Supplementary Information

Quantitative chemical extraction for arsenic speciation in rice grains

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11 Pages, 9 Figures and 1 Table

Experimental

Reagents and materials

Arsenate (As(V)) and arsenite (As(III)) were obtained as standard solutions (1 g L^{-1}) from Merck (Darmstadt, Germany) and Fluka (Buchs, Switzerland). Monomethylarsonic acid (MMA) with purity ≥97% was purchased from Argus Chemicals (Vernio, Italy). Dimethylarsinic acid (DMA) (≥97%), ammoniumdihydrogenphosphate ((NH$_4$)H$_2$PO$_4$) (≥99%) and tetramethylammonium hydroxide (TMAH) were from Merck. *Streptomyces griseus* (protease XIV) was received from Sigma (Steinheim Germany) and *Bacillus subtilis* (α-amylase) was obtained from Fluka (Steinheim, Germany). Ammonia solution, hydrochloric acid, nitric acid and all solvents with analytical grade were obtained from Merck. Deionized water, used throughout the work, was purified in Milli-Q system (Milford, MA, USA). Individual stock solutions (50 mg As L^{-1}) of As(III), As(V), MMA and DMA, were prepared with de-ionized water and stored at 4 ^{o}C in the dark. A multi-species working solution of As(III), As(V), MMA and DMA with a total concentration of 20, 40, 80, 200 or 300 μg As L^{-1} was prepared before each use by diluting the stock solutions with de-ionized water.
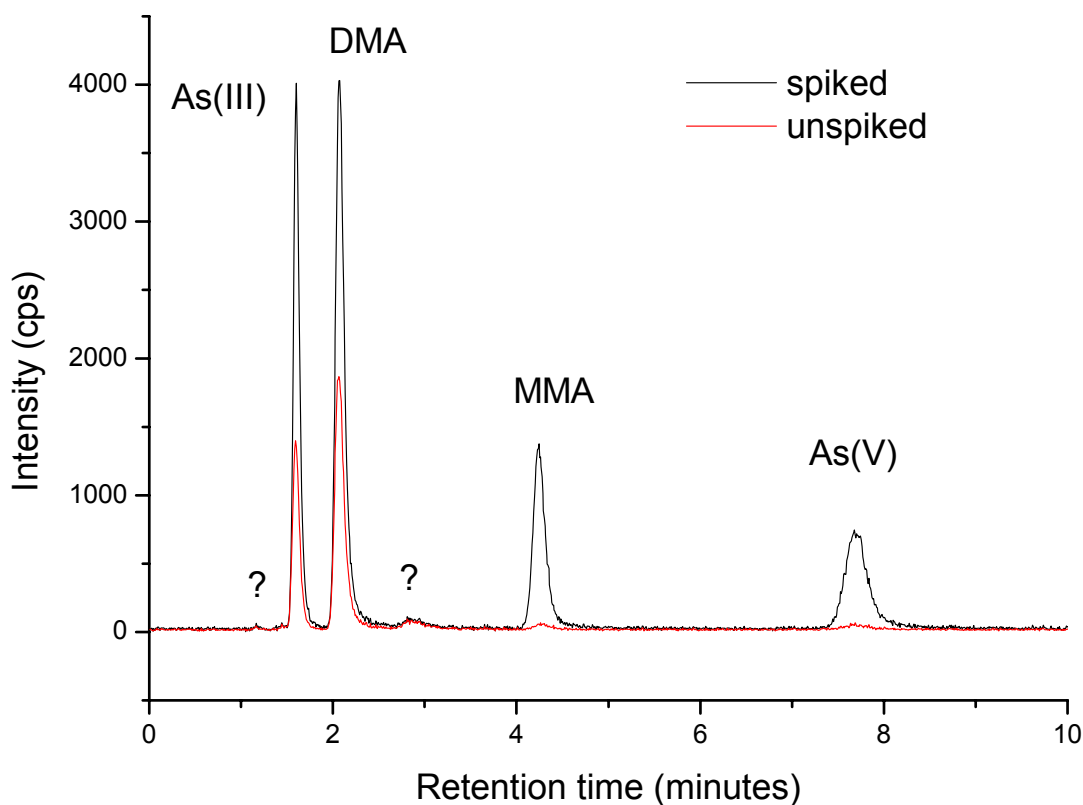
Instrumentation

A standalone high performance liquid pump (Spectra System P2000, Thermo Separation Products, Dreieich, Germany), a capillary PEEK tubing (0.25 mm i. d.), a 20-μL injection loop (Stainless Steel), and a HPLC autosampler Basic Marathon (Spark Holland, Emmen, Holland) were connected to an ion exchange column and coupled to an ICP-MS (Thermo Element 2, Bremen, Germany). ICP-MS was equipped with a concentric nebulizer (Micromist, Glass Expansion, West Melbourne, Australia) and a cyclon spray chamber (Glass Expansion). The ion intensity at m/z 75 (^{75}As) was monitored in a low resolution mode.

Speciation of As(III), As(V), MMA and DMA were performed on a PRP-X100 anion-exchange column (250 × 4.1 mm, 10 μm) from Hamilton Company (Reno, NV, USA) at 40°C with a mobile phase (1.5 mL min^{-1}). The mobile phase solution is made of 20 mM NH$_4$H$_2$PO$_4$ with a pH 5.6. Quantification was done by external calibration with standard solutions. Arsenic speciation in extracts was conducted immediately after extraction. Identification of As species was confirmed by spiking real extracts with a mixture of standard solutions (Fig. S1). Detection limits for As speciation were estimated from the average of instrumental blank values three times.

Extraction procedure

All rice grains were grounded or obtained as fine powders. A 1.5 g rice powder was extracted with 15 mL of different extractants in 50 mL polypropylene with heating blocks (Digi PREP Jr, SCP Science) or microwave ovens (Multiwave 3000, Anton Paar). The microwave oven is equipped with a pT sensor to measure the temperature inside the extraction vessel. The pT sensor of was calibrated to read the correct temperature in the range of 80~120°C. The temperature control of the DigiPrep heating device was controlled using a temperature sensor filled with water. A part of the resulting suspensions was filtered to 0.45 μm (Teflon) for

As speciation by HPLC-ICP-MS. Another part of suspensions was 1:1 mixed with 65% HNO_3 in a High Pressure Asher (HPA, Anton Paar, Austria) by heating up to 280°C for 90 minutes. The resulting clear supernatants were analyzed using ICP-MS. Additionally, the milled rice grains were extracted right after Sanz et al., 2005 and Narukawa et al., 2008 for As speciation.
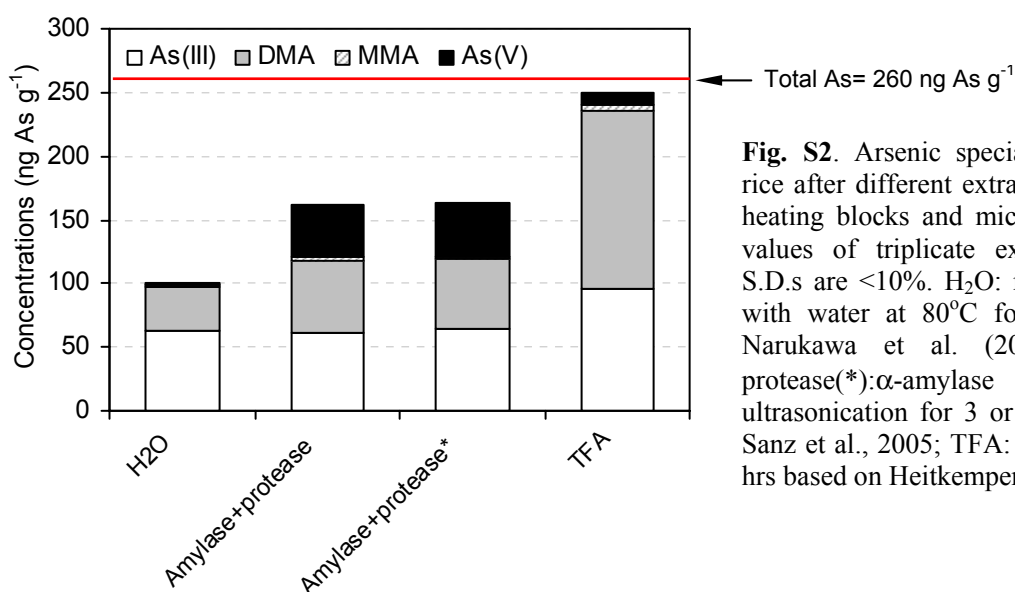

Fig. S1. Chromatograms of whole grain rice extracted with 0.28 M HNO_3 at 95°C for 1.5 hours without (—) and with (—) spiking 15 µg As L^{-1} of As(III), DMA, MMA and As(V) standards. ?: unknown arsenic species.

Results and discussion

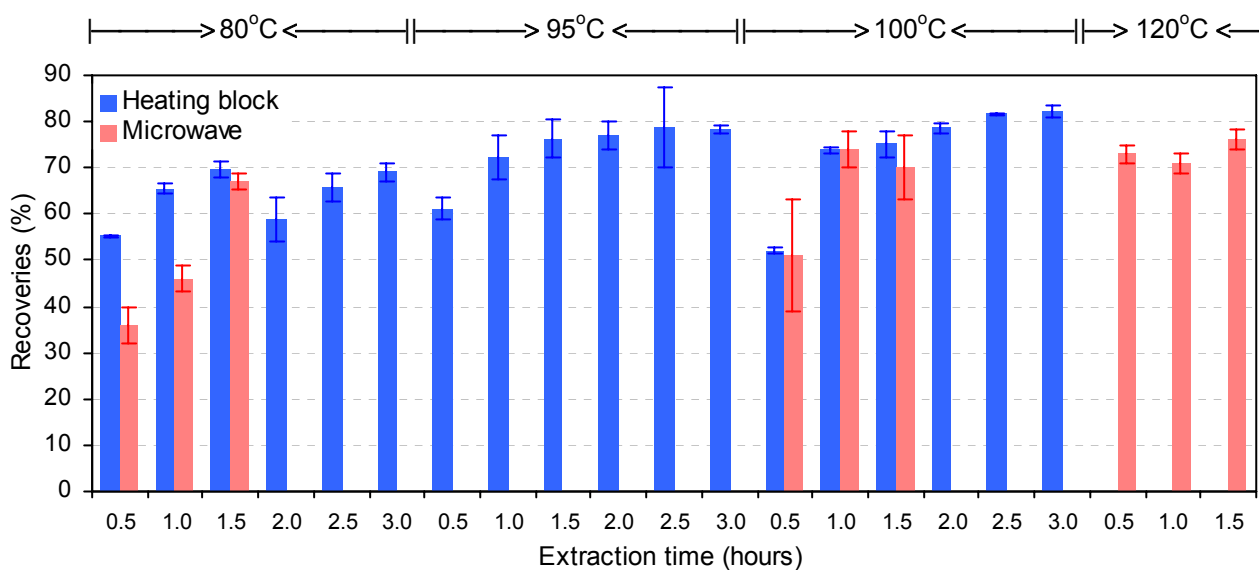*State of knowledge about extraction methods for arsenic speciation in rice grain*

Among most existing extraction methods, very few of them are developed for rice grains. None of these methods was able to quantitatively recover As from rice (Pizarro et a., 2003; Baba et al., 2008). Arsenic species may undergo redox transformation (Schoof et al., 1999; Heitkemper et a., 2001; Baba et al., 2008) (Table S1). Extraction based on water and methanol usually keeps As speciation. However, recovery rate of total As is generally low even with the assistance of microwave oven heating or ultrasonication (Pizarro et a., 2003; D'amoto et al., 2004; Baba et al., 2008). At room temperature, the solution with 50% water and 50% methanol may improve the recovery to 63 and 74% of total As from milled rice grains after 6 hour shaking. Pizzaro et al. (2003) extracted CRM and NIST 1568a rice flours with water, 50% and 90% methanol and 50% methanol followed by 90% methanol at 55°C for 10 hours. This allowed recovery up to 62~80% of total

As. The recoveries first improved to 86-96% with one extraction repetition. Recovery rates of spiked As(III), DMA, MMA and As(V) were higher than 90% (Pizarro et al., 2003). With accelerated solvent extraction, 95-105% of total As in NIST 1568a was recovered with 25~100% methanol at room temperature and de-ionized water at 100°C (Heitkemper et al., 2001). Nevertheless, the recoveries of total As in long grain white rice were 0–36% using the same extraction methods, suggesting the extraction efficiency was influenced by the rice grain types (Heitkemper et al., 2001).

Fig. S2. Arsenic speciation in whole grain rice after different extraction treatments with heating blocks and microwave ovens. Mean values of triplicate extraction are shown. S.D.s are <10%. H_2O: microwave extraction with water at 80°C for 30 min, based on Narukawa et al. (2008); Amylase and protease(*):α-amylase + protease XIV, ultrasonication for 3 or 6 min (*), based on Sanz et al., 2005; TFA: 2 M TFA at 100°C 6 hrs based on Heitkemper et al., 2001.

Narukawa et al. (2008) found that microwave extraction with water at 80°C for 30 minutes made it possible to recover As quantitatively (97–106%) from NIST 1568a, NIES no.10s rice grains and white rice flour without species transformation. However, the extraction from Narukawa et al. (2008) only recovered 39% of total As in whole grain rice from our preliminary experiments (Fig. S2). Especially the recovery of DMA was only 23%. These results indicate Narukawa's method strongly is influenced by the rice grain types, where the differences in extraction efficiency are not clearly known. In comparison, TFA method recovered 96% of total As from whole grain rice, showing little dependence (Fig. S2, Heitkemper et al., 2001). Additionally, the resulting extracts from Narukawa et al. (2008) were not able to pass through the filter for HPLC (Table S1). The issue with filtration was solved with addition of α-amylase. However, extraction with water and α-amylase at 80, 95 and 100°C with heating blocks or microwave ovens recovered only 60-80% amount of As from whole grain rice (Fig. S3). Extraction based on water and α-amylase showed apparent increase of recoveries with time at constant temperatures. This shows enzymatic extraction is sensitive to the extraction time. After 1.5 hours, the recovery changes little. The recovery of As did not improve with the temperature increase from microwave extraction at 120°C (Fig. S3). The filtration was also difficult in this condition.
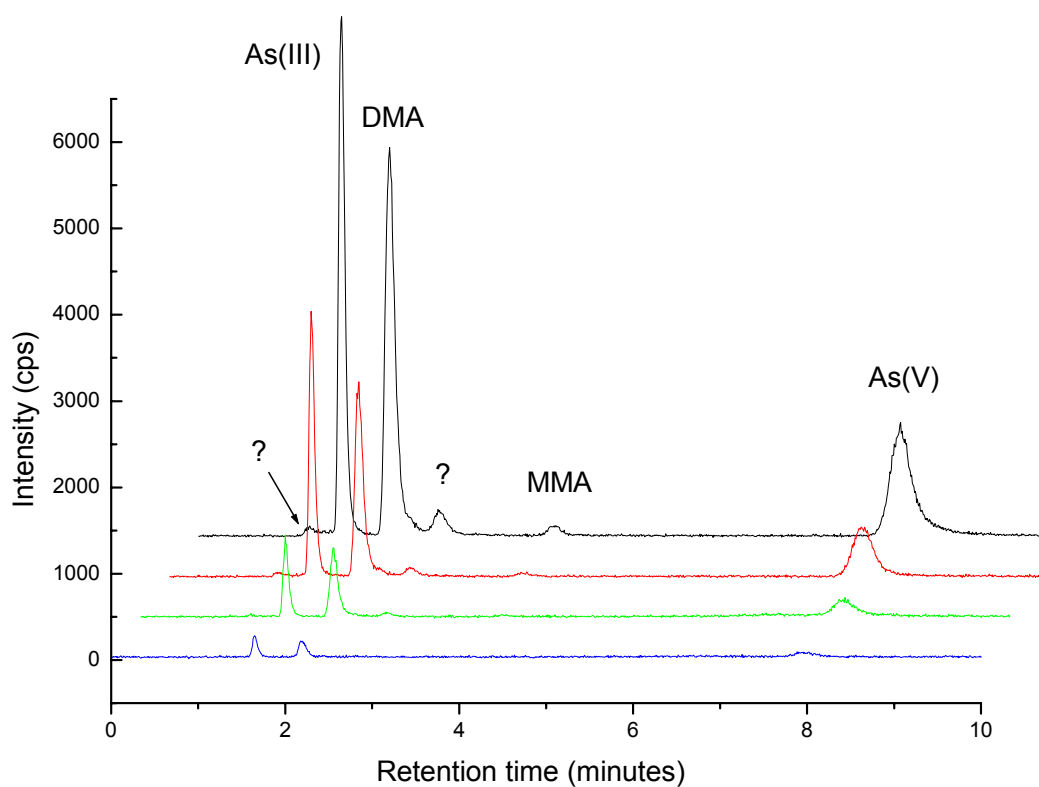
Fig. S3. Recoveries of total arsenic from whole grain rice extracted with water and α-amylase during different periods of time and at different temperatures with either heating blocks or microwave ovens. Mean values and S.D.s of triplicate extraction are shown

In comparison, the use of relatively strong extractants like TFA and concentrated HNO_3 and HCl may recover higher amounts of As from rice grains (Schoof et al., 1999; Heitkemper et a., 2001; Baba et al., 2008). Nevertheless, inorganic As species (As(V) or As(III)) underwent either reduction (TFA, Heitkemper et a., 2001) or oxidation (68% HNO_3, Baba et al., 2008), while organic As species were usually resistant against these relatively strong extractants (Heitkemper et a., 2001, Baba et al., 2008) (Table S1). Schoof et al. (1999) investigated the stability of As specie in 2 M HCl solution at 80°C with 21 different food. The recovery of spiked As(V) was slightly lower than other As species. This indicates As(V) loss during HCl treatment. Hence, As speciation in rice based on TFA and concentrated acid extraction was usually categorized into different individual organic species along with inorganic fraction without differential determination of As(III) and As(V) (Schoof et al., 1999; Sun et al., 2008; 2009; Meharg et al., 2008, 2009; Torres-Escribano et al., 2008). This results in ignorance of the much higher toxic effects of As(III) than As(V).

To our knowledge, quantitative extraction of As species without species transformation could show the combination of α-amylase and protease XIV in ultrasonication (Sanz et al., 2005) or microwave ovens (Guzmán Mar et al., 2009). However, both enzymatic methods showed large variation of total As recoveries from rice grains in different types. The microwave enzymatic extraction recovered 98 and100% of total As from NIST 1568a and jasmine rice, whereas the recoveries were 74 and 80% in basmati and white rice (Guzmán Mar et al., 2009). Similarly, the ultrasonication assisted method extracted around 90% of total As from R4-II, Spanish white and Indian basmati rice grain, but 82% of total As from SRM 1586a. We extracted whole grain rice after Sanz et al., 2005 and recovered 62 and 63% of total As (Fig. S2). Quantitative

extraction of total As with enzymatic extraction is suggested dependent on the type of rice grains. On the other hand, the control of the pH and temperature is usually essential during enzymatic treatment, although the authors declared no special care required (Sanz et al., 2005). Sanz et al. (2005) suggested that the presence of bacteriological activity during extraction might affect the enzymatic rupture of rice components. Guzmán Mar et al. (2009) observed trace amounts of As(V) in the protease XIV reagent. We also identified significant amount of As(V) in protease XIV with concentration of 0.44 ± 0.04 μg As g^{-1}. This affects the accuracy in detecting As species especially when As concentration is low in rice grain. Potential interference of the extraction matrixes on HPLC-ICP-MS analysis may occur in the enzymatic methods. We have observed different ICP-MS signal intensities from exactly the same extract (Fig. S4). It is known that addition of C such as methanol may elevate signal intensities in ICP-MS detection (Huang et al., 2004). It is suggested that the bacterial matrix may result in similar effects. Furthermore, enzymatic extraction is not favoured due to the high-cost of both enzymes.
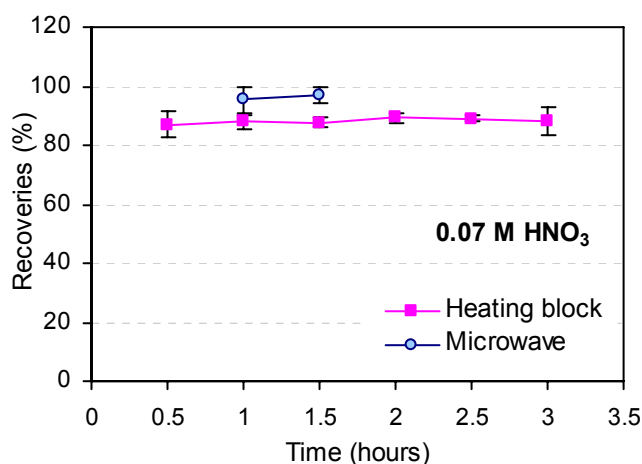

Fig. S4. Four replicate chromatograms of arsenic species from the same extract of whole grain rice extracted with α-amylase and protease XIV based on Sanz et al., 2005, showing the influence of enzymatic extraction matrix on ICP-MS signals.

Table S1. Comparison of different extraction methods for arsenic speciation in rice grains

Methods	Recoveries of total As	As(III) & As(V) Preservation	Organic As Preservation	Interference with further analysis	Rice type examined	References
Water or 10-90% MeOH, 55°C 10 h + 5 min sonication; one repetition	86-96%	Yes	Yes	No information available	NIST 1568a (long grain), CRM	Pizarro et al., 2003
Water, 80°C microwave 0.5 h	97-106%	Yes	Yes	No information available	NIST 1568a (long grain), NIES no10 (japonica)	Narukawa et al., 2008
Water, 80°C microwave 0.5 h (after Narukawa et al., 2008)	60-80%	Not tested	Not tested	Unfilterable	Whole grain rice	This study
50% MeOH, sonication 2.5 h	31-76%	Not tested	Not tested	No information available	3 types: long grain, long grain brown, wild	Heitkemper et al. 2001
0-100% MeOH, Accelerate solvent extraction, room temperature to 100°C	24-100%	Not tested	Not tested	No information available	3 types: long grain, long grain brown, wild	Heitkemper et al. 2001
Water or 50% MeOH, room temperature 6 hrs shaken	63-73%	Not tested	Not tested	No information available	1 type	Babe et al., 2008
50% MeOH, sonication 2 h	87%	Not tested	Not tested	No information available		Polatajko & Szpunar, 2004
Cold or 100°C water 15 min	8-41%	Not tested	Not tested	No information available	2 types: japonica, indica rice	Mihucz et al., 2007
2 M TFA, 100°C 6 h	92-112%	Partial As(V) reduction	Yes	Dilution done	3 types: long grain, long grain brown, wild rice	Heitkemper et al. 2001
2 M TFA, 90°C 6 h + 50% MeOH 1 hr shaken	97%	Not tested	Yes	No information available	1 type	Babe et al., 2008
68% HNO_3, 100°C 4 h	97%	As(III) oxidation	Yes	Dilution suggested	1 type	Babe et al., 2008
0.28 M HNO_3, 95°C 1.5 h	89-106%	Yes	Yes	No	6 types: japonica, round-sticky, long-grain, risotto, wild, whole grain rice (6)	This study
2 M HCl, 80°C 16 h	No information available	Not tested	Not tested	No information available	No information available	Shoof et al., 1999
α-amylase, 37°C overnight shaken + 50% MeOH sonication 1.5 h	59-97%	Not tested	Not tested	Dilution done	3 types: long grain, long grain brown, wild	Heitkemper et al. 2001
Protease XIV and α-amylase, ultrasonication, 3 min	82-91%	Yes	Yes	No information available	3-4 types: R4-II, NIST 1568a, Spanish white, Indian basmati	Sanz et al., 2005;
Protease XIV and α-amylase, microwave, 37°C, 30 min	74-100%	Yes	Yes	Dilution done	2-3 types: NIST 1568a (long grain), basmati, jasmine, white rice	Guzm´an Mar et al., 2009
Protease XIV and α-amylase, ultrasonication, 3 min (after Sanz et al., 2005)	62%	Not tested	Not tested	Yes	whole grain rice	This study
Protease XIV and α-amylase, ultrasonication, 3 min, one repetition (after Sanz et al., 2005)	63%	Not tested	Not tested	Yes	whole grain rice	This study

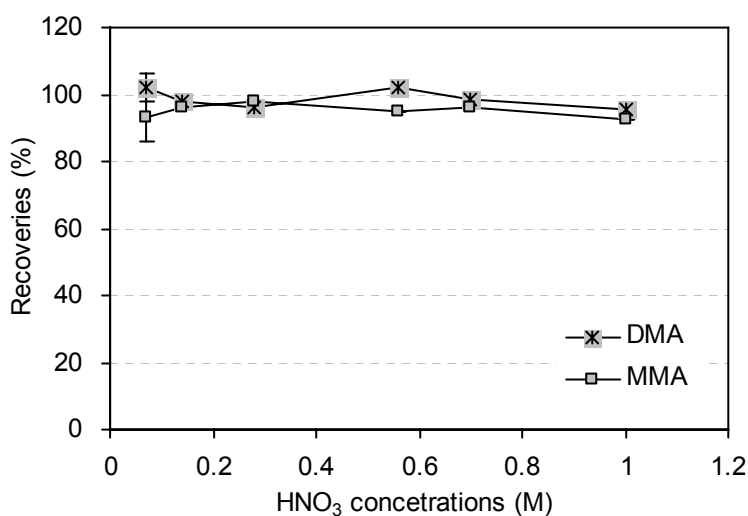*Development of method*

Optimization of extraction time

Fig. S5. Recovery of total arsenic from whole grain rice extracted with 0.07 M HNO_3 at 95°C with heating blocks and 100°C with microwave ovens for different length of extraction time. Mean values and S.D.s of triplicate extraction are shown.

Optimization of extraction temperature

The extraction efficiency for As(III), MMA and DMA increased with temperatures based on the results from microwave-assisted extraction (Fig. S7). However, it is not feasible to conduct extraction at the temperature higher than 100°C with heating blocks. 95°C was used for our proposed method.
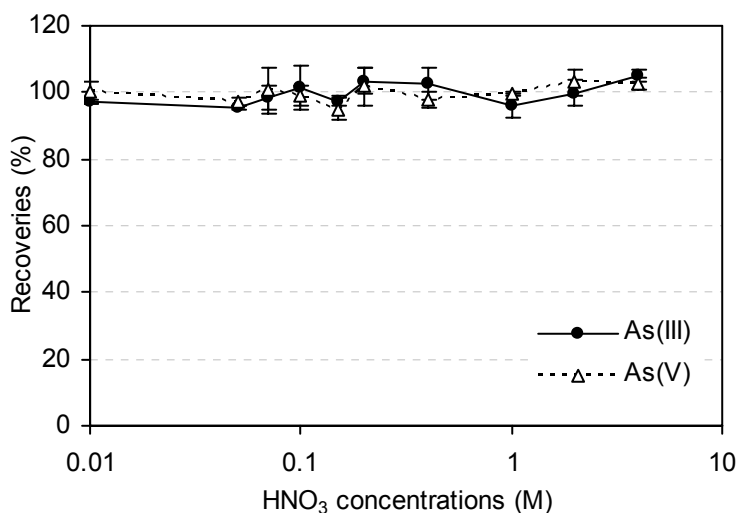
Optimization of HNO_3 concentration: arsenic redox transformation during rice extraction

To preserve As(III) and As(V) from redox transformation during rice grain extraction is challenging. As shown in Fig 1, satisfactory recovering spiked As(III) or As(V) during extraction with heated HNO_3 was achieved only from 0.28 to 0.70 M. We observed remarkable reduction of As(V) when HNO_3 concentration is smaller than 0.28 M. On the other hand, there is slightly but significant As(III) oxidation when HNO_3 concentration is larger than 0.70 M. These findings indicate the increasing oxidizing conditions with increasing concentrations of HNO_3, following well with the prediction based on Nernst equation.
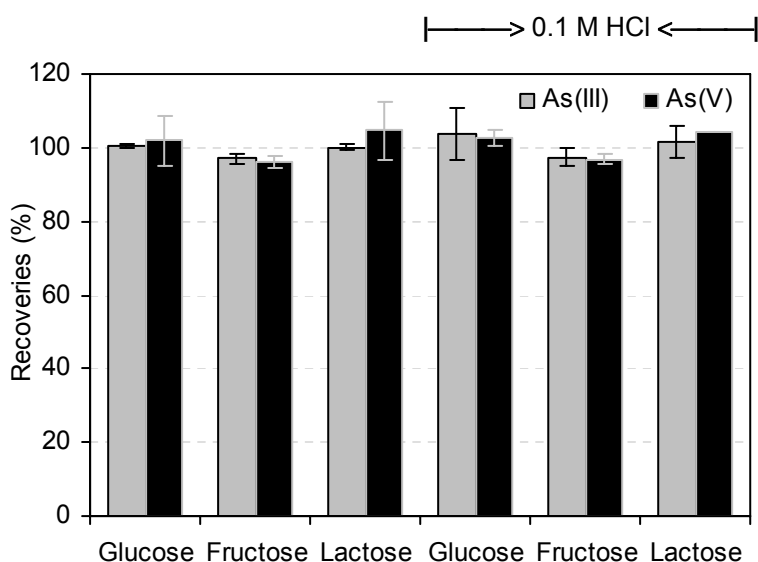

Fig. S6. Recoveries of individually spiked DMA and MMA at 50 μg As L^{-1} during extraction of whole grain rice using different concentrations of HNO_3 at 95°C for 1.5 hours. Mean values and S.D.s of triplicate extraction are shown.
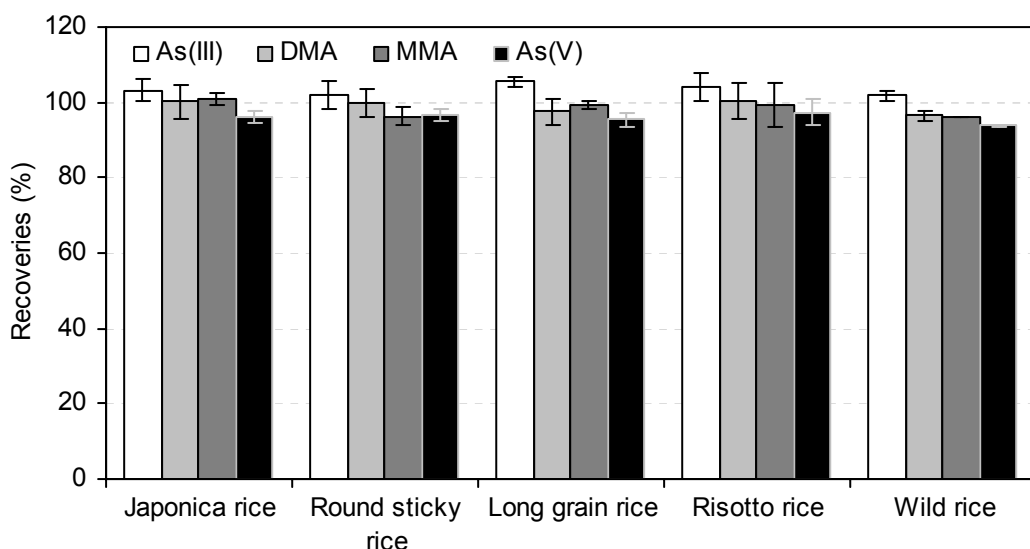
Fig. S7. Recoveries of As(III) and As(V) spiked at 15 μg As L^{-1} during treatments at 95 °C for 1.5 hours with different concentrations of HNO$_3$. Mean values and S.D.s of triplicate extraction are shown.

Fig. S8. Recoveries of As(III) and As(V) spiked at 15 μg As L^{-1} in 1 g L^{-1} glucose, fructose or lactose during treatments at 95 °C for 1.5 hours with and without 0.1 M HCl. Mean values and S.D.s of triplicate extraction are shown.

Validation tests

We have demonstrated that the resulting matrix during rice extraction may lead to As redox transformation. This suggests the type of rice may affect the extraction. Heitkemper et al. (2001) have also studied the extraction recoveries with different types of rice. Thus, we tested the stability and recoveries of As species in six different representative types of rice, including japonica, round-sticky, long-grain, risotto, wild and whole grain rice, with 0.28 M HNO$_3$ extraction at 95°C. The recoveries As ranged from 89 to 106% among different types of rice (Table S2). The results of recovery tests demonstrated the validation of the proposed extraction method for As speciation in rice grains, independent of types.

Fig. S9. Recoveries of simultaneously spiked As(III), DMA, MMA and As(V) at 50 μg As L^{-1} each during extraction of rice grains of different types using 0.28 M HNO$_3$ at 95°C for 1.5 hours. Mean values and S.D.s of triplicate extraction are shown. Data of whole grain rice is integrated in Fig 3 and Fig S8.

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