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Speciated urinary arsenic as a biomarker of dietary exposure to inorganic arsenic in residents living in high-arsenic areas in Latium, Italy*

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Abstract: Current knowledge indicates that total urinary arsenic is not a suitable biomarker of exposure to toxic, i.e., inorganic, arsenic (iAs), whereas measurement of iAs and its methylated metabolites in urine using speciation analysis provides much more reliable estimates of exposure. The relative proportions of urinary iAs, monomethylarsonate (MA), and dimethylarsinate (DMA) can be used as a measure of methylation capacity, provided that there are no confounding factors such as consumption of food rich in DMA or containing As compounds metabolized to DMA.

We analyzed by gradient elution anion-exchange HPLC-ICP-MS (high-performance liquid chromatography-inductively coupled plasma-mass spectrometry) urine samples from 153 residents in Latium (central Italy) chronically exposed to iAs via water and food. Excluding 26 subjects that excreted high concentrations of arsenobetaine (AB) (≥50 µg As/L) due to seafood consumption, iAs and related metabolites summed up about 75 % of total urinary As as measured by ICP-MS. AB and other organoarsenic compounds were detected at low concentrations in all urine samples. Considering all subjects, the sum of iAs and metabolites ranged 2–72 µg/L and relative proportions were iAs 14 %, MA 13 %, and DMA 72 % (median values), with a wide individual variability.

In addition to the above arsenocompounds, the analytical method used in this study enabled the detection of dimethylthioarsinic acid (DMTA), which was found to be present in 33 % of the samples at concentrations ranging mostly from trace amounts to ~6 µg As/L. We found that part of the certified DMA content of human urine reference material SRM 2669 was present as DMTA. Four unknown arsenicals were also detected as minor species in a small proportion of samples.

Keywords: arsenic; biomarkers; dietary exposure; speciation; urine.

INTRODUCTION

Inorganic arsenic (iAs) is a well-established human carcinogen [1], and oral exposure is associated with a wide range of adverse health effects, in addition to cancer, such as developmental toxicity, neuro-

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toxicity, cardiovascular diseases, abnormal glucose metabolism, and diabetes [2,3]. There is emerging evidence of negative impacts on fetal and infant development, and susceptibility to the toxic effects may vary considerably between individuals depending on inter-individual variations in As metabolism [2,3].

Tens of millions of people are exposed world-wide via As-contaminated groundwater, used as drinking water, for crop irrigation, and in food preparation and cooking [4,5]. For populations not exposed to As-contaminated drinking water, food is the major contributor to the intake of inorganic As. Cereal grains and cereal-based products, followed by food for special dietary uses, bottled water, coffee and beer, rice grains and rice-based products, fish, and vegetables were identified as largely contributing to the iAs daily exposure in the general European population [2]. High consumers of rice and algae-based products are estimated to have a daily dietary exposure of iAs considerably above the average [2].

Ingested iAs is largely biotransformed and excreted mainly as monomethylarsonate (MA) and dimethylarsinate (DMA, see Table 1 for structures of relevant As species). Available data suggest that humans generally excrete fairly constant ratios of 10–30 % iAs, 10–20 % MA, and 60–80 % DMA [6]. Urinary DMA percentage has been regarded as an indicator of methylation efficiency. Some authors calculate the primary methylation index defined as the ratio between MA and iAs (arsenate + arsenite) level, and secondary methylation index as the ratio between DMA and MA to assess the As methylation capacity of the first and second methylation step, respectively [2]. Marked inter-individual variations in As metabolism have been observed depending on such factors as age, life stage, gender, nutritional status, and genetic polymorphism in the regulation of enzymes responsible for As biotransformation [2,7–9].

Table 1 Structure of As species relevant to this study.

Name	Abbreviation	Chemical structure
Arsenite	AsIII	$As(O^-)_3$
Arsenate	AsV	$O{=}As(O^-)_3$
Arsenobetaine	AB	$(CH_3)_3As^+CH_2COO^-$
Arsenocholine	AC	$(CH_3)_3As^+CH_2CH_2OH$
Monomethylarsonate	MA	$CH_3AsO(O^-)_2$
Dimethylarsinate	DMA	$(CH_3)_2AsO(O^-)$
Dimethylthioarsinate	DMTA	$(CH_3)_2AsS(O^-)$
Trimethylarsine oxide	TMAO	$(CH_3)_3AsO$

Inorganic arsenic is the component of real concern within the overall intake of As. Thus, total urinary As is not a suitable biomarker of exposure to dietary iAs. Seafood can contain high concentrations of organic As compounds, in particular AB presenting no toxicological concern, and consumption of even small amounts of such food may markedly increase the total urinary As concentrations, thereby leading to an overestimation of the exposure to iAs [10,11]. Specific measurement of the iAs and its methylated metabolites in urine (MA and DMA) provides much more reliable estimates of iAs exposure. In European reference populations with no occupational As exposure, no seafood consumption at least 48 h prior to urine sampling and As concentrations in drinking water far below 10 µg/L, mean concentrations of urinary iAs and related metabolites were around 5–6 µg/L [10,12–14]. Assuming excretion of 1–2 L urine a day, a concentration of, e.g., 5 µg/L iAs and related metabolites would correspond to an intake of about 5–10 µg iAs/day. Several studies from Europe, the United States, South America, and southeast Asia have indicated a roughly 1:1 ratio between the sum of the concentrations of iAs and related metabolites in urine and the concentrations of iAs in water in cases where the As intake from water exceeds that from food [5,7,9,15–17]. However, when levels of

As in drinking water are lower, the ratio of the sum of urinary iAs and related metabolites in urine to iAs in water is higher than 1.

The sum of iAs and related metabolites in urine may be affected by the ingestion of DMA contained in food. DMA is normally present at relatively low levels in most food, but rice as well as some algae and seafood are notable exceptions. In populations consuming large quantities of rice, the higher dietary intake of DMA alters the DMA/MA ratio in urine and such ratio should be used with caution as an indication of methylation capacity [18]. Also, the ingestion of the arsenosugars and arsenolipids present in seafood, which are metabolized into DMA, may affect the speciation of the different metabolites of iAs used to evaluate the efficiency of As metabolism [19]. Therefore, it is still recommended that the individuals under study refrain from seafood consumption a few days before sampling even when speciated urinary As is used to assess iAs exposure.

In Latium (central Italy), As concentrations exceeding the regulatory limit of 10 μg/L for drinking water have been found in groundwater from three provinces encompassing a large area of volcanic origin [20–22]. High As concentrations have been detected also in topsoil and subsoil, whereas a recent survey showed that—at least in part of the area—geogenic As in agricultural soils is largely phytoavailable and enters the food chain [23]. As a result, the local population may be exposed to iAs via water, used mainly for food preparation and cooking, and also through consumption of locally grown food with higher than background As concentrations. As part of a study investigating iAs exposure and metabolism in non-occupationally exposed residents living in this high-As area, we report the analysis of 153 urine samples by high-performance liquid chromatography-inductively coupled plasma-mass spectrometry (HPLC-ICP-MS) and discuss the need for analytical approaches directed toward the specific determination of the entirety of toxicologically relevant urinary metabolites in studies on As biomonitoring and toxicology.

METHODS

Study population and sample collection

This study is part of a survey aimed at assessing iAs exposure and metabolism in non-occupationally exposed residents living in an As-rich area of Latium (central Italy). On the whole, the population residing in the As affected area consists of ca. 814 000 persons. The overall study comprised subjects (n = 269) recruited in three different provinces in Latium, namely, Rome, Viterbo, and Latina, between November 2010 and March 2011. The study areas within each province were selected based on their levels of As in drinking water identified through analyses carried out before and during biological sample collection. Each prospective participant was first interviewed by a local recruiter to ascertain age, duration of residence, drinking water source, and interest in participation. They were asked to participate if they had lived in the study area at least for the last two months, used local tap or well water for drinking and/or cooking, and if they were in good health. In order to assess total dietary exposure to iAs and identify the main contributors to the exposure, an integrated approach was adopted combining biomarkers of exposure, As speciation in local food and duplicate diets, and a study of the impact of As-rich water in food processing and cooking. The study was carried out in adherence with the guidelines of the Ethical Committee of the Istituto Superiore di Sanità, and volunteers gave informed consent to participate.

All subjects provided samples of the water(s) used for drinking or cooking and were asked to refrain from seafood and mushroom consumption four days before urine sampling. They filled in a questionnaire detailing water use, food habits, smoking status, and other relevant information. First morning voided (FMV) urine samples were collected in polypropylene tubes and transported at ambient temperature to the laboratory where they were stored at –80 °C until analysis. For this study, a total

of 153 urine samples, with varying concentrations of total As (≤20 μg As/L, 37 %; 20–50 μg As/L, 44 %; ≥50 μg As/L, 20 %), were selected. The characteristics of the participants are shown in Table 2.

Table 2 General characteristics of study subjects.

No. of subjects	153
Males (%)	69 (45 %)
Females (%)	84 (55 %)
Mean age (range)	42.3 (1.1–87.0)
Smokers (%)	21 (14 %)
Alcohol consumption (%)	78 (51 %)
BMI (range)	24.1 (13.8–39.0)
Water use	
All purposes	74 (48 %)
Cooking only	79 (52 %)

Instrumentation

An Elan DRC II ICP mass spectrometer (PerkinElmer, Norwalk, CT) was used for total As determinations and as element selective detector for As speciation. Chromatographic separations were performed using a PerkinElmer Series 200 metal-free HPLC system. The exit of the column was directly connected by means of PEEK capillary tubing to the Meinhard quartz concentric nebulizer of the ICP-MS, used in conjunction with a PC3 Peltier-cooled quartz cyclonic spray chamber (Elemental Scientific Inc., Omaha, NE) set at 2 °C. Sample handling was carried out in a laminar flow box (Spetec GmbH, Erding, Germany).

Standard solutions and reagents

Analytical reagent grade chemicals and ultrapure deionized water obtained by a Milli-Q Element System (Millipore, Molsheim, France) were used throughout unless stated otherwise.

Calibrants and internal standards (Rh and Y) used for total As measurements were obtained from certified solutions of 1 mg mL^{-1} (High Purity Standard, Charleston, SC) by dilution with acidified (HNO_3 Ultrapure grade, Carlo Erba Reagenti, Rodano, Italy) water as necessary. For As speciation, stock solutions of 1 mg mL^{-1} (expressed as As) were prepared by dissolving in water adequate amounts of disodium hydrogen arsenate heptahydrate (AsV), DMA (both from Fluka, Dorset, UK), MA, trimethylarsine oxide (TMAO), arsenocholine bromide (AC) (from Tri Chemical Laboratories Inc., Yamanashi, Japan). Arsenic trioxide (AsIII, Fluka, Dorset, UK) was dissolved in KOH (0.5 % w/w). A certified standard solution (BCR CRM 626, IRMM, Geel, Belgium) was used for AB. Dimethylmonothioarsinic acid (DMTA), also known as thio-dimethylarsinic acid, was provided by Prof. K. A. Francesconi (Karl-Franzens University, Graz, Austria). Standard stock solutions were stored at –20 °C, and the exact concentrations were ascertained by ICP-MS analysis. Working standard solutions were prepared daily, and their purity for speciation analysis was constantly checked by HPLC-ICP-MS.

For the preparation of the HPLC mobile phases ammonium carbonate (Merck, Darmstadt, Germany), ammonia solution (25 % Suprapur grade), and methanol (HPLC grade) were used. The mobile phases were degassed and filtered (0.22 μm) before use.

Determination of total arsenic and arsenic species

Urine specimens for total As determination were diluted with a solution (1 % v/v HNO_3) containing Rh and Y (10 μg L^{-1}) as internal standards, filtered through 0.22 μm PVDF syringe filters and analyzed by inductively coupled plasma-dynamic reaction cell-mass spectrometry (ICP-DRC-MS). The total As concentration was determined in the DRC mode using H_2 as reaction gas (10 % in Ar, flow 0.35 mL min^{-1}, RPq 0.45) and ^{75}As as the analytical mass. Quantification was performed by six points matrix-matched calibration in the range 1–50 μg L^{-1}. Averaging the results obtained with ^{89}Y and ^{103}Rh normalization improved the accuracy of measurements of about 2 %.

For As speciation analysis, 25 μL of 0.22 μm PVDF-filtered urines were injected on a strong anion-exchange column (ICSep ION-120, 120 × 4.6 mm i.d., 10 μm particles; Transgenomics, San Jose, CA). Mobile phases A and B were 0 and 100 mM $(NH_4)_2CO_3$, respectively, prepared in aqueous 3 % (v/v) methanol adjusted to pH 10.3 with aqueous ammonia. Analytical conditions used for chromatography were slightly modified from Sloth et al. [24]. Briefly, the operating temperature was set at 35 °C, and the gradient used for elution was as follows: 0–5 min 95 % A, flow 0.7 mL/min; 5–10 min 70 % A, flow 1 mL/min; 10–14 min 50 % A, flow 1 mL/min; 14–15 min 95 % A, flow 1 mL/min; 15–17 min 95 % A, flow 1.5 mL/min; 17–18 min 95 % A, flow 0.7 mL/min. Arsenic species were identified by retention time matching with the standard substances spiked to the samples. Quantitative calculations, based on peak areas, were accomplished by the method of single-point standard addition. A pooled urine sample, obtained from the combination of individual samples (n = 40) was used throughout for matrix-matched calibration. ArCl interference on m/z 75 was assessed by monitoring m/z 37 and 77. The method limits of quantifications for the different As species were in the range 0.06–0.12 μg As/L.

Because of the inter- and intra-individual variations in dilution of urine (depending on, e.g., fluid intake) the concentrations of As in urine need to be adjusted. Specific gravity (*SG*) was preferred to creatinine excretion for normalizing data [25] and measured in FMV urine samples by a hand refractometer (ATAGO, Japan). Urinary As and As species (*U-As*) were adjusted to the overall mean *SG* value of 1.020 g mL^{-1} of the study group according to the following equation: $U\text{-}As\ SG = U\text{-}As \times (1.020 - 1)/(measured\ SG - 1)$. Analytical results are expressed accordingly throughout.

Analytical quality control

Average intra- and inter-day repeatability based on consecutive analysis of the same sample were determined for both total and speciation measurements and turned out to be <7 %.

Trueness assessment of total As determination was carried out using the control material Seronorm Urine blank (Sero, Billingstad, Norway), which was included in each analytical batch. The found value (±95 % c.i.) was 82.3 ± 0.8 μg As/L and compared well with the reference value of 85 ± 5 μg As/L. A reference material with certified values of As species at two different concentration levels, SRM 2669 Arsenic Species in Frozen Human Urine (Gaithersburg, MD, USA), was used for quality control in speciation analysis.

In order to calculate mass balances, the recovery of As from the chromatographic column was assessed by comparing the sum of As species eluting from the column with the amount of As injected on the column, i.e., total As measured by ICP-MS.

Statistics

For descriptive analysis, the sum of detected urinary As metabolites, the concentration of AB, iAs, MA, and DMA, the percentages of iAs and metabolites present as one of the three species, the ratios MA:iAs (primary methylation index) and DMA:MA (secondary methylation index) were calculated for all subjects and for the two groups exhibiting different AB excretion (low-AB and high-AB). The mean and median of each group were derived by first calculating the individual percentage or ratio and then aver-

aging over each category. Differences among groups were checked by *t*-test using log-transformed data (due to departures from normality) and, if not significant, using nonparametric analysis. In fact, the latter (Mann–Whitney U Test) in some instances may offer greater power to reject the null hypothesis than the *t*-test. We investigated various factors, such as sex and age, by univariate comparisons in relation to their possible association with metabolite distribution using log-transformed data. Indicator (dummy) variables were used for dichotomous variables (e.g., sex). Statistical analyses were computed using SPSS 17.0 software.

RESULTS AND DISCUSSION

Sources of inorganic arsenic exposure

In the study area, As-rich groundwater is the major source of exposure to iAs for the local population. Since the regulatory limit of 10 μg As/L in the water intended for human consumption has been enacted (Directive 98/83/EC transposed into Italian legislation with the Legislative Decree 31/2001), derogations up to 50 μg As/L and recently 20 μg As/L have been issued due to the lack of alternative water supplies. Public awareness led to widespread use of bottled water for drinking purposes, whereas the water of the public water supply and private wells have been devoted mainly to other household uses including food preparation and cooking. The mean and median As concentrations in the drinking water of the volunteers included in this study were 11.1 and 9.2 μg/L, respectively, with a range of 0.1–35.2 μg/L. The mean and median As concentration in the water used for cooking were 15.7 and 14.7 μg/L, respectively, with a range of 0.2–65.2 μg/L. Therefore, food cooking and preparation in the household and consumption of food with higher than background As concentrations represented additional sources of dietary iAs exposure, as confirmed by speciation analysis of local food (unpublished results).

Identification of arsenic species

The main aim of the present work was to determine As metabolites in urines of people exposed to dietary iAs. Figure 1 shows an HPLC-ICP-MS chromatogram of a mixture of AB, AsIII, AsV, MA, and DMA standards, whereas Fig. 2 shows a real urine sample containing the same compounds.

AB, a species of no toxicological concern abundant in fish and most seafood, is excreted unchanged and occurs frequently in human urine. In the conditions used in this study, AB coelutes with other cationic organoarsenic compounds, which are not retained on the anion-exchange column such as TMAO and AC. However, AB is the predominant species while other cationic species are present only as minor constituents, and thus it can be assumed that the first eluting peak largely corresponds to AB. In the conditions of this study, chlorine eluted after AsV, and thus the $^{40}Ar^{35}Cl$ interference, was resolved chromatographically from the As species (Fig. 2).

Analysis of human urine reference material SRM 2669 revealed the presence of a peak with a retention time of ca. 8 min. The unknown peak disappeared completely after addition of H_2O_2, and the signal for DMA increased concomitantly. This led to the hypothesis that the unknown metabolite was DMTA, a thioanalogue of DMA, which was verified by cochromatography with the standard substance (Fig. 3). Similar spiking experiments revealed that DMTA was present in one-third of the samples of the present study at concentrations ranging from trace amounts to ~6 μg As/L, representing up to 34 % of the sum of the detected species.

Four unknown arsenicals with the approximate retention times of 2.5, 3.7, 6.1, and 10.5 min were also detected as minor species in a small proportion of samples.

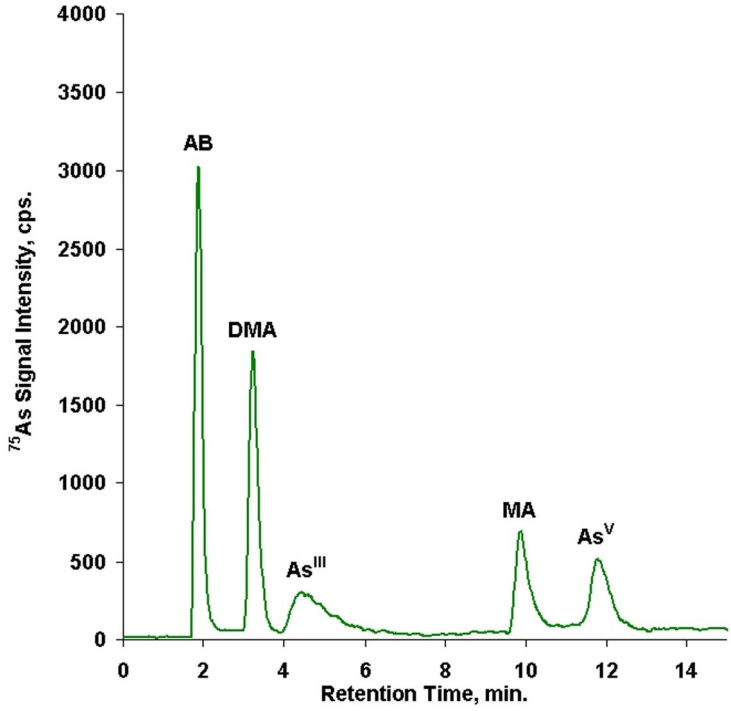

Fig. 1 HPLC-ICP-MS chromatogram of mixture of standards of As species at a concentration of 10 µg As/L.

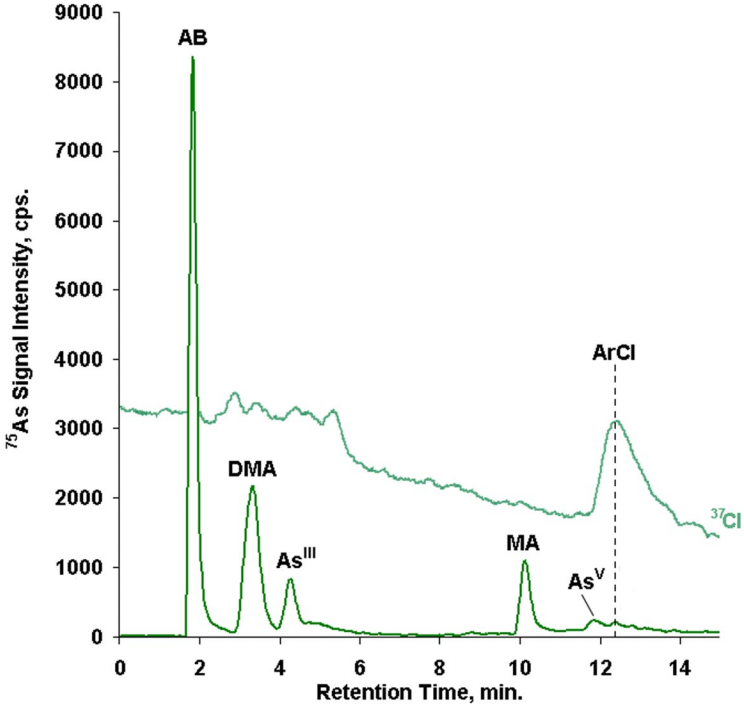

Fig. 2 HPLC-ICP-MS chromatogram of a real urine sample.

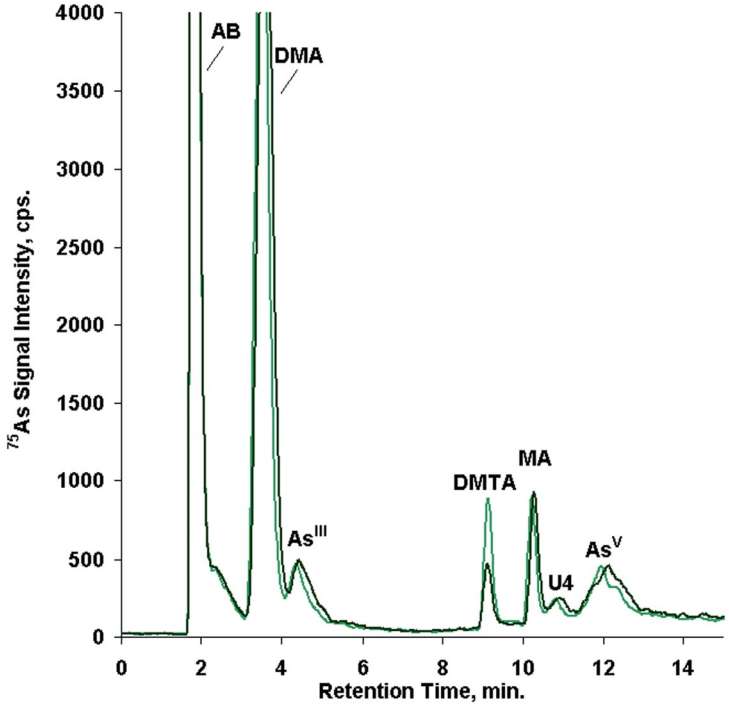

Fig. 3 HPLC-ICP-MS chromatogram of a real urine sample spiked with DMTA.

Analysis of human urine reference materials and mass balance

The results from the As speciation analysis of SRM 2669 (levels I and II) are given in Table 3. Interconversion of DMTA and DMA is known to be dependent on the analytical method, including sample treatment [26,27], and no DMTA was reported in the certificate of the reference material. Therefore, in order to allow comparison with the certified DMA concentration reported therein, the sum of the concentrations of the oxo- and thio-analogue was computed. Measured DMTA concentrations were 1.7 and 2.2 µg/L as As for levels I and II, respectively. Partial interconversion of the labile inorganic species, AsIII and AsV, was also detected as compared to the relative amounts determined in the certification exercise and thus the sum of the two species, i.e., iAs, is shown in Table 3.

Table 3 Results from As speciation analysis of SRM 2669 (levels I and II).[a]

Level	AB[b]		iAs[c]		MA		DMA[d]		∑species	
	Found	Certified	Found	Certified	Found	Certified	Found	Certified	Found[e]	Certified[f]
I	14.5 ± 0.5	13.9 ± 1.9	4.3 ± 0.5	3.88 ± 0.32	2.2 ± 0.2	1.87 ± 0.39	2.7 ± 0.2	3.47 ± 0.41	23.7	22.2 ± 4.8
II	5.7 ± 0.2	7.11 ± 0.45	8.7 ± 0.8	11.19 ± 1.0	7.1 ± 0.5	7.18 ± 0.56	27.6 ± 1.6	25.3 ± 0.7	49.1	50.7 ± 6.3

[a]Concentrations in µg/L as As. Found values given as mean ± s.d (n = 9). Certified values given as mean ± expanded uncertainty.
[b]Sum of AB, AC, and TMAO. Certified values given as mean ±combined expanded uncertainty of the concentration of the three species.
[c]iAs. Certified values given as mean ±combined expanded uncertainty of the concentration of AsIII and AsV.
[d]Sum of oxo- and thio-analogue (DMTA).
[e]Sum of all detected species in HPLC-ICP-MS.
[f]Reference values for total As.

According to the certificate, SRM 2669 was prepared by pooling urine collected from volunteers and adjusting to target levels the different As species with addition of appropriate amounts of the standard substances. Level II contains higher concentrations of iAs and related metabolites compared to level I, whereas AB is present at low concentrations along with some AC and TMAO. As mentioned above, with the analytical method used in this study the three trimethylated species coelutes and therefore a single concentration value is reported for them (Table 3).

Overall, the found concentrations for AB (along with other cationics), DMA, MA, and iAs were 92, 93, 109, and 95 % of the certified values, respectively (levels I and II pooled recoveries). Found values were slightly lower than the certified values for DMA in level I and for AB and iAs in level II.

For the 153 urine samples of this study, the sum of the chromatographed As species amounted on average to 104 % of the total As, as measured by ICP-MS, demonstrating that the As injected on the column was completely recovered thereafter.

Arsenic speciation in urine of volunteers

Very high concentrations of total As were measured in urine of few subjects. It was hypothesized that these volunteers did not refrain from seafood and mushroom consumption, and indeed high concentrations of AB were detected. In particular, 26 subjects excreted concentrations of AB ≥ 50 µg/L and 18 subjects ≥ 100 µg/L (up to 2188 µg/L). Levels and relative distributions of urinary As species are shown in Table 4.

Table 4 Levels and relative distributions of urinary As species.

	AB[a]	iAs[a,b]	MA[a]	DMA[a,c]	∑iAs[a,d]	∑species[a,e]	iAs %[f]	MA %[f]	DMA %[f]
Subjects with urinary AB < 50 µg/L (n = 127)									
Mean	9.6	2.4	2.0	10.3	14.6	24.3	16	13	71
Median	4.5	1.8	1.7	8.4	12.0	22.1	14	14	71
Min	0.3	0.1	0.2	1.9	2.9	4.0	1	2	28
Max	48.1	9.9	7.7	28.8	40.4	66.8	46	27	95
Subjects with urinary AB ≥ 50 µg/L (n = 26)									
Mean	260	2.1	2.7	16.7	20.9	281	14	12	74
Median	141	1.5	1.3	12.2	13.8	160	10	12	74
Min	59	0.2	0.3	1.7	1.7	63	2	5	39
Max	2188	6.6	14.3	51.5	72.4	2248	47	21	91
All subjects (n = 153)									
Mean	52.1	2.3	2.1	11.4	15.7	68.0	16	13	71
Median	6.8	1.8	1.6	8.6	12.4	25.1	14	13	72
Min	0.3	0.1	0.2	1.7	1.7	4.0	1	2	28
Max	2188	9.9	14.3	51.5	72.4	2248	47	27	95

[a]Concentrations in µg/L as As (*SG* normalized).
[b]iAs.
[c]Sum of oxo- and thio-analogue (DMTA).
[d]Sum of iAs, MA, and DMA.
[e]Sum of all detected species in HPLC-ICP-MS (including unknowns).
[f]Ratio of iAs, MA, and DMA to the sum of the three species (∑iAs).

Excluding the subjects who excreted high concentrations of AB (≥50 µg As/L), iAs and its methylated metabolites summed up 75 % of the sum of the As species (median value) and relative proportions were unmethylated As 14 %, MA 14 %, and DMA 71 % (median values). In the population

sample of this study, relative proportions of these species appears to be almost unaffected by ingestion of food that can alter urinary DMA (i.e., excreted as iAs metabolite). The diet is wheat-based with marginal consumption of rice, and we demonstrated that wheat and wheat products contain almost exclusively iAs [23,28]. Only a very weak effect of seafood consumption on median DMA percentage was apparent when the high-AB group (urinary AB ≥ 50 µg/L, Table 4) was compared to the low-AB group, with a median DMA percentage of 74 vs. 71 % ($p = 0.04$, Mann–Whitney U Test). Further inspection of data by comparing samples containing ≥50 % and <50 % of the sum of the As species as AB ($n = 58$ and 95, respectively), confirmed a weak difference in urinary DMA percentage (median value 74 vs. 71 %, $p = 0.03$). We then investigated factors such as sex and age in relation to their possible association with methylation efficiency as reflected by iAs metabolite distribution. The greatest differences observed were a higher percentage of iAs in males ($p = 0.03$) and the positive association of MA percentage with age. However, the latter was almost exclusively due to males ($p = 0.01$), and accordingly the DMA percentage and the DMA:MA ratio were negatively associated with age in males ($p = 0.01$ and 0.004, respectively). The age-dependent changes in MA and DMA percentages (positive and negative association, respectively) have been detected in other studies, and together with gender differences indicate an effect of sex hormones on As methylation [9]. A high fraction of MA in urine is a risk-modifying factor for several As-induced health effects and men are more susceptible than women due to a less efficient As methylation, as defined by a higher fraction of MA and lower fraction of DMA in the urine [29].

Interindividual differences in percent iAs, MA, and DMA in the study sample were marked (Table 4). The same was true for the ratios MA:iAs (mean 1.4, median 0.9, range 0.1–12.5) and DMA:MA (mean 6.6, median 5.3, range 1.0–47.3). Both the relative percentages (iAs and related metabolites) and the methylation indexes covered a wide range as compared to other studies on populations from several different countries, of diverse ethnicities and with widely varying As exposures [7–9,30–31]. Overall, a wide individual variability was found in the study population reflecting differences in As methylation efficiency, which will be further investigated in the future.

It is worth noting that other (i.e., mainly trimethylated) organoarsenic compounds, which can be assumed to be essentially AB, were detected at low concentrations in all urine samples. This finding confirms the need for determining speciated As as urinary biomarker of dietary exposure to iAs; on the other hand, it reinforces questions that have been raised about the diffusion of AB in so far unidentified terrestrial food sources or the presence and excretion in human body. With regard to the latter, it has been recently speculated that either accumulated AB in human tissues is slowly released over time (making it detectable also several days after seafood consumption) or that AB is a human metabolite of DMA or iAs from food [32]; clearly, this issue deserves further investigation.

The widespread occurrence of DMTA in urine from the subjects of this study is another issue of interest. It is known that DMTA can be present in food [27,33], and it is currently considered as a (generally minor) human urine metabolite of iAs and arsenosugars [26]. Thiolated As species can be formed in the human intestine, due to the highly reducing conditions and the high sulfur concentrations, or be the result of endogenous metabolism of mammalian cells; the relative importance of intestinal and endogenous thiolation and possible significance in As-induced toxicity are still matter of speculation [34]. It has been hypothesized that DMTA can be simply formed from DMA and sulfane sulfur generated in the body, hence being an occasional byproduct of DMA formation [26]. Regardless of how DMTA is biosynthesized, it usually escapes detection even when it is specifically searched for [31,35]. In As speciation analysis, the stability of DMTA is highly dependent on the urine matrix and interconversion of DMTA and DMA is likely to occur depending on storage conditions and the analytical method used [26,27]. It cannot be ruled out that DMTA is often determined as DMA in urine speciation studies, however, it is clear that most studies still fail to detect this thio-arsenical as such in urine besides its more stable oxidation product. From a practical point of view, in this study DMTA was considered along with DMA for the estimation of the pool of dimethylated metabolites likely resulting from iAs exposure of the studied population and calculation of the secondary methylation index.

In the present study the mean, median, and range of the sum of iAs and metabolites were 15.7, 12.4, and 1.7–72.4 μg/L, respectively. A previous study of a reference population of 148 Italian subjects with no occupational As exposure and low As concentrations in drinking water found that urinary iAs and related metabolites averaged 5.9 ± 2.9 μg/L (range 2–21 μg/L [12]), and a reference range of 2–15 μg/L has been proposed for the Italian population [36]. In the present study, values exceeding 15 μg/L were found in 41 % of the subjects, highlighting dietary As exposures above the average of the general population. The recent assessment of As human exposure in European countries revealed that average dietary exposure to iAs is high compared to health reference points and should be reduced [2].

CONCLUSIONS

We reported our experience in measuring urinary As metabolites in a sample of a population exposed to iAs via water and food. The analytical approach enabled sensitive detection of target species, i.e., iAs, MA, and DMA, in all samples. This is particularly useful since relative proportions of these species and related methylation indexes could be accurately calculated, thus providing insight into the inter-individual variability in iAs metabolism. Arsenicals unrelated to iAs exposure (essentially AB) were determined in all samples, which highlights the need for determining speciated As as urinary biomarker of dietary exposure to iAs. Since the population studied had limited consumption of seafood, this also raises questions about the presence of AB in other dietary sources or possible formation/metabolism inside the human body. The ability of the analytical method used in this study to detect DMTA is an important feature since this compound appears to escape detection in most biomonitoring studies, whereas it was found to be present in one-third of the samples accounting for up to 34 % of the sum of the detected As species.

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