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Simultaneous Extraction of Arsenic and Selenium Species From Rice Products by Microwave-Assisted Enzymatic Extraction and Analysis by Ion Chromatography-Inductively Coupled Plasma-Mass Spectrometry

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A microwave-assisted enzymatic extraction (MAEE) method was developed for the simultaneous extraction of arsenic (As) and selenium (Se) species in rice products. The total arsenic and selenium content in the enzymatic extracts were determined by inductively coupled plasma mass spectrometry (ICP-MS), while the speciation analysis was performed by ion chromatography coupled to inductively coupled plasma-mass spectrometry (IC-ICP-MS). The main factors affecting the enzymatic extraction process were evaluated in NIST SRM-1568a rice flour. The optimum extraction conditions were 500 mg of sample, 50 mg of protease XIV, and 25 mg of α-amylase in aqueous medium during 40 min at 37 °C. The extraction recoveries of total As and Se reached 100 ± 3 and 80 ± 4%, respectively. The species stability study during the MAEE process did not show transformation of the target species in rice products. The results of As speciation obtained for SRM-1568a were in agreement with previous studies of As speciation performed on the same reference material. The proposed method was applied to the determination of As and Se species in rice and rice-based cereals. Arsenite [As(III)], arsenate [As(V)], dimethylarsinic acid (DMA), and selenomethionine (SeMet) were the predominant species identified in rice products.

KEYWORDS: Arsenic (As); selenium (Se); speciation; microwave-assisted enzymatic extraction (MAEE); rice; rice-based cereal; ion chromatography (IC); inductively coupled plasma-mass spectrometry (ICP-MS)

INTRODUCTION

Rice (*Oryza sativa*) in its grain and flour forms constitutes a staple food in many countries owing its nutritional value. Among other nutrients, rice serves as an important source of mineral elements (*1, 2*). Hence, it is necessary to determine the content of trace elements and heavy metals as well as their species existing in rice for evaluating their nutritional value.

Rice is particularly susceptible to arsenic (As) accumulation compared with other cereals. Arsenic is a highly toxic element and a number of studies have reported its presence in rice in concentration ranging from 10 to 2050 μg As/kg (*3*). It has been estimated that inorganic As species [As(III) and As(V)] can vary from 10 to 90% of the total As in rice with the remainder as dimethylarsinic acid (DMA) (*2*). Inorganic species of As have been linked to an increased risk of cancer, while methylated forms such as monomethylarsonic acid (MMA) and DMA are significantly less toxic than the inorganic forms (*4*). The Food and Agriculture Organization (FAO) branch of the World Health Organization (WHO) has recommended a provisional tolerable weekly intake (PTWI) of not more than 15 μg of inorganic As/kg body weight (*5*). On the other hand, rice is one of the major sources of selenium (Se) in most diets (*6*). Se is an essential micronutrient for humans in a very narrow concentration range; outside of this range deficiency or toxicity occurs. The recommended dietary allowance (RDA) is 55 mg of Se/day. The toxicity of Se again depends to a large extent on its chemical forms, with inorganic Se compounds being more toxic than organoselenium compounds (*6, 7*). The predominant form of Se in rice is selenomethionine (SeMet) (68−81%). Selenite [Se(IV)], selenate [Se(VI)], and selenocysteine (SeCys) compounds exist in smaller proportions (*7*). Several studies have revealed toxicologically relevant antagonistic interactions between Se and heavy metals such as Hg, Pb, and As (*8*). Se has been shown to reduce oxidative damage caused by As toxicity (*9*). Therefore, there is an increasing demand to develop analytical methods which enable the simultaneous determination of different species of As and Se in rice products. Speciation

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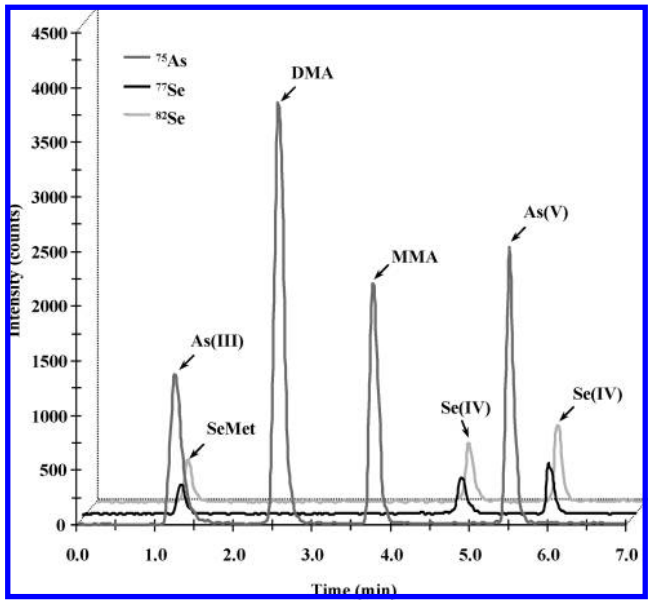

Figure 1. A chromatogram of a mixed standard solution of As and Se species containing 10 μg/L (as element) of DMA, Se(IV), Se(VI), and SeMet and 5 μg/L (as As) of As(III), As(V), and MMA. The chromatographic conditions are listed in **Table 1**.

studies are mandatory to provide detailed understanding of the nutritional significance of As and Se in various foodstuffs. There are few studies of elemental speciation in cereals that represent the main constituent of the diet, mostly pertaining to As speciation in rice (*5, 10*−*12*) and cereals (*13*) and Se speciation in cereals (*8*), wheat flour (*14*), and selenized-rice (*15*); however, to the authors' knowledge, there is no reported work for the simultaneous speciation of As and Se in rice products.

A number of techniques have been developed for As and/or Se speciation in biological materials, and the coupling of liquid chromatography (LC) with inductively coupled plasma mass spectrometry (ICP-MS) is nowadays the most commonly used technique for the determination of As and/or Se speciation in biological samples, for its advantage of high sensitivity, wide linearity, low detection limit, and the multielement capability (*4, 16*). Among the various LC techniques, ion chromatography (IC) has frequently been used.

Speciation analysis usually requires the extraction of the species of interest into the solvent before they can be measured and identified. Reliable and practical extraction methods for the determination of As and Se species in complex biological matrices are of considerable interest. Treatment of rice samples with protease XIV and α-amylase (*5*) and water−methanol mixture (*17*) have been commonly reported in the literature for the extraction of As species. Other extraction solvents such as trifluoroacetic acid (TFA) have been used for the extraction of As species from rice and infant food products (*10, 18*); however, TFA has led to the partial reduction of As(V) to As(III), making the method unable to distinguish between As(III) and As(V) species. Conversely, acid hydrolysis (*19*) and enzymatic extraction using protease XIV (*14*) and pronase E (*8*) have been applied to Se speciation studies in cereals and wheat flour samples. Among the procedures reported in the literature for the extraction of As or Se, enzymatic extraction is of interest due to the usage of moderate conditions of temperature and pH which prevents elemental losses by volatilization and minimizes organometallic species degradation; however, the main drawback of enzymatic hydrolysis is the large time required to complete the hydrolysis process. Extraction times of 6−24 h have typically been reported in the literature (*8, 14*); therefore, the development of new methods to accelerate the enzymatic extraction procedures, guaranteeing quantitative releases and species stability, are mandatory. Microwave-assisted extraction (MAE) using a closed vessel system allows the automation of the extraction process with significant reduction in the extraction time, diminution of the solvent consumption, and simplicity and increase of sample throughput. Despite the relatively large number of microwave-assisted extraction methods for speciation analysis reported in recent years (*20*), the application of microwave extraction to the acceleration of enzyme hydrolysis procedures in speciation analysis is scarce. The use of a focused microwave oven for Se speciation in selenized yeast was recently reported by Peachey et al. (*21*).

The main objective of this study was to establish and optimize an extraction method for the simultaneous extraction of As and Se species from rice products prior to their analysis by ion chromatography-inductively coupled plasma mass spectrometry (IC-ICP-MS). After a comparison of several common extraction

Table 1. IC and ICP-MS Operating Conditions

Operational Conditions of HP-4500 ICP-MS	
RF power	1475 W
plasma gas flow	Ar, 15.1 L/min
auxiliary gas flow	Ar, 1 L/min
sample and skimmer cones	Ni, 1.1 and 0.8 mm, respectively
Measurement Parameters of HP-4500 ICP-MS	
monitoring isotopes	$^{75}As^{a,b}$ $^{77}Se^{a,b}$ and $^{82}Se^{a,b}$
acquisition mode	spectrum[a] and time resolved analysis[b]
integration time per mass, s	0.30[a] and 0.20[b]
replicates	5[a] and 1[b]
total analysis time, s	34.45[a] and 718[b]
IC Conditions	
column	Metrosep Anion Dual 3 column, 100 × 4.0 mm, 6 μm and Metrosep Anion Dual 3 guard column, 1.7 × 3.5 mm, 0.2 μm (Metrohm Peak, LLC.)
column temperature	ambient
injection volume	100 μL
flow rate	1 mL/min
mobile phase	A: 5 mM NH_4NO_3, and B: 50 mM NH_4NO_3, 2% (v/v) methanol, pH 8.2
gradient program	(1) 0 min, 0% B, (2) 2 min, 0% B, (3) 7 min, 100% B, (4) 9 min, 100% B, (5) 9.5 min, 0% B and (6) 12 min, 0% B

[a] For total As and Se analysis. [b] For As and Se speciation analysis.

Table 2. Total As and Se Concentration Found in Rice Flour SRM-1568a and Rice Products by ICP-MS after Microwave Digestion Using EPA Method 3052

sample	As concentration (μg/kg)[b]	Se concentration (μg/kg)[b]
SRM 1568a Rice flour (NIST, USA)[a]	290 ± 9	381 ± 5
basmati rice (India)	275 ± 6	673 ± 16
jasmine rice (Thailand)	140 ± 6	68 ± 6
white rice (Texas, USA)	320 ± 10	142 ± 8
rice-based cereal (Italy)	228 ± 7	62 ± 7
rice-based cereal (USA)	301 ± 9	322 ± 13
rice-based cereal (Canada)	259 ± 4	441 ± 18

[a] Certified values of total As and Se in SRM-1568a rice flour are 290 ± 30 and 380 ± 40 μg/kg, respectively. [b] The values are the means of three determinations ± 95% CL.

Table 3. Comparison of the Extraction Efficiency of Different Extraction Methods on Total As and Se from SRM-1568a Rice Flour ($n = 3$)[a,b]

extraction medium	extraction conditions	As concentration, μg/kg	Se concentration, μg/kg	reference
50% (v/v) methanol in water	500 mg sample and 10 mL of 50% (v/v) methanol in water, 2 h extraction at room temperature in an ultrasonic bath	253 ± 7 (87 ± 2)	28 ± 7 (7.3 ± 0.5)	*24*
2 M TFA	500 mg sample and 5 mL of 2 M TFA, 6 h extraction at 100 °C in a water bath	289 ± 4 (100 ± 2)	268 ± 4 (70 ± 1)	*11, 18*
protease XIV and α-amylase	500 mg sample, 10 mg α-amylase and 5 mL of H_2O. Ultrasound-assisted extraction with ultrasonic probe for 1 min. Addition of 50 mg protease XIV to the mixture. Extraction for 2 min.	240 ± 6 (82 ± 3)	145 ± 4 (38 ± 4)	*5*
protease XIV and α-amylase	500 mg sample, 10 mg α-amylase, 50 mg protease XIV and 10 mL of H_2O. Microwave-assisted extraction at 37 °C for 30 min.	278 ± 8 (96 ± 3)	261 ± 9 (69 ± 5)	this study

[a] Extraction recovery as ratio of total content in the extract to certified value in SRM-1568a reference material. [b] Certified values of total As and Se in SRM 1568a rice flour are 290 ± 30 and 380 ± 40 μg/kg, respectively.

procedures used for As or Se in rice and cereal samples, a rapid and reliable extraction method using microwave energy was developed and optimized. The method was based on the application of a mixture of protease XIV and α-amylase. The procedure was validated by the analysis of SRM-1568a rice flour reference material and then applied to the speciation of As and Se in rice products purchased from local markets.

MATERIALS AND METHODS

Reagents and Standards. The double-deionized water (DDI water, resistivity > 18 MΩ cm) from Barnstead NANOpure Water System (Dubuque, IA) was used for the preparation of aqueous solutions and dilutions. Analytical reagent grade nitric acid and optima grade methanol were purchased from (Fisher Scientific, Pittsburgh, PA). Ammonium hydroxide solution (20−22% NH_3) and ammonium nitrate (Sigma-Aldrich St. Louis, MO) were used for the IC eluent. The prepared mobile phase was filtered through a MF-Millipore filter (0.45 μm) and degassed in an ultrasonic bath for 15 min. Hydrogen peroxide 35% (w/v) in water and ammonium phosphate dibasic were purchased from Acros Organics (Bridgewater, NJ). Ammonium phosphate monobasic, protease type XIV from *Streptomyces griseus* and α-amylase from *Bacillus subtilis* were obtained from Sigma-Aldrich (St. Louis, MO).

A 500 mg/L stock solution, expressed as metal, of MMA and DMA, were prepared by dissolving adequate amounts of $CH_3AsNa_2O_3 \cdot 6H_2O$ (MMA) from (ChemService,West Chester, PA) and $C_2H_6AsNaO_2 \cdot 3H_2O$ (DMA), from Sigma-Aldrich (St Louis, MO), respectively. A 1000 mg/L stock solution of As(III) in 2% HCl and As(V) in H_2O were purchased from SPEX CertiPrep (Metuchen, NJ).

A 1000 mg/L stock solution, expressed as metal, of Se(IV) and Se(VI), were prepared by dissolving adequate amounts of Na_2SeO_3 [Se(IV)] and Na_2SeO_4 [Se(VI)] from Sigma-Aldrich (St Louis, MO). A 500 mg/L stock solution of SeMet was prepared by dissolving adequate amounts of $C_5H_{11}NO_2Se$ (SeMet) from Sigma-Aldrich (St Louis, MO). All stock solutions were kept at 4 °C and stored in high-density polyethylene (HDPE) bottles until use and the working solutions were prepared daily.

Samples. For method validation, the NIST SRM-1568a rice flour from National Institute of Standards and Technology (NIST, Gaithersburg, MD) was analyzed. The applicability of the method to real samples was demonstrated by the analyses of two different rice products: rice and rice-based cereals. The rice (basmati from India, jasmine from Thailand, and white rice from Texas, USA) and rice-based baby cereals (Italy, USA, and Canada) were purchased from local markets. The samples were grounded, sieved through a 250 μm mesh, homogenized, and stored in high-density polyethylene bottles at room temperature until analysis.

Instrumentation. A microwave labstation (Ethos 1, Milestone Srl, Italy) was used for total digestion and extraction of the samples. Perfluoroalkoxy Teflon (PFA) vessels of 100 mL internal volume equipped with a Milestone temperature control system (model ATC-400CE) were employed under temperature adjusted conditions. The microwave system provides stirring capability in individual vessels for homogenization of the digest/extract during the MAE digestion/extraction procedure.

The chromatography system (Metrohm-Peak, LLC, Houston, TX) consisted of two 818 IC pumps, a 762 IC interface, and an 838 IC autosampler. The separation center enclosure included a 6-port injection valve with a 100 μL sample loop. The separation was performed on an anion exchange column (Metrosep Anion Dual 3, Metrohm-Peak, LLC, Houston, TX) equipped with a guard column (Metrosep Anion Dual 3, Metrohm-Peak, LLC, Houston, TX). The outlet of the chromatographic column was directly connected to the concentric nebulizer of the ICP-MS with a small piece of perfluoroalkoxy (PFA) tubing. The determination of As and Se was carried out with a HP-4500 ICP-MS from Agilent Technologies (Palo Alto, CA, and Yokogawa Analytical Systems Inc., Tokyo, Japan) equipped with a concentric nebulizer. The settings of ion lens system, gas flow rates, and other parameters were tuned daily to obtain maximum sensitivity by using 10 μg/L tuning solution (Agilent Technologies, Palo Alto, CA) containing Li, Y, and Tl in 2% (v/v) HNO_3 solution. The ion intensity at m/z 75 was used to monitor As and the signal at m/z 77 and 82 were selected to monitor Se. Data were collected using *Spectrum* mode for direct analysis and *Time Resolved Analysis (TRA)* mode for speciation analysis. The chromatographic conditions and the instrumental parameters used for IC and ICP-MS are summarized in the **Table 1**.

A FAM-40 vacuum unit (Milestone, Sorisole, Italy) was used to filter the extracts. Centrifugation was carried out to separate the supernatant from the sample extracts by a Fisher Scientific Co. centrifuge model 225 (Pittsburgh, PA).

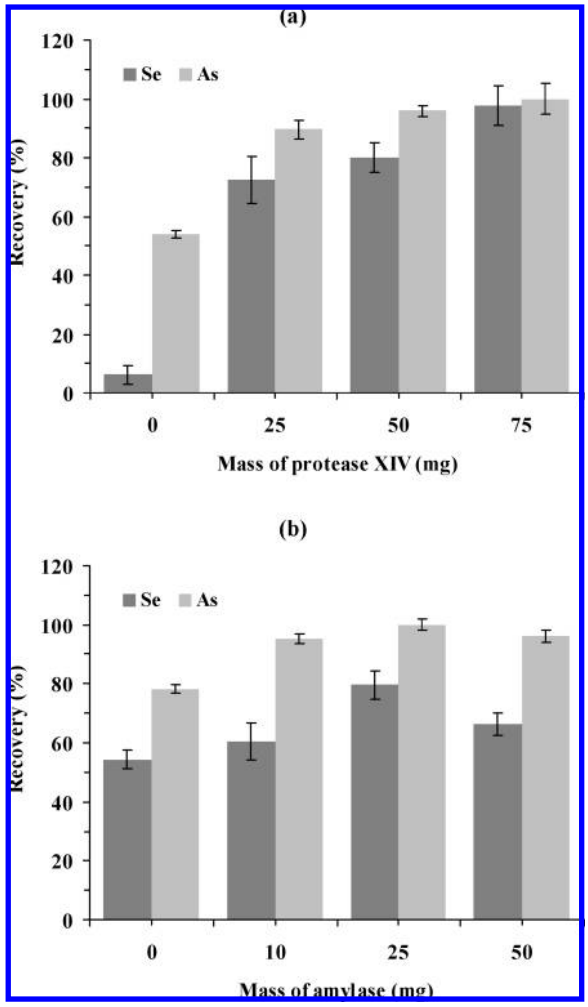

Figure 2. Optimization of sample to enzyme ratio: (a) protease XIV and (b) α-amylase. Conditions: 500 mg of SRM-1568a rice flour, 10 mL of DDI water at 37 °C ($n = 3$). In (a), the amount of α-amylase is 10 mg and in (b), the amount of protease XIV is 50 mg.

Microwave Digestion Method. Digestion of the samples was carried out in a microwave oven using U.S. EPA method 3052 (*22*). Briefly, approximately 500 mg of representative sample was weighed into microwave vessels, and 9 mL of concentrated HNO_3 and 0.5 mL of H_2O_2 as well as a magnetic stir bar were added to each vessel. Vessels were sealed and microwave irradiated at 180 ± 5 °C for 10 min. After digestion, the sample aliquots were filtered using a 1.0 μm Millipore glass-fiber filter, diluted to 25 mL with DDI water, and stored in a cold location at 4 °C until analysis. For analysis, three subsamples and blanks were prepared in parallel.

Microwave-Assisted Enzymatic Extraction Procedure. Approximately 500 mg of sample (SRM-1568a, rice or rice-based cereal) was accurately weighed into a PTFE vessel along with 25 mg of α-amylase and 50 mg of protease XIV, and 10 mL of double deionized water and a magnetic stir bar were then added. The vessels were capped and placed in the rotor of a microwave extraction oven, and the operating program of the system was implemented as follows: the temperature was raised from room temperature to 37 °C over 5 min, then held at 37 °C for 40 min. After each extraction program, the samples were allowed to cool to room temperature and centrifuged for 10 min at 4000 rpm. Finally, the supernatant was filtered (0.45 μm Millipore glass-fiber filter) and diluted to 20 mL with DDI water. This procedure was performed in triplicate. Blank tests were performed in parallel. Extracts were analyzed for total As and Se content using ICP-MS and for speciated As and Se using IC-ICP-MS.

Stability of As and Se Species during the Extraction Procedure. As and Se species stability during microwave assisted enzymatic extraction (MAEE) was assessed using the extraction reagent spiked with individual As and Se species (As(III), As(V), DMA, MMA, Se(IV), Se(VI), and SeMet) at a concentration of 5 μg/L ($n = 3$). Finally the spiked standards were analyzed by IC-ICP-MS in the same way as the samples to verify their stabilities during the extraction procedure.

Total As and Se Determination. The total As and Se concentration in both the original samples and the extract solution ($n = 3$), were all determined by ICP-MS. The extraction/digestion solutions were 5-fold diluted with DDI water before the analysis. The total As and Se determination was verified by the analysis of the certified reference material SRM-1568a. The operating parameters used are given in **Table 1**. Quantification was performed by external calibration using matrix-matching.

Determination of As and Se Species. The online coupling system of IC-ICP-MS was applied to analyze As and Se species in the extracts. The rice extracts were injected without further dilution. The As and Se species were separated in an anion exchange column (Metrosep Anion Dual 3). The chromatographic conditions are given in **Table 1**. A chromatogram of a mixed standard solution of As and Se species is shown in **Figure 1**. Data processing was performed using the PlasmaChem software supplied with the HP-4500 instrument, and quantification was performed based on peak areas by external calibration.

RESULTS AND DISCUSSION

Determination of Total As and Se in Rice Products by ICP-MS. Total As and Se concentrations in SRM-1568a rice flour, rice samples, and rice-based cereals were determined after microwave acid digestion (EPA Method 3052) by ICP-MS under conditions given in **Table 1**. The results are shown in **Table 2** ($n = 3$). Correction for any interference from argon chloride on As at *m*/*z* 75 was not found to be necessary for the samples analyzed. This assumption was supported by data obtained in the analysis of the certified reference material. For Se quantification, the ^{82}Se monitoring ion signal was used. Similar results were obtained for the ^{77}Se monitoring ion signal. The results for As and Se in SRM-1568a certified reference material using the proposed digestion procedure were in good agreement with the certified values at 95% CL. The As content in different rice and rice-based cereals tested during this study varied within the range from 140 ± 6 to 320 ± 10 μg/kg. Those results were in agreement with a recent survey of As in rice grain (*3*). On the other hand, total Se concentration in rice and rice-based cereal products ranged from 62 ± 7 to 673 ± 16 μg/kg. Cereals and cereal products contain a wide range of Se concentrations, most being between 10 and 550 μg/kg on a fresh weight basis (*23*).

Chromatographic Separation. In order to develop a rapid chromatographic separation of As and Se species in rice products, an anion-exchange column (Metrosep Anion Dual 3) was chosen in combination with a gradient elution profile. Several mobile phases were tested, and the best separation of As(III), As(V), MMA, DMA, Se(IV), Se(VI), and SeMet was achieved within 12 min (including 3 min for equilibration of the column) using NH_4NO_3 with concentrations ranging from 5 to 50 mM at pH 8.2. The addition of 2% (v/v) methanol to the mobile phase resulted in a significant improvement in the sensitivity of As and Se species. A summary of the optimum IC operating conditions is listed in **Table 1**. A typical chromatogram of a solution containing 10 μg/L (as element) for DMA, Se(IV), Se(VI), and SeMet and 5 μg/L (as As) for As(III), As(V), and MMA is shown in **Figure 1**.

The limits of detection (LOD) were evaluated for each of the As and Se species. LOD under optimum conditions, calculated as 3σ of the baseline noise based on peak height, were 0.09 ± 0.02 μg/L for As(III), 0.06 ± 0.02 μg/L for DMA, 0.04 ± 0.01 μg/L for MMA, 0.04 ± 0.01 μg/L for As(V), 1.1 ± 0.1 μg/L for SeMet, 0.57 ± 0.03 μg/L for Se(IV), and 0.63

Table 4. Effects of Extraction Medium and Microwave Irradiation Time on As and Se Extraction Recovery from SRM-1568a Rice Flour[a]

extraction medium (at 37 °C for 40 min)			extraction time (DI water at 37 °C)		
extraction medium	As	Se	time (min)	As	Se
DI water (pH 6.85)	92 ± 1	64 ± 9	10	96 ± 2	50 ± 5
50 mM NH_4PO_3 buffer (pH 7.00)	100 ± 2	80 ± 7	20	96 ± 3	61 ± 6
50 mM NH_4PO_3 buffer (pH 7.25)	97 ± 2	81 ± 4	30	97 ± 2	66 ± 9
50 mM NH_4PO_3 buffer (pH 7.50)	85 ± 3	60 ± 9	40	100 ± 3	79 ± 6
			60	96 ± 2	68 ± 6

[a] Uncertainties are expressed at 95% CL, $n = 3$. The results are expressed in percent recovery.

Table 5. Total As and As Species with Microwave-Assisted Enzymatic Extraction from Rice Products and SRM-1568a Rice Flour[a]

	concentration found (μg/kg as As)						
As compounds	SRM-1568a rice flour	basmati rice (India)	jasmine rice (Thailand)	white rice (Texas, USA)	rice-based cereal (Italy)	rice-based cereal (USA)	rice-based cereal (Canada)
total As	291 ± 2 (100 ± 3)[b]	204 ± 5 (74 ± 4)[c]	137 ± 3 (98 ± 3)[c]	255 ± 6 (80 ± 6)[c]	217 ± 3 (95 ± 5)[c]	267 ± 4 (89 ± 4)[c]	235 ± 4 (91 ± 4)[c]
As(III)	55 ± 6	105 ± 4	86 ± 4	161 ± 5	119 ± 4	115 ± 2	83 ± 3
DMA	166 ± 6	43 ± 2	18 ± 1	48 ± 3	69 ± 2	88 ± 5	73 ± 2
MMA	10 ± 2	n.d.[e]	n.d.[e]	n.d.[e]	< LOD[f]	< LOD[f]	< LOD[f]
As(V)	41 ± 3	37 ± 3	25 ± 1	8 ± 4	14 ± 9	40 ± 6	65 ± 6
sum of As species	272 ± 4 (93 ± 3)[d]	185 ± 3 (91 ± 3)[d]	129 ± 2 (94 ± 3)[d]	217 ± 4 (85 ± 5)[d]	202 ± 4 (93 ± 5)[d]	243 ± 3 (91 ± 5)[d]	221 ± 3 (94 ± 4)[d]

[a] The values are the means of three determinations ± 95% CL. [b] In parentheses, the extraction recovery based on total As certified values. [c] In parentheses, the extraction recovery based on the total As determined by microwave-assisted total digestion (EPA Method 3052). [d] In parentheses, total As speciation recovery based on total As extracted. [e] Not detected. [f] Method detection limits for As(III), DMA, MMA, and As(V) in rice products are 4 ± 1, 2.2 ± 0.5, 1.8 ± 0.4 and 1.4 ± 0.3 μg/kg, respectively.

± 0.03 μg/L for Se(VI). The calibration curves based on peak areas were linear for As and Se species in the range from 0.5 to 10 μg/L and from 1 to 20 μg/L, respectively. The percent RSDs of seven replicates were calculated. At the 5 μg/L concentration level of As and Se species, the percent RSDs were less than 5% for all the species investigated.

Selection of the Extraction Method for As and Se Determination. Several procedures for extracting As or Se from rice were evaluated in the SRM-1568a. The purpose of this study was to obtain the maximum extraction yield for both As and Se while preserving the original species signature present during the extraction procedure. The extraction methods that were evaluated includes solvent extraction using 50% (v/v) methanol in water with sonication (*24*), acid extraction using 2 M TFA (*10*, *18*), enzymatic extraction using ultrasonic-assisted extraction with ultrasound-probe (*5*), and a modification of the previous extraction procedure using closed-vessel MAE as an alternative to ultrasonic-assisted extraction. In each case, extracts were analyzed for total As and Se by using ICP-MS, and then speciation analysis was carried out by IC-ICP-MS. The extraction efficiencies of As and Se were defined as the percent recovery of the corresponding certified values. The obtained results are summarized in **Table 3**.

Among the four extraction procedures used during this evaluation, 50% (v/v) methanol in water and enzymatic extraction with ultrasonic probe using protease XIV and α-amylase were inefficient and ineffective for the simultaneous extraction of As and Se in rice samples. Sonication with 50% (v/v) methanol in water and a mixture of protease XIV and α-amylase showed similar As recoveries in SRM-1568a reference material. The observed extraction recoveries for As were 87 ± 2 and 82 ± 3%, respectively. The results obtained for As recovery using a water/methanol mixture were comparable to those obtained by Heitkemper et al. (*10*), whereas the results by enzymatic extraction using ultrasonic probe were lower than the reported value by the original method (*5*). The extraction efficiency of As in SRM-1568a reported by Sanz et al. was 99 ± 1%. For Se, the extraction recoveries were 7.3 ± 0.5% using 50% (v/v) methanol in water procedure and 38 ± 4% using enzymatic extraction with ultrasonic probe, respectively. Enzymatic extraction using protease XIV lead to improve Se extraction efficiency from SRM-1568a rice flour compared with water/methanol mixture extraction; however, the results obtained were lower than those reported in cereals (*8*) and wheat flour (*14*), where a conventional extraction procedure using proteolytic enzymes was used. The quantitative extraction recoveries of total As using the TFA extraction procedure or MAEE using the mixture of protease XIV and α-amylase has also been shown in **Table 3**. The extraction recoveries of As were 100 ± 2 and 96 ± 3%, respectively, for each procedure. Although quantitative results of As extraction recovery were obtained using TFA, transformation of As(V) to As(III) was detected during IC-ICP-MS analysis and allowed only to quantify the inorganic As species [As(III) + As(V)]. Similar results were reported by Heitkemper et al. (*10*). On the other hand, the attained extraction recoveries for Se were up to 70 ± 1% using 2 M TFA and 69 ± 5% for MAEE using protease XIV and α-amylase. On the basis of these results, MAEE in combination with enzymatic treatment was suitable for the simultaneous extraction of As and Se species from rice products. A comparison between MAEE and ultrasonic-assisted extraction showed that the former method was a more effective method for extracting simultaneously As and Se from rice. Additionally, interconversion of As species were not detected by MAEE compared with TFA extraction procedure.

Optimization of Extraction Conditions for MAE. Simultaneous extraction of As and Se species from rice using MAEE was demonstrated in the previous section. MAEE is based on the use of two enzymes: α-amylase and protease XIV. While α-amylase hydrolyzes starch, the major rice component (up to 78%), and assists in the increase in solubility of proteins, protease hydrolyzes those proteins (7−8% in rice) to peptides and amino acids (*25*−*27*). For the MAEE, the following parameters were investigated: sample to enzyme ratio, extraction medium, and irradiation time. The reference material, SRM-

Table 6. Total Se and Se Species with Microwave-Assisted Enzymatic Extraction from Rice Products and SRM-1568a[a]

	concentration found (μg/kg as Se)						
Se compounds	SRM-1568a rice flour	basmati rice (India)	jasmine rice (Thailand)	white rice (Texas, USA)	rice-based cereal (Italy)	rice-based cereal (USA)	rice-based cereal (Canada)
total Se	304 ± 6 (80 ± 4)[b]	543 ± 7 (81 ± 6)[c]	48 ± 8 (70 ± 7)[c]	124 ± 8 (88 ± 8)[c]	51 ± 9 (82 ± 8)[c]	291 ± 10 (90 ± 9)[c]	390 ± 11 (88 ± 10)[c]
SeMet	303 ± 9	507 ± 7	46 ± 6	116 ± 7	46 ± 8	259 ± 7	341 ± 12
Se(IV)	n.d.[e]	n.d.[e]	n.d.[e]	n.d.[e]	n.d.[e]	n.d.[e]	n.d.[e]
Se(VI)	<LOD[f]	n.d.[e]	<LOD[f]	<LOD[f]	n.d.[e]	<LOD[f]	<LOD[f]
sum of Se species	303 ± 9 (100 ± 9)	507 ± 7 (93 ± 6)	46 ± 6 (96 ± 8)	116 ± 7 (93 ± 7)	46 ± 8 (90 ± 7)	259 ± 7 (88 ± 8)	341 ± 12 (87 ± 11)

[a] The values are the means of three determinations ± 95% CL. [b] In parentheses, the extraction recovery based on total Se certified values. [c] In parentheses, the extraction recovery based on the total Se determined by microwave-assisted total digestion (EPA Method 3052). [d] In parentheses, total Se speciation recovery based on total Se extracted. [e] Not detected. [f] Method detection limits for SeMet, Se(IV) and Se(VI) in rice products are 41 ± 2, 23 ± 1 and 25 ± 1 μg/kg, respectively.

1568a rice flour, was used throughout this study. The standard temperature of 37 °C was selected for the extraction of As and Se in rice products. These conditions were based on information provided by the enzyme supplier. The minimum amount of solvent recommended by the manufacturer of the microwave system was used in this study (10 mL). Extraction efficiency was calculated by comparison with the total certified values of As and Se in the sample.

(a) Influence of the Amount of Enzyme Used. Different amounts of protease XIV (0, 25, 50, and 75 mg) and α-amylase (0, 10, 25, and 50 mg) were separately tested in order to evaluate the effect of the amount of enzyme on the extraction of As and Se from SRM-1568a. The results are shown in **Figure 2**. The extraction conditions were 500 mg of sample, 37 °C extraction temperature, 30 min irradiation time, and 10 mL of DDI water as the extracting medium. Procedural blanks were used as a control. After analysis of the blank, it was observed that trace amounts of As(V) were detectable in thet protease XIV reagent. Similar results were reported by Camara et al. (*5*). The blank levels of As were subtracted from the sample results to obtain correct results corresponding to each sample. In case of Se, the two enzymes used for the extraction did not contain detectable amounts of Se. However, a careful control of Se levels in commercially available enzymes was recommended in a recent study carried out by Cuderman et al. (*28*) where the presence of Se was detected in common enzymes used for Se speciation analysis. It was evident from **Figure 2a** that concentrations of As and Se increased significantly as the mass of protease XIV increased from 0 to 75 mg, indicating that those compounds are associated with the protein structure. For As, no statistically significant difference in the extraction recovery of As was noted between 50 and 75 mg of protease XIV. The extraction recoveries were 96 ± 2 and 100 ± 5, respectively. On the other hand, the extraction recoveries of Se for 50 and 75 mg of protease XIV were 80 ± 5 and 98 ± 7%, respectively. The use of 50 mg of protease XIV for 500 mg of subsample (protease XIV to sample ratio of 1:10) was selected for the final quantitation of As and Se in rice products. This value was chosen as a compromise between satisfactory extraction recovery of both As and Se in the rice reference material and the reduction of the levels of As(V) in the procedural blank as well as the diminution of the experiment cost.

The extraction recoveries of As and Se using different masses of α-amylase are shown in **Figure 2b**. As can be seen from **Figure 2b** that the extraction recovery of As and Se was enhanced with an increase of the amount of α-amylase from 0 to 25 mg (in all the cases the amount of protease XIV was 50 mg). For As, the extraction recovery remained constant when the amount of α-amylase was from 10 to 50 mg. The extraction recovery of As was 96 ± 2%. For Se, the maximum extraction efficiency was observed when the procedure was performed using 25 mg of α-amylase. The extraction recovery was 80 ± 5%. Therefore, the optimum amount of α-amylase was chosen to be 25 mg and was used in all the further studies with MAEE.

(b) Extraction Medium. DDI water or 50 mM phosphate buffer (pH 7.00, 7.25, or 7.50) were used as an extraction medium in order to optimize the sample preparation for the 500 mg of sample. The results of the extraction recoveries of As and Se in SRM-1568a rice flour are reported in **Table 4**. An increase in the extraction efficiency of As using 50 mM phosphate buffer at pH 7.00 was obtained in comparison to those using DDI water (without pH control). The extraction recoveries of As were 100 ± 2 and 92 ± 1%, respectively. Similar results in the extraction recovery of Se were obtained using 50 mM phosphate buffer at pH 7.00 and DDI water extraction medium. The extraction recoveries of Se were 80 ± 7 and 64 ± 9%, respectively. Although the simultaneous extraction of As and Se from SRM-1568a was enhanced using 50 mM phosphate buffer at pH 7.00, the distribution of As species in the SRM-1568a extract changed compared to reported As speciation data in this reference material (*5*, *17*, *29*, *30*). Transformation of DMA to As(III) was detected during IC-ICP-MS analysis; thus, DDI water was used as the extraction medium during MAEE.

(c) Extraction Time. It has been shown that extraction time is one of the crucial parameters for an enzymatic extraction procedure. Using a microwave oven, the effect of irradiation time on the extraction efficiency of As and Se was evaluated from 10 to 60 min using the following conditions: 500 mg of SRM-1568a, 25 mg of α-amylase, 50 mg of protease XIV, 37 °C and 10 mL of DDI water. As can be seen from **Table 4**, the total As content in the extracts determined by ICP-MS indicated no statistically significant difference in extraction efficiency when the extraction time was increased from 10 to 60 min. The extraction recovery was the greatest, 100 ± 3%, at 40 min extraction time. In the case of Se, a slight increase in the recovery was observed with an increase in extraction time; however, longer extraction times did not significantly increase the extraction yield. The extraction recovery of Se was 79 ± 6% at 40 min. It was, therefore, decided to use this extraction time as the optimum time for the MAEE procedure. The optimum conditions for the simultaneous extraction of As and Se species from rice using MAEE were selected as follows: 500 mg of sample, 25 mg of α-amylase, 50 mg of protease XIV along with 10 mL of DDI water and then heated in a microwave system at 37 °C for 40 min.

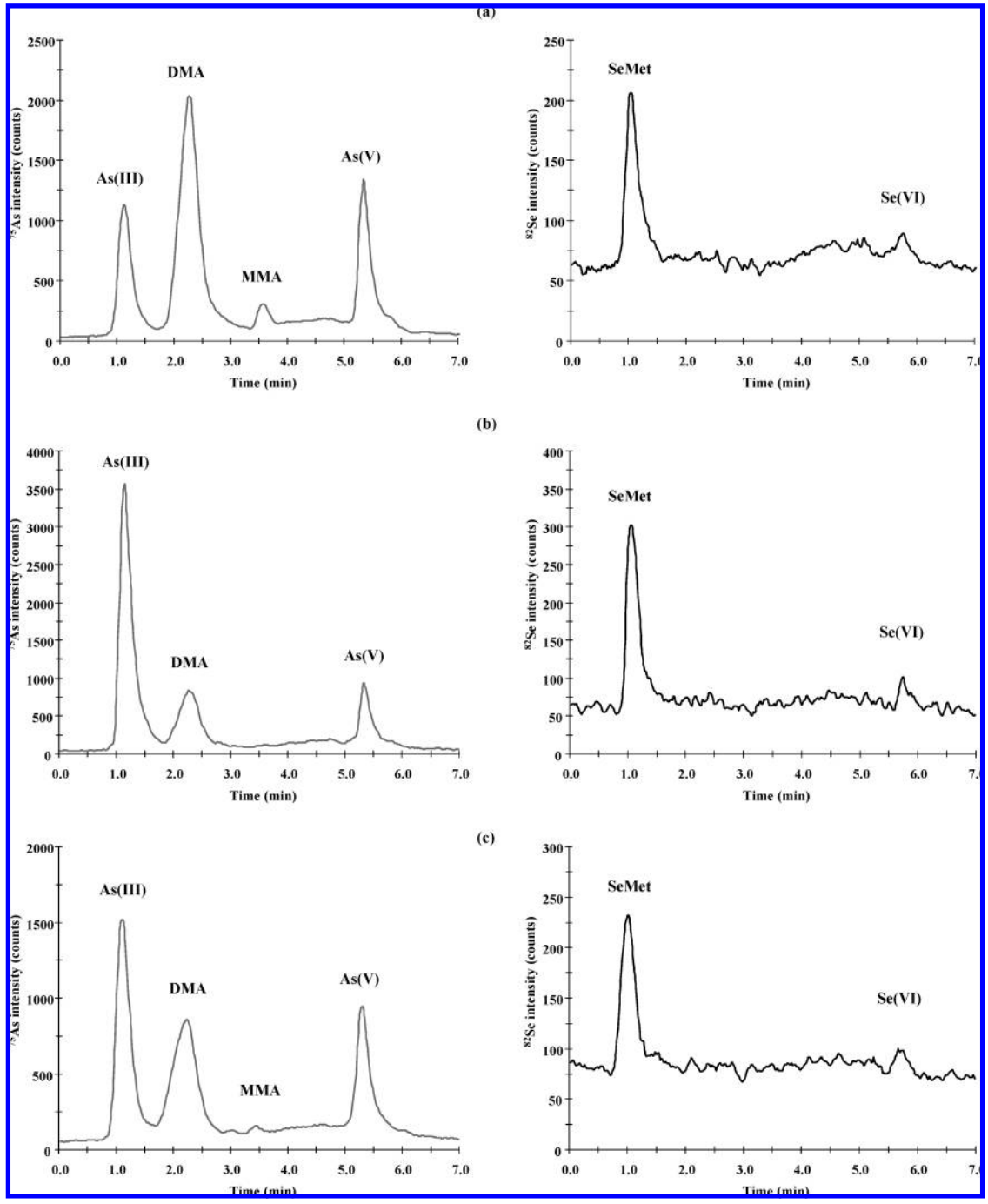

Figure 3. Ion-selective intensity chromatogram for the separation of As and Se compounds using IC-ICP-MS after microwave-assisted enzymatic extraction. (a) NIST SRM-1568a rice flour, (b) white rice (Texas, USA), and (c) rice-based cereal (Canada). Chromatographic conditions are listed in **Table 1**.

(d) Stability of As and Se Species during the MAEE. Recoveries of As (III), As(V), MMA, DMA, Se(IV), Se(VI), and SeMet were investigated using the above extraction procedure ($n = 3$). Single peaks were identified for all the species following IC-ICP-MS analysis with extraction recoveries in the range 97−102% of the original spiked standard solutions. The developed MAEE protocol is an ideal combination for a simple, fast, and simultaneous extraction procedure for As and Se species in rice products without alteration in the relative composition of the species extracted.

Validation of MAEE Method. The accuracy of the results was checked by analyzing SRM-1568a rice flour reference material. This reference material is certified for total As and Se. The samples were analyzed in triplicate. The concentration obtained for individual species and total content are shown in **Table 5** and **6**. The extraction efficiencies of As and Se were verified by comparing the total content in the extracts with the certified values. The recoveries of total As and Se were 100 ± 3 and 80 ± 4%, respectively. As shown, the total concentration of As and Se in the extract were in good agreement with sum of the species, supporting the reliability of the speciation data. The IC-ICP-MS chromatogram of the enzymatic extract of SRM-1568a after MAEE is shown in **Figure 3a**. The species of As and their concentrations were as follows: As(III), 55 ± 6 μg/kg; As(V), 41 ± 3 μg/kg; DMA, 166 ± 6 μg/kg; and MMA: 10 ± 2 μg/kg. Although NIST SRM-1568a is not certified for arsenic species, there are sufficient reported speciation values available from the literature (*5*, *17*, *29*, *30*). According to these values, the As content in the different species varies between: As(III), 27−68 μg/kg; As(V), 20−77 μg/kg; DMA, 116−200

μg/kg; and MMA: 8−15 μg/kg. The speciation distribution of As species in rice flour reference material after MAEE was comparable to those results reported in the literature. With respect to Se, SeMet was the major species in SRM-1568a. The concentration of SeMet was 303 ± 9 μg/kg and accounted for approximately 80% of the total Se present in the rice flour reference material. No data relating to the speciation of Se in rice flour reference material could be found in the literature.

Determination of As and Se Species Content in Rice and Rice-Based Cereals. The developed MAEE methodology was applied to the extraction and quantitation of As and Se species in rice and rice-based cereals. The IC-ICP-MS chromatograms of two rice products are presented in **Figure 3b** and **3c**. The concentrations obtained for the individual As species and total As in rice products using the MAEE methodology are shown in **Table 5**. The total As extracted ranged from 74 ± 2 to 98 ± 3%. The sum of the concentration of individual As species and total As in the extract matched with the total As content in the extracts. The main arsenic species detected in the rice products extract were As(III), followed by DMA and As(V). Traces of MMA were also detected in rice-based cereals. The average concentration of inorganic As [As(III) + As(V)] in rice products was 143 ± 16 μg/kg, which corresponds to approximately 67% of the total As extracted. This result was in agreement with a recent survey carried out by Zavala et al. (*12*). The average concentration of inorganic As reported in that study was 103 ± 45 μg/kg. Average concentration of DMA varied between 36 ± 4 μg/kg (rice) and 77 ± 6 μg/kg (rice-based cereals). The difference in concentration of DMA between these two groups could be related to the components of rice-based cereals. The major ingredient of rice-based cereals was rice flour.

For Se, the concentrations obtained for Se-compounds and total Se in rice products using the MAEE are shown in **Table 6**. The total extraction yields of Se were between 70 ± 7 and 90 ± 9%. The predominant species of Se in rice products was SeMet. Trace amounts of Se(VI) were detected in some rice and rice-based cereals varieties. The content of SeMet in rice products ranged from 46 ± 6 μg/kg (jasmine rice) to 507 ± 7 μg/kg (basmati rice).

Conclusions. An enzymatic treatment (mixture of protease XIV and α-amylase in aqueous medium) in conjunction with microwave-assisted extraction was shown to provide a fast, simple, and novel extraction method for simultaneous speciation of As and Se in rice products without promoting species interconversion. One of the two significant advantages of the proposed method was the reduction of the extraction time to 40 min compared to conventional enzymatic extraction procedures (6−20 h). The second advantage was the increase of the total As and Se extracted in comparison with enzymatic-ultrasound probe assisted extraction, most likely because the action of microwave enhances the cleavage of proteins and complex structures. Additional attributes of the developed procedure were high sample throughput, adequate temperature control, minimum risk of contamination, and loss of the analyte since the reaction takes place in a closed system. The extraction recoveries obtained in rice products were between 74 and 100% for As and 70 and 90% for Se, respectively. As(III), DMA, As(V), and SeMet were the predominant species of As and Se identified in rice products. While the concentration of inorganic As [As(III) + As(V)] was similar in rice and rice-based cereals investigated (average concentration, 143 ± 16 μg As/kg), DMA concentration was higher in rice-based cereals.

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