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ORIGINAL PAPER

Methods for the separation and quantification of arsenic species in SRM 2669: arsenic species in frozen human urine

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Abstract Two independent liquid chromatography inductively coupled plasma-mass spectrometry (LC/ICP-MS) methods for the separation of arsenic species in urine have been developed with quantification by standard additions. Seven arsenic species have been quantified in a new NIST frozen human urine Standard Reference Material (SRM) 2669 Arsenic Species in Frozen Human Urine, Levels 1 and 2. The species measured were: arsenite (As(III)), arsenate (As(V)), monomethylarsonate (MMA), dimethylarsinate (DMA), arsenobetaine (AB), arsenocholine (AC), and trimethylarsine oxide (TMAO). The purity of each arsenic standard used for quantification was measured as well as the arsenic species impurities determined in each standard. Analytical method limits of detection (L_D) for the various species in both methods ranged from 0.2 to 0.8 μg L^{-1} as arsenic. The results demonstrate that LC/ICP-MS is a sensitive, reproducible, and accurate technique for the determination of low-level arsenic species in urine. Measurements of the arsenic species 3 years after initial production of the SRM demonstrate the stability of the arsenic species in the urine reference material.

Keywords ICP mass spectrometry · Arsenic speciation · Reference materials · Urine

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Introduction

Arsenic is a significant global environmental toxicant, and contamination of ground and drinking water is a problem threatening human health around the world. Humans are exposed to arsenic through the intake of air, food, and water, and occupational exposure occurs in several industries including gold mining [1, 2], smelting operations [3], and manufacturing of pharmaceuticals, glass, and microelectronics [4, 5]. Historically, inorganic arsenic compounds are often regarded as the most dangerous forms of arsenic and methylated arsenicals as the least harmful [6–8]. Recent studies suggest that methylated metabolites of inorganic arsenic, especially in the trivalent state, may be more toxic than the parent inorganic arsenic compounds [9–14]. Ingestion or inhalation of large doses of inorganic arsenic can result in a variety of adverse health effects, including skin lesions; cardiovascular and neurological disorders; cancers of the liver, bladder, kidneys, prostate, and lung; and rapid death [15, 16]. In humans, inorganic arsenic is methylated to monomethylarsonic acid and dimethylarsinic acid by alternating reduction of arsenic from the pentavalent to the trivalent oxidation state and addition of a methyl group or groups [6–8]. Hence, the methylation of inorganic arsenic is considered a detoxification reaction [10].

The dietary (i.e., fish, shellfish, or seaweed) arsenic species of arsenobetaine, arsenocholine, and the arsenosugars, are generally considered to be less toxic because of rapid clearance from the body in urine. Urinary levels of total arsenic are generally regarded as a good measure of exposure [17], although measurements of total arsenic in urine and other matrices do not contain information concerning the arsenic species, thereby complicating the assignment of toxicity and potential health risk. To

successfully identify toxic species, the determination of the associated elemental complex or oxidation state in addition to the quantitative determination of the amount of a specific element is particularly important. An ideal solution to the problem of species-specific detection of arsenic in urine is the combination of powerful separation techniques, such as liquid chromatography (LC), with compatible detection modes, such as inductively coupled plasma-mass spectrometry (ICP-MS) [18–22]. For this reason, speciation methods are considered essential for drawing accurate conclusions in arsenic toxicological, exposure, and risk assessment studies.

The National Institute of Standard and Technology (NIST) has issued a variety of clinical and biological-matrix Standard Reference Materials (SRMs). Natural-matrix SRMs have been issued as freeze-dried, sterilized powder, and fresh-frozen materials exhibiting excellent long-term compositional stability when properly stored and handled. At present, fresh-frozen materials may represent the best approach to maintain the integrity of organic, inorganic, and organometallic compounds that are volatile, unstable, and subject to degradation. This class of SRMs truly mimics samples that are actually analyzed in laboratories, and therefore, provides optimum commutability of results. Development and validation of clinical and analytical methods require reference materials in a variety of matrices and at varying concentrations. The work reported here describes two independent methods for chromatographic separation and quantification of seven organic and inorganic arsenic compounds, viz. arsenous acid (As(III)), arsenic acid (As(V)), monomethylarsonic acid (MMA), dimethylarsinic acid (DMA), arsenobetaine (AB), arsenocholine (AC), and trimethylarsine oxide (TMAO) in SRM 2669 Arsenic Species in Frozen Human Urine. SRM 2669 consists of two separate urine materials designated Levels 1 and 2 and containing different levels of each arsenic compound.

Experimental

All chemicals for purity assessments, separation method development, and quantification of arsenic species were purchased separately and performed independently at the NIST Charleston (Method 1) and Gaithersburg (Method 2) laboratories.

Safety warning Certain aspects of the sample preparation scheme required working with human urine, as well as strong oxidizing acids under conditions of elevated temperature and pressure, all of which required the use of extracting fume hoods, special personal protective equipment, and adherence to biohazard handling procedures.

Reagents and chemicals

Separate arsenic standard compounds were purchased independently, and stock solutions of the individual arsenic species were prepared gravimetrically from arsenious acid (Sigma Aldrich, St. Louis, MO—Method 1, Spex Certiprep, Metuchen, NJ—Method 2), dimethylarsinic acid (Sigma Aldrich—Methods 1 and 2), arsenic acid (Fluka, Germany—Method 1, Spex Certiprep—Method 2), methylarsonic acid (Chem Service, West Chester, PA—Methods 1 and 2), trimethylarsine oxide (Argus Chemicals, Vernio Italy—Methods 1 and 2) and arsenocholine (Argus Chemicals—Methods 1 and 2) and BCR-626 arsenobetaine (AB) solution (IRMM, Geel, Belgium PA—Methods 1 and 2). The mobile phase for ion-exchange chromatography (IC) was prepared by using high-purity nitric acid (Fisher Scientific, Suwanee, GA), Omni-Trace Ultra acetic acid (EMD Chemicals, Gibbstown, NJ), and reagent-grade sodium acetate (Sigma Aldrich). The liquid chromatography mobile phases were prepared from ACS-grade pyridine and formic acid (Fisher Scientific Fair Lawn, NJ), reagent-grade ammonium nitrate and ammonium sulfate (Mallinkrodt, Paris, KY), Ultrex grade ammonium hydroxide, and HPLC-grade methanol (JT Baker, Phillipsburg, NJ).

Separation of arsenic species

Method 1 (NIST Charleston)

An ion chromatography (IC) system (Dionex, Sunnyvale, CA) consisted of a GP50 gradient pump and six-port injector equipped with a 50-μL injection loop. The separation of all seven arsenic species was performed with a Dionex IonPac AS7 (4 mm×250 mm) column protected by a Dionex IonPac AG7 (4 mm×50 mm) guard column. The mobile-phase composition and chromatographic method details are listed in Table 1. The IC system was coupled to a X7 ICP-MS (Thermo Elemental, Winsford, Cheshire, UK) with a standard, low-volume, glass impact bead spray chamber (Peltier-cooled at +3 °C) and concentric glass nebulizer operating in collision cell mode (cell gas of 8% H_2 in He) to minimize ion spectral overlap from argon chloride on arsenic ($^{40}Ar^{35}Cl^+$ on ^{75}As).

Method 2 (NIST Gaithersburg)

A LC system (Perkin-Elmer, Shelton, CT) consisted of a Peltier-cooled Series 200 autosampler and a Series 200 quaternary pump equipped with a 50 μL injection loop. Separations of As(III), MMA, DMA, AB, AC, and TMAO were accomplished using a Nucleosil 100-5 SA cation-exchange column (Macherey-Nagel, Bethlehem, PA). The

Table 1 Mobile-phase compositions and chromatographic programs for separation of arsenic species

Method 1	
Columns	
Guard	Dionex IonPac AG7 (4 mm×50 mm)
Analytical	Dionex IonPac AS7 (4 mm×250 mm)
Mobile phase	
A: 0.25 mmol/L acetic acid/sodium acetate	
B: 25 mmol/L nitric acid	
Gradient program (1.0 mL/min)	
0 min to 3 min	100% A
3 min to 28 min	100% A linear gradient to 100% B
28 min to 32 min	100% B
Method 2	
LC method conditions for separations of As(III), MMA, DMA, AB, AC, and TMAO.	
Columns	
Guard	CC 8/4 Nucleosil 100-5 SA (4 mm×8 mm)
Analytical	Nucleosil 100-5 SA (4 mm×250 mm)
Mobile phase	
A: 0.10 mmol/L pyridine+2% methanol in deionized water, pH 6.2	
B: 30 mmol/L pyridine+2% methanol in deionized water, pH 3.0	
Gradient program (1.0 mL/min)	
0 min to 4 min	100% A
4 min to 5 min	100% A linear gradient to 100% B
5 min to 17 min	100% B
LC method conditions for As(V) separations.	
Columns	
Guard	PEEK guard column for PRP X-100 PEEK
Analytical	PRP X-100 PEEK (4.6 mm×150 mm, 3 μm)
Mobile phase	
10 mmol/L NH4NO3+10 mmol $(NH_4)_2SO_4$ 2% methanol in deionized water, pH 10.0	
Isocratic program (1 mL/min)	
0 min to 7 min	100% A

separation of As(V) was accomplished using a separate chromatographic method utilizing a PRP X-100 (3 μm) anion exchange column (Hamilton, Reno, NV). The mobile-phase composition and chromatographic method details are listed in Table 1. The LC system was coupled to an Elan DRC II ICP-MS operating in standard mode.

Purity analysis

Of the seven arsenic standards to be characterized, AB, As(III), and As(V) are available as Certified Reference Materials that are certified for total As mass fraction. The four commercial arsenic compounds not certified were utilized as primary reference standards after proper characterization. The characterization of each commercial arsenic compound was performed by determining the total arsenic and the amounts of arsenic-containing impurities. Total arsenic was determined independently using instrumental neutron activation analysis (INAA) and microwave digestion inductively coupled plasma optical emission spectrometry (ICP-OES). The arsenic-containing impurities were assessed by using LC/ICP-MS.

Total arsenic by microwave digestion ICP-OES (standards used in Method 1)

For the standards of As(III), As(V), MMA, DMA, AC, and TMAO used in Method 1, the total arsenic concentrations were determined by standard additions with SRM 3103a Arsenic Standard Solution and utilizing yttrium as an internal standard. Specifically, the sample dissolution procedure utilized microwave digestion (Perkin-Elmer Multiwave) in 3 mL of concentrated HNO_3 and 0.5 mL of

H_2O_2. Six individual aliquots of each arsenic stock solution were digested along with three procedural blanks. All aliquots were weighed by difference into pre-cleaned quartz microwave vessels, and spiked with a yttrium internal standard. Prior to digestion, three of the specimens were spiked with arsenic using working standards gravimetrically prepared from SRM 3103a. The resultant digests were vented, diluted with 18.2 MΩ cm water, and stored at 2 °C for 24 h before analysis. One aliquot of spiked and one of unspiked digests from each stock solution were analyzed by LC/ICP-MS (Method 1) to confirm complete digestion by the absence of the original compound. Samples were then measured using ICP-OES (Thermo Jarrell Ash Iris Advantage). Total arsenic purity is defined as the mass of arsenic measured by ICP-OES divided by the mass of arsenic expected from a 100% pure chemical and the results are listed in Table 2.

Total arsenic by INAA (standards used in method 2)

The total arsenic in MMA, DMA, AC, and TMAO was determined by INAA. A solution containing approximately 14 μg/g arsenic in deionized water was prepared from each individual arsenic compound. Three specimens of each chemical were prepared by depositing 0.14 g aliquots of this solution onto 42.5 mm Whatman (Middlesex, UK) # 41 filters. The filters were dried under an infrared lamp for 15 min. Three filters containing arsenic derived from SRM 3103a were prepared similarly to serve as calibration standards for the INAA measurements. The total arsenic purity is defined as the mass of arsenic found by INAA divided by the mass of arsenic expected of a 100% pure chemical, and the results are listed in Table 2.

Determination of arsenic-containing impurities

Arsenic from arsenic-containing impurities in each of the arsenic compounds was assessed independently by using the separation methods (Method 1 and Method 2) described previously. Stock solutions of the individual arsenic compounds were prepared and analyzed to determine the presence of possible arsenic impurity species. These were quantitatively determined by standard additions with internal standard and the results are listed in Table 3.

Quantification of arsenic species in SRM 2669 arsenic species in frozen human urine—Method 1

Level 1 analysis

The quantification of individual arsenic species was accomplished by the method of single-point standard addition with use of an internal standard. Cryovials of the urine were removed from a −80 °C freezer and placed inside a class 100 HEPA filtered workstation until the samples reached room temperature. The vials were gently shaken to homogenize the urine samples before transfer of the contents to pre-cleaned 2.0-mL microcentrifuge tubes and centrifugation at 14,000 rpm for 5 min at 10 °C to remove any residual sediment. Duplicate sub-samples (≈0.6 g, exact mass known) from six vials were weighed by difference into pre-cleaned vessels, a known aliquot of AC (not present in Level I) was added as an internal standard, and the mixture was diluted to ≈5 g (exact mass known). One of the duplicate sub-samples was spiked with a mixture of As(III), As(V), MMA, DMA, and AB resulting in six single-point standard addition pairs. The standard addition and internal standard spikes were prepared daily from the stock solutions and the samples were processed in batches of three unspiked/spiked pairs and analyzed the day of preparation to minimize species transformation and/or decomposition.

Individual arsenic species were identified by comparison of retention times to standard solutions. Arsenic species were measured by monitoring *m*/*z* 75 while potential interference due to the polyatomic ion $^{40}Ar^{35}Cl^{+}$ was determined by

Table 2 Results of determinations of total arsenic in arsenic compound reference materials

As compound	INAA Purity as mass fraction of As (%)	ICP-OES Purity as mass fraction of As (%)	ICP-MS[a] Purity as mass fraction of As (%)
As(III)	–	98.7±0.5	98.4±0.4
As(V)	–	97.5±0.2	97.2±0.5
MMA	81.5±0.7	95.3±0.4	95.6±0.4
DMA	99.1±0.3	98.3±0.7	98.1±0.6
AC	86.6±0.9	92.5±1.4	92.2±0.4
TMAO	94.7±0.6	95.9±1.0	95.4±0.5

The uncertainties associated with the average measured values are expressed as expanded uncertainties, U, at the approximately 95% level of confidence

[a] Results from SRM 2669 stability testing

Table 3 Results of determinations of impurity species in arsenic compound reference materials

As standard	As impurity	Method 1 Impurity as As (%)	Method 2 Impurity as As (%)	Method 1[a] Impurity as As (%)
As(III)	As(V)	–	1.0±0.5	–
MMA	As(V)	3.4±0.5	11.2±0.2	9.8±0.4
TMAO	MMA	2.3±0.8	5.3±1.2	6.5±0.7

The uncertainties associated with the average measured values are expressed as expanded uncertainties, U, at the approximately 95% level of confidence

[a] Results from SRM 2669 stability testing

measuring *m*/*z* 77 at dwell times of 250 ms. The time-resolved analysis mode was employed to quantify arsenic species by measuring peak area ratios, i.e., As(V) peak area/internal standard peak area, calculated in the PlasmaLab instrument software. The average calculated mass fraction of the arsenic species was converted from micrograms per kilogram to micrograms per liter using the measured density of the urine.

Level 2 analysis

Samples of SRM 2669 Level 2 were processed as described for the Level 1 analysis with the exception that the samples were spiked with a mixture of As(III), As(V), MMA, DMA, TMAO, and AC. Because Level 2 contains all seven arsenic species at detectable levels, the AB present in the Level 2 urine was used as an internal standard. The AB was quantified in a separate analysis using As(V) present in the Level 2 urine as an internal standard. The standard addition spike was prepared daily from the stock solutions and the samples were processed in batches of three unspiked/spiked pairs and analyzed the same day.

Quantification of arsenic species in SRM 2669 arsenic species in frozen human urine—Method 2

Determination of As(III), MMA, DMA, AB, AC, and TMAO

Level I analysis

Calibration for the measurement of arsenic species was accomplished by the method of single-point standard addition with use of an internal standard. Six cryovials of Level 1 urine were removed from a −80 °C freezer and placed on the laboratory bench until the samples reached room temperature. The vials were gently shaken to homogenize the urine samples before a 0.7-mL aliquot from each vial was transferred into a 1.7-mL centrifuge tube. The samples were centrifuged for 10 min at 15,000 rpm at room temperature to remove any residual sediment. Two aliquots of 200 μL were transferred from each centrifuge tube into two 1.5-mL LC sample vials each containing 200 μL of AC internal standard. The spiked sample was prepared by adding 200 μL of a spike solution containing As(III), MMA, DMA, and AB to one of the vials, and the unspiked sample was prepared by adding 200 μL of deionized water to the other vial.

Individual arsenic species were identified by comparison of retention times to standard solutions. The time-resolved analysis mode was employed to quantify arsenic species by measuring peak area ratios. The average calculated mass fraction of the arsenic species was converted from micrograms per kilogram to micrograms per liter using the measured density of the urine.

Level 2 analysis

Samples of SRM 2669 Level 2 were processed as described for the Level 1 analysis with the exception that the analytes were measured in two runs. In the first run, samples were spiked with a mixture of As(III), MMA, DMA, and AB. AC was added to each sample as an internal standard before spiking. In the second run, samples were spiked with a mixture of TMAO and AC. MMA was added to each sample as an internal standard before spiking.

Determination of As(V) in SRM 2669 arsenic species in frozen human urine

Urine samples were prepared as previously described for both Levels 1 and 2 with the exception that a spike solution containing As(V) and DMA was utilized as the internal standard. The As(V) in the urine samples was determined using the same instrument configuration, except a PRP X-100 (3 μm) anion exchange column (Table 1) was used instead of the cation exchange column.

Uncertainty contributions

The uncertainty of the arsenic species determinations was assessed according to the ISO and NIST guidelines [23, 24]. The major uncertainty components included the

standard deviation of the mean of the individual arsenic species measurements (replication), the total arsenic purity determinations of the quantification standards, the arsenic species impurities in the quantification standards, the density determination, uncertainty of weighing, the standard uncertainty of primary calibrant SRM 3103a, and the standard uncertainty of each arsenic species concentration in the spike solution used in a determination.

Stability measurements of SRM 2669

To verify the stability of the arsenic species in SRM 2669, a second round of purity analysis for both total arsenic content and arsenic species was performed 3 years after the production of the SRM. The determinations of total arsenic in the standards followed the procedure outlined in Method 1 with the exception that ICP-MS was utilized for the total arsenic determinations. The species impurities in the standards and the determination of mass fraction of the individual arsenic species also followed the methods described for Method 1. Vials from ten randomly selected units across the production lot of each SRM level were used in the stability measurements resulting in ten spiked and ten unspiked pairs from each vial.

Results and discussion

Purity analysis

The purity of the standard used for calibration contributes to the uncertainty associated with any quantitative measurement. Therefore, when suitable reference standards are unavailable, a detailed purity analysis of the "quantification standard" should be performed. Table 2 details the results of separate analysis of the arsenic standards which were used as quantification standards in an effort to establish traceability of the commercial standards used for quantification to the SI through NIST SRM 3103a Arsenic Standard Solution. Comparison of the total arsenic purity assessments between INAA (Gaithersburg) and ICP-OES (Charleston) show significant differences for both MMA and AC. Although the individual arsenic species were purchased separately and at different times, identical chemical lots of MMA, DMA, AC, and TMAO were used for the analysis at the Gaithersburg and Charleston laboratories. Preparation and storage of stock solutions were very similar at both locations; therefore, the differences are most likely related to inhomogeneity of the arsenic compounds lots.

In addition to the total arsenic purity, it was important to determine the arsenic species impurities of the quantification standards. BCR-626 arsenobetaine standard solution is known to contain impurities of DMA and TMAO [25] at relatively low levels. The results from the arsenic standards prepared at NIST Charleston and analyzed by standard additions with IC/ICP-MS described in Method 1 are significantly different (Table 3) from results for the standards prepared at NIST Gaithersburg and analyzed by LC/ICP-MS described in Method 2. Different standards of As(III) and As(V) were used at the two locations, but the significant difference in the percentage of the As(V) impurity found in the MMA is most likely not related to instrumental differences.

Because of the significant TMAO impurity in DMA calibrant and As(V) in MMA calibrant, the determinations of the seven arsenic species were performed in three runs with Method 2. MMA, DMA, and AB were determined together in the first run using AC as an internal standard with the cation exchange method. TMAO and AC were determined in the second run using MMA as an internal standard, also with the cation exchange method. By determining TMAO separately from DMA, the adverse effects of the TMAO impurity in the DMA calibrant were avoided. As(V) was determined alone with the anion exchange method. Determining As(V) alone with the method not only avoided the interferences from chlorine (see below), but also avoided the potential complication of As(V) impurity in MMA calibrant.

Separation of arsenic species

The Centers for Disease Control and Prevention (CDC) has identified As(III), As(V), MMA, DMA, AB, AC, and TMAO as the arsenic species to be monitored in urine for the National Health and Nutrition Examination Survey (NHANES) [22, 26]; therefore, separation and quantification of these arsenic species in a single run is of great importance to provide accurate arsenic exposure assessment measurements in the largest quantity of urine samples feasible.

Figures 1a–c shows typical separations of arsenic species in an unspiked sample of SRM 2669 Level 2. Baseline resolution was obtained for all arsenic species in contrast to the method used for quantification in the NHANES measurements in which AB and TMAO are not completely separated [22]. The fact that all seven arsenic species are baseline resolved in a single chromatographic run suggest that NIST Method 1 (Fig. 1a) is inherently superior to the separation used for quantification in the NHANES measurements in which AB and TMAO are not completely separated [22, 26].

The diluted urine samples prepared and analyzed utilizing Method 1 in combination with the use of the collision cell showed no detectable chloride interference on the transient arsenic signal. Chromatograms for the cation exchange separation used in Method 2 (Fig. 1b) show an

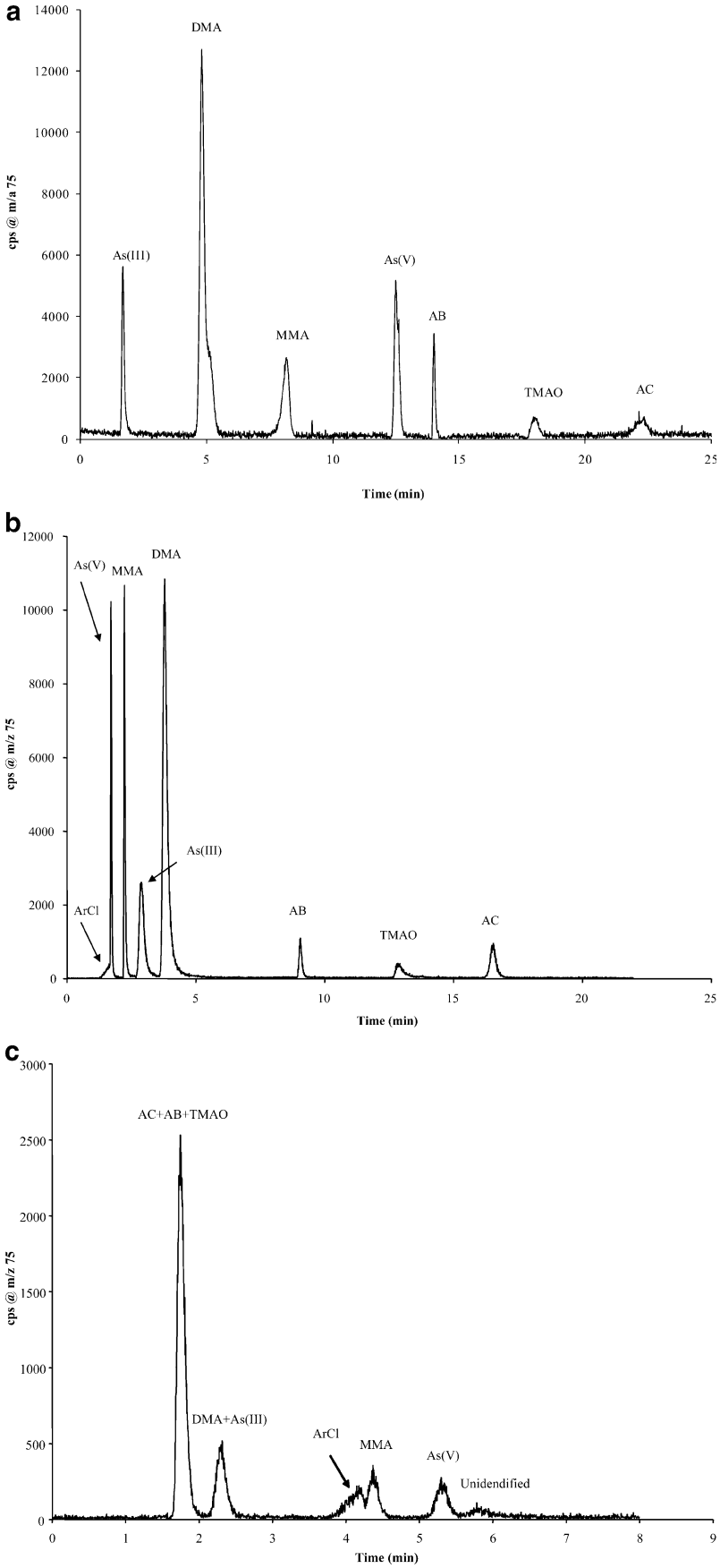

Fig. 1 LC/ICP-MS Chromatograms of SRM 2669 Level 2; **a** Method 1—ion exchange, **b** Method 2—cation exchange, **c** Method 2—anion exchange

elution of the chloride in SRM 2669 Level 2 in the void volume along with As(V). Because no other arsenic species were affected by the chloride interference, the ICP-MS was operated in the normal (no reaction gas) mode for better sensitivity. For the anion exchange separation and analysis of As(V), chloride was eluted between the DMA internal standard and As(V). The ICP-MS was also operated in the normal mode for better sensitivity. As(III), MMA, DMA, AB, AC, and TMAO were separated with the cation exchange separation; hence, a complete separation of arsenic species (Fig. 1c) was unnecessary. Therefore, a simple isocratic method was used for the analysis of As(V) to reduce analysis time.

Method validation

All seven arsenic species can be separated and quantified with one column utilizing Method 1, suggesting that the method may be used by itself or as an alternative in comprehensive arsenic exposure assessment that requires measurements of inorganic (As (III), As (V)), organic (AB, AC), and metabolite (MMA, DMA, TMAO) arsenic species. To assess the robustness of the method, the separation and instrument repeatability was verified with 50 replicate injections of a 5 ng g^{-1} standard containing all seven arsenic species in addition to 50 replicate injections of a vial of SRM 2669 Level 2 prepared as described above. The repeatability of retention time at peak maximum for each arsenic species was less than 2% RSD over 30 h of chromatographic run time. The repeatability of retention time for SRM 2669 Level 2 was not as good with the retention time for As(V) gradually shifting from 14.2 to 12.0 min and the retention time for TMAO gradually shifting from 15.8 to 15.0 min during the 30 h. However, the repeatability of retention times for As(III), MMA, DMA, AB, and AC in SRM 2669 Level 2 were all <3% RSD. Even with shifts of retention times of both As(V) and TMAO, all arsenic species remained completely separated and baseline resolved. The instrument repeatability over the 50 injections resulted in the integrated peak areas varying less than 2% RSD and 6% RSD for the arsenic species in the standard solution and in SRM 2669 Level 2, respectively, without the use of an internal standard.

Quantification of arsenic species

Certification of mass fraction values and estimation of uncertainty often present serious challenges and involve varying levels of complexity. Typically, an analyte can be certified when the value is confirmed by several analysts or laboratories working independently using either one primary method (i.e., isotope dilution) or at least two fully evaluated methods with known and independent sources of uncertainty. The selected approach depends on the required traceability, limits of uncertainty, capabilities of analytical techniques, and compliance with the NIST certification criteria [27]. Arsenic, being monoisotopic, rules out the use of a primary quantification method such as speciated isotope dilution, and the inherent matrix effects of analyzing urine rule out the use of external calibration. While the two methods presented are based on a simple 'dilute and shoot' sample preparation approach and quantification by standard additions, the separations utilized different columns and mobile phases resulting in different chromatographic specificities for the arsenic species.

To ensure a linear instrument response, acceptable column loading, and good separation efficiency, the linear range for standard addition quantification of each arsenic species was verified using an external calibration covering several orders of magnitude using mixtures of arsenic.

The results of arsenic species determinations (as arsenic) in SRM 2669 Arsenic Species in Frozen Human Urine for the two NIST analytical methods, together with their respective expanded uncertainties, based on a 95% level of confidence, are listed for Levels 1 and 2 in Tables 4 and 5, respectively. The concentrations of AC and TMAO in Level 1 samples are less than the calculated detection limits, L_D, for both methods. Therefore, the L_D values are listed in the tables. The L_D values for all seven of the arsenic species ranged from between 0.2 μg L^{-1} and 0.8 μg L^{-1} and were calculated from the standard addition measurements of the arsenic species in Level 2 [28]. The reproducibility of the six individual mass fraction determinations in Levels 1 and 2 were typically under 6% RSD for both methods. Despite the differences in the total arsenic and the arsenic species purity

Table 4 Quantification results for SRM 2669 arsenic species in frozen human urine Level 1 with expanded uncertainties (95% level of confidence; all values are in micrograms per liter as As)

As compound	NIST Method 1	NIST Method 2	NIST Method 1[a]
As(III)	1.42±0.18	1.49±0.15	1.43±0.18
As(V)	2.52±0.18	2.25±0.28	2.22±0.15
MMA	1.67±0.15	1.86±0.09	1.57±0.15
DMA	3.27±0.18	3.26±0.30	3.31±0.20
AB	11.7±0.8	11.1±0.3	11.6±0.8
AC[b]	0.7	0.7	0.7
TMAO[b]	0.8	0.8	0.8

The uncertainties associated with the average measured values are expressed as expanded uncertainties, U, at the approximately 95% level of confidence

[a] Results from SRM 2669 stability testing

[b] Estimated L_D values given because the measured results were less than L_D

Table 5 Quantification results for SRM 2669 arsenic species in frozen human urine Level 2 with expanded uncertainties (95% level of confidence; all values are in micrograms per liter as As)

As compound	NIST Method 1	NIST Method 2	NIST Method 1[a]
As(III)	4.94±0.71	5.14±0.93	5.13±0.49
As(V)	6.89±0.69	5.59±0.20	5.78±0.31
MMA	7.05±1.39	7.01±0.50	7.35±0.62
DMA	25.8±1.4	25.5±1.5	25.0±0.6
AB	1.49±0.17	1.39±0.11	1.39±0.07
AC	3.40±0.28	3.96±0.34	3.54±0.23
TMAO	1.94±0.27	1.72±0.25	2.05±0.14

The uncertainties associated with the average measured values are expressed as expanded uncertainties, U, at the approximately 95% level of confidence

[a] Results from SRM 2669 stability testing

results, the results between the two NIST laboratories, the analysis of both levels of arsenic species in SRM 2669 are not statistically different, in that, the expanded uncertainties (95% confidence) overlap. The one exception is the results for the As(V) mass fraction in Level 2, which is discussed below.

Stability measurements of SRM 2669

Stock solutions of each arsenic species have been stored at −20 °C since the original certification measurements. The solutions were thawed and the total arsenic and species impurities were assessed prior to the quantitative determinations. Total arsenic determinations of the individual arsenic compounds were in good agreement with both original certification analyses (Table 2). The arsenic impurities results show a significant increase in both the As(V) impurity in MMA and the DMA impurity in TMAO (Table 3). Taking into account the increased arsenic impurities in the standard addition spikes, the results from the stability measurements are in good agreement with the preliminary analysis by both methods (Tables 4 and 5). The mass fraction of As(V) in Level 2 is also in good agreement with the original value determined by Method 2. A comparison of the determined As(V) mass fraction between the original certification measurement and the stability measurement by Method 1 shows that the expanded uncertainties are 0.01 g mL^{-1} from overlapping. The difference between the two results from Method 1 suggests there might be unknown sources of uncertainty. However, the method used to calculate the certified values will combine results from additional contributing laboratories, and will account for the potential for between-method and between-laboratory deviations by counting them as components of uncertainty in the certified values [29].

Conclusions

Independent analytical methods were developed for the determination of total arsenic purity and arsenic species impurities in commercially available compounds used in the quantification of arsenic species. Independent separation methods were developed for identification and quantification of seven species in a new frozen urine SRM. The values for the arsenic species determined at NIST detailed in this manuscript will be combined with additional measurements from outside expert laboratories to calculate certified values for SRM 2669 resulting in the first fresh-frozen urine reference material with certified mass fractions of the arsenic species [30]. The low mass fractions of the arsenic species in the urine are at biologically relevant concentrations and will be of use to researchers, who routinely analyze fresh urine for bio-monitoring and toxicological studies. The low levels of arsenic species demonstrate the need for sensitive analytical methods and instrumental techniques. The individual arsenic species have been found to be stable over the first 3 years, and the stability will continue to be monitored on a periodic basis. Future work will focus on development of a LC/MS/MS method for quantification of arsenic species in urine as well as other NIST SRMs.

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