Enzymatic Digestion−High-Pressure Homogenization Prior to Slurry Introduction Graphite Furnace Atomic Absorption Spectrometry for Selenium Determination in Plant and Animal Tissues

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Homogenization using a new flat valve homogenizer in combination with enzymatic digestion with a crude protease was investigated as a means of releasing Se compounds from zoological and botanical matrixes prior to slurry introduction GFAAS. Timed trials with four zoological certified reference materials (CRMs), three botanical reference materials (RMs), and a food crop indicated that Se release into 5% (v/v) ethanol−0.03 M TRIS containing 20 mg of protease was quantitative after homogenization or became quantitative within 1 h of digestion at 60 °C. For each of the zoological RMs (whole egg powder, dogfish muscle, and dogfish liver), three passes through the homogenizer in the presence of protease provided a quantitative release of selenium, and incubation with the enzyme was not necessary. No separation of the Se between the liquid phase and the particulate phase was evident even after several days of subsequent storage at 4 °C. The botanical matrixes (three milled wheat RMs and a rapeseed sample) were more resistant to selenium release and required up to 1 h of digestion with protease at 60 °C. Alternatively, 10 passes through the homogenizing valve (in the presence of the enzyme) resulted in the quantitative release of analyte.

Keywords: *Selenium determination; enzymatic digestion; slurry introduction graphite furnace atomic absorption spectrometry; botanical and zoological certified reference materials*

INTRODUCTION

Conventional sample preparation of biological materials prior to atomic spectrometry involves complete solubilization of the analyte and complete destruction of organic matrix, which is achieved typically by oxidative mineralization (Mincwewski et al., 1982; Sansoni and Panday, 1983; Novozamski et al., 1995). However, digestion procedures can be labor-intensive, time-consuming, and prone to contamination errors. As a consequence, there is a continuing interest in the development of simplified sample preparation techniques. The introduction of slurried samples into graphite furnace atomic absorption spectrometry (GFAAS) continues to attract considerable interest because of the ease with which these quasi-stable preparations can be generated and their compatibility with the instrument's automated liquid handling procedures (Miller-Ihli, 1992, 1993).

A variety of sample pretreatment procedures and additives (Thompson and Allen, 1981; Hoenig and Van Hoeyweghen, 1986; Tsalev et al., 1990; López-García et al., 1996) have been described/evaluated for the production of quasi-stable suspensions of samples prior to analysis by atomic spectrometry. Alternatively, suspensions with a tendency to segregate rapidly have been sampled reproducibly by using ultrasonic agitation (Miller-Ihli, 1989), air or argon bubbling, vortex mixing (Miller-Ihli, 1988), or magnetic stirring (Lynch and Littlejohn, 1989). Partial digestion to produce carbonaceous slurries has also been successfully applied to the analysis, by inductively coupled plasma−atomic emission spectrometry (ICP−AES), of a series of standard reference materials of biological origin (Fagioli et al., 1986). Various alkylammonium hydroxide formulations have been used extensively to solubilize tissue (Hansen and Bush, 1967; Jackson et al., 1972; Murthy et al., 1973; Uchida et al., 1992), particularly those of zoological origin.

Recently, high-pressure homogenization has been evaluated for the preparation of quasi-stable dispersions suitable for Fourier transform infrared (Dion et al., 1994) or GFAAS (Tan et al., 1996a,b). The advantages of this approach to sample preparation were as follows: the ease and speed of the slurry preparation, which required less than 1 min; and the fact that analyte metals were extracted quantitatively into the liquid phase during the preparation. No analyte segregation was detected within the slurry even after several days of standing. Certified reference materials (CRMs), frozen liver and kidney, and dried animal feeds of botanical origin were analyzed successfully for Cd, Cr, Cu, Mn, Ni, and Pb but not for Se. The principal limitation of the high-pressure homogenization technique was the quantities of contaminating analyte metals introduced into the sample by the homogenizing operation. Contamination was reduced appreciably, but not eliminated entirely, by capping the flat face of the stainless steel homogenizing valve with a ruby disk or with various ceramic materials (Tan et al., 1996b).

A previous study (Tan and Marshall, 1997) had established that a high-pressure homogenization of solid

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samples in the presence of aqueous tetramethylammonium hydroxide (TMAH)–ethanol did not release all of the selenium analyte into the aqueous phase. Determinations performed on slurries of eight different CRMs consistently underestimated the concentration of Se in these materials. However, if the homogenization was performed in the presence of protease and the resulting slurry was digested at 37 °C for 4–8 h, recoveries in the resulting digests became quantitative. Whereas zoological CRMs required up to 4 h of enzymatic hydrolysis in TRIS buffer–ethanol, botanical CRMs proved to be more resistant and required up to 8 h of hydrolysis. The addition of the protease to the sample suspension prior to homogenization (i) accelerated the hydrolysis somewhat relative to additions of protease post homogenization and (ii) resulted in less variation (decreased relative standard deviation) among replicate analyses. Since determinations of the supernatant fraction after several days of storage at 4 °C provided estimates that agreed with the certified concentration in these reference materials, it was suggested that the analyte had been transferred quantitatively to the liquid phase. In this paper, reference is made to the release of the analyte into a form that can be detected by the GFAAS technique without implying whether the analyte has been transferred quantitatively to the liquid phase or whether a fraction remains bound to solids.

There have been few reports of the determination of Se in slurried samples (Ebdon and Parry, 1988; Bradshaw and Slavin, 1989; Wagley et al., 1989; Bendicho and Sancho, 1993). Recent reports (Cabrera et al., 1995; López-García et al., 1996) indicate that the approach is promising for this analyte as well. Prolonged enzymatic digestion with a crude protease has been used (Gilon et al., 1995) to liberate component selenoamino acids from proteins. This approach seemed promising as a post-homogenization sample preparation. The objectives of the current studies were to evaluate the performance of a new homogenizing instrument and to develop a more rapid sample preparation for the determination of Se in biological materials by GFAAS.

EXPERIMENTAL PROCEDURES

Reagents. Tris(hydroxymethylamino)methane (TRIS) and tetramethylammonium hydroxide (TMAH) were purchased from Aldrich Chemical Co. (Milwaukee, WI) and aqueous Se solution (sodium selenite, 1000 μg/mL, traceable to a NIST primary standard) was purchased from SCP Chemical Co. (St-Laurent, Québec).

Samples. CRMs were purchased from the National Research Council of Canada (DOLT-2, DORM-2, TORT) or the U.S. National Institute of Science and Technology (Durham wheat flour, SRM 1538; whole egg powder, SRM 8415). The rapeseed sample and two flour reference materials (QIV and QV) that had been subjected to round-robin Se determinations by six laboratories was kindly supplied by B. Gowalko, Canadian Grain Commission, Winnipeg, Mannitoba.

Sample Preparation. Reference materials (RMs) were stored in a desiccator until use. Rapeseed was ground, to pass a 0.5-mm screen, in a Tecator Cyclotec sample mill Tecator AB, Höganäs, Sweden). Sample (approximately 0.2 g of CRM or plant tissue) weighed to the nearest 0.1 mg was added directly to 10 mL of 5% (v/v) ethanol–0.03 M TRIS containing 20 mg of crude protease (Type XIV, ex *Streptomyces griseus*, Sigma Catalog No. P-5147; Sigma Chemical Co., St. Louis, MO). The resulting suspension was sonicated at low power for 15 min and then processed three times through the flat valve homogenizer (EmulsiFlex Model C5, Avestin, Inc., Ottawa, ON) capable of developing 137.9 MPa (20 000 psi) when provided with compressed air (689.5 KPa, 100 psi). This unit was capable of recirculating the processed fluid back through the homogenizing valve. The slurries, in 50-mL Erlenmeyer flasks, were then digested for up to 4 h at 60 °C with gentle agitation every 15 min. Alternatively, the solid sample was suspended in 90% (v/v) water–ethanol containing 0.25% (w/v) tetramethylammonium hydroxide. The suspension was sonicated at low power for up to 15 min and then processed as above.

Table 1. Furnace Operating Parameters[a] for Determinations of Selenium

	selenium
wavelength (nm)	196.0
lamp current (A)	10
slit width (nm)	1.0
injection T (°C)	60
pre-injection	yes
temp of last dry step (10 s) (°C)	250
charring sequence	10 s ramp to 1400 °C, 40-s hold
cool down	none
atomization	0.6 s ramp to 2400 °C, 5.0 s hold
measurement time	5.6 s
matrix modifier	5 μL [0.5% (m/m) $Pd(NO_3)_2$ + 2.5% (m/m) citric acid] for 10 μL of sample

[a] Each step of the furnace programs (with the exception of the read step) was performed in the presence of Ar purge gas (3 L min^{-1}).

GFAAS. Selenium determinations were performed using a hot injection technique on a Varian model 300 GFAAS system equipped with an autosampler, pyrolytically coated platform graphite tubes, a conventional Se hollow cathode lamp, and Zeeman effect background correction. Ashing–atomization curves were generated for sodium selenite in the presence/absence of co-injected bovine serum albumin. Furnace operating parameters are presented in Table 1.

Calibration. GFAAS quantification was performed by both the method of external standards (ES) and by standard additions (SA). ES consisting of appropriately diluted processed solvent blank plus protease. Three or four levels of standard were prepared automatically by the sample introduction device. Background-corrected peak area responses, resulting from three replicate injections of each diluted standard, were used to define the optimal regression equation as determined by the method of least squares. For standard addition (SA) calibrations, 10-μL aliquots of processed fluid were amended with 2, 5, or 10-μL of aqueous standard sodium selenite chosen to result in a range of peak areas including signals that were one-half and at least twice the signal for the unamended sample. The data were modeled by least-squares linear regression. Quantification was performed by dividing the Y-intercept of the regression equation by the slope of that equation and the overall standard error of estimate (SE_{est}) was calculated from

$$SE_{est} = (SE^2_{Y-int} + SE^2_{slope})^{1/2} \qquad (1)$$

Particle Size Determinations. Particle size/shape characterizations were performed with a Galai CIS-100 instrument. The distributions of the numbers of particles as a function of length/area/volume in TRIS buffer–ethanol suspensions of three CRMs before and after processing through the homogenizer were recorded. To detect concentration-dependent associations/agglomerations of the particles, the sample suspension was diluted 10-fold with TRIS buffer–ethanol and processed through the homogenizer, and the determination was repeated (as above). After a second 10-fold dilution of the crude suspension followed by homogenization, the distribution of particle sizes was determined a third time.

RESULTS AND DISCUSSION

Graphite Furnace Operation. At temperatures less than 2300 °C, the Se atomization signal was somewhat

Table 2. Means of the Length of Particles, Their Cross-Sectional Areas, and Volumes for Three CRMs before and after Homogenization at 103.4 MPa

matrix (before or after 1, 5, or 10 passes through homogenizer)	mean length (μm ± 1 SD)[a]	mean area (μm^2 ± 1 SD)	mean volume (μm^3 ± 1 SD)
DORM-2			
before	8 ± 20	140 ± 98	206 ± 121
after 1 homogenization	4 ± 4	12 ± 8.2	18 ± 11
DWF			
before	17 ± 33	140 ± 89	203 ± 107
after 1 homogenization	8 ± 20	92 ± 65	137 ± 88
after 5 homogenizations	3 ± 6	42 ± 32	67 ± 30
after 10 homogenizations	4 ± 3	14 ± 14	28 ± 22
egg			
before	38 ± 44	90 ± 58	162 ± 78
after 1 homogenization	8 ± 20	62 ± 42	103 ± 31

[a] SD, standard deviation.

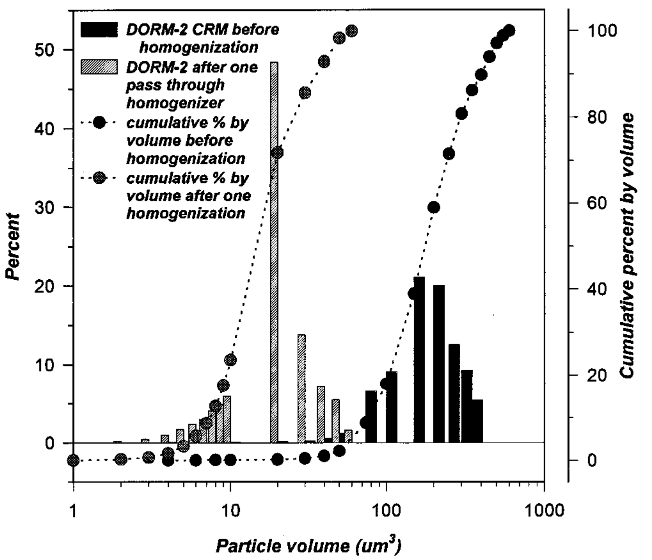

Figure 1. Distributions of the volumes of particles and the cumulative particle volume (expressed as a percentage) as a function of particle length (μm) in the dogfish muscle (DORM-2) certified reference material before and after a single pass through the homogenizing valve.

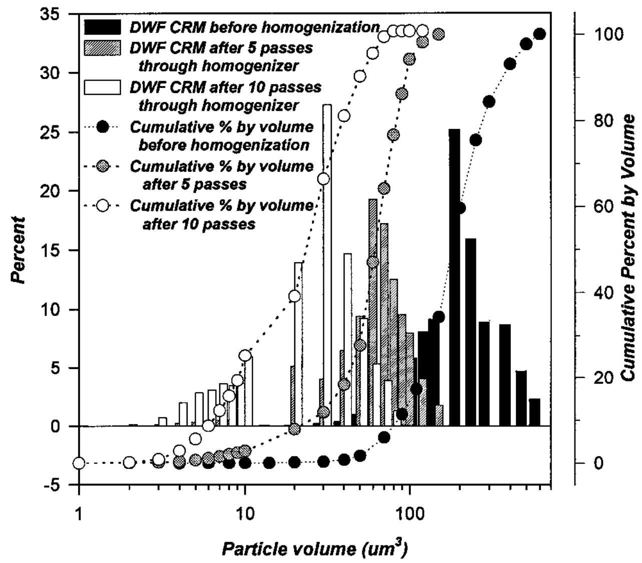

Figure 2. Distributions of the volumes of particles and the cumulative particle volume (expressed as a percentage) as a function of particle length (μm) in the Durham wheat flour (DWF) standard reference material before and after a single, 5 or 10 successive passes through the homogenizing valve.

broadened by the presence of biological materials but was sharpened (and did not tail) for atomizations at 2400 °C. In the presence of the palladium−citric acid modifier, no loss in the Se signal was observed at an ashing temperature equal to or less than 1400 °C.

High-Pressure Homogenizer. The homogenizing instrument (EmulsiFlex Model C5) posessed several desirable features (Avestin Corporation, 1997) that were not available in the prototype model that had been used for previous studies. These included (i) the ability to recirculate the product dispersion back through the homogenizing valve, (ii) a higher operating pressure [137.9 MPa (20 000 psi) vs 68.9 MPa (10 000 psi)], and (iii) a ceramic homogenizing valve (to minimize metal contaminations within the final product).

Particle Size Characterizations. An initial characterization of the influence of high-pressure homogenization on the sizes of particles was performed using laser granulometry. The Galai CIS-100 instrument was capable of determining the length, cross-sectional area, and volume of irregularly shaped particles that fell within the range of 0.5−1200 μm in length. The distribution of the volumes of the particles within the sample was of special interest since it was considered that volume (rather than mean length or mean cross-sectional area) would relate more closely to mass and therefore to the concentrations of individual analytes. It was possible that much of the mass of the sample might be included in a relatively few particles of larger volume. Arbitrarily, three CRMs (DORM-2, DWF, and egg) were chosen for analysis by granulometry. Homogenization at 103.4 MPa produced a dispersion that was characterized with respect to the mean size, mean cross-sectional area, and mean volume of the particles. Distributions of the numbers of particles as a function of length, area, and volume were also recorded. The means of these parameters for each of the three CRMs before and after processing are recorded in Table 2. Homogenization appreciably reduced the mean length, the area, and the volume of the particles in all three matrixes. However, particles within the flour sample proved to be more resistant to size reduction. The lack of any appreciable change in the form of the distribution can be observed in Figures 1−3 that present distributions of the numbers of particles as a function of particle volume for the dogfish muscle (DORM-2), Durham wheat flour, and the whole egg powder samples, respectively. Plots of the cumulative percent by volume vs particle length that are included in each of these figures also indicate an approximately normal distribution (symmetrical s-shaped plots) for the egg and DORM-2 matrixes. Ten passes of the flour sample through the

Table 3. Mean Percent Recoveries of Selenium (± 1 SD Based on Three Replicate Samples) from Certified Zoological Reference Materials As Determined after 15 min of Sonication at Low Power Followed by Three Sequential High-Pressure Homogenizations and 0, 0.5, 1.0, 2.0, or 3.0 h of Enzymatic Digestion[a] (or Following Sonication, Digestion, and Several Days of Storage at 4 °C)

matrix	hours of digestion at 60 °C (%) 0	0.5	1.0	2.0	3.0 h	certified concn (μg/g ± 1 SD)
whole egg powder	108 ± 9	104 ± 5	106 ± 4	108 ± 8		1.39 ± 0.17
	88 ± 3%	98 ± 6%		88 ± 3%		
DOLT-2		100 ± 2	100 ± 5		95 ± 2 (2 d)	6.06 ± 0.49
		103 ± 4 (6 d)	104 ± 2 (6 d)	103 ± 6 (6 d)		
DORM-2	96 ± 6	100 ± 7	96 ± 8			1.40 ± 0.09
TORT	110 ± 4	103 ± 5	100 ± 6 (3 d)			6.9 ± 6.8
	110 ± 9 (1 d)	106 ± 5 (1 d)	100 ± 7 (1 d)			

[a] Each sample, slurried in 5% (v/v) ethanol−0.03 M tris(hydroxymethyl)aminomethane, was processed through the homogenizer three times. Homogenized blank slurry, composed of solvent plus protease enzyme, contained 23 ± 8 ng/10 mL.

Table 4. Mean Percent Recoveries (± 1 SD Based on Three Replicate Samples) of Se from DORM-2 or DOLT-2 CRM That Had Been Suspended in TRIS or TMAH Solvent and Then Sonicated, at Low Power, and Subjected to 5, 8, or 10 Sequential Passes through the Homogenizing Valve in the Absence of Protease Enzyme

matrix	homogenization (%) 5 passes	8 passes	10 passes	solvent	sonication before homogenization [time (min)]
DORM-2[a]	77 ± 6[b]	81 ± 9[b]	71 ± 4[b]	TMAH[c]	0
		76 ± 2[b]		TMAH	5
			64 ± 8[b]	TRIS[d]	15
			66 ± 3[b]		
DOLT-2[a]	86 ± 4[b]	72 ± 10[b]	80 ± 7[b]	TMAH	5
		82 ± 4[b]	85 ± 1[b]		
			83 ± 4[b]	TRIS	15

[a] Certified Se concentrations of CRMs are quoted in Table 3.. Homogenized blank slurry, composed of solvent plus protease enzyme, contained 23 ± 8 ng/10 mL. [b] Significantly different from the certified concentration at the 95% level of confidence. [c] TMAH = 90% (v/v) water−ethanol containing 0.25% (m/v) of tetramethylammonium hydroxide. [d] TRIS = 5% (v/v) ethanol−0.03 M tris(hydroxymethyl)aminomethane.

homogenizer did result in some broadening of the particle volume distribution, yet the distribution remained approximately Gaussian, and the mean volume was reduced to approximately one-eighth by the processing. Each suspension, before and after processing, was diluted either 10- or 100-fold with solvent and then re-analyzed. Since the resulting particle size/cross-sectional area/volume distributions remained essentially unchanged by the dilution, there was no evidence for concentration-dependent particle agglomeration or flocculation within the suspension.

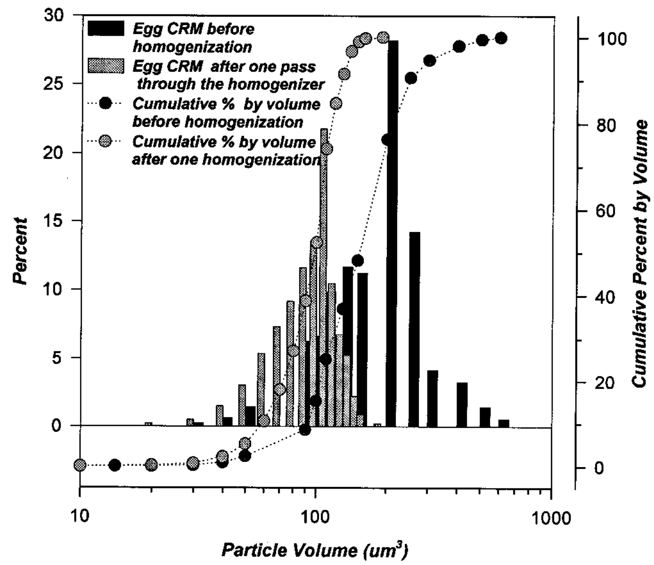

Figure 3. Distributions of the volumes of particles and the cumulative particle volume (expressed as a percentage) as a function of particle length (μm) in the whole egg standard reference material before and after a single pass through the homogenizing valve.

Enzymatic Digestions. Because crude enzyme isolates tend to be more resistant to heat-induced denaturation, this enzyme isolate offered the possibility that the rate of hydrolysis might be accelerated by performing the incubation at elevated temperature. In preliminary trials with 0.02 g of enzyme suspended in TRIS buffer, sufficient hydrolytic activity remained in the

Table 5. Mean Percent Recoveries of Selenium (± 1 SD Based on Three Replicate Samples)[a] from Botanical Reference Materials Determined after 15 min Sonication at Low Power and Three Successive Passes[b] or Continuous Homogenization during 2 min[c] Followed by 0, 0.5, 1.0, 2.0, or 4.0 H of Enzymatic Digestion at 60 °C (or Following Sonication, Homogenization, and Enzymatic Digestion plus Several Days of Storage at 4 °C)

matrix	hours of digestion at 60 °C (%) 0	0.5	1.0	2.0	4.0 h	certified concn (μg/g ± 1 SD)
Durham wheat flour (DWF)	82 ± 2[b,d]	87 ± 5[b]	92 ± 9[b]	97 ± 3[b]		1.23 ± 0.09
	86 ± 8[b]	91 ± 6[b]	86 ± 8[b]	90 ± 4[b]		
	117 ± 9[c] (10)	107 ± 13[c] (10)	107 ± 8[c] (10)	91 ± 6[c] (10)		
	92 ± 11[c]	92 ± 11[c]	97 ± 9[c]			
milled wheat flour (QCIV)	52 ± 11[b,d]	77 ± 16[b,d]	87 ± 13[b]	94 ± 7[b]	91 ± 8[b] (2)	0.87 ± 0.02
		95 ± 8[c]	92 ± 9[c]	92 ± 5[c]		
	91 ± 15[c]	91 ± 7[c]	100 ± 9[c]	108 ± 10[c]		
	101 ± 2[c]	109 ± 6[c]	109 ± 6[c]	105 ± 5[c]		
milled wheat flour (QCV)	55 ± 14[b,d]	60 ± 12[b,d]	90 ± 5[b]	115 ± 9[b]	115 ± 7[b] (3)	1.11 ± 0.07
	77 ± 20[b]	71 ± 20[b,d]	105 ± 13[b]	105 ± 8[b]		
	99 ± 12[c]	86 ± 17[c]	92 ± 4[c]	102 ± 4[c]		
	73 ± 3[b,d]	86 ± 17[b]	87 ± 10[b]			
rapeseed	99 ± 12[b]	104 ± 10[b]	92 ± 4[b]	102 ± 4[b]		0.23 ± 0.25

[a] SD, standard deviation. Homogenized blank slurry, composed of solvent plus protease enzyme, contained 23 ± 8 ng/10 mL. [b] Samples were subjected to three successive passes through the homogenizing valve. [c] Samples subjected to continuous homogenization during 2 min. [d] Significantly different from the certified concentration at the 95% level of confidence.

suspension after 2 h incubation at 75 °C to cause the quantitative release (at 37 °C) of the selenium from DORM-2 and egg CRM. Somewhat arbitrarily, in subsequent trials, suspensions containing 0.2-g samples in TRIS buffer plus 20 mg of protease enzyme were sonicated for 15 min and then homogenized three times. The resulting slurries were digested at 60 °C for 0, 0.5, 1, 2, or 3 h. For 0-h trials, the crude sample suspension was sonicated, homogenized, and then analyzed immediately by GFAAS. Sonication for 15 min at low power setting served to completely wet the sample matrix. For all four zoological matrixes (egg, DORM, DOLT, and TORT), the quantitative release of Se was achieved by sonication followed by three passes through the homogenizer (Table 3). Relative to the previous model, the higher operating pressure of the current homogenizer and the three sequential passes resulted in the quantitative liberation of Se from each of the four zoological matrixes, and subsequent incubation with the enzyme was not necessary. It is unclear whether the analyte was released into the liquid phase quantitatively or whether processing reduced the particle size sufficiently to permit efficient pyrolysis/atomization of analyte directly from the solid phase. Within experimental error, all of the Se analyte in the TORT matrix was detected after 1 day of storage of the slurry at 4 °C. Similarly a quantitative recovery of Se in the DOLT-2 slurry that had been digested enzymatically for 0.5 h was observed even after 6 days of storage of the slurry at 4 °C. In both trials, storage caused no apparent segregation of the analyte between the liquid and solid phases, suggesting that the analyte had been transferred quantitatively to the liquid phase.

Companion sample preparations were also generated with two zoological matrixes (DORM-2 and DOLT-2) in the absence of the protease enzyme. The results for Se determinations in CRMs that had been diluted with either of two solvents and then homogenized in the absence of the protease (Table 4) indicated that the recovery of Se was not influenced by the number of passes through the homogenizing valve, by the duration of the sonication procedure (0, 5, or 15 min) prior to homogenization, or by either of the two solvents [90% (v/v) water−ethanol containing 0.25% (m/v) of tetramethylammonium hydroxide or 5% (v/v) ethanol−0.03 M tris(hydroxymethyl)aminomethane]. In all cases, the recovery of Se from these reference materials remained significantly less than the certified concentration. Quantitative recoveries were achieved only if the enzyme was present during the homogenization (Table 3). The reason for the beneficial effect of the added enzyme on Se recovery is not known.

In contrast to the zoological reference materials, the botanical RMs proved to be somewhat more resistant to the homogenization-induced release of Se. Three passes through the homogenizing valve in the presence of protease released Se quantitatively from the rapeseed sample but did not release Se efficiently from any of the three flour RMs (Table 5). By contrast, homogenization coupled with enzymatic digestion at 60 °C during 1 h resulted in a quantitative liberation of the analyte from all three flour samples. Quantitative recoveries from each of the flour samples was also achieved if the sample was processed through the homogenizer several times. This was accomplished conveniently by operating the homogenizer in the recycle mode during 2 min (<10 passes). For the DWF matrix that had been homogenized continuously during 2 min, 10 days of storage at 4 °C did not result in any decrease in Se in the supernatant fraction. Similarly, once complete enzymatic release from the QCIV and QCV samples had been achieved (1 h), subsequent storage at 4 °C for 2 or 3 days did not result in any segregation of the Se between the supernatant fraction and the solids.

CONCLUSIONS

High-pressure homogenization provides a rapid sample preparation technique for soft biological materials prior to GFAAS. Reliable determinations of the selenium concentration of the resulting slurry can be achieved provided that the particle volume has been reduced sufficiently and that the matrix is homogeneous. This work has also demonstrated that enzymatic digestion with a crude protease can be accelerated by incubation at 60 °C to liberate Se from biological matrixes. However, Se release can be achieved more rapidly by reducing the size of the particles within the sample matrix. Multiple passes through the homogenizing valve generated quasi-stable slurries that could be sampled reliably for Se even after several (1−10) days of storage at 4 °C post-preparation.

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