JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Analysis of Bioavailable Arsenic in Rice with Whole Cell Living Bioreporter Bacteria

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A multiwell plate bioassay was developed using genetically modified bacteria (bioreporter cells) to detect inorganic arsenic extracted from rice. The bacterial cells expressed luciferase upon exposure to arsenite, the activity of which was detected by measurement of cellular bioluminescence. The bioreporter cells detected arsenic in all rice varieties tested, with averages of 0.02−0.15 μg of arsenite equivalent per gram of dry weight and a method detection limit of 6 ng of arsenite per gram of dry rice. This amounted to between ≈20 and 90% of the total As content reported by chemical methods for the same sample and suggested that a major proportion of arsenic in rice is in the inorganic form. Calibrations of the bioassay with pure inorganic and organic arsenic forms showed that the bacterial cells react to arsenite with highest affinity, followed by arsenate (with 25% response relative to an equivalent arsenite concentration) and trimethylarsine oxide (at 10% relative response). A method for biocompatible arsenic extraction was elaborated, which most optimally consisted of (i) grinding rice to powder, (ii) mixing with an aqueous solution containing pancreatic enzymes, (iii) mechanical shearing, (iv) extraction in mild acid conditions and moderate heat, and (v) centrifugation and pH neutralization. Detection of mainly inorganic arsenic by the bacterial cells may have important advantages for toxicity assessment of rice consumption and would form a good complement to total chemical arsenic determination.

KEYWORDS: ***Escherichia coli*; ArsR; bacterial luciferase; bioreporter**

INTRODUCTION

Arsenic, a toxic but odorless and nearly tasteless element, is a natural component of many rocks and soils. Under certain geochemical conditions and upon microbiological activity arsenic is dissolved from minerals and can contaminate groundwater. This phenomenon is particularly pronounced in the Bengal Basin, where high arsenic levels in groundwater used for drinking water pose considerable health hazards (*1*, *2*). As a result of extensive well-drilling programs, which started in the 1970s, some 35−50 million people in Bangladesh and West Bengal are nowadays using arsenic contaminated groundwater as their source of drinking water (*2*). In the most severely affected districts, people are exposed to arsenic in potable water at up to 2 mg of As/L (*3*), as compared to the provisional WHO guideline value of 10 μg of As/L. In such areas, arsenic-related health effects such as skin lesions and skin and internal cancers are common and widespread.

Although the full extent of the arsenic threat in Bangladesh and West Bengal (India) is becoming relatively detailed, it is increasingly recognized that arsenic pollution is not restricted to this area only (*4*). Water quality monitoring programs have demonstrated that neighboring countries, such as China, Vietnam, Thailand, and Taiwan, also suffer from arsenic contamination in potable water sources (*5*). In many cases arsenic levels in drinking water considerably exceed the WHO guidelines or prevailing national standards (often, 50 μg of As/L).

Unfortunately, arsenic pollution does not stop at the level of potable water sources. Recent surveys have suggested that arsenic is entering into food sources, in particular, rice. This is mainly due to the use of arsenic-rich groundwater for the irrigation of paddy fields, which effectively is resulting in an increase of arsenic in soils and rice plants (*6*, *7*). Although relatively high arsenic concentrations in rice are not unusual, concentrations of arsenic in rice varieties collected in affected areas in Bangladesh are often higher than 1 μg of As/g of dry material (*7*, *8*). Subsequent cooking of rice in contaminated water may even boost its arsenic level by an additional 10−35% (*9*, *10*).

Although rice can contain a wide variety of arsenicals, the inorganic arsenic forms, arsenite [As(III)] and arsenate [As(V)], and the methylated organic forms, such as dimethylarsinic acid (DMAA), are in general predominant (*11*). Interestingly, the proportion between inorganic and organic arsenic species can vary considerably from sample to sample and thus is an important parameter to measure (*11*). Exact data on the mode of action of arsenicals on human health are limited, but it is generally considered that inorganic arsenic forms are substantially more toxic than the organic forms (*12*, *13*). This may

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10.1021/jf0631676 CCC: $37.00
Published on Web 02/21/2007

explain why the provisional maximum tolerable daily intake for arsenic given by the FAO/WHO Expert Committee on Food Additives is based on inorganic arsenic intake and not on total arsenic intake.

Because arsenic analysis in water and food samples via chemical means is particularly cumbersome and involves quite sophisticated instruments and harsh chemical conditions, we evaluated whether a simple whole cell bacterial bioassay could be adopted to measure arsenic in rice. This bioassay, which is based on arsenic detection by genetically modified *Escherichia coli* bacteria expressing bioluminescence, was previously developed and extensively validated for measuring arsenic in potable water sources (*14*−*16*). Similar bacterial bioassays for arsenic detection have been developed by others and shown their usefulness under a variety of conditions (*17*−*19*). An additional importance of a bioassay could be that arsenic availability to bacterial cells is representative of human arsenic toxicity and thus can be used instead of or complementary to a total chemical arsenic analysis (*20*). Here we modified and optimized arsenic extraction procedures from rice to be compatible with the bioassay. Our data strongly suggest that the bacterial cells in the bioassay discriminate between inorganic and organic arsenic, reacting only to the inorganic forms.

MATERIALS AND METHODS

Reagents and Standards. Arsenite calibration standards were prepared from a commercial stock solution of 0.05 mol of As(III)/L (3.75 g of As/L) (Merck, Darmstadt, Germany). Stock solutions of 0.05 M arsenate [As(V)], 11.1 mg/L arsenocholine (AsChol), 9.8 mg/L arsenobetaine (AsBet), 9.35 mg/L dimethylarsinic acid (DMAA), 12.6 mg/L monomethylarsonic acid (MMAA), 12.3 mg/L tetramethylarsonium iodide (TMAI), and 10.4 mg/L trimethylarsine oxide (TMAO) were kindly provided by Daniel Hammer, Nestlé Research Center (NRC), Vers-chez-les-Blanc, Switzerland. Pancreatin powder (from hog pancreas) and 65% nitric acid (HNO_3, ultrapure) were purchased from Fluka (Buchs, Switzerland). All reagents were stored at 4 °C in the dark, and standards were diluted fresh from concentrated stock solutions before every measurement. Except when described otherwise, all solutions were prepared with ultrapure deionized water obtained from a Milli-Q system (Millipore, Molsheim, France).

Reference Materials and Rice Samples. Certified reference rice flour (NIST SRM 1568a) was purchased from the U.S. Department of Commerce (National Institute of Standards and Technology, Gaithersburg, MD). Samples of different rice varieties were kindly provided by Daniel Hammer (designated "N") or Paul Williams and Andy Meharg (University of Aberdeen, Aberdeen, U.K.; designated "M"). Samples were stored as dry grains in screw-cap polypropylene tubes at room temperature.

Sample Preparation. The reference rice flour was used without any further preparation. All other rice samples were milled in an A11 Basic IKA Analytical Mill (IKA-Werke GmbH & Co., Staufen, Germany) to obtain a dry powder. The rice powder was then used in a mild extraction protocol involving one or more of the following steps: extraction with nitric acid, mechanical disruption with glass beads, incubation with a pancreatin mixture, moderate heating, and neutralization with phosphate buffer. For extraction with nitric acid, we accurately weighed about 0.1 g of rice powder in a 2 mL screw-cap polypropylene tube (Sarstedt, Nömbrecht, Germany) and added 1.5 mL of 15 mM HNO_3. In case of mechanical disruption, addition of rice powder was followed by the addition of an approximately equal volume of glass beads (106 μm and finer, acid washed, Sigma-Aldrich, Basel, Switzerland) and 1.5 mL of 15 mM HNO_3, after which the samples were processed in a FastPrep FP 120 cell disrupter (BIO 101 Savant Instruments, Holbrook, NY) according to the following protocol: mixing for two periods of 45 s at speed 6, cooling of the samples on ice for about 15 min, mixing for two periods of 45 s at speed 6. By accident we discovered that some batches of glass beads (Braun) were contaminated with arsenic, and their use needs to be avoided. Sample tubes were placed in hybridization bottles that were incubated overnight (approximately 14 h) at 55 °C in a rotisserie hybridization oven turning at approximately 20 rpm. The next day, the tubes were centrifuged in a microcentrifuge (Heraeus Biofuge Pico, Kendro, Geneva, Switzerland) at 13000 rpm for 1 min. Subsequently, 1 mL of each supernatant fluid was transferred to a fresh 1.5 mL polypropylene tube. Sample pH of the supernatants was adjusted at 7 with 0.1 mL of phosphate buffer (0.67 M sodium phosphate, pH 7) and used for the bioreporter assay.

Table 1. Different Arsenic Forms Used in the Bioassays

organic arsenic compd	abbrev	concn of stock solution (mg/L)	concn in the assay (mg/L)
arsenocholine	AsChol	11.1	0.111
arsenobetaine	AsBet	9.8	0.098
dimethylarsinic acid	DMAA	9.35	0.094
monomethylarsonic acid	MMAA	12.6	0.126
tetramethylarsonium iodide	TMAI	12.3	0.123
trimethylarsine oxide	TMAO	10.4	0.104

In the case of pancreatin incubation, rice powder samples were weighed and glass beads added as before, but complemented with 1.4 mL of 15 mM HNO_3 in the screw-cap polypropylene tubes. After the cell disrupter treatment, samples were allowed to cool to room temperature and then supplied with another 0.1 mL of 15 mM HNO_3 containing about 15 mg of pancreatin powder. Samples were now first incubated at 37 °C (for 8 h) and then at 55 °C (overnight or ≈14 h). The next day, sample tubes were centrifuged as before, and 1 mL of each supernatant fluid was transferred to a fresh 1.5 mL Eppendorf tube and boiled in a water bath for 15 min to inactivate the pancreatin. After cooling to room temperature, the sample pH was adjusted to 7 with 0.1 mL of phosphate buffer.

Bioreporter Assay. The basic principles of the bioreporter assay have been described by Stocker et al. (*14*) and Trang et al. (*15*). In brief, cell suspensions of *E. coli* DH5α (pJAMA-arsR) are incubated with an aqueous sample solution. The bacterial cells express the ArsR transcription repressor that functions as a sensory protein for the intracellular presence of arsenite. In the absence of arsenite, ArsR represses expression of the genes for bacterial luciferase via direct promoter−operator interaction, but when arsenite is encoutered by ArsR, it loses affinity for the operator (*21*) and unleashes luciferase expression. The bioluminescence production from bacterial luciferase is proportional to the concentration of arsenite within a certain range and can be easily recorded by a luminometer. Arsenate is detected via the intracellular conversion of arsenate to arsenite via arsenate reductase.

Bioreporter cell stocks were prepared by inoculating 5 mL of Luria broth (Biolife, Milan, Italy) supplemented with 50 μg/mL ampicillin with one colony of *E. coli* (pJAMA-arsR) from a freshly streaked plate (LB agar with 50 μg/mL ampicillin). After overnight incubation at 37 °C, 1 mL of this culture was transferred into 50 mL of LB medium. Cells were grown at 37 °C until the turbidity at 600 nm reached 0.6. Subsequently, the culture was placed on ice for about 15 min, supplemented with 10 mL of ice-cold sterile glycerol [87% (v/v)], and thoroughly mixed. This mixture was divided into aliquots of 0.65 mL that were stored in sterile 1.5 mL polypropylene tubes at −80 °C.

Bioreporter assays were performed in 96-well microtiter plates (Microlite 1, Catalys AG, Wallisellen, Switzerland). For each microtiter plate, four Eppendorf tubes with frozen bioreporter cells were thawed at room temperature, centrifuged (13000 rpm, 1 min), and decanted without removal of the cell pellet. Each pellet was resuspended in 0.4 mL of LB medium, and the bioreporter cell suspensions were pooled in a fresh 2 mL polypropylene tube.

Assay mixtures were directly prepared in the microtiter plates. First, 20 μL of 10-fold concentrated LB medium was pipetted into each well. Then, 170 μL of sample (or arsenite standard solution) and 10 μL of bioreporter cell suspension were added, after which the solution was mixed with the pipet. Arsenite standard series ranged from 0 to 0.40 μM As(III) [0−30 μg As(III)/L]. To correct for matrix interference, standard series were prepared as similar as possible to the rice extracts. For such standards the arsenite stock solution was diluted in 15 mM HNO_3, after which 10 vol % of phosphate buffer (e.g., 0.1 mL of buffer/mL of sample) was added to adjust the pH to 7.

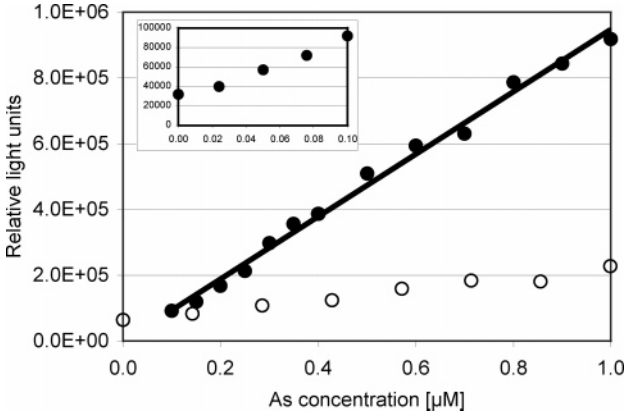

Figure 1. Light response of the bacterial cells in the bioassay as a function of arsenic concentration (micromolar): (solid symbols) arsenite [As(III)]; (open symbols) arsenate [As)V)]. (Inset) Light production at very low arsenite concentrations.

Plates were covered with Parafilm and incubated at 30 °C in a rotary shaker at 500 rpm (Thermostar, BMG Labtech, Offenburg, Germany) for about 1 h. After incubation, 20 μL of *n*-decanal substrate solution (2 mM in 50% v/v ethanol in water) was added to each well. The solutions were again mixed by pipetting and incubated at room temperature for about 3 min. Subsequently, bioluminescence production was measured in a Microlumat LB960 luminometer (Berthold Technologies, Bad Wildbad, Germany) as the integrated value over a 1 s period.

Validation of the Bioassay Specificity. To verify whether organic arsenicals would interfere with the bioreporter response, calibration assays were carried out either with arsenite alone, with arsenite plus one of the organic arsenic forms (**Table 1**) in different concentrations, or with organic arsenic compounds individually. Recovery efficiencies were examined by spiking samples with 0.2 μM As(III).

RESULTS

Calibration of the Luciferase Bioreporter Assay. Given the expected low arsenic level in rice, we first optimized the arsenic calibration curve in the range of arsenite concentrations between 0 and 0.1 μM. In **Figure 1**, the light emission from the *E. coli* DH5α (pJAMA-arsR) cells is plotted as a function of the arsenite concentration in the assay, ranging from 0 to 1.0 μM As(III). Between 0 and 0.1 μM arsenite, the data can be fitted to a logarithmic regression line (see inset), whereas between 0.1 and 1.0 μM the luciferase output was essentially linearly proportional to the As(III) concentration, as had been demonstrated before (*14*, *15*). Calibration curves were produced from the averages of three replicates. Error bars are smaller than the size of the used symbol and therefore not visible. Arsenic concentrations in unknown samples were interpolated from the logarithmic (0−50000 light units) or the linear relationships (>50000 light units). The method detection limit calculated from the interpolated arsenite equivalent concentration at a light emission of that of the blank average plus 3 times the average deviation in the blank was 5.4 nM in aqueous solution, which corresponds to 6.0 ng of arsenite/g of rice dry weight via the extraction procedure ($n = 9$, range = 2−12 ng/g).

Influence of Different Arsenic Forms on the Bioreporter Response. To determine which of the arsenic forms (organic and inorganic) the bioreporter cells were detecting, we performed calibration series with both inorganic and organic arsenic compounds individually and in combination with arsenite. **Figure 1** shows that the highest response of the bioreporter is elicited with arsenite, As(III). The bioassay response is reduced

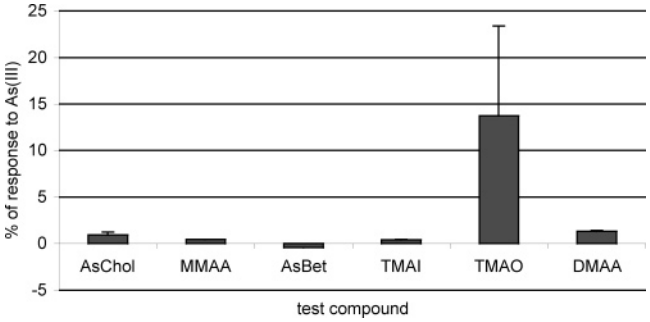

Figure 2. Response of the bioreporter cells to individual organoarsenic compounds. All compounds were added at ≈100 μg/L. Response is given relative to 1 μM As(III) solution (=78 μg/L). Abbreviations: AsChol, arsenocholine; MMAA, monomethylarsinic acid; AsBet, arsenobetaine; TMAI, tetramethylarsonium iodide; TMAO, trimethylarsine oxide; DMAA, dimethylarsinic acid.

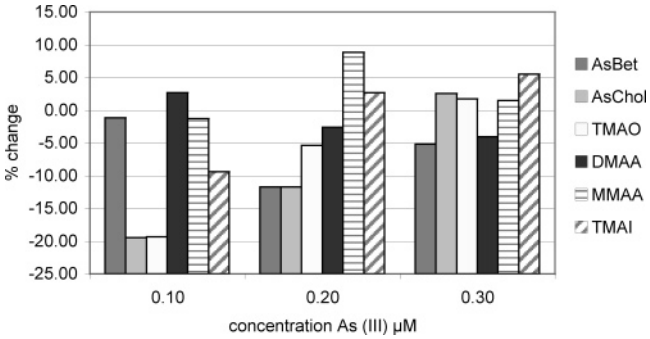

Figure 3. Influence of organoarsenicals on the bioreporter response to arsenite, tested at three different concentrations (0.1, 0.2, and 0.3 μM). Organoarsenicals were added at concentrations of ≈80 μg/L [at 0.1 μM As(III)], 50 μg/L [at 0.2 μM As(III)], and 25 μg/L [plus 0.3 μM As(III)].

to about 25% with equivalent concentrations of arsenate, As(V). Of the organoarsenicals tested, only trimethylarsine oxide (TMAO) at 100 μg/L caused a response $^1/_{10}$ of that of an equivalent concentration of As(III). None of the other organic arsenic compounds gave a response at 100 μg/L significantly different from the control (**Figure 2**). Higher concentrations were not tested.

Subsequently, we tested if the simultaneous presence of organoarsenicals could inhibit or enhance the response of the cells with As(III). At ≈80 μg/L, TMAO and arsenocholine reduced the response of the cells to 0.1 μM As(III) (8 μg/L) by 20% (**Figure 3**). Concentrations of 50 μg/L of arsenobetaine and arsenocholine reduced the response to 0.2 μM of As(III) by 10%. This indicates that the biosensor (i) essentially reacts only to inorganic arsenic, with a high preference for As(III), and (ii) may underestimate the "true" inorganic arsenic concentration when high concentrations of TMAO, AsChol, or AsBet are present. Samples with a high proportion of arsenate to arsenite will be undervalued when the bioreporter response is expressed as "arsenite-equivalent" concentration.

Optimization of the Arsenic Extraction Procedure from Rice. The most difficult step during arsenic analysis in rice is to extract the various arsenic species in their original form from the sample. Various extraction techniques for rice have been reported in the literature (*10*, *22*, *23*). Chemical-based extractions using trifluoroacetic acid or water−ethanol and water−methanol mixtures have been shown to provide good extraction efficiency without internal species conversion. In this study, however, "gentler" methods had to be developed, which would not directly

Table 2. Arsenic Content after Nitric Acid Extraction with or without Mechanical Disruption

				biosensor assay	
sample	total As (μg of As/g)	total inorg As (as reported in the literature) (μg of As/g)	% of total	μg of As equiv/g[a]	% of total
NIST CRM 1568a	0.29	0.08 ± 0.014[b]	28	0.034	12
		0.101[c]	35	0.045[d]	16
N 010	0.245[e]			0.088	36
N 058	0.132[e]			0.055	42
N 061	0.053[e]			0.017	32
N 077	0.092[e]			0.062	67

[a] Values from the biosensor assay are reported as concentrations equivalent to the response with arsenite. [b] Reference *8*. [c] Reference *10*. [d] Mechanical cell disruption plus extraction with HNO_3. [e] Communicated by Daniel Hammer, NRC.

inhibit the bacterial cells in the bioassay. On the basis of the literature data on chemical arsenic analysis of rice, we tested combinations of (i) grinding rice to powder, (ii) extraction with water or mild acid conditions (HNO_3), (iii) digestion of the rice matrix with pancreas enzymes, (iv) mechanical disruption, and (v) heat. In all cases, the procedure had to be optimized to allow the bacterial reporter cells to be active in the final aqueous rice extract. When using pancreatin, it appeared to be necessary to inactivate the enzymes by heating at 100 °C. Without this inactivation step, the pancreatin, being a mixture of lipase, protease, and amylase, inhibited the bacterial cells in the subsequent bioreporter assay (not shown).

Compared to the NIST standard rice, extraction with nitric acid at 55 °C for 16 h and subsequent neutralization resulted in arsenic contents of 0.034 μg of As(III) equivalent/g of rice. On the basis of the assumption that this is only arsenite, this would be equivalent to between 32 and 43% of the reported total inorganic As content of NIST (**Table 2**).

For several other rice samples the reporter cells measured between 36 and 67% inorganic available arsenite equivalent in extracts made with HNO_3 at 55 °C compared to the total arsenic concentration (values not corrected for the extraction efficiency based on the NIST standard). When mechanical disruption with glass beads was included in the protocol, between 46 and 58% of the total inorganic arsenic in NIST rice was recovered (**Table 2**), which was significantly higher than without disruption.

On the basis of the results of extractions under mild acidic conditions and mechanical disruption, it was decided to add another step involving digestion of the rice matrix by pancreatic enzymes. By including this step, the recovery of inorganic detectable arsenic from NIST could be increased to between 69 and 92% (**Table 3**). Consequently, also the detectable inorganic arsenic content from the four unknown rice samples increased to between 54 and 100%, suggestive of the fact that some rice had mainly inorganic arsenic. By analyzing another set of rice samples of which the inorganic arsenic had previously been measured by traditional chemical methods, we could determine that the overall inorganic arsenic recoverable fraction from unknown rice samples is on the order of 50−100%, probably fluctuating depending on rice matrix factors that we could not resolve in our protocol. Precisions obtained were between 4.8 and 6.1 (RSD as percent of average, $n = 7$, each with triplicate light measurements), with 6.0% for rice with a total As content of 0.05 μg/g (range = 3.6−8.2), 3.6% for that with 0.11 μg/g (range = 2.5−4.7), and 4.9% for rice with 0.29 μg/g (range = 3.9−5.9).

Table 3. Arsenic Rice Content after Mechanical Disruption, Pancreatic Digestion, and Nitric Acid Extraction at 55 °C, As Determined by the Bioassay

				bioassay		
sample	total As (μg of As/g)	total inorg As (lit.) (μg of As/g)	% of total	μg of As equiv/g[a]	% of total	% of inorg
NIST	0.29	0.08 ± 0.014[b]	28	0.069 ± 0.005	24	68–75
		0.101[c]	35			
N 010	0.245[d]	–[e]	–	0.133 ± 0.006	54	
N 058	0.132[d]	–	–	0.118 ± 0.014	89	
N 061	0.053[d]	–	–	0.055 ± 0.004	104	
N 077	0.092[d]	–	–	0.097 ± 0.007	105	
NIST	0.29	0.08 ± 0.014	28	0.055 ± 0.002	19	55–69
		0.101	35			
M 01	0.232	0.072	30	0.060 ± 0.002	26	85
M 02	0.052	0.032	60	0.015 ± 0.002	30	50
M 11	0.142	0.062	43	0.030 ± 0.002	21	50
M 15	0.112	–	–	0.040 ± 0.003	36	
M 25	0.292	0.122	41	0.073 ± 0.005	25	61

[a] Values from the biosensor assay are reported as concentrations equivalent to the response with arsenite. [b] See **Table 2**. [c] As for **Table 2**. [d] As for **Table 2**. [e] No data reported.

Table 4. Effect of Shortening the Extraction Period

		bioassay (ng of As equiv/g)		
sample	total As[a] (ng of As/g)	procedure 1[b]	procedure 2[b]	procedure 3[b]
N 003	212	108	102	59
N 010	245	133	113	65
N 023	151	56	49	39
N 027	340	115	114	65
N 043	77	45	41	37
N 066	43	28	25	21
N 077	92	97	92	53
N 084	110	95	94	59
N 091	33	39	29	30
N 098	366	152	152	105

[a] Communicated by Daniel Hammer. [b] Procedure 1: 8 h at 37 °C, 16 h at 55 °C; procedure 2, 16 h at 55 °C; procedure 3, 8 h at 55 °C.

Influence of Extraction Duration on Arsenic Extraction Efficiency. As the overall extraction procedure of mechanical disruption−enzymatic digestion−heat acid extraction took around 24 h, we decided to test the effect of reducing the time span of the steps. These results (**Table 4**) indicate that a long extraction time at 55 °C is optimal and should not be shortened with the risk of extraction losses, but that the long digestion time at 37 °C contributes only to some 10% more of extracted arsenic from the sample. It is thus clear that the rapidity of the procedure is hampered by the extraction of arsenic from the sample. A further increase of the extraction temperature from 55 to 65 °C resulted in only 2% more detectable arsenic. On the contrary, some samples became gelatinous and much more difficult to handle.

With the most optimal procedure we tested the recovery of externally added arsenic to the rice (spiking). As can be seen from **Table 5**, spiking recoveries equaled between 87 and 114%, which can be contributed to an inaccuracy of determination of values from the calibration curve. It shows that no major losses of arsenite via adsorption to the rice matrix or via other steps in the extraction procedure are taking place.

Table 5. Spiking Recoveries

sample	As(III) recovery (as % of the amount spiked)
N 010	87
N 058	114
N 061	113
N 070	104
NIST CRM 1568a	89

Table 6. Possible Bioassay Decision Scheme

if bioassay outcome *X* [μg of As(III) equiv]	then	"true" inorg As concn	action
$X > T$[a]	unsafe	*T* or higher	avoid usage
$0.25T < X < T$	probably unsafe	$0.25T < X < 4T$	determine total As
$X < 0.25T$	possibly safe	$<T$	determine total As, if desired

[a] T = predefined threshold concentration.

DISCUSSION

Our results clearly demonstrated that it is possible to use a bioassay with bioreporter bacteria to detect inorganic arsenic in rice. As far as we are aware, this is the first instance of application of a bioreporter assay to measure As in a rice matrix and one of the very few others involving bacteria based gene reporter assays for toxicants in foodstuffs (*24*−*27*). Compared to detecting arsenic in water samples, rice poses a more difficult challenge due to its organic matrix. We adapted a procedure involving (i) grinding of rice to powder, (ii) mixing with an aqueous solution containing pancreatic enzymes, (iii) mechanical shearing, (iv) extraction in mild acid conditions and moderate heat, and (v) centrifugation and pH neutralization. The whole procedure of extraction took around 1 day. The subsequent bioassay (i.e., mixing the cells with the aqueous extract, incubating, and reading the light emission) is a procedure that takes around 2 h. Shortening parts of the extraction procedure or leaving out certain treatments resulted in a significant decrease in the amount of arsenic detected and thus is not recommended. Unless a radically different method for extraction of arsenic from rice or complete destruction of the rice matrix is achieved within short time, the determination of arsenic in rice remains relatively complex and time-consuming. On the other hand, the bioassay itself allows processing multiple samples at the same time and does not involve harsh chemicals, and several extraction steps run automatically, thus liberating time for other activities and reducing the time investment per sample.

The bioassay with bacterial bioreporters was highly repeatable, which can be seen from the small standard error in triplicate determinations and the overall precision (4.8−6.1%). The bacterial cells were not greatly disturbed by the remnants of the rice matrix in the aqueous extract, which could be concluded from spiking assays. This also means that no factors are extracted from the rice, which could positively falsify the signal obtained from the bacterial cells, such as growth factors or nutrients. Not unsurprisingly, the cells were sensitive to low pH, when pH neutralization was omitted, and to pancreatic enzymes, when these were not inactivated by heat.

The second important result from our bioassays was that arsenic was readily detectable in all rice samples tested. The sensitivity of the bioassay method (6 ng of As/g of rice dry weight) is in the range of reported method detection limits for standard chemical analysis (2−12 ng of As/g of rice) (*6*−*8*, *11*) and, therefore, quite competitive. This means that the sensitivity of the bacterial bioreporter cells was sufficient to detect inorganic arsenic at low levels. Typical values that were determined in the bioassay were 0.02−0.15 μg of As(III) equiv/g of dry weight. This amounted to between ≈20 and 90% of the total As content reported by chemical methods for the same sample. Because we could show that the bioreporter bacteria essentially detect preferably arsenite, followed by arsenate (at 25% sensitivity at the same concentration) and trimethylarsenoxide (at ≈10%), this means that bioassay values must be interpreted differently from chemical data. For this reason, we propose the use of "arsenite equivalent" concentrations, based on interpretation from the calibration curve simultaneously (but arbitrarily) carried out with arsenite.

For those samples for which simultaneous literature data existed on the composition of inorganic and organic arsenic forms (e.g., **Table 3**), we can see that the arsenite equivalent concentration determined in the bioassay was greater than 50% of the inorganic arsenic content determined by chemical methods. This suggests that a little bit less than 40% of the inorganic arsenic in rice is in the form of arsenite (detectable with 100% efficiency by the bioassay) and 60% as arsenate (detectable at 25% efficiency). The generality of this proportion can clearly be better substantiated when a larger sample set is analyzed both by chemical methods and with the bioassay, similar to a recently carried out analysis of potable water samples (*15*). Among those samples of which we only knew the total arsenic content (**Tables 2** and **4**), some contained 100% arsenite equivalent concentrations in the bioassay, suggesting arsenite to be the major species in those rice samples. On the other hand, none of the samples reacted with less than 25% arsenic equivalent concentrations in the bioassay, indicating that all rice varieties contain arsenite and arsenate in detectable forms.

It is obvious that the bioassay cannot be used for exact quantification of arsenic species in rice, but because of the selectivity of the cells in the bioassay for arsenite, one could envision a first rapid sample screening to determine an inorganic arsenic threshold or arsenic accessibility. By incorporating a safety factor of 4 (based on the 25% lowest arsenite equivalent concentration found here for some samples), one could define corresponding categories as "possibly safe", "probably unsafe", "unsafe" and define actions for further testing by chemical means (**Table 6**). Alternatively, chemical methods could be employed to convert all inorganic arsenic to arsenite before sample addition to the bioassay. Unfortunately, the standard chemical pretreatment for reduction of arsenate to arsenite by incubation with 1% KI and 0.5% ascorbate at low pH (10% HCl) appeared to be incompatible with the bioassay due to inhibitory effects of salt concentration, KI, and ascorbate (not shown). Because the bacteria react to only a fraction of the total arsenic, namely, the fraction formed by arsenite, arsenate, and TMAO, and in a genuinely biological manner, that is, involving transport, enzymatic modification, and expulsion from the cell, it might be that the bacterial response is actually a good representation for the arsenic toxicity in higher organisms. This may be particularly relevant because we used the assay in combination with the enzymatic pretreatment of rice, which was proposed to mimic release processes in the human intestine (*10*). This concept and hypothesis, however, will require further study and careful calibrations.

The major bottleneck for arsenic analysis in rice for the moment remains the extraction procedure. Despite optimization

procedures, the extraction procedure adaptable for the bioassay took 16−24 h, which is too long for a very quick assay. Further time reduction here must come from improvements in sample extraction. The main advantages of the assay itself are its simplicity, the fact that harsh chemicals are not needed, and the fact that bacterial cells differentiate clearly between inorganic and organic forms, which could be a feature to exploit further. The requirement for small sample volumes makes the bioassay an ideal method of choice for high sample throughput analysis.

SAFETY

Use of the genetically engineered *E. coli* DH5α bacterium is permitted under the lowest biological risk category in GLP laboratories.

ABBREVIATIONS USED

AsChol, arsenocholine; MMAA, monomethylarsinic acid; AsBet, arsenobetaine; TMAI, tetramethylarsonium iodide; TMAO, trimethylarsine oxide; DMAA, dimethylarsinic acid.

ACKNOWLEDGMENT

We greatly appreciate the support of Daniel Hammer, Andrew Meharg, and Paul Williams in providing rice samples, arsenic standards, and total arsenic analysis data.

LITERATURE CITED

(1) Nickson, R.; McArthur, J; Burgess, W.; Ahmed, K. M.; Ravenscroft, P.; Rahman, M. Arsenic poisoning of Bangladesh groundwater. *Nature* **1998**, *395*, 338.

(2) Smedley, P. L.; Kinniburgh, D. G. A review of the source, behaviour and distribution of arsenic in natural waters. *Appl. Geochem.* **2002**, *17*, 517−568.

(3) Tondel, M.; Rahman, M.; Magnuson, A.; Chowdhury, I. A.; Faruquee, M. H.; Ahmad, S. A. The relationship of arsenic levels in drinking water and the prevalence rate of skin lesions in Bangladesh. *Environ. Health Perspect.* **1999**, *107*, 727−729.

(4) Nordstrom, D. K. Worldwide occurrences of arsenic in ground water. *Science* **2002**, *296*, 2143.

(5) Berg, M.; Tran, H. C.; Nguyen, T. C.; Pham, H. V.; Schertenleib, R.; Giger, W. Arsenic contamination of groundwater and drinking water in Vietnam: a human health threat. *Environ. Sci. Technol.* **2001**, *35*, 2621−2626.

(6) Abedin, M. J.; Cresser, M. S.; Meharg, A. A.; Feldmann, J.; Cotter-Howells, J. Arsenic accumulation and metabolism in rice (*Oryza sativa* L.). *Environ. Sci. Technol.* **2002**, *36*, 962−968.

(7) Meharg, A. A.; Rahman, M. D. M. Arsenic contamination of Bangladesh paddy field soils: implications for rice contribution to arsenic consumption. *Environ. Sci. Technol.* **2003**, *37*, 229−234.

(8) Williams, P. N.; Islam, R.; Raab, A.; Hossain, S. A.; Zhu, Y. G.; Feldmann, J.; Meharg, A. A. Increase in rice grain arsenic for regions of Bangladesh irrigating paddies with elevated in arsenic groundwaters. *Environ. Sci. Technol.* **2006**, *40*, 4903−4908.

(9) Misbahuddin, M. Consumption of arsenic through cooked rice. *Lancet* **2003**, *361* (9355), 435−436.

(10) Ackerman, A. H.; Creed, P. A.; Parks, A. N.; Fricke, M. W.; Schwegel, C. A.; Creed, J. T.; Heitkemper, D. T.; Vela, N. P. Comparison of a chemical and enzymatic extraction of arsenic from rice and an assessment of the arsenic absorption from contaminated water by cooked rice. *Environ. Sci. Technol.* **2005**, *39*, 5241−5246.

(11) Williams, P. N.; Price, A. H.; Raab, A.; Hossain, S. A; Feldmann, J.; Meharg, A. A. Variation in arsenic speciation and concentration in paddy rice related to dietary exposure. *Environ. Sci. Technol.* **2005**, *39*, 5531−5540.

(12) Lewis, R. J., Tatken, R. L., Eds. *Registry of Toxic Effects of Chemical Substances*; U.S. Department of Health, Education and Welfare, Public Health Service, Center for Disease Control, National Institute for Occupational Safety and Health; U.S. Government Printing Office: Washington, DC, 1978.

(13) National Research Council of Canada (NRCC). *Effects of Arsenic in the Canadian Environment*; NRCC Publication 15391; NRCC: Ottawa, Canada, 1978.

(14) Stocker, J.; Balluch, D.; Gsell, M.; Harms, H.; Feliciano, J. S.; Daunert, S.; van der Meer, J. R. Development of a set of simple bacterial biosensors for quantitative and rapid field measurements of arsenite and arsenate in potable water. *Environ. Sci. Technol.* **2003**, *37*, 4743−4750.

(15) Trang, P. T. K.; Berg, M.; Viet, P. H.; Mui, N. V.; van der Meer, J. R. Bacterial bioassay for rapid and accurate analysis of arsenic in highly variable groundwater samples. *Environ. Sci. Technol.* **2005**, *39*, 7625−7630.

(16) Wells, M.; Gösch, M.; Rigler, R.; Harms, H.; Lasser, T.; van der Meer, J. R. Ultrasensitive reporter protein detection in genetically engineered bacteria. *Anal. Chem.* **2005**, *77*, 2683−2689.

(17) Corbisier, P.; Ji, G.; Nuyts, G.; Mergeay, M.; Silver, S. *LuxAB* gene fusions with the arsenic and cadmium resistance operons of *Staphylococcus aureus* plasmid pI258. *FEMS Microbiol. Lett.* **1993**, *110*, 231−238.

(18) Ramanathan, S.; Shi, W.; Rosen, B. P.; Daunert, S. Sensing antimonite and arsenite at the subattomole level with genetically engineered bioluminescent bacteria. *Anal. Chem.* **1997**, *69*, 3380−3384.

(19) Tauriainen, S.; Karp, M.; Chang, W.; Virta, M. Recombinant luminescent bacteria for measuring bioavailable arsenite and antimonite. *Appl. Environ. Microbiol.* **1997**, *63*, 4456−4461.

(20) Harms, H.; Wells, M. C.; van der Meer, J. R. Whole-cell living biosensors—are they ready for environmental application? *Appl. Microbiol. Biotechnol.* **2006**, *70*, 273−280.

(21) Wu, J.; Rosen, B. P. Metalloregulated expression of the ars operon. *J. Biol. Chem.* **1993**, *268*, 52−58.

(22) Chun-gang, Y.; Gui-bin, J.; Bin, H. Evaluation of the extraction methods for arsenic speciation in rice straw, *Oryza sativa* L., and analysis by HPLC-HG-AFS. *J. Anal. At. Spectrom.* **2005**, *20*, 103−110.

(23) Heitkemper, D.; Vela, N. P.; Stewart, K. R.; Westphal, C. Determination of total and speciated arsenic in rice by ion chromatography and inductively coupled plasma mass spectrometry. *J. Anal. At. Spectrom.* **2001**, *16*, 299−306.

(24) Hansen, L. H.; Sørensen, S. J. Detection and quantification of tetracyclines by whole cell biosensors. *FEMS Microbiol. Lett.* **2000**, *190*, 273−278.

(25) Pellinen, T.; Bylund, G.; Virta, M.; Niemi, A.; Karp, M. Detection of traces of tetracyclines from fish with a bioluminescent sensor strain incorporating bacterial luciferase reporter genes. *J. Agric. Food Chem.* **2002**, *50*, 4812−4815.

(26) Reunanen, J.; Sanis, P. E. Microplate bioassay for nisin in foods, based on nisin-induced green fluorescent protein fluorescence. *Appl. Environ. Microbiol.* **2003**, *69*, 4214−4218.

(27) Kurittu, J.; Lonnberg, S.; Virta, M.; Karp, M. Qualitative detection of tetracycline residues in milk with a luminescence-based microbial method: the effect of milk composition and assay performance in relation to an immunoassay and a microbial inhibition assay. *J. Food Prot.* **2000**, *63*, 953−957.

Received for review November 3, 2006. Revised manuscript received January 7, 2007. Accepted January 11, 2007. This work was supported by a grant from the Swiss Development Council.

JF0631676