL-methionine (Sigma) in 50 cm^3 of (1 + 1) distilled water/methanol. This solution contained 100 $\mu g/cm^3$ Se; all working standard selenium solutions were prepared by serial dilution immediately before use.

Digestion mixtures: HNO_3/H_2SO_4. Concentrated sulphuric acid and 65% nitric acid were mixed in 1:3 v/v ratio; HNO_3/H_3PO_4: concentrated phosphoric acid and 65% nitric acid were mixed in 1:5 v/v ratio; $HNO_3/K_2S_2O_8$: potassium persulphate (4% w/v) and 65% nitric acid were mixed in 4:5 v/v ratio.

Potassium hexacyanoferrate(III) solution (10% w/v) was prepared by dissolving 10 g of $K_3Fe(CN)_6$ in 100 cm^3 of distilled water.

Sodium tetrahydroborate solution was prepared by dissolving $NaBH_4$ powder (Fluka) in 1% NaOH solution to a final concentration of 2% (w/v). The solution was stirred for 10 min and filtered before use. This solution was freshly prepared for daily work.

The National Institute for Environmental Studies (NIES, Japan) certified reference material (CRE) No. 6 mussel, with a selenium reference content of 1.5 $\mu g/g$ was used. This is a provisional value given by NIES. No standard deviation is available.

Digestion procedures

The microwave digestion of fish tissue or reference material was carried out using four replicates of about 100 mg of the dried samples weighed into dried cleaned PTFE bombs. Calibration graphs were prepared by the addition of known amounts of standard selenite solutions to the PTFE bombs with or without sample matrix and were subjected to the same acid digestion bomb treatments as the sample digestion procedures. The sample digestion procedures were also applied to the blank determination.

For HNO_3/H_2O_2 (Method A) digestion, a 5 cm^3 aliquot of HNO_3 (65%) was added to each digestion bomb. Eight tightly capped digestion bombs, together with one small plastic beaker filled with 30 cm^3 of water were placed in a lidded plastic box, which was then placed in the oven. The digestion programme consisted of three heating stages as follows. (1) The plastic

box was heated for 6 min at 330 W (55% power), and then removed from the microwave oven. After cooling to the room temperature in a cold water-bath, the PTFE bombs were opened to release the pressure. (2) An aliquot of 0.2 cm^3 of H_2O_2 was added to each of the PTFE bomb, recapped tightly, and then placed in plastic box, followed by heating for 4 min at 450 W (75% power). Then, the cooling and pressure release sequence were repeated as in Step 1. (3) Another portion of 0.2 cm^3 of H_2O_2 was added and the content heated for another 4 min at 600 W (100% power).

The digestion procedures described above were also used for the other digestion media: $HNO_3/H_2SO_4/H_2O_2$ (Method B); $HNO_3/H_3PO_4/H_2O_2$ (Method C); and $HNO_3/K_2S_2O_8/H_2O_2$ (Method D). All the procedures were the same except using 5 cm^3 of each of the digestion mixtures (HNO_3/H_2SO_4; HNO_3/H_3PO_4; and $HNO_3/K_2S_2O_8$) instead of 5 cm^3 of HNO_3, respectively.

Reduction of the Se(VI) to Se(IV)

The digest obtained from the various methods described above was transferred into a glass tube, 2 cm^3 of 4 mol/dm^3 HCl was added and then placed in a boiling water-bath for 20 min to reduce any selenate to selenite. After cooling to room temperature, the solution was quantitatively transferred into a 25 cm^3 volumetric flask, 2.5 cm^3 of 10% potassium hexacyanoferrate(III) was added and made up to volume with 1 mol/dm^3 HCl.

Determination of the selenium by HG–AAS

A 10 cm^3 aliquot of the above sample solution was pipetted into a reaction vessel connected to the MHS-10 system. The $NaBH_4$ solution in reservoir was pumped into the reaction vessel for 30 sec, during which time the hydride selenide generated was swept with argon into the quartz cuvette. The selenium absorption signal was measured at 196.0 nm, using a 2 nm band pass.

RESULTS AND DISCUSSION

Choice of digestion reagents with microwave heating

In previous studies on the microwave digestion methods of biological samples with acids in an open or closed system, combined acid mixtures containing nitric acid as the main digestion reagent have commonly been used.[10–15] Hydrogen peroxide has been applied as an additive to promote ashing and is often combined with nitric and/or sulphuric acids.[11,21] In a recent study, potassium persulphate was used successfully as the main decomposition reagent with microwave oven digestion to decompose all arsenicals to arsenate and then measured by HG–AAS.[22] Although a number of studies have reported the use of phosphoric acid as a reagent during microwave heating for various analysis,[23–24] no literature on phosphoric acid as a digestion acid for the dissolution of biological materials by microwave heating is available. However, in conventional wet digestion methods, the $HNO_3/H_3PO_4/H_2O_2$ digestion media have been successfully applied for the decomposition of some biological samples.[25–26] Thus, digestion methods employing HNO_3/H_2O_2, $HNO_3/H_2SO_4/H_2O_2$, $HNO_3/H_3PO_4/H_2O_2$, and $HNO_3/K_2S_2O_8/H_2O_2$ digestion media were chosen for comparison. Methods requiring the use of perchloric acid were not included as this acid can be dangerous, particularly in the presence of organic materials, requires extreme caution and special venting facilities in its use.[27]

Microwave digestion procedures

A wide-mouthed plastic box (for microwave oven) with a lid was used to house the PTFE bombs so as to minimize the release of the acid fumes to the microwave oven. This would also prolong the life span of the electronics of the oven and the magnetron. During digestion, the presence of 30 cm^3 of water (in a plastic cup) was found to be very effective for protecting the magnetron from damage in the case of small sample loads and small volumes of liquid in the oven. It also reduced the intense heat generated during the reaction of the solid sample with the digestion acids.

Although the PTFE bombs can be used at temperature up to 250°C, it is not recommended for use above 200°C.[9] Furthermore, pressure rather than temperature was more often the limiting parameter for the combinations of digestion media with sample matrix.[11,28] Unfortunately, in our experiments, as most digestion methods with domestic microwave oven,[14,29–33] no real-time monitoring accessory of temperature and pressure is available. Therefore, in order not to subject the PTFE bomb to unduly high temperature and pressure, the microwave digestion procedures were performed at three stages as described in the Experimental section.

Table 1. Determination of selenium in the NIES CRM No. 6 mussel*

Method	Digestion media	Se Content† ($\mu g/g$)
A	HNO_3/H_2O_2	0.92 ± 0.06§
B	$HNO_3/H_2SO_4/H_2O_2$	1.45 ± 0.05
C	$HNO_3/H_3PO_4/H_2O_2$	1.36 ± 0.05‡
D	$HNO_3/K_2S_2O_8/H_2O_2$	1.12 ± 0.08§

*Reference value: 1.5 $\mu g/g$. The difference between results obtained and the reference value was analyzed by two-tailed Student's t-test.

†Mean value $\pm$ S.D. ($n = 4$), ‡$p < 0.05$, §$p < 0.01$.

Only small amounts of sample (100 mg) were used.

Comparison of four microwave digestion methods

The results of the determination of selenium in the NIES CRM No. 6 mussel by using the four digestion methods are given in Table 1. It is seen that rather low values of selenium were obtained using the HNO_3/H_2O_2 and $HNO_3/K_2S_2O_8/H_2O_2$ digestion media, both values obtained are significantly different from the reference value at $p < 0.01$. Although relatively higher value of selenium was obtained by using the digestion media of $HNO_3/H_3PO_4/H_2O_2$, the data obtained were significantly different from the reference value at $p < 0.05$. The best result was obtained by using the $HNO_3/H_2SO_4/H_2O_2$ digestion media, where no statistical difference was observed when compared with the reference value $p > 0.1$.

The four microwave digestion methods of fish tissue were further compared by recovery experiments. Known amounts of selenate, selenite and seleno-DL-methionine were added to the prepared sample solutions. The four digestion methods were then used to determine the amount of selenium present. The recoveries of selenite, selenate and seleno-DL-methionine are listed in Table 2. It can be seen that the conclusions obtained from Table 2 were in accord with that obtained from Table 1. Lower recoveries were obtained for methods A, C, and D when compared with that obtained from method B.

In traditional wet digestion procedures, low recoveries and poor precision have previously been reported for the HNO_3 only digestion of biological samples and subsequent determination by HG–AAS[34] and stripping voltammetric methods.[27] For the latter method, similar conclusion was also reported when $HNO_3/K_2S_2O_8$ digestion mixture was chosen for the decomposition of animal muscle.[27] Our results indicated that the dissolution of fish tissue by HNO_3/H_2O_2 and $HNO_3/K_2S_2O_8/H_2O_2$ digestion media in conjunction with microwave heating technique gave a clear digestion solution. However, the recoveries of the selenium were not satisfactory. The reason may be due to incomplete destruction of some of the organic selenium compounds.[35]

Table 2. Recoveries obtained by adding different selenium species into sample solutions

	Selenium content ($\mu g/g$ dry mass)						Recovery† (%)			
			Found†							
Sample	Present*	Added	A	B	C	D	A	B	C	D
Yellow tail	1.64	0.00	1.01	**1.60**	1.39	1.21	62	**98**	85	74
	1.64	1.00‡	1.77	**2.69**	2.06	1.85	67	**102**	78	70
	1.64	2.00‡	2.48	**3.44**	2.78	2.44	68	**94**	76	63
	1.64	1.00§	1.51	**2.66**	1.80	1.66	57	**101**	68	63
	1.64	2.00§	1.96	**3.40**	2.64	2.36	54	**93**	72	65
	1.64	1.00‖	1.27	**2.54**	1.64	1.56	48	**96**	62	59
	1.64	2.00‖	1.67	**3.28**	2.03	1.82	46	**90**	56	50
Fancy carp	0.49	0.00	0.30	**0.50**	0.44	0.38	61	**102**	90	78
	0.49	0.25‡	0.54	**0.72**	0.65	0.50	73	**97**	88	68
	0.49	0.50‡	0.68	**0.95**	0.85	0.72	69	**96**	86	73
	0.49	0.25‖	0.39	**0.70**	0.50	0.44	53	**94**	68	59
	0.49	0.50‖	0.47	**0.98**	0.63	0.51	47	**99**	64	52
Tilapia	0.74	0.00	0.49	**0.74**	0.65	0.56	66	**100**	88	76
	0.74	0.50‡	0.83	**1.26**	1.03	0.96	67	**102**	83	77
	0.74	1.00‡	1.20	**1.69**	1.45	1.24	69	**97**	83	71
	0.74	0.50‖	0.69	**1.18**	0.89	0.82	56	**95**	72	66
	0.74	1.00‖	0.85	**1.77**	1.12	0.94	49	**102**	64	54

*Determined according to the proposed digestion method (Method B).

†A: HNO_3/H_2O_2; B: $HNO_3/H_2SO_4/H_2O_2$; C: $HNO_3/H_3PO_4/H_2O_2$; D: $HNO_3/K_2S_2O_8/H_2O_2$ digestion media.

‡Standard selenite solutions were used for standard addition.

§Standard selenate solutions were used for standard addition.

‖Standard seleno-DL-methionine solutions were used for standard addition.

Table 3. Calibration curve regression equations of different conditions

Curve No.	Sample matrix	Masking agent*	Digestion media(B)‡	Regression equation§ (Regression coefficient)	95% Confidence Intervals of slope
a	None	None	None	$X = 0.062C + 0.011$ ($R = 0.998$, $n = 6$)	[0.057, 0.067]
b	None	Yes	None	$X = 0.063C + 0.012$ ($R = 0.999$, $n = 6$)	[0.059, 0.066]
c	None	None	Yes	$X = 0.039C + 0.004$ ($R = 1.000$, $n = 6$)	[0.038, 0.040]
d	None	Yes	Yes	$X = 0.039C + 0.004$ ($R = 0.999$, $n = 6$)	[0.037, 0.041]
e	Yes	None	Yes	$X = 0.029C + 0.099$ ($R = 0.999$, $n = 5$)	[0.028, 0.030]
f	Yes	Yes	Yes	$X = 0.038C + 0.122$ ($R = 0.999$, $n = 5$)	[0.037, 0.039]

*Potassium hexacyanoferrate(III).
‡$HNO_3/H_2SO_4/H_2O_2$ digestion media.
§X Absorption values; C selenium concentration S (unit: $\mu g/dm^3$).

In this study, the result obtained by using the $HNO_3/H_3PO_4/H_2O_2$ digestion media was better than that by the HNO_3/H_2O_2 and $HNO_3/K_2S_2O_8/H_2O_2$ digestion media. However, it is apparent that the recoveries obtained are obviously low and variable. By using $HNO_3/H_2SO_4/H_2O_2$ digestion media, the recoveries of the spiked samples investigated ranged from 90 to 102%. The most likely explanation for satisfactory results obtained by method B is that the combination of the H_2SO_4 with HNO_3 increases oxidation potential and boiling point temperature, and results in complete destruction of the organic selenium compounds while maintaining the selenium in a more stable oxidizing state. In Table 2, except the method B, all other methods (methods B, C, and D) show that the recoveries obtained by adding standard seleno-DL-methionine solutions into the prepared sample solutions are obviously lower than that obtained by adding standard selenite/selenate solutions into the same sample solutions. These results support the above explanation.

To sum up, we propose that the microwave digestion procedures using $HNO_3/H_2SO_4/H_2O_2$ digestion media be used for the determination of selenium in fish tissue by HG–AAS.

Elimination of matrix interference and application of the proposed method

Interference and calibration. Table 3 shows the various calibration regression equations obtained for the determination of selenium. Equations (a) and (b) are for selenite in dilute hydrochloric acid, without the $HNO_3/H_2SO_4/H_2O_2$ digestion media, sample matrix, and microwave treatment. Equations (c) and (d) are for selenite in the digestion media of $HNO_3/H_2SO_4/H_2O_2$ with microwave treatment but without sample matrix. Comparing either equation (a) with equation (b) or equation (c) with equation (d), it is clear that in the absence of sample matrix, there is no effect from the addition of potassium hexacyanoferrate(III). By comparing equations (a) and (b) with equations (c) and (d), it is seen that the $HNO_3/H_2SO_4/H_2O_2$ digestion media do suppress the selenium absorption signal. Real samples with the $HNO_3/H_2SO_4/H_2O_2$ digestion media and microwave treatment were used for the construction of equations (e) and (f). When comparing equation (e) [without the addition of potassium hexacyanoferrate(III)] with equation (f) [with addition of potassium hexacyanoferrate(III)] it is seen that the selenium absorption signal is obviously enhanced with the addition of

Table 4. Contents of selenium in fish tissue as determined by HG–AAS

Sample	Selenium content* ($\mu g/g$ dry mass)	R.S.D. (%)
Batang	2.61 ± 0.09	3.4
Black pomfret	2.64 ± 0.07	2.6
Boon tong	1.76 ± 0.13	7.4
Chupak	2.07 ± 0.07	3.3
Fancy carp	0.49 ± 0.02	4.1
Flower grouper	1.50 ± 0.05	3.3
Grey trevally	2.56 ± 0.09	3.5
Ikan kuning	1.81 ± 0.06	3.3
Ikan selar	1.86 ± 0.10	5.4
Red grouper	1.69 ± 0.09	5.3
Red snapper	1.77 ± 0.09	5.1
Sea bream	1.91 ± 0.05	2.6
Silver belly	1.83 ± 0.12	6.6
Silver Jew fish	2.35 ± 0.18	7.7
Tilapia	0.74 ± 0.03	4.0
Trevally	2.90 ± 0.08	2.8
White pomfret	1.64 ± 0.10	6.1
Yellow tail	1.64 ± 0.05	3.0

*Mean value $\pm$ S.D. ($n = 4$).

potassium hexacyanoferrate(III). Moreover, the calibration curve with sample matrix and potassium hexacyanoferrate(III) (equation (f)) is parallel to the calibration curves without sample matrix (equations (c) and (d)), indicating that interference from sample components was eliminated with the addition of potassium hexacyanoferrate(III). It means that potassium hexacyanoferrate(III) as a masking agent for eliminating matrix interferences in fish tissue is suitable.

Analytical characteristics. For the instrumental conditions used in real sample analyses, the calculated detection limit, defined as the selenium concentration corresponding to three times the standard deviation of the blank, according to IUPAC recommendations,[36] was 0.12 $\mu g/dm^3$. The calibration graph was linear up to about 15 $\mu g/dm^3$. Correspondingly, the limit of detection for selenium was 0.03 $\mu g/g^3$ dry mass for a 100 mg sample.

Sample analysis. The flesh of 18 of the common fish species from the local market was analyzed. The contents of selenium ranged from 0.49 to 2.90 $\mu g/g$ and the relative standard deviation (R.S.D.) ranged from 2.6 to 7.7%. The results obtained and the relative standard deviations are given in Table 4. The results from the real sample analysis indicate that the limit of detection, sensitivity and precision of the proposed method are adequate. Therefore, the $HNO_3/H_2SO_4/H_2O_2$ digestion method with microwave heating can be applied to any routine laboratory analysis for selenium in fish tissue. By using this digestion method, a good agreement was encountered between the values found by the HG–AAS and DPP (Differential Pulse Polarography) techniques for the determination of selenium in fish tissue. The comparison of the above two techniques have been carried out and reported in our previous paper,[15] which confirmed the suitability of both the techniques for the determination of selenium in fish tissue.

Acknowledgements—W. G. Lan wishes to thank the National University of Singapore for the award of a research studentship to read for a Ph.D. degree. This study was supported by a grant from the National University of Singapore (RP 920604).

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