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SPECIAL ISSUE PAPER

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Selenomethionine contents of NIST wheat reference materials

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Abstract Values of the total selenium and selenomethionine (Semet) content of four wheat-based reference materials have been obtained by gas chromatography-stable isotope dilution mass spectrometry methods. The total Se method is an established one, and the results obtained with it are consistent with previously-assigned values. The Semet method (previously reported by our laboratory) is based on reaction with CNBr. Our data indicate that the four wheat samples (wheat gluten, durum wheat, hard red spring wheat, and soft winter wheat), though having a 30-fold range in total Se content, all have about 45% of their total Se values in the form of selenomethionine. Investigation of the CNBr-based method suggests that additional experiments are needed to verify that all selenomethionine in the wheat samples is accounted for, but also indicates that the values obtained are within 15% of the true values. As the form in which Se occurs in foods and dietary supplements is important from a nutritional perspective, adding information about Se speciation to total Se values in appropriate reference materials makes these materials more valuable in relevant analytical work.

Keywords Selenomethionine · Selenium · Mass spectrometry · Speciation

Introduction

Reference materials (RMs) are crucial in establishing the validity of methodology and measurements in chemical analysis. Development of a RM, such as those available from the National Institute of Standards and Technology (NIST, Gaithersburg, MD, USA) [1], requires considerable time and resources. As new analytes become of interest and new methodology is developed, new matrix reference materials are needed to satisfy these advances. Often it is not feasible to produce entirely new RMs to meet these needs. In many cases, adding new information or assigning new certification values to presently available materials is sufficient to meet these needs, and greatly diminishes the cost and time required in comparison to producing suitable, entirely new materials. The availability of published information on additional components in currently-available homogeneous, stable materials can be of significant value, even without the full certification process, especially in newly-emerging areas of measurement. In this work, we present measured values of selenomethionine (Semet) content for four wheat-based reference materials from NIST for which the total Se values are already assigned.

Selenium is an important trace nutrient, having antioxidant properties and a variety of other health effects [2, 3, 4], and it has been the subject of increasing analytical interest over the past few years [5, 6, 7, 8, 9, 10, 11]. There is evidence that the bioavailability and activity of Se depend upon the particular form in which it is ingested [12]. Se species in foods and also in dietary supplements are not well-characterized, but Se may occur in various organic and inorganic forms. Selenoamino acids, such as selenomethionine (Semet), selenocysteine (Secys), and Se-methylselenocysteine (Mesecys) are common organic forms. Selenoamino acids may occur within the sequence of a protein or in non-protein-bound forms, such as various amino acid secondary metabolites. The distribution of Se species across food types varies, and may also be influenced by growing conditions. For example, the major form of Se in an enriched yeast sample was found to be Semet, while a Se-enriched garlic sample was found to contain γ-glutamyl-mesecys as the major form [12]. The predominant form in a broccoli sample was found to be Mesecys [13].

Grains, including wheat, are an important source of Se in the diet. It is therefore of interest to obtain information about Se speciation across a range of wheat and other grain samples. The four NIST wheat-based RMs (see "Experimental" section) are all from sources in Canada,

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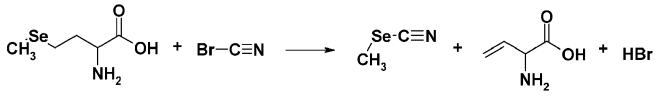
but they are a sampling of different types of wheat flour: soft winter wheat, hard red spring wheat, durum wheat, and wheat gluten. NIST-assigned total Se values for these RMs range from less than 0.1 μg/g to over 2.5 μg/g.

The previously-reported method that we use for Semet measurements [11] is based upon the textbook reaction of cyanogen bromide (CNBr) with methionine (Met), which is used to cleave peptide bonds on the C-side of Met residues [14]. Similar chemistry occurs for Semet. CNBr will also react with free Met and Semet [11, 15]. For Semet, the reaction can be depicted as shown in Scheme 1.



Whether the reaction occurs with bound or free Semet, the species of interest in our measurement is the reaction product methylselenocyanide, CH_3SeCN, which is sufficiently volatile to allow analysis by gas chromatography-mass spectrometry (GC-MS). Isotopically-enriched ^{74}Se-labeled Semet is added to our samples and also reacts with CNBr, allowing Semet measurement by stable isotope dilution (SID) techniques. We report total Se measurement results for the four RMs, by a previously reported [16] SID-GC-MS method utilizing isotopically-enriched ^{82}Se metal, for direct comparison to our Semet measurements, as well as to the assigned NIST total Se values.

Experimental

Samples

The four wheat reference materials obtained from the National Institute of Standards and Technology (NIST), Gaithersburg, MD are: RM 8418 Wheat Gluten, RM 8436 Durum Wheat Flour, RM 8437 Hard Red Spring Wheat Flour, and RM 8438 Soft Winter Wheat Flour. USP Reference Standard selenomethionine was obtained from US Pharmacopoeia, Rockville, MD. ^{74}Se-labeled selenomethionine (77.7% ^{74}Se) and ^{82}Se (96.8% ^{82}Se) solutions were obtained from C. Veillon, USDA, ARS, BHNRC, Beltsville, MD. The isotopically-enriched solutions were previously-calibrated by reverse isotope dilution against pure Se metal (99.99%). Validity of the calibrations was checked by isotope dilution measurements of gravimetrically-prepared solutions of the USP Reference Standard selenomethionine.

Sample preparation and analysis

All sample solutions were prepared gravimetrically. Sample preparation for determination of Semet by reaction with CNBr has been previously described [11]. In brief, sample and ^{74}Se-labeled spike are weighed into a conical vial, and 1.0 mL of a 2% by weight solution of $SnCl_2$ (Aldrich) in 0.1 M HCl is added. The vials are then held at 37 °C for up to 24 h. 200 μL of 3 M CNBr in $CHCl_2$ (Aldrich) is added, and the vials are again maintained at 37 °C for up to 24 h. The product CH_3SeCN is then extracted with chloroform. Samples were analyzed by GC-MS as described below.

For determination of total Se, sample and ^{82}Se spike are weighed into a Kjeldahl flask. Acid digestion yields inorganic Se^{4+}, which is then chelated with 4-trifluoromethyl-o-phenylenediamine (TFMPD). The chelate is then extracted with chloroform [16]. Samples were analyzed by GC-MS as described below.

CH_3SeCN determinations were obtained on a Hewlett-Packard (HP) 6890 GC with a HP 5973 Mass Selective Detector. The GC was equipped with an Agilent HP-5MS 15 m capillary column. The GC was run in pulsed splitless and constant flow (1.7 mL/min) modes, with temperature programming (45 °C for 1 min, 10 °C/min to 90 °C, 30 °C/min to 225 °C, hold 2 min). Retention time for the peak of interest was 5.3 min. For all quantitative work, MS was done in negative chemical ionization (CI) and selected ion monitoring (SIM) modes. CH_3SeCN loses the methyl group prior to detection, so that the ions of interest were of mass-to-charge ratio (m/z) 106 and 100, corresponding to ^{80}SeCN and ^{74}SeCN, respectively.

Total Se samples were analyzed using a Shimadzu QP 5050A system. The GC was equipped with a J&W DB-5MS 30 m capillary column and was run at a flow rate of 1.5 mL/min with a pressure ramp and temperature programming (35 °C for 1 min, 25 °C/min to 250 °C, hold 2 min). A spilt ratio of 33:1 was used. Retention time for the peak of interest was 6.5 min. MS analysis was in negative ion mode. The ions of interest were of m/z=252 and 254, corresponding to the TFMPD chelates of ^{80}Se and ^{82}Se, respectively.

In SID-MS, the analyte level is determined by measuring the isotopic abundance ratio for a sample containing a known amount of an isotopically-enriched analogue of the analyte [17, 18]. Abundance ratios are calculated using the appropriate peak areas for the natural and enriched analogues. It is often the case that instrumental bias cannot be ignored when determining the abundance ratios. We apply a correction for instrumental bias based on measurements of the abundance ratio for a standard of the natural analogue. A blank correction is also applied.

Results and discussion

Values of Semet and total Se content obtained with our methods are given in Table 1. Se as Semet values are averages of three samples run on the HP instrument. Total Se values are averages of two samples run on the Shimadzu instrument, except for the value of soft winter wheat RM 8438, which is an average of four samples. Analytical results, with uncertainties expressed as 95% confidence limits, listed in Table 1 are for dry weight, corrected for moisture content obtained on separate samples. Expected total Se values, with 95% confidence limits, are assigned values for the NIST RMs (the uncertainty for RM 8418 is a range based on accepted results rather than a 95% confidence limit) [19]. The total Se method is capable of high precision and accuracy [16]. The moisture-corrected results we obtained show good precision and are in excellent agreement with the NIST values for total Se content of these RMs.

The Semet values listed for RM 8436, RM 8437, and RM 8438 are the first reported values for these materials. We have previously reported values of the Semet content of RM 8418 [11] and noted some variation in the values obtained by different analysts. The result for RM 8418 in Table 1 above is, within the bounds of the stated uncertainties, consistent with the lower of the two earlier values. The measured Semet content as a percentage of total Selenium is close to 45% for all four of the samples, despite the variation in wheat variety and the 30-fold range of magnitude difference in level of total Se. Further studies are underway to ascertain if this close agreement holds up for representative samples of the variety of types of wheat commercially available.

Table 1 Measurements of Se as Semet and total Se for the four wheat reference materials

Sample	Se_{Semet} (μg/g)[a] *N*=3	Se_{total} (μg/g)[a] *N*=2 (except where stated)	Expected Se_{total}[b] (μg/g)	Se_{Semet}/Se_{tot} (%)	Moisture (%) *N*=4
RM 8418 Wheat gluten	1.21±0.03	2.71±0.02	2.58±0.19	44.6	6.88±0.35
RM 8436 Durum wheat	0.59±0.04	1.26±0.08	1.23±0.09	47.0	8.60±0.22
RM 8437 Hard red spring wheat	0.26±0.04	0.56±0.02	0.56±0.04	45.6	8.76±0.57
RM 8438 Soft winter wheat	0.032±0.003	0.071±0.006 (*N*=4)	0.076±0.009	45.4	8.38±0.95

[a] Uncertainties are 95% confidence limits. Dry weight values are corrected for moisture content (last column). Samples (0.2 to 0.5 g) of each RM were dried in an oven for 4 h at 85 °C per certificate; [b] assigned value from NIST

Although the variance associated with the Semet results in Table 1 is somewhat higher than that of the total Se method, relative standard deviations (RSD) are still only in the range of 1% to 6%. Also considering the earlier reported discrepancy in results from different analysts [11], it was of interest to test the reliability of the method and to explore factors that may affect precision and accuracy.

Variation in sample size

The recommended sample size stated on the certificate for these materials is 500 mg. This size is too large for our Semet method and we routinely use much smaller sample sizes of about 50 mg for these determinations. For total Se content we used sample sizes of 0.5 to 2 g, obtaining precisions of less than 1% RSD for the three highest level materials and 5% for the RM 8438 sample with total Se content below 0.1 μg/g. For the smaller sample sizes used for Semet determinations, we saw precisions that were between 1 and 6% RSD, which are still well within the uncertainty expressed in the NIST assigned values for total Se content of these materials. Our analytical values would include not only method precision, but also any added inhomogeneity due to small sample size. Therefore we feel that the smaller sample size was not significant.

The method was tested for consistency over other sample sizes for Semet analysis of wheat gluten. Samples of size 25 mg to 100 mg were analyzed, keeping all other reagent amounts and conditions constant, and the results are presented in Fig. 1. Although precision is not as good as in the results above, the plot of measured Se as Semet versus amount of sample is linear (in other words, as the amount of sample is increased, the amount of measured Semet increases in a proportional manner). The slope value of 1.2 μg/g is in agreement with the value for RM 8418 reported in Table 1. The achievement of a zero intercept for the plot in Fig. 1 is also consistent with complete reaction of Semet in the wheat gluten samples. It rules out problems that would result in fixed measurement errors, such as a limitation in reactants. It does not, however, rule out problems that would give multiplicative measurement errors (for which a certain percentage of the total Semet present is consistently missed at all sample sizes).

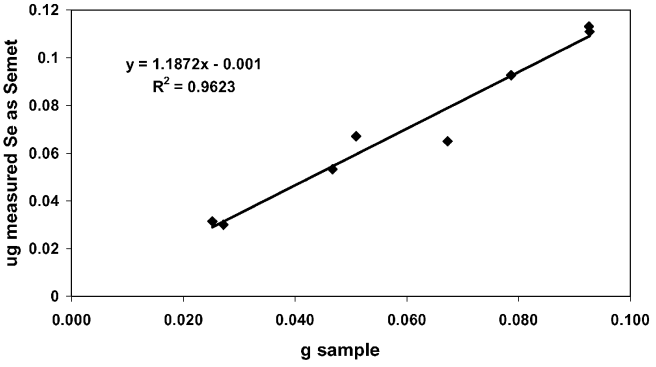


Fig. 1 Measured μg Se as Semet in RM 8418 versus grams of sample used

Standard additions

Results of a standard additions experiment on RM 8418 are presented in Fig. 2. Additions of a standard Semet solution (USP standard) were made to 50 mg wheat gluten samples at five levels: 0%, 25%, 50%, 75%, and 100% of the expected Semet content of the wheat gluten, based on the value in Table 1. Samples were run in duplicate. The plot of measured Se as Semet content in μg/g versus that based on the amount of the standard additions (μg added Se as Semet/g of RM 8418) is linear, and the *y*-intercept value of 1.24 μg/g, corresponding to the measured Semet content of the wheat gluten sample with no addition, is consistent with the value for RM 8418 of 1.21 μg/g given in Table 1.

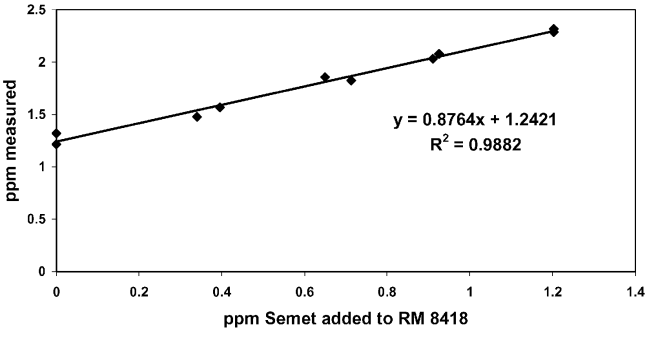


Fig. 2 Method of Additions for RM 8418: μg/g measured Semet versus μg/g added Semet

The standard additions analysis raises a question about whether the value in Table 1 may slightly underestimate the true value. For complete recovery of added analyte, the slope of the standard additions curve should be 1.0. In our experiment, the slope of the regression equation, 0.8764, suggests that about 12% of the added Semet is not detected. 95% confidence limits for the slope are 0.799 to 0.954, indicating a high probability that the true slope is not unity. Setting y=0 in the regression equation and solving for x gives a value of 1.417 µg/g Se as Semet. Using the y-intercept value as a fulcrum about which the slope is varied over its confidence limits gives a range of values for the standard additions Semet content of 1.30 µg/g to 1.56 µg/g. A slope value other than one, however, does raise a question about the absolute value obtained from the standard additions. Is this due to only the added Semet, or is the bound Semet in these materials also affected? The obtained slope of less than 1.0 possibly suggests that there may be some difference in reaction efficiency between the added free natural ^{80}Semet and the added ^{74}Se-labeled Semet and the ^{80}Se isotopic analogues of endogenous protein-bound Semet, but such an effect requires additional experiments to verify.

Effects of $SnCl_2$ and predigestion

Our usual procedure for determining Semet includes overnight treatment of samples in 0.1 M HCl at 37 °C in order to denature proteins, and so promote reaction efficiency. Oxidation of Met to methionine sulfoxide and methionine sulfone can occur under conditions used for routine protein hydrolysis and digestion [20, 21], and Semet is subject to analogous oxidation [15, 22]. The oxidized forms do not react with CNBr, and so our overnight treatment includes 2% by weight of the reducing agent $SnCl_2$. An earlier report on the use of CNBr for Semet determination suggests that free Semet is more prone to oxidation than bound Semet [15]; and in fact claimed that $SnCl_2$ was not necessary for determination of bound Semet in a yeast sample.

The effects of added $SnCl_2$ and the predigestion step were examined for the wheat gluten sample and for a standard solution of free Semet. For each of these, a factorial experiment [23] was run using two levels of $SnCl_2$ and two levels of predigestion. A two-level factorial allows one to measure the individual effects of $SnCl_2$ and predigestion and also to test for their interaction (in other words, for whether the predigestion step influences the effect of $SnCl_2$ and vice versa). The factor levels are given in Table 2.

Three samples were run for each of the factor combinations, giving a total of 12 samples each for the Semet standard and the wheat gluten materials. All samples were run as part of a single, randomized experiment, in which CNBr was added to all samples at essentially the same time and reaction times were uniform (about 12 h). A nominally uniform, weighed amount of the ^{74}Semet spike was added to all samples prior to treatment.

Responses examined were peak areas for m/z=106 and m/z=100 and µg/g Se as Semet. The peak areas were corrected for molar amounts of sample and spike and for the isotopic distributions of the spike and of natural SeCN, so that the corrected response at m/z=106 is the molar response due to the natural source only (USP standard solution or RM 8418) and the corrected response at m/z=100 is the molar response due to the ^{74}Semet spike only. In making the corrections involving RM 8418, the Semet value listed in Table 1 was used. Peak areas were not corrected for instrument bias, but the area ratio used to determine µg/g Se as Semet was corrected as described above. Each response was modeled as follows:

$$\text{Response} = \mu + \alpha + \beta + \alpha\beta + \varepsilon$$

Here μ is the overall mean response, α is the effect of $SnCl_2$, β is the effect of predigestion, $\alpha\beta$ is the $SnCl_2$-predigestion interaction effect, and ε is an error term that includes all sources of random variation. The significance of the modeled effects was tested by analysis of variance

Table 2 Factor levels for the predigestion-$SnCl_2$ factorial experiment

Factor	− level	+ level
Predigestion	Addition of 0.1 M HCl concurrently with addition of CNBr (no overnight treatment)	Overnight treatment in 0.1 M HCl prior to addition of CNBr
$SnCl_2$	No use of $SNCl_2$	0.1 M HCl used in predigestion levels, includes 2% by weight $SnCl_2$

Table 3 Analysis of variance (ANOVA) for the Semet standard

Response[a]	Effect	MS_{effect}[b]	MS_{error}[c]	F value	Prob>F
Area 106	$SnCl_2$	1.15×10^{11}	4.15×10^{7}	2773	<0.0001
	predigestion	8.37×10^{4}		0.002	0.9653
	$SnCl_2$* predig	1.00×10^{5}		0.002	0.9620
Area 100	$SnCl_2$	9.79×10^{10}	1.13×10^{8}	865	<0.0001
	predigestion	4.94×10^{5}		0.004	0.9489
	$SnCl_2$* predig	7.06×10^{6}		0.06	0.8091
µg/g Se	$SnCl_2$	0.0036088	0.00006169	58.5	<0.0001
	predigestion	0.00002054		0.33	0.5798
	$SnCl_2$* predig	0.00015052		2.44	0.1569

[a] Area responses corrected for molar amounts and isotopic distributions; [b] mean square associated with effect; [c] mean square of error

Table 4 Analysis of variance (ANOVA) for wheat gluten

Response[a]	Effect	MS_{effect}[b]	MS_{error}[c]	*F* value	Prob>*F*
Area 106	$SnCl_2$	4.46×10^9	1.90×10^8	23.5	0.0013
	predigestion	1.20×10^9		6.30	0.0364
	$SnCl_2$* predig	4.78×10^7		0.25	0.6294
Area 100	$SnCl_2$	8.05×10^9	1.25×10^8	64.4	<0.0001
	predigestion	1.46×10^9		11.6	0.0092
	$SnCl_2$* predig	1.00×10^7		0.08	0.7842
µg/g Se	$SnCl_2$	0.06705075	0.00095433	70.3	<0.0001
	predigestion	0.00957675		10.0	0.0132
	$SnCl_2$* predig	0.00639408		6.70	0.0322

[a] Area responses corrected for molar amounts and isotopic distributions; [b] mean square associated with effect; [c] mean square of error

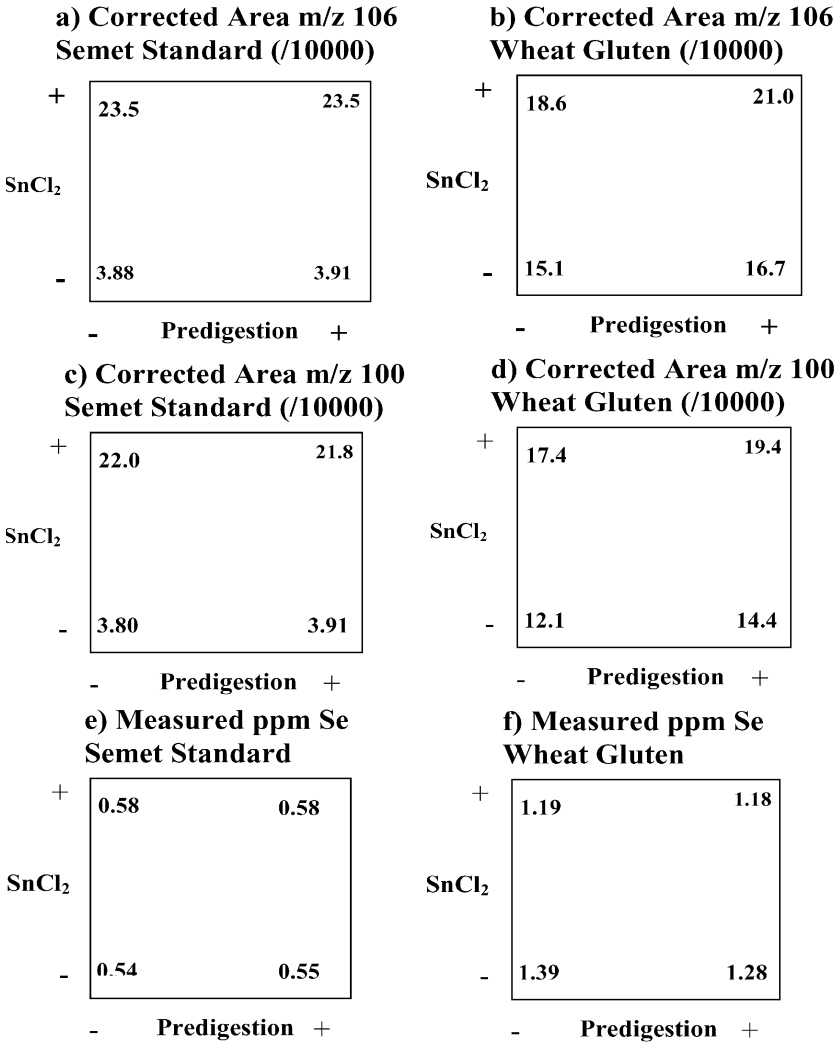


Fig. 3 Two-dimensional representation of the results of the factorial experiment

(ANOVA), and the results are given in Table 3 and Table 4. A two-dimensional graphical view of the results of the factorial experiment is given in Fig. 3.

Based on the above considerations, $SnCl_2$ would be expected to have a strong effect on both area responses of the standard Semet solution and on the *m*/*z*=100 area response of the wheat gluten sample, but less effect on the *m*/*z*=106 area response of the wheat gluten sample. The measured Semet value obtained for the wheat gluten sample with no $SnCl_2$ would be expected to be in error, due to the difference in effect on the bound natural Semet and the added free ^{74}Semet spike. Likewise, the predigestion step would be expected to have different effects on bound and free Semet, having some effect on the *m*/*z*=106 area response of the wheat gluten, but no effect on any of the free Semet area responses. Therefore, one might expect an error in the measured amount of Se in the wheat gluten samples with no predigestion step.

Considering the standard Semet solution first, it is clear from Table 3 and Figs. 3a and 3c that the presence of

$SnCl_2$ strongly affects the strength of response of free natural ^{80}Semet and labeled ^{74}Semet. Not using $SnCl_2$ reduces the corrected area responses by about 85%. No effect of the predigestion step and no interaction between $SnCl_2$ and predigestion were detected for any of the three responses (Figs. 3a, 3c, and 3e). Although there is no reason to expect that the isotopically-enriched Semet should behave any differently than the natural form with respect to the CNBr reaction, a statistically-significant effect of $SnCl_2$ on the measured Se as Semet content was also observed for the Semet standard. Using no $SnCl_2$ gave a value about 6% lower than when utilizing $SnCl_2$ (Fig. 3c), the value from the latter being in agreement with the expected value for the standard solution. The Semet determination depends on the ratio of the *m*/*z*=106 (from the natural) and *m*/*z*=100 (from the labeled) responses, so interpretation is not straightforward. It is apparent from figures 3a and 3c that the values obtained with no use of $SnCl_2$ reflect reactions that did not approach completion. Any difference in reaction rate or level of completion, whether inherent or due to some difference in the starting Semet solutions (such as level of oxidation), would influence the ratios, and so the Semet values obtained.

$SnCl_2$ also had an effect on the responses of *m*/*z*=106 and *m*/*z*=100 for the wheat gluten samples (Table 4 and Figs. 3b and 3d), but this was not as strong as for the standard Semet solution. A somewhat stronger effect was observed for *m*/*z*=100 (Fig. 3d, reduction of 25% to 30% with no $SnCl_2$) than for *m*/*z*=106 (Fig 3b, about 20%). Presumably a significant portion of the wheat gluten Semet occurs in a protein-bound form, which may account for the observed difference. It is notable, however, that the effect on the free ^{74}Semet spike is much weaker than was observed in the standard solution samples (Fig. 3d versus Fig 3c). The wheat gluten matrix in some way promotes reaction of free Semet with CNBr in the absence of $SnCl_2$ (experiments with wheat gluten samples spiked with the USP standard solution indicate that it does so for both ^{80}Semet and ^{74}Semet).

Also in contrast to the results with the standard Semet solution, there is an effect of predigestion on the *m*/*z*=106 and *m*/*z*=100 area responses of the wheat gluten samples. The interpretation would seem to be that predigestion helps to make more Semet available for reaction by breaking down the wheat gluten sample and denaturing the proteins, except that the effect occurs for both the naturally-occurring Semet and for the spike of free ^{74}Semet. The explanation is therefore not so straightforward, but the results again imply that added free Semet has a strong interaction with the wheat gluten matrix.

As determined by *t*-test at the 95% confidence level, the means of the corrected responses of ^{74}Semet in the standard solution and in the wheat gluten matrix when both predigestion and $SnCl_2$ are used are not different (the responses in Figs. 3c and 3d for which both predigestion and presence of $SnCl_2$ are at their + levels, signified by "++" hereafter), but they are near the borderline of statistical significance. The same holds for the corrected response of ^{80}Semet (++ responses of Figs. 3a and 3b). There is therefore some possibility that small, true differences do exist. However, in each case (*m*/*z*=106 and *m*/*z*=100) the nominal difference in average response under ++ conditions is about 10%, so that there is no indication that the (*m*/*z*=106)/(*m*/*z*=100) ratio measurement, upon which the Semet measurement depends, is affected by the wheat gluten matrix. We should recall, though, that the molar correction for ^{80}Semet in the wheat gluten uses the value of 1.207 µg/g Se as Semet, as given in Table 1. If this value is in error, that would obviously affect the ++ area response for *m*/*z*=106 listed in Fig. 3b. Taken together, the observation that the *m*/*z*=100 area response under ++ conditions changes little or not at all in the different matrices, and the observation that the *m*/*z*=106 response in the standard solution is close to that obtained for the wheat gluten, provide evidence that any error in our value of Se as Semet content for RM 8418 given in Table 1 (obtained under ++ conditions) is not likely to be large. We should also note that when the area responses are corrected for instrument bias (see "Materials and Methods" section), which allows comparison of the *m*/*z*=106 and *m*/*z*=100 responses, all four area responses under ++ conditions (Figs. 3a, 3b, 3c, and 3d) are statistically equivalent.

No predigestion-$SnCl_2$ interaction was detected for the *m*/*z*=106 and *m*/*z*=100 responses in wheat gluten, but such an interaction was noted for the µg/g Se response, along with significant main effects of $SnCl_2$ and predigestion. None of the effects on µg/g Se as Semet are strong (Fig. 3f). $SnCl_2$ has the strongest effect, its presence resulting in a lower measured value than is obtained in its absence, and also giving a value in agreement with that in Table 1. The higher values obtained when no $SnCl_2$ is used may be due to a favored reaction of protein-bound Semet over free Semet in the absence of $SnCl_2$. Predigestion has an effect only when $SnCl_2$ is absent, which also gives rise to the interaction effect. Perhaps the predigestion facilitates some component of the wheat gluten in assisting in the reaction of free Semet. As was the case for the standard Semet solution above, Se as Semet values, obtained with no use of $SnCl_2$, reflect incomplete reaction, and so such values are suspect. Skipping predigestion also results in incomplete reaction, but, as mentioned above, has no effect on measured Semet content when $SnCl_2$ is present.

Conclusions

Although there is some evidence that our current GC-SIDMS method based upon the reaction of Semet with CNBr may underestimate the Semet content of the wheat reference materials, the values obtained appear to be no more than 12–15% lower than the true values, as shown by the standard additions recovery. Investigation of the method has provided information about the CNBr reaction and matrix effects that should help to refine the method and so increase its accuracy and precision. Problems related to oxidation of Semet are one concern with the method. Use of the reducing agent $SnCl_2$ eliminates this concern in the case of free Semet solutions, but it can't be ruled out as a

problem in the case of the wheat RMs, even though $SnCl_2$ also enhances Semet response in them. Accessibility to all Semet incorporated in proteins in the wheat matrices is another potential problem. Overnight treatment of samples in 0.1 M HCl at 37 °C is intended to denature proteins and so make Semet accessible, but whether additional digestion steps are needed remains to be tested. The acidic pretreatment of the wheat matrices appears to have a more complex role than simply denaturing proteins, as it was found to affect the response of added free Semet as well as that of the endogenous Semet. The wheat gluten matrix was found to influence the response of added free Semet in other ways as well, indicating a strong interaction between the two. Although such interaction was not expected, it is probably analytically advantageous, as accuracy of the method requires complete "exchange" between the endogenous Semet and the added spike of free ^{74}Semet. In the USP solutions of free Semet, full exchange occurs before reaction with CNBr. In the wheat matrices and other samples containing protein-bound Semet, exchange of the ^{74}Semet label occurs only after reaction with CNBr. There is good evidence that significant exchange with natural ^{80}Se occurs in the wheat matrices, but further probing of the method is required to show that it is complete.

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