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Speciation of Selenium Compounds from High Selenium Broccoli Is Affected by the Extracting Solution

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The speciation of selenium compounds from high selenium broccoli (876 μg/g) depends on the extraction conditions. Twenty-seven extraction conditions were explored involving nine different buffering systems between pH 1 and pH 9. In nonbuffered extractions of broccoli, more than 40% of the spiked Se-methylselenocysteine was not recovered in the filtered solution. However, in buffered extractions, losses for Se-methylselenocysteine ranged from 10 to 20%. Mass balance indicated that approximately 30% of naturally occurring selenium in broccoli samples was volatilized and lost to the atmosphere when buffered extractions were made. Solid phase extractions indicated that the polarity of selenium compounds in solution was also dependent on the extracting solution. High-pressure liquid chromatography coupled to an inductively coupled plasma mass spectrometer was used to show that selenium compounds extracted from broccoli reacted with the extracting solution. Compound identities were assigned by matching retention times to standards of selenite, selenate, methylseleninic acid, Se-methylselenocysteine, selenomethionine, and the selenonic acids of Se-methylselenocysteine and selenomethionine. Changes in speciation were analyte-, pH-, and buffer-dependent, but generally, a higher pH resulted in more highly oxidized selenium compounds. For valid conclusions to be drawn from the analytical data, the extraction conditions should match the conditions present in the matrix or be specified for a particular application such as a simulated gastrointestinal digestion.

KEYWORDS: Selenomethionine; Se-methylselenocysteine; methylseleninic acid; HPLC; ICP-MS; plant

INTRODUCTION

In 1996, Clark et al. reported that dietary supplementation of 200 μg of Se per day, administered as high Se yeast, significantly reduced lung, prostate, and colorectal cancer in a human population (*1*). It is established that the chemical form of Se determines its metabolism and biological activity (*2*−*12*); therefore, determining the chemical form of Se in a food is essential to understanding the metabolism of Se from that food. Plants such as *Astragalus bisulcatus* (two-grooved milkvetch or locoweed) and *Brassica oleracea* (broccoli) may accumulate Se concentrations greater than 6500 and 500 μg/g, respectively (*2*, *3*). Rats fed supranutritional amounts of Se as broccoli (1−2 μg Se/g diet) exhibited greater colon cancer protection than rats fed low Se broccoli supplemented with the same amount of selenite or selenate (*2*, *13*).

Consequently, the speciation of Se compounds in various foods is an important step in understanding the cancer protective properties of foods such as high Se broccoli. Unfortunately, reliable methodology for the extraction and separation of Se compounds from most natural matrixes is lacking. As a result, identification and quantification of Se in most situations are incomplete (*14*).

To determine the concentration of the different Se compounds found in a food, the extraction must preserve the species while being quantitative. Chemical reactions that take place during an extraction may result in misidentifications and skewed extraction efficiencies. Because conditions in the extracting solution may be different from the conditions in the matrix, the analyte may react with the extracting solvent, matrix components, or gases in the headspace of the extraction vessel. The reactivity is strongly dependent on temperature and pH (*15*, *16*). Temperature and pH are easily controlled using either climate control or buffered extracting phases; however, the use of buffered extracting phases for Se speciation is rarely reported in the literature (*10*, *17*−*23*). A compound that acts as a buffer in aqueous solution exchanges protons with the bulk solution; consequently, the buffer may be able to alter the different extracted species. This is because the moiety that allows for proton exchange also has the potential to enter into reactions

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with the Se compounds. The matrix may also affect extraction conditions; therefore, one must consider the reactions that can take place between the buffer and the matrix components. For example, the solvent or buffer may liberate Se-Met or cleave methylselenol from a protein. Liberated matrix components can also react with extracted Se compounds. Because of the lability of certain Se compounds, it is necessary to define strict extracting phase conditions in order to improve reproducibility and to simplify the identification of compounds. Setting such conditions was a primary objective of this study.

Se is isoelectronic with sulfur, and Se analogues are found in much lower concentrations than their sulfur counterparts; therefore, it is necessary to use a detector that is both sensitive and selective (*14*, *16*, *24*). Electrospray ionization mass spectrometry (ESI-MS) has been used as an LC detector in elucidating the structure of organoselenium compounds in standard solutions and complex matrixes (*21*, *22*, *25*−*28*). However, sample complexity and relatively high limits of detection make compound identification challenging. The ICP-MS is both sensitive and selective for Se (*14*). Unlike ESI-MS, no structural information is gained using ICP-MS. Identification must be made solely by retention time matching to known standards. HPLC-ICP-MS has enjoyed a number of successes in Se speciation including Se compounds of high Se garlic and yeast (*10*, *21*, *22*, *29*−*32*).

The present paper utilized a number of common buffers in an attempt to stabilize extracting phase pH. These buffers have been used in a pH range of 1−9. We report that Se compounds are greatly affected by the extracting phase conditions as demonstrated by IPC and elemental analysis (EA).

EXPERIMENTAL PROCEDURES

Extractions. High Se broccoli samples (876 μg/g total Se) were grown in-house, and the raw broccoli was ground to powder following lyophilization. Broccoli powder (0.4 g) was placed in a clean, dry 50 mL beaker with a stir bar and 10 mL of extracting phase. The beaker was covered with Parafilm and allowed to stir overnight at 60 rpm. The slurry resulting from the extraction was centrifuged and filtered, first with a 1.0 μm and then a 0.2 μm PTFE syringe filter (Chromtech, Apple Valley, MN). Filtered solutions were placed in plastic screwtop test tubes and stored at 4 °C. All glassware and stir bars were scrubbed with Alconox, rinsed with deionized water, soaked in a 50% nitric acid bath for 2 h, rinsed five times with Milli-Q water, and dried at 60 °C prior to extractions.

Extracting Phases. Hydrochloric acid, phosphoric acid, glacial acetic acid, chloroacetic acid, and ascorbic acid were purchased from J. T. Baker (Phillipsburg, NJ). Sodium bicarbonate, pyridine, Trizma HCl, Trizma base, and citric acid were purchased from Sigma-Aldrich (St. Louis, MO). Sodium hydroxide and diammonium citrate were from Alfa-Aesar (Ward Hill, MA). All buffers were prepared in Milli-Q water (18.2 MΩ cm^{-1}) (Millipore, Bedford, MA). Unless otherwise specified, buffer solutions were made so that the total concentration of the acid and conjugate base was 430 mM. Buffered solutions were adjusted to integer values of pH (**Figure 1**) by using concentrated HCl or 4 M NaOH and a Beckman Φ32 pH meter (Fullerton, CA) with an Accumet double junction, Ag/AgCl reference electrode (Fisher Scientific, Pittsburgh, PA). For example, phosphate buffer was made at pH 1.0, 2.0, 3.0 and 6.0, 7.0, and 8.0.

C-18 Trapping. Two milliliters of extract was applied to a cartridge and rinsed with 5 mL of an aqueous solution of 0.6% HFBA (Sigma-Aldrich). The packing was removed and digested for EA. Trapping efficiencies were normalized for cartridge bed weight. Extracting phases used in trapping studies were glycine/HCl at pH 3, phosphate at pH 7, and phosphate at pH 3, as well as nonbuffered water.

Elemental Analyses. Elemental analyses were carried out as described by Finley et al. (*33*).

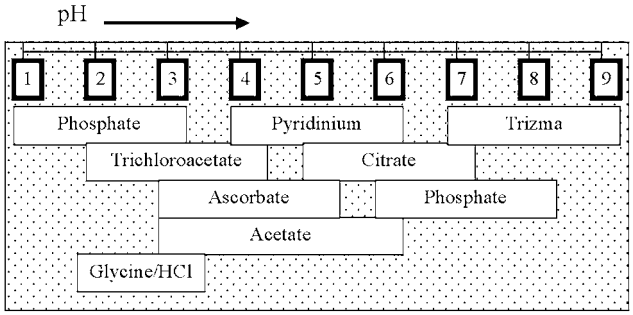

Figure 1. Buffers used in extraction experiments were both organic and inorganic in nature and covered a wide range of buffering chemistries. All buffers were made to contain 430 mM in the buffering component and adjusted to integer values using either 4 M NaOH or concentrated HCl.

Spiking Studies. Spiking studies were carried out to determine the effects of the matrix and extracting conditions on the reactivity and relative hydrophobicity of standard Se compounds. Mass balance of broccoli extractions and standards placed in the extracting phase and run through the extraction process used the described EA method. Quantitative mass calculations determined total Se recovery.

Matrix Interactions. Organoselenium compounds may contain hydrophobic moieties; oxidation may decrease the hydrophobicity while reduction may increase the hydrophobicity. Spiking analytes into the matrix may shift the extraction equilibria or perturb the redox chemistry. These effects were studied by using standards put through the extraction procedure with or without broccoli. Extracts were applied to C-18 cartridges, and trapping efficiencies were determined.

HPLC-ICP-MS. HPLC separations were an adaptation of Kotrebai et al. (*21*). An Isco model 2360 gradient programmer and an Isco (Lincoln, NE) model 2350 pump were used to deliver the mobile phase for HPLC separations. Separations took place on a 5 μm Symmetry Shield RP-8 column, 150 mm × 3.9 mm (Waters Corporation, Milford, MA). The mobile phase was 0.6% HFBA at a flow rate of 1 mL/min. The eluate from the LC was introduced to the ICP-MS via a CETAC U-5000 AT Ultrasonic Nebulizer (CETAC, Omaha, NE). A VG Elemental VGA 430 ICP-MS was used for the detection of all separations (Fisons Instruments, Danvers, MA). Operating conditions for the ICP-MS were as follows: torch flow, 0.8 L/min; auxiliary flow, 0.6 L/min; cooling gas flow, 16 L/min; dwell time, 10.24 ms; averaging 5 points/peak; lag time, 0.31 ms; *m*/*z* monitored 74, 76, 77, 78, and 82.

Standards. Standards were prepared in Milli-Q water and stored at 4 °C. Periodic separations of standards were used to check for degradation. Selenic acid, selenious acid, and Se-Cys were purchased from Sigma-Aldrich. Se-Me-Cys, Se-Met, and MeA were purchased from PharmaSe (Lubbock, TX).

Oxidized Standards. Hydrogen peroxide was used to oxidize standards so that retention times for the respective selenoxides could be determined. A 600 μL aliquot of a 2 mM solution of the standard was introduced to 900 μL of 30% hydrogen peroxide. After 1 h, Se-Me-Cys was converted to two other products, but if the reaction was allowed to proceed over a 24 h period, a single more oxidized compound resulted. Block et al. used NMR and ESI-MS to identify these oxidized, earlier eluting species as the selenonic acids of the respective standards (*25*). On the basis of the separation mechanisms of IPC, the retention times of the selenonic acids of the different amino acids studied were expected to be similar. When the oxidized reaction mixture was injected onto the column for separation after 1 h instead of 24 h, the later eluting oxidation peaks were determined to be the intermediate hydrates.

HPLC Separation of Spiked Samples. Broccoli was spiked so that the extracting solution contained 0.2 mM Se standard for the determination of reactions of specific compounds with matrix components under different extracting conditions.

RESULTS AND DISCUSSION

pH. *pH Changes During Extraction.* Nonbuffered extractions of high Se broccoli had an average final pH of 4.96 (±0.01)

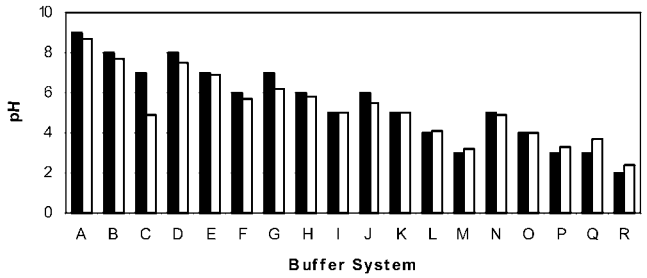

Figure 2. Final pH of extractions carried out in 430 mM buffers was stabilized; solid bars indicate original pH, and empty bars indicate final pH. Identities correspond to (**A**) Trizma, (**B**) Trizma, (**C**) Trizma, (**D**) phosphate, (**E**) phosphate, (**F**) phosphate, (**G**) citrate, (**H**) citrate, (**I**) citrate, (**J**) acetate, (**K**) acetate, (**L**) acetate, (**M**) acetate, (**N**) ascorbate, (**O**) ascorbate, (**P**) ascorbate, (**Q**) glycine/HCl, and (**R**) glycine/HCl.

for three trials. Neither 140 nor 240 mM buffer concentrations stabilized the pH of the extraction, and all of the extractions approached a final pH of approximately 4.9. However, a 430 mM phosphate buffer at pH 7 stabilized the pH to within 0.1 pH units over the course of the extraction. Therefore, all further extractions were carried out in the presence of a 430 mM buffer and changes in pH during extractions were minimized (**Figure 2**).

Elemental Analyses. *Extraction Efficiency Depends on the Extracting Phase.* Mass balance of total Se was determined using EA of the original broccoli, the postextraction solids, and the filtered liquid (**Figure 3**). In general, buffered extractions removed more Se from the matrix (**Figure 3A**) but did not affect the amount that was extracted into the final aqueous phase (**Figure 3B**). Mass balance (**Figure 3C**) indicated that a nonbuffered aqueous phase resulted in a more quantitative extraction whereas ascorbate, phosphate, or glycine/HCl buffered extractions accounted for less than 70% of the total Se. The remaining Se was either adsorbed to the walls of the extraction vessel and the stir bar or vaporized; odor from the extraction suggested that the Se was volatilized. Therefore, reduction or disproportionation of organoselenium compounds to volatile forms such as dimethylselenide and dimethyldiselenide was suspected (*25*). Whether reduction was an artifact of the buffer systems or if the nonbuffered extracting phase oxidized the vaporized forms so that they stayed in solution was not clear and is beyond the scope of this paper. However, it is clear that reactions in the extraction process may affect speciation and may result in misidentifications and inaccurate values if analyses are reported as quantitative.

Polarity of Extracted Se Compounds Depends on the Extracting Phase. Because of visual differences in the postextraction slurries that appeared to be pH- and buffer-dependent, we considered that the buffers may have promoted reactions that changed the polarity of the organoselenium compounds. C-18 cartridges used to partition hydrophobic compounds into the octadecyl coated packing were analyzed for total Se content to determine the overall hydrophobic character of the extracted Se compounds. Extractions carried out in water had the lowest total Se trapped on the C-18 cartridge (4000 (±483) ng Se/g (mean ± standard deviation, $n = 3$)) and were the least reproducible with 12% relative standard deviation (% RSD). A Glycine/HCl, pH 3, buffer allowed for trapping of the greatest amount of Se with 6700 (±363) ng Se/g while trapping efficiency was the most reproducible with a phosphate, pH 7, buffer (4% RSD). Because the trapping efficiencies were pH- and buffer-dependent, the question arose, which compounds

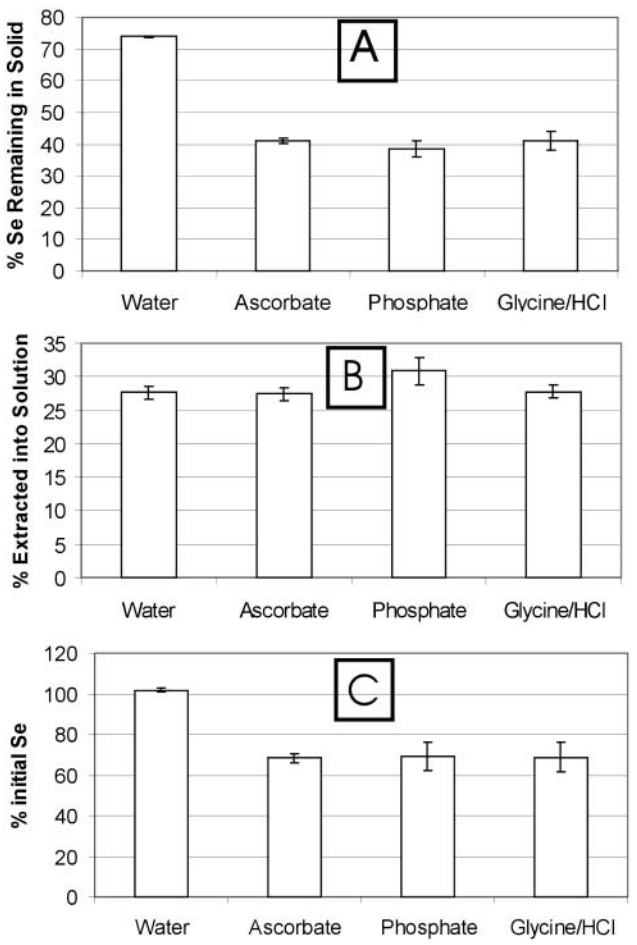

Figure 3. Not all of the Se is accounted for in buffered extractions. Ascorbate and glycine/HCl buffers are initially set at pH 3.0, while phosphate buffer is set at pH 7.0. (**A**) % of Se remaining in the solid after the extraction; (**B**) % of Se found in the extracting solution; and (**C**) total Se accounted for in the postextraction liquid and the solid. Error bars indicate standard deviations, $n = 3$.

from the broccoli are being trapped on the C-18 cartridges and which hydrophobic Se compounds are sufficiently volatile so that they are lost during the extraction?

Organoselenium Compounds Can Partition into the Matrix. Mass balance showed significant losses from the aqueous phase (**Figure 4**). When Se-Me-Cys was spiked into a glycine/HCl (pH 3.0) buffer, only about 60% of the Se was recovered. However, in the same buffer, 98% of Se from a Se-Cys spike was recovered.

We examined whether spiked Se may be lost by sorption to the matrix solids. Nonbuffered extractions potentially had more than a 25% increase in final Se concentrations in the post-extraction solid (MeA) or as little as 0.06% increase (Se-Me-Cys) when a pH 3 glycine/HCl buffer was used (**Figure 5**). Ascorbate buffered solutions (pH 3) gave the most uniform results, while pH 7 phosphate and pH 3 glycine/HCl allowed relatively little sorption of Se-Cys and Se-Me-Cys. Recall that extractions carried out in nonbuffered extracting phases had the poorest extraction efficiencies from the solid, while they tended to have the greatest spike sorbing capacity for all analytes tested, except Se-Cys.

Losses of Se-Me-Cys and Se-Cys from the liquid in pH 7 phosphate buffered solutions were likely the consequence of volatilization during the extraction because an increase in Se concentrations in the postextraction solids was not observed.

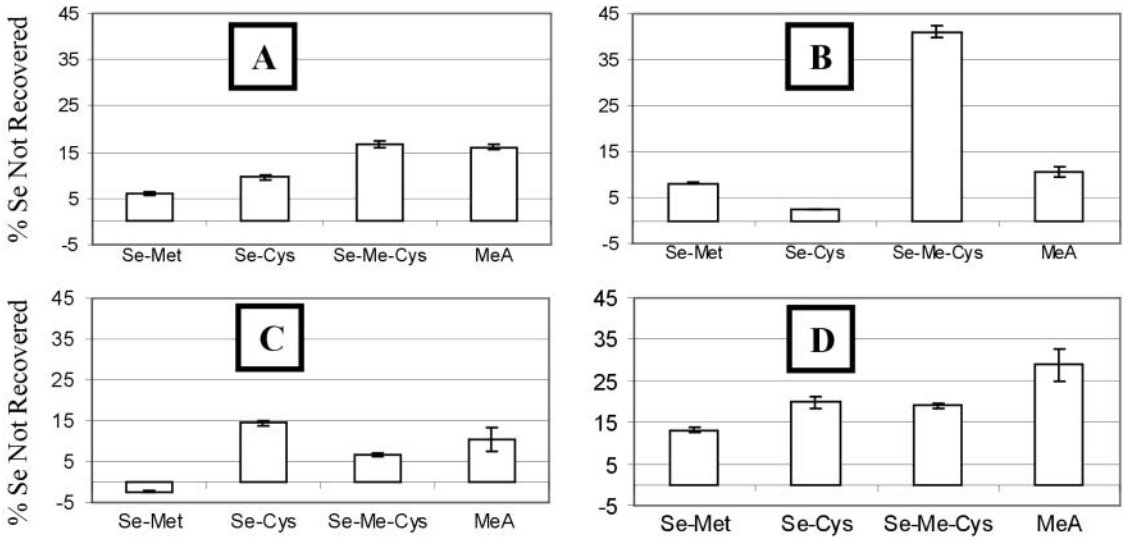

Figure 4. Spiked Se standards were lost from the extracting solutions in all but one case, pH 3 glycine/HCl. (**A**) Phosphate, pH 7; (**B**) nonbuffered; (**C**) glycine/HCl, pH 3; and (**D**) ascorbate, pH 3. Error bars indicate standard deviations, $n = 3$.

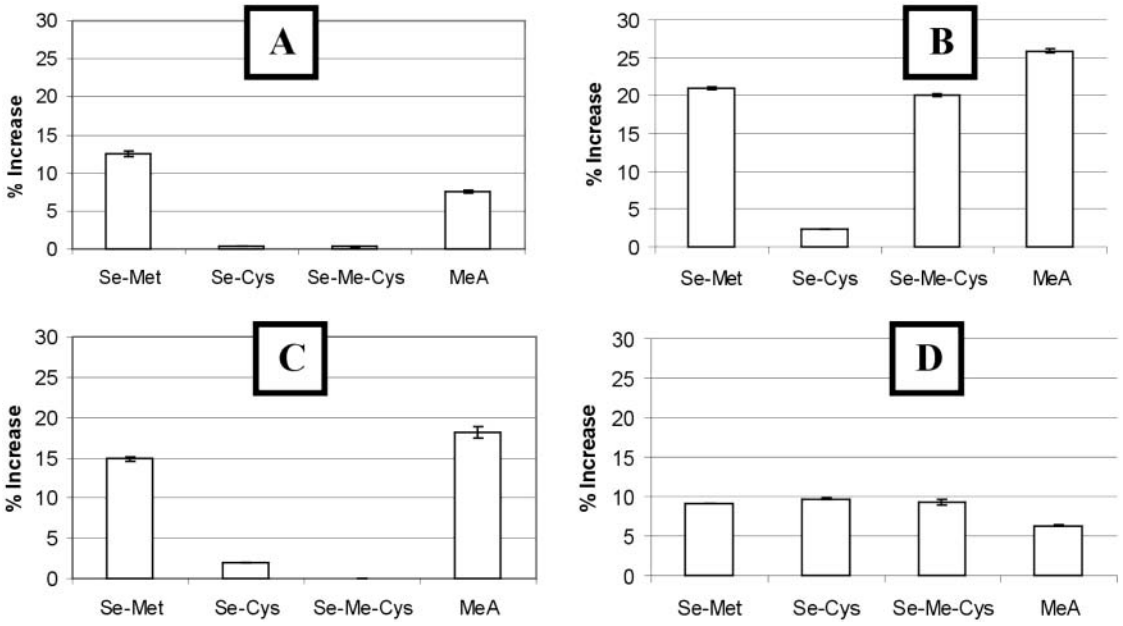

Figure 5. Spiked Se standards increased the total Se in the postextraction solid; the amount of increase was compound- and receiving phase-dependent. (**A**) Phosphate, pH 7; (**B**) nonbuffered; (**C**) glycine/HCl, pH 3; and (**D**) ascorbate, pH 3. Error bars indicate standard deviations, $n = 3$.

Conversely, Se-Met and MeA both exhibited considerable sorption as shown by their increased concentration in the postextraction slurry. Se concentrations increased in the postextraction solids of nonbuffered solutions for samples spiked with Se-Met, MeA, and Se-Me-Cys indicating sorption; however, Se from Se-Cys did not sorb to the solid phase. Extractions with glycine/HCl (pH 3) resulted in volatilization of Se-Me-Cys as shown by both a significant loss from the liquid and negligible increases in the slurry concentration. Se-Met and MeA sorbed to the solids when glycine/HCl was used as the buffer. There was an increase in both the liquid and the solid Se concentrations when Se-Met was added to the extractions using pH 3 glycine/HCl, suggesting that less Se may have been volatilized. These data show that each buffer system should be assessed individually because standards and buffering systems each have their own interactions with the matrix. A detailed description of each particular system is beyond the scope of this study.

Matrix Interactions Determine Trapping Efficiency. Discrepancies in trapping efficiency were compound and buffer specific (**Figure 6**). More Se was trapped on the cartridges than expected in several cases. The most pronounced was Se-Met in a nonbuffered extraction followed by Se-Met in pH 7 phosphate, Se-Cys in nonbuffered water, Se-Cys in pH 3 ascorbate, MeA in pH 7 phosphate, and Se-Me-Cys in pH 7 phosphate and pH 3 ascorbate. The greater than expected amounts of Se trapped on the C-18 cartridges can mean that (i) the matrix reduced the spiked analyte, (ii) the spiked analyte reduced the Se containing components in the matrix, or (iii) the spike reacted with the matrix to reduce the overall polarity of the species in solution, i.e., esterifications. Therefore, an absence of changes in the trapping efficiency does not necessarily mean that the species in solution were the ones found in the matrix. Rather, it indicated that once the analytes entered the bulk extracting solution the average hydrophobicity remained the same. Separation and online specific detection of Se compounds is necessary to

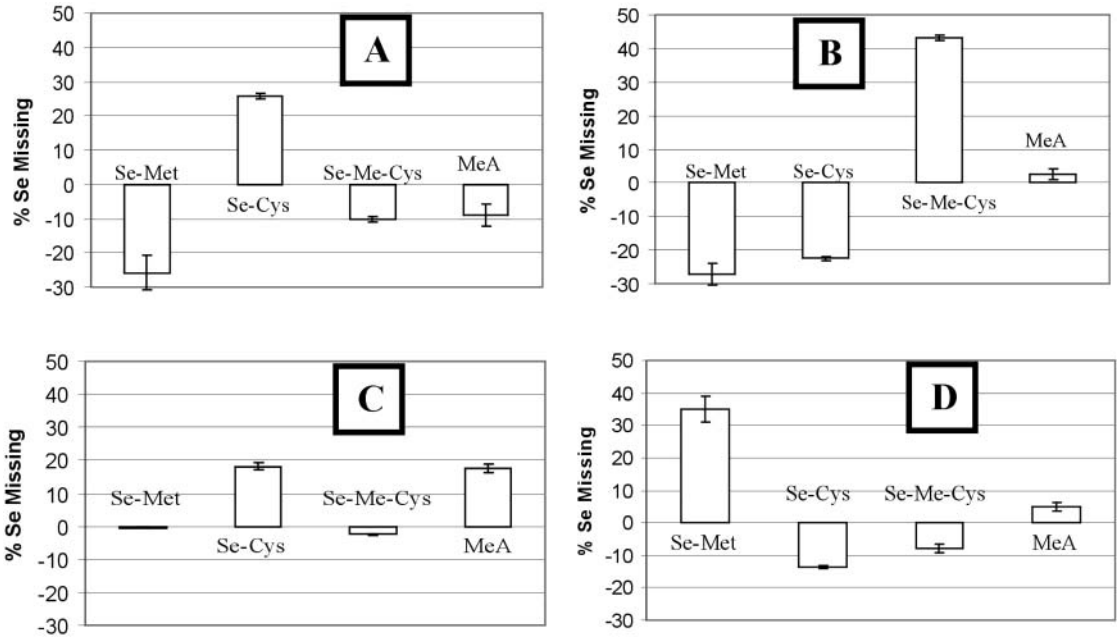

Figure 6. Recovery of spiked Se compounds on C-18 cartridges was compound specific and depended on the receiving phase conditions. (**A**) Phosphate, pH 7; (**B**) nonbuffered; (**C**) glycine/HCl, pH 3; and (**D**) ascorbate, pH 3. Error bars indicate standard deviations, $n = 3$.

determine whether there are changes in the Se compounds being extracted.

Chromatography. *Buffer Type and pH Affect Speciation.* Retention mechanisms in IPC rely on both hydrophobicity and charge. Later eluting compounds are either more hydrophobic or pair more strongly with the ion pairing reagent whereas earlier eluting compounds are more hydrophilic or pair to a lesser extent. Extractions carried out at lower pH generally resulted in more compounds at greater concentrations with longer retention times, indicating less oxidation during the extraction. Therefore, two scenarios can be put forth. (i) Compounds were extracted but some of them reacted with the buffers or liberated matrix components to produce the different compounds observed in the separation. (ii) Different buffers selectively extracted various Se compounds.

At this time, we are unaware of any papers of the selective solubilization of organoselenium compounds by the buffers used in this study. Moreover, mass balance experiments (**Figure 3**) showed that the total Se extraction efficiency did not depend on the buffered extracting phase, only whether a buffering system was present. Thus, Se compounds probably were extracted in a homogeneous manner but reactions took place in the extracting phase and caused the differences in the identities of Se compounds in solution.

Buffer- and pH-dependent influences on Se compounds found in extracted solutions have been observed. Differences between a phosphate buffer used at pH 8 and pH 1 are shown in **Figure 7**. Peak number 1 always contained selenite and selenate; when the extractions were carried out at pH 8, peak number 2 was MeA but MeA was not observed at pH 1. The oxidation of a monomethylated Se compound in the pH 8 extractions was most likely the source for the MeA. This is important because it has been theorized that the active cancer chemopreventive species is a monomethylated Se compound (*6*). Oxidation products of Se-Me-Cys, Se-Cys, or Se-Met all eluted with retention times that corresponded to peak number 3. Peak number 4 was not identified while peak number 5 matched retention times with Se-Met. Peak 6 was identified as Se-Me-Cys. Depending on extraction conditions, a number of unidentified peaks were observed.

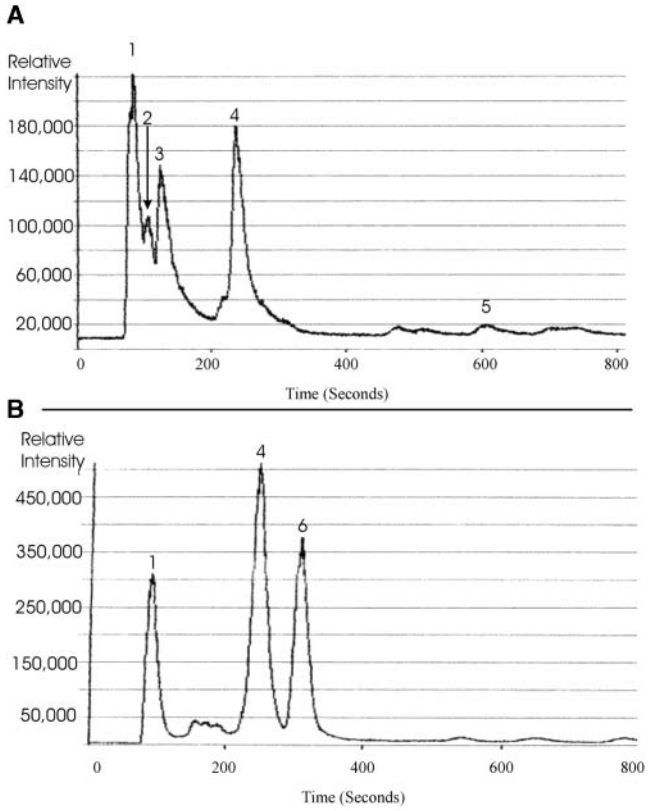

Figure 7. Chromatograms of extractions carried out in 430 mM phosphate buffer at pH 8 (**A**) and pH 1 (**B**). Peak identities are discussed in the text.

Different extracting phases resulted in different chromatograms. The number of peaks and their respective areas had a strong dependence on the buffer used for the extraction. For example, chromatograms from Trizma, phosphate, and citrate buffered extractions at pH 7 are all different (**Figure 8**). Points of difference occurred primarily at retention times corresponding to MeA and the selenonic acids. Solvent conditions controlled the extent of oxidation and other reactions that involved matrix components.

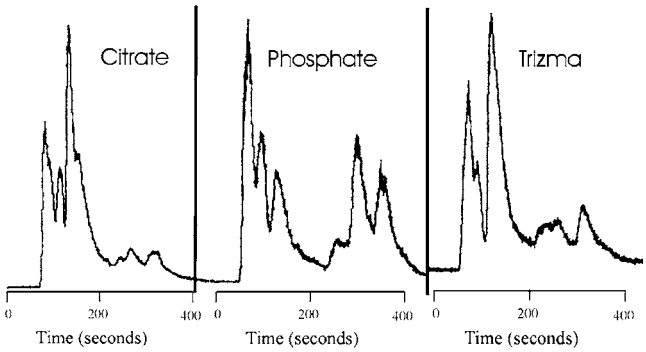

Figure 8. Differences in chromatograms were caused by using different buffers for extracting solutions at pH 7.

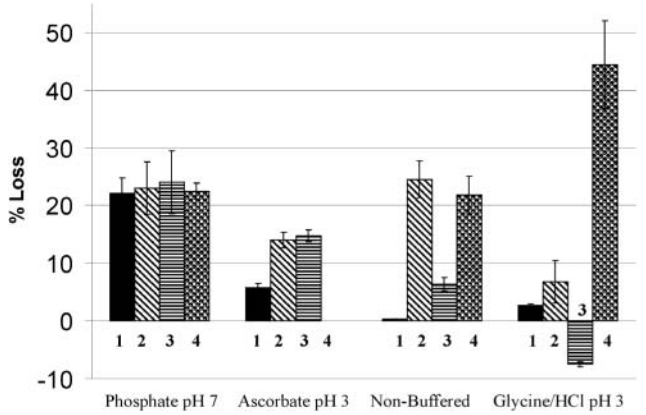

Figure 9. Spiked Se compounds were lost because of interactions with the matrix, not the extracting solution. Expected ICP-MS responses for spiked compounds were lost when separated by IPC. (**1**) Se-Met; (**2**) Se-Cys; (**3**) Se-Me-Cys; and (**4**) MeA. Error bars indicate standard deviations, $n = 3$.

Recovery of Spiked Analytes. To study changes in analyte composition that occur in the buffer during the extraction, spiking studies were carried out (**Figure 9**). MeA in the pH 3 ascorbate buffer is not shown because it was completely volatilized. Extractions carried out in phosphate buffer at pH 7 were the most reproducible. Extracting phases composed of the pH 3 glycine/HCl buffer showed the least sample loss for Se-Met, Se-Cys, and Se-Me-Cys; however, 44% of the MeA was lost. Extractions that were not buffered showed significant sample loss as well.

Retention times of standards did not change when spiked into the buffered extracting phase and run through the extraction. However, standards spiked into the broccoli extracts were often transformed or lost. This indicated that standards are stable in the buffered extracting phases; yet, components present in the broccoli caused pH- and buffer-dependent changes in the composition of the organoselenium compounds or sorbed to the broccoli matrix.

SUMMARY

Accurate speciation is necessary for determining the biochemical role of Se because different forms of Se can give very different biological results. For example, selenite or selenate are readily incorporated into selenoproteins (*34*) whereas Se-Met can substitute randomly for methionine in general body proteins.

Se speciation reported in the literature makes no effort to stabilize the extraction conditions (*10*, *17*−*23*). Changes in extraction conditions can change the species and lead to results that are not indicative of the original sample. Stabilizing conditions minimize the variability that arises from the extraction process. However, buffers used to stabilize pH may lead to changes in the speciation as well. Samples maintained at high pH are generally more oxidized than those at low pH. Overall, a glycine/HCl buffer at pH 3 may be the best buffer to use if the intended speciation information is for studies of Se availability to animals. When determining the species present, it is important that the buffer be matched to the matrix as closely as possible; in the case of broccoli, a pH 7 phosphate buffer would be recommended.

Labile Se compounds exist not only in the laboratory but also in nature as well. The pH of an extracting solution can play a major role in the reproducibility of quantitative and qualitative results. Stabilizing the pH with buffered extracting solutions removes one variable from the analysis. To ensure analyte preservation, methods for the extraction of these compounds must take into consideration the chemical environment of the extracting solution and the matrix. For valid conclusions to be drawn from the analytical data, the extraction conditions should match the conditions present in the matrix or else be specified for a particular application, i.e., a simulated gastrointestinal digestion; extractions carried out under dissimilar conditions may not produce comparable results.

ABBREVIATIONS USED

Se, selenium; IPC, ion pairing chromatography; ICP-MS, inductively coupled plasma mass spectrometer; HPLC, high-pressure liquid chromatography; HFBA, heptafluorobutyric acid; Se-Cys, selenocystine; Se-Me-Cys, Se-methylselenocysteine; Se-Met, selenomethionine; MeA, methylseleninic acid.

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