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Green and Simple Extraction of Arsenic Species from Rice Flour Using a Novel Ultrasound-Assisted Enzymatic Hydrolysis Method

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Abstract: It is well established that arsenic (As) has many toxic compounds, and in particular, inorganic As (iAs) has been classified as a type-1 carcinogen. The measuring of As species in rice flour is of great importance since rice is a staple of the diet in many countries and a major contributor to As intake in the Asian diet. In this study, several solvents and techniques for the extraction of As species from rice flour samples prior to their analysis by HPLC-ICP-MS were investigated. The extraction methods were examined for their efficiency in extracting various arsenicals from a rice flour certified reference material, NMIJ-7532a, produced by the National Metrology Institute of Japan. Results show that ultrasound-assisted extraction at 60 °C for 1 h and then heating at 100 °C for 2.5 h in the oven using a thermostable α-amylase aqueous solution was highly effective in liberating the arsenic species. The recoveries of iAs and dimethylarsinic acid (DMA) in NMIJ-7532a were 99.7% ± 1.6% (n = 3) and 98.1% ± 2.3% (n = 3), respectively, in comparison with the certificated values. Thus, the proposed extraction method is a green procedure that does not use any acidic, basic, or organic solvents. Moreover, this extraction method could effectively maintain the integrity of the native arsenic species of As(III), As(V), monomethylarsonate (MMA), DMA, arsenobetaine (AsB) and arsenocholine (AsC). Under the optimum extraction, chromatography and ICP-MS conditions, the limits of detection (LOD) obtained were 0.47 ng g^{-1}, 1.67 ng g^{-1} and 0.80 ng g^{-1} for As(III), As(V) and DMA, respectively, while the limits of quantification (LOQ) achieved were 1.51 ng g^{-1}, 5.34 ng g^{-1} and 2.57 ng g^{-1} for As(III), As(V) and DMA, respectively. Subsequently, the proposed method was successfully applied to As speciation analysis for several rice flour samples collected from contaminated areas in China.

Keywords: arsenic speciation; rice flour; enzymatic hydrolysis; ultrasound-assisted extraction; HPLC-ICP-MS

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1. Introduction

Arsenic (As) is toxic and has been classified as a human carcinogen by the International Agency for Research on Cancer (IRAC) [1]. There are several naturally occurring chemical forms of arsenic, such as inorganic compounds of As(III) and As(V) and organic species of methylarsonic acid (MMA), dimethylarsinic acid (DMA), arsenobetaine (AsB), arsenocholine (AsC), etc. Of these species, inorganic arsenic (iAs) is classified as a type-1 carcinogen, with lethal dose 50 (LD_{50}) values in the range of 15–42 mg kg^{-1} for As(III) and 20–200 mg kg^{-1} for As(V); following that, the organic forms of arsenic MMA and DMA are possibly carcinogenic to humans (type 2B), with LD_{50} values in the range of

700–1800 mg kg^{-1} and 1200–2600 mg kg^{-1}, respectively [2,3]. Therefore, the Food and Agriculture Organization/World Health Organization (FAO/WHO) have recommended a provisional tolerable weekly intake (PTWI) of iAs at 15 μg kg^{-1} of body weight [4].

Arsenic is gradually accumulated in crops from the water, soil and air owing to human industrial activities [5,6]. As a staple of the diet in Asia, rice contributes more arsenic to the Asian diet than all other agricultural products [7]. Moreover, it has been reported that the arsenic concentration in rice is 10 times higher in comparison with that in other crops under flooded soil conditions [8,9]. As the largest rice-producing and -consuming country in the world, China has regulated the iAs concentration in rice to be not more than 0.2 mg kg^{-1} through its National Medical Products Administration [10,11]. Thus, it is necessary to investigate the concentrations of arsenic species in rice to ensure that rice can be safely consumed.

The studies of arsenic speciation analysis have grown rapidly in recent years and are generally conducted using high-performance liquid chromatography coupled to inductively coupled plasma mass spectrometry (HPLC-ICPMS) [12–16]. Prior to that, speciation analysis usually requires the extraction of the species from complex matrices into solvents before they can be identified and measured. This is also one of the key steps for accurate determination of As species in rice flour samples, as incomplete extraction of species would lead to erroneous results [17]. In previous studies, a number of procedures utilizing different extraction solvents, such as water, diluted nitric acid, methanol–water or nitric acid–hydrogen peroxide mixtures, with the extraction techniques of mechanical agitation, sonication, microwave heating or pressurized liquid, have been implemented for extraction of As species from rice samples [18–23]. Narukawa et al. have reported the extraction of As from rice using water at 90–100 °C. However, the extraction efficiency of As(III) varied depending on the rice sample analyzed; thus, the total arsenic extracted was in the range from 80% to 100% of the arsenic present. Therefore, in order to achieve 100% extraction of As species, it is still necessary to optimize the extraction conditions for rice samples. Moreover, the use of acidic or organic solvents would increase occupational risk and the amount of hazardous waste, which are the main disadvantages of other reported extraction methods [24].

In the present study, we developed and optimized a method based on ultrasound-assisted enzymatic hydrolysis, which provided for the quantitative, green and simple extraction of arsenicals in rice flour samples, and hence facilitated As speciation analysis using HPLC-ICP-MS. Several extraction media and techniques were evaluated based on the total arsenic extracted and the extraction efficiencies for different chemical forms of native As species in a rice flour reference material NMIJ-7532a. The optimum method is based on hydrolyzing the starch and other components in rice by mainly using α-amylase. Ultrasonic and heating techniques were implemented to enhance the extraction efficiency and shorten the time consumption. Once optimized, the method was further validated by analyzing two other rice flour reference materials, NMIJ-7501a and NIST SRM 1568b. Subsequently, arsenic speciation in a variety of real rice flour samples was carried out.

2. Experimental Section

2.1. Reagents and Standards

High-purity nitric acid was purchased from Sigma-Aldrich Inc. (St. Louis, MO, USA) and used for sample digestion. Ultra-pure water with a resistivity of 18.2 MΩ·cm was produced by a Milli-Q system (Millipore Corp., Burlington, MA, USA) and used for the preparation of all samples and standard solutions. The thermostable α-amylase from Sigma-Aldrich Inc. (St. Louis, MO, USA) was used. For the preparation of the mobile phase, dibasic ammonium hydrogen phosphate (purity ≥ 99.0%) was supplied by Sigma-Aldrich Inc. (St. Louis, MO, USA). HPLC-grade methanol was purchased from Thermo Fisher Scientific (Waltham, MA, USA). The mobile phase was degassed and filtered before use. The syringe filter (0.22 μm) PTFE PVDF was from Jinteng Experimental Equipment (Tianjin, China).

The certified reference materials of arsenite (As(III), GBW08666), arsenate (As(V), GBW08667), monomethylarsonic acid (MMA, GBW08668), dimethylarsinic acid (DMA, GBW08669), arsenobetaine (AsB, GBW08670) and arsenocholine bromide (AsC, GBW08671) were all produced by the National Institute of Metrology (Beijing, China). The concentrations of all standard solutions are SI-traceable.

Certified reference materials of As species in rice flour, NMIJ-7532a, from the National Metrology Institute of Japan (NMIJ, Tsukuba, Japan) were selected for method development. The information and certified values of the total As, iAs and DMA were 0.320 ± 0.010 mg/kg, 0.298 ± 0.008 mg/kg and 0.0186 ± 0.0008 mg/kg, respectively.

2.2. Intrumentation

A Mars5 microwave system (CEM, Matthews, NC, USA) was used for the extraction of the total arsenic. Ultrasound-assisted extractions were carried out by an ultrasonic bath (KQ-500GDV, Kun Shan Ultrasonic Instruments Co., Ltd, Kunshan, Jiangsu, China). Shaking was performed on a WS20 shaking incubator (Wiggens, Straubenhardt, Germany). A DKN612C forced convection oven (Yamato Scientific Co., Ltd., Tokyo, Japan) was utilized as the heating device during digestion. A universal 320R centrifuge (Hettich, Tuttlingen, Germany) was employed for the centrifugation of the extracts obtained from the samples.

An ICP-MS (8800, Agilent Technologies, Santa Clara, CA, USA) equipped with a collision cell, a Scott double pass spray chamber and a PFA nebulizer was used for the determination of As. To reduce some polyatomic molecular interferences ($^{40}Ar^{35}Cl^+$, $^{59}Co^{+16}O^+$) in $^{75}As^+$ analysis, the collision cell gas of O_2 was set as 25%. In this case, the mass monitored was *m/z*= 91 for AsO^+. The spray chamber temperature, collision gas flow rate and $^{91}AsO^+$ signal integration time were optimized to obtain the best limit of detection (LOD) of As. Typical operating parameters for the ICP-MS are summarized in Table 1.

Table 1. Operating conditions of the HPLC-ICP-MS system.

ICP-MS
RF powder: 1500 W Carrier gas: 1.0 mL/min Reaction gas: O_2, 25% Isotope monitored: $^{91}AsO^+$ Integration time: 0.1 s (spectrum) per point Points per peak: 3
HPLC
Column: PRP-X100 anion exchange Dimensions: 250 mm × 4.1 mm, particle size: 10 μm Mobile phase: 20 mM $(NH_4)_2HPO_4$, pH 6.0 Injection volume: 20 μL Flow rate: 1.2 mL/min Mode: Isocratic

For arsenic speciation, chromatographic separations were performed by an Agilent 1290 HPLC system (Santa Clara, CA, USA). A Hamilton PRP-X100 anion exchange column (Grace, Belgium) was used as the stationary phase. Guard columns of the same stationary phase were connected in front of the separation columns. All the columns were preconditioned according to the manufacturer's instructions before use. The pH of the mobile phase used in LC was adjusted by a Mettler Toledo FiveEasy Plus pH meter (Zurich, Switzerland).

2.3. Samples

Rice samples were obtained by pooling individual samples (*n* = 3) of different varieties grown in contaminated areas in China. The rice flour certified reference materials were NMIJ-7502a, or brown rice obtained by NMIJ (NMIJ, Tsukuba, Japan), and NIST SRM

1568b, obtained by the National Institute of Standards and Technology (NIST, Gaithersburg, MD, USA).

2.4. Total Arsenic Determination

The total arsenic in the reference material NMIJ-7532a and the test samples was determined in accordance with the following procedures. Three replicate portions of each sample (0.5 g dry weight) were weighed into Teflon microwave vessels. High-purity nitric acid (5 mL) was added into the vessels, and the mixtures were predigested for 6 h or overnight in a clean air hood at an ambient temperature (20 °C). This step could effectively prevent the samples with high starch from bumping during digestion. After that, the samples were placed in the microwave digestion system. The microwave program was as follows: 15 min ramp to 150 °C, held for 5 min at 150 °C; then, 10 min ramp to 175 °C, held for 5 min at 175 °C; finally, 10 min ramp to 195 °C, held for 35 min at 195 °C. The digested samples were cooled to room temperature and then transferred to 50-mL polypropylene test tubes, which were gravimetrically diluted with ultra-pure water for ICP-MS analysis. For sample digestion, each digestion batch consisted of two procedural blanks and triplicates of the reference material NMIJ-7532a.

2.5. Evaluation of Extraction Methods

Table 2 shows the extraction methods evaluated in this study. The list includes acidic and enzymatic extractions. Different extraction temperatures, times and devices were also evaluated to provide an optimum extraction procedure for the analysis of As species in rice flour. The reference material of rice flour, NMIJ-7532a, was used to examine their efficiencies in extracting iAs and DMA. The sample weight was 0.5 g for all procedures. Each extraction procedure was repeated at least three times. Procedural blanks were included in each extraction batch. Samples were kept in the refrigerator after filtration prior to HPLC-ICP-MS measurements.

Table 2. Extraction conditions evaluated in this study.

Extraction	Procedure	Solvent/Solution	Extraction Temperature and Time	Heating Device
Acidic	A-1	10 mL 1% (*v*/*v*) HNO_3	stand overnight; 80 °C, 2.5 h	Oven
	A-2	10 mL 1% (*v*/*v*) HNO_3	stand overnight; 90 °C, 2.5 h	Oven
	A-3	10 mL 1% (*v*/*v*) HNO_3	stand overnight; 100 °C, 2.5 h	Oven
	A-4	10 mL 1% (*v*/*v*) HNO_3	stand overnight; 100 °C, 0.5 h	Oven
	A-5	10 mL 1% (*v*/*v*) HNO_3	stand overnight; 100 °C, 1.5 h	Oven
	A-6	10 mL 1% (*v*/*v*) HNO_3	stand overnight; 100 °C, 2.5 h	Oven
	A-7	10 mL 1% (*v*/*v*) HNO_3	stand overnight; 100 °C, 3.5 h	Oven
Enzymatic	B-1	5 mL 10 mg/mL α-amylase	60 °C, overnight	Shaking incubator
	B-2	5 mL 10 mg/mL α-amylase	60 °C, overnight; 100 °C, 2.5 h	Shaking incubator Oven
Ultrasound-assisted enzymatic	C-1	5 mL 10 mg/mL α-amylase	60 °C, 1 h	Ultrasonic bath
	C-2	5 mL 10 mg/mL α-amylase	60 °C, 1 h; 100 °C, 2.5 h	Ultrasonic bath Oven

Procedure A (Acidic extraction)—A total of 10 mL of 1% (*v*/*v*) HNO_3 was added to the samples, which were then predigested overnight in a clean air hood at an ambient temperature. The next day, the samples were placed in the oven for digestion and shaken every 30 min. Different extraction times of 0.5 h to 3.5 h under various temperatures from 80 °C to 100 °C were examined. After each extraction, the samples were centrifuged at 8000 rpm for 10 min under 4 °C. The supernatants were then filtered through a 0.22 μm filter.

Procedure B (Enzymatic extraction)—A total of 5 mL of 10 mg/mL α-amylase aqueous solution were added to the samples, which were then treated in a shaker at 60 °C overnight. The next day, the samples were placed in the oven for 2.5 h under 100 °C for digestion (shaken every 30 min). The extracts were centrifuged and filtered as described in procedure A.

Procedure C (Ultrasound-assisted enzymatic extraction)—A total of 5 mL of 10 mg/mL α-amylase aqueous solution were added to the samples, which were then placed in ultrasound equipment for 1 h at 80 W. Then, further digestion was performed in the oven for 2.5 h at 100 °C (shaken every 30 min). The extracts were treated as described above.

2.6. Determination of Arsenic Species

Arsenic species were determined by HPLC-ICP-MS using a strong anion exchange column fitted with a matching guard column filled with the identical phase. The operating conditions of the HPLC-ICP-MS system are shown in Table 1. The arsenic species were identified by retention time matching with the mixed standard solution consisting of As(III), As(V), MMA, DMA, AsB and AsC substances as external standards.

2.7. Analyte Quantification

For the total arsenic analysis using ICP-MS, the standard additions method was carried out to correct the matrix effect. The calibration standards were prepared by serially diluting the 1 mg kg^{-1} arsenic stock solution in 6% HNO_3 to obtain final concentrations of 0.5 to 5 ng g^{-1}. For quantifying As species by HPLC-ICP-MS, the external calibration method was used with a standard solution containing 10 ng g^{-1} of each arsenicals in 1% HNO_3. All calibration standards were prepared gravimetrically and daily before use. Data acquisition, chromatographic peak integration and quantification were all carried out using the Agilent MassHunter software. Microsoft Excel was used for further calculations.

3. Results and Discussion

3.1. Determination of Total Arsenic by Microwave Digestion

The determinations of the total arsenic in the certified reference materials and rice flour samples were performed using ICP-MS after microwave digestion. The results are reported in Table 3. The total As (n = 3) determined in reference materials NMIJ-7532a, NMIJ 7502a and NIST SRM 1568b were 0.316 ± 0.012 mg kg^{-1}, 0.112 ± 0.003 mg kg^{-1} and 0.288 ± 0.006 mg kg^{-1}, respectively, which were in good agreement with their certified values. The total As concentrations of the three rice flour samples (n = 3) were 0.157 ± 0.003 mg kg^{-1}, 0.482 ± 0.008 mg kg^{-1} and 0.378 ± 0.008 mg kg^{-1}, respectively. These values were further used for calculating the recoveries of the total As during sample analysis under the optimum extraction procedure, since no reference value was available for them.

Table 3. Total arsenic in the certified reference materials and rice flour samples determined by ICP-MS after MW.

Code	Description	Total As (mg kg^{-1})	Certified Value (mg kg^{-1})
NMIJ 7532a	Rice flour from NMIJ	0.316 ± 0.012 [a]	0.320 ± 0.010
NMIJ 7502a	Rice flour from NMIJ	0.112 ± 0.003	0.109 ± 0.005
NIST 1568b	Rice flour from NIST	0.288 ± 0.006	0.285 ± 0.014
S1	Chinese brown rice flour	0.157 ± 0.003	-
S2	Chinese brown rice flour	0.482 ± 0.008	-
S3	Chinese brown rice flour	0.378 ± 0.008	-

[a] The standard deviation has been obtained for n = 3.

3.2. Determination of Arsenic Species

The As speciation was analyzed by the HPLC-ICP-MS method. The chromatograms obtained with the mixed standard solutions (100 ng g^{-1} as As) are displayed in Figure 1. As can be seen, the six arsenic species of As(III), As(V), MMA, DMA, AsB and AsC were well separated on the PRP-X100 column using an isocratic gradient (conditions see Table 1). Quantification was based on peak area measurements.

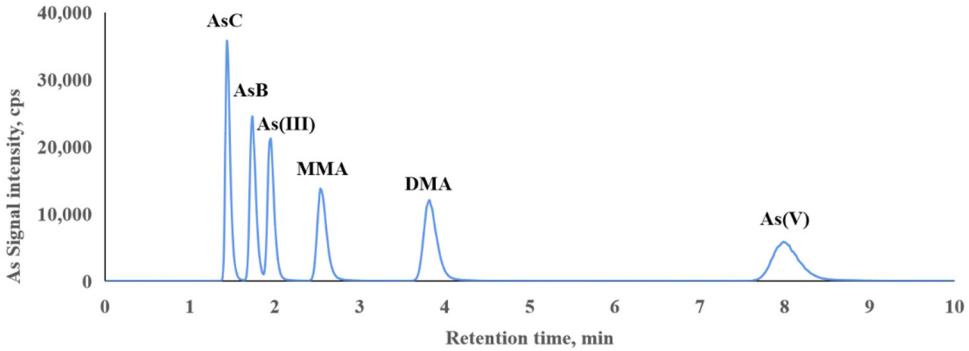

Figure 1. HPLC-ICP-MS chromatogram of a mixture of standards at a concentration of 100 ng g^{-1} as As.

In the measurement of As by ICP-MS, it is well known that $^{40}Ar^{35}Cl^+$ and $^{59}Co^{16}O^+$ with *m/z*= 75 causes interferences for $^{75}As^+$ analysis. Therefore, oxygen was used as reaction gas and resulted in detection of arsenic as AsO^+ at *m/z*= 91 in this study. The reproducibility of peak areas for the mixture of standards at a concentration of 10 ng g^{-1} as As was measured over a period of approximately 2 h (n = 10). The relative standard deviations were 2.28%, 1.56%, 2.14% and 2.29% for As(III), As(V), MMA and DMA, respectively.

3.3. Optimization of Extraction Methods

The rice flour certified reference material NMIJ-7532a was used to compare the extraction efficiencies of the two solvents and three extraction techniques. The speciation analysis of arsenic was carried out by HPLC-ICP-MS. The concentrations of the extracted arsenic species and total As were directly compared with the values stated in their certificates. The obtained results are reported in Table 4.

Table 4. Results of arsenic speciation of NMIJ 7532a.

Procedure	iAs (mg kg^{-1})	DMA (mg kg^{-1})	Total (mg kg^{-1})
A-1	0.253 ± 0.005 (84.8%) [a]	0.0163 ± 0.0003 (87.9%)	0.269 ± 0.005 (84.1%)
A-2	0.284 ± 0.006 (95.3%)	0.0171 ± 0.0003 (92.1%)	0.301 ± 0.006 (94.1%)
A-3	0.299 ± 0.006 (100.3%)	0.0182 ± 0.0004 (97.8%)	0.317 ± 0.006 (99.1%)
A-4	0.264 ± 0.006 (88.6%)	0.0170 ± 0.0003 (91.4%)	0.281 ± 0.006 (87.8%)
A-5	0.288 ± 0.006 (96.6%)	0.0190 ± 0.0004 (102.2%)	0.307 ± 0.006 (95.9%)
A-6	0.300 ± 0.006 (100.7%)	0.0190 ± 0.0004 (102.2%)	0.319 ± 0.006 (99.7%)
A-7	0.298 ± 0.006 (100.0%)	0.0180 ± 0.0004 (96.8%)	0.316 ± 0.006 (98.8%)
B-1	0.220 ± 0.005 (73.7%)	0.0133 ± 0.0003 (71.4%)	0.233 ± 0.005 (72.8%)
B-2	0.240 ± 0.005 (80.4%)	0.0145 ± 0.0003 (78.2%)	0.254 ± 0.005 (79.4%)
C-1	0.278 ± 0.006 (93.2%)	0.0163 ± 0.0003 (87.5%)	0.294 ± 0.006 (91.9%)
C-2	0.297 ± 0.006 (99.7%)	0.0182 ± 0.0004 (98.1%)	0.315 ± 0.006 (98.5%)
Certified value	0.298 ± 0.008	0.0186 ± 0.0008	0.320 ± 0.010

[a] Recovery displayed in parentheses.

3.3.1. Extraction by Acidic Solvent

Diluted solutions of HNO_3 have been widely used to extract arsenic from samples in various matrices. As illustrated in GB/5009.11-2014, published by the National Health Commission of China [25], the optimum extraction conditions for arsenic in rice is using 1% HNO_3 at 90 °C for 2.5 h. However, different extraction temperatures and times were reported in the literature [18,19,21,26]. For verifying the optimum extraction protocol for arsenic species in rice flour, the extraction efficiencies of 1% HNO_3 were evaluated at different heating temperatures using a forced convection oven (80 °C, 90 °C and 100 °C) and extraction times of 0.5 to 3.5 h in this study. As can be seen from Table 4, under the same extraction time of 2.5 h, the extraction yields increased from 84.8% to 100.3% for iAs and from 87.9% to 97.8% for DMA when the heating temperature increased from 80 °C to 100 °C. It was also possible to achieve the complete extraction of iAs and DMA in rice flour in 2.5 h at 100 °C. No significant advantages in extraction efficiencies were observed for either iAs or DMA when the extraction time increased from 2.5 h to 3.5 h under 100 °C. Hence, the extraction of arsenic species in rice flour with 1% HNO_3 under 100 °C is recommended instead of 90 °C for 2.5 h.

3.3.2. Enzymatic Extraction by Shaking

As reported in the literature, α-amylase hydrolyses the α-1,4-linkage of starch, which is the major component in rice of up to 78% [27]. This process could increase the solubility of the proteins by liberating the starch-bound proteins and hence facilitating the arsenic extraction. In order to establish an effective extraction method for As species in rice flour using α-amylase, the extraction efficiency was first evaluated with α-amylase aqueous solution by shaking treatment. As shown in Table 4, the extractions of iAs and DMA were only 73.7% and 71.4%, respectively, after being treated with α-amylase aqueous solution and left in a shaking incubator at 60 °C overnight. Increased extraction efficiencies were obtained of 80.4% and 78.2% for iAs and DMA, respectively, when the extracts were further heated in the oven under 100 °C for 2.5 h, although the method for enzymatic extraction using α-amylase still needs to be optimized for the effective extraction of As species in rice flour.

3.3.3. Ultrasound-Assisted Enzymatic Extraction

In order to enhance the extraction efficiencies of As species and reduce sample treatment time, the ultrasonic technique was employed to accelerate the enzymatic hydrolysis activity. The results are summarized in Table 4. The detected iAs and DMA were increased to 93.2% and 87.5%, respectively, after 1 h of sonication with α-amylase aqueous solution as the extraction media. Extraction efficiencies of approximately 100% could be achieved for both iAs and DMA when the samples were further heated in the oven for 2.5 h under 100 °C after ultrasound-assisted extraction. These results suggested that the reactivity of α-amylase was enhanced with physical processing by acoustic cavitation, and hence, accelerated the reaction rates of enzymatic hydrolysis. Moreover, the heating process is necessary for achieving the complete extraction of As species in rice flour.

The optimum conditions for ultrasound-assisted enzymatic extraction were as follow: 5 mL of 10 mg/mL α-amylase as the extraction solvent, sonication treatment for 1 h at 60 °C; then, heating in the oven for 2.5 h under the extraction temperature of 100 °C. Thus, an effective method for extracting As species from rice flour was developed, which was more green, rapid and simple in comparison with the conventional acidic extraction method.

3.3.4. Stability of Arsenic Species

The maintenance of the native chemical forms of As is an essential requirement for As speciation analysis during the extraction process. Hence, to evaluate the preservation of As species by the ultrasound-assisted enzymatic extraction method, the recoveries of As(III), As(V), MMA, DMA, AsB and AsC were investigated. The rice flour reference material NMIJ-7532a was spiked with each of the As species' standards (10 and 100 ng g^{-1} as As),

and their recoveries were determined following the proposed optimum extraction and quantification procedures. As displayed in Table 5, the recoveries of all these species were nearly 100%. These results demonstrated that no chemical alteration happened to these As species during the proposed ultrasound-assisted enzymatic extraction process.

Table 5. Recoveries of As species during ultrasound-assisted enzymatic extraction.

Concentration of Spiked Standards	Recovery (%)					
	As(III)	As(V)	MMA	DMA	AsB	AsC
10 ng g^{-1}	99.6	100.7	99.5	98.5	99.4	98.0
100 ng g^{-1}	100.2	101.3	100.3	98.9	100.1	99.2

3.3.5. Limits of Detection and Quantification

The limits of detection (LOD) and quantification (LOQ) of the optimized protocols were determined based on the standard deviation of replicate (n = 10) analyses of a blank solution of 1% HNO_3. The results are listed in Table 6.

Table 6. Limits of detection and quantification of arsenic species (ng g^{-1}).

Species	As(III)	As(V)	MMA	DMA	AsB	AsC
LOD	0.47	1.67	0.71	0.80	0.32	0.21
LOQ	1.51	5.34	2.26	2.57	1.01	0.66

3.4. Speciation Analysis of Arsenicals in Rice Flour Samples

The ultrasound-assisted enzymatic extraction was applied to determine the arsenic species in five rice flour samples in this study, including two rice flour reference materials and three real rice samples (S1 to S3) collected from the contaminated areas in China. The obtained results are summarized in Table 7. For reference materials NMIJ-7502a and NIST SRM 1586a, the detected amounts of iAs, MMA and DMA and the total As recovered show no significant differences from their certificated values. These results further validated the reliability of the proposed enzymatic extraction method. In addition, for all of the three real rice samples, the sums of each As species were in good agreement with the total arsenic concentrations that were determined by ICP-MS after microwave-assisted digestion.

Table 7. Results of Arsenic species in rice flour samples determined using ultrasound-assisted enzymatic extraction.

Code	iAs (mg kg^{-1})	MMA (mg kg^{-1})	DMA (mg kg^{-1})	Sum (mg kg^{-1})
NMIJ 7502a	0.096 ± 0.003 (98.0%) [a]	- [b]	0.0130 ± 0.0004 (100.7%)	0.109 (100.0%)
NIST 1568b	0.089 ± 0.003 (96.7%)	0.0118 ± 0.004 (101.7%)	0.181 ± 0.006 (100.5%)	0.282 (98.9%)
S1	0.149 ± 0.005	-	0.0061 ± 0.0002	0.155 (98.7%)
S2	0.440 ± 0.015	0.0032 ± 0.0002	0.041 ± 0.001	0.484 (100.4%)
S3	0.365 ± 0.012	-	0.0092 ± 0.0003	0.374 (98.9%)

[a] The recovery displayed in parentheses for each As species was calculated by dividing its certified value; the recovery displayed in parentheses for the sum of As was obtained by dividing the certified value or total digestion of As. [b] Not detected.

As the results show in Table 7, the relative amount of iAs ranged from 90.9% to 97.6% of the sum of the As species in rice samples S1–S3. Most of the remaining arsenical was DMA. In samples S1 and S3, MMA was not detected. These results confirm that rice is a bio-accumulative plant for the more toxic As species. Furthermore, the concentrations

of iAs in samples S2 and S3 were 0.440 ± 0.015 mg kg^{-1} and 0.365 ± 0.012 mg kg^{-1}, respectively, which are higher than the nationally set safety standards of China for iAs in rice (0.2 mg kg^{-1}). Thus, the importance of monitoring the contents of As species in rice should be further addressed to ensure that rice can be safely consumed.

4. Conclusions

In this study, various conditions for extracting arsenic species from rice flour samples were investigated. HPLC-ICP-MS was used as the analytical method for the determination of As species. Enzymatic hydrolysis based on the use of α-amylase in aqueous media, in conjunction with ultrasound treatment, has been found to be a green, rapid and simple extraction method for the determination of As species in rice flour. The extraction efficiencies, 96.7~99.7% for iAs and 98.1~100.7% for DMA, were obtained by analyzing three rice flour reference materials (NMIJ-7532a, NMIJ-7502a and NIST SRM 1568a). Moreover, the proposed ultrasound-assisted enzymatic extraction method showed advantages in preserving the chemical integrity of native arsenic species of As(III), As(V), MMA, DMA, AsB and AsC with nearly 100% recoveries.

The novel extraction method could also meet the analytical needs for accurate quantification of As species in real rice samples. Our results indicated that more than 90% of As was found to be present in its inorganic form, which is more toxic, in the tested rice samples from China, whereas the remainder was mainly DMA. In this instance, persistent monitoring of the content of As species in rice products is necessary to prevent the dietary exposure to iAs for populations consuming a predominantly rice-based diet.

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