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Concentrations of UV-filters (benzophenones) and arsenic in urine samples from participants in the dietary survey Riksmaten adolescents 2016-17

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Sammanfattning

Inom ramen för Horizon 2020-projektet HBM4EU analyserade 300 urinprover från deltagare i matvane-undersökningen Riksmaten ungdom 2016-17 avseende UV-filter (bensofenoner) samt arsenik och arsenikföreningar. I denna rapport redovisas resultaten från dessa analyser och dessutom undersöks skillnader i exponering mellan pojkar och flickor och mellan åldersgrupper (årskurs 5, årskurs 8 och årskurs 2 på gymnasiet).

Bensofenon-1 (BP-1) och bensofenon-3 (BP-3) kunde detekteras i nästan alla prover (292 respektive 283 av 300), medan bensofenon-2 och bensofenon-7 låg under detektionsgränsen i mer än 90% av proverna. Medianhalten för BP-1 var 0,67 ng/ml (0,49 µg/g kreatinin) och för BP-3 1,2 ng/ml (0,92 µg/g kreatinin). Halterna låg på ungefär samma nivå som i andra europeiska studier av barn och ungdomar. Flickor hade signifikant högre halter av BP-1 och BP-3 i urin än pojkar. Skillnaden kan bero på att flickor använder mer produkter innehållande bensofenoner (kosmetika och hudvårdsprodukter) än pojkar. Halterna av bensofenoner skiljde sig inte åt mellan åldersgrupperna.

Förutom total arsenik (total As) analyserades oorganisk arsenik (arsenit, AsIII och arsenat, AsV), metaboliterna monometylarsonsyra (MMA) och dimetylarsinsyra (DMA) samt den organiska formen arsenobetain i urin. Samtliga föreningar kunde kvantifieras i 91-100% av proverna. Medianhalten var 21 ng/ml (16 µg/g kreatinin) för total As, 5,4 ng/ml (4,0 µg/g kreatinin) för summan av AsIII, AsV, MMA och DMA samt 11 ng/ml (8,5 µg/g kreatinin) för arsenobetain. Summan av AsIII, AsV, MMA och DMA låg på liknande nivå som i andra studier av barn och ungdomar. Pojkar hade signifikant högre urinhalter av arsenobetain än flickor, troligen på grund av att pojkar som deltog i Riksmaten ungdom 2016-17 konsumerade mer fisk än flickor. Det fanns inga tydliga skillnader i arsenikexponering mellan åldersgrupperna, men deltagare i årskurs 8 hade högre urinhalter av MMA än deltagare i gymnasiets årskurs 2.

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INTRODUCTION

The Swedish Food Agency (SFA) regularly conducts national dietary surveys to gather information on food consumption in the Swedish population. The collected data are used to calculate nutrient and energy intake and to estimate exposure to unwanted substances via food. In the most recent dietary survey, Riksmaten Adolescents 2016–17 (RMA), the collection of dietary data was supplemented by a biomonitoring part, where blood and urine samples were collected in a subgroup of the participants. The samples have been used for analyses of markers for nutritional status (Livsmedelsverket, 2018b) and a large number of contaminants (Livsmedelsverket & Naturvårdsverket, 2020).

This report summarizes the results of analyses of UV-filters (benzophenones) and arsenic/arsenic species in 300 urine samples from participants in RMA. Differences in exposure between boys and girls and between age groups are also investigated. The chemical analyses were partly financed by HBM4EU (Human Biomonitoring for Europe), a European Horizon 2020 co-funded biomonitoring initiative. One of the aims with HBM4EU is to prioritize chemicals for human biomonitoring and thereafter collect biomonitoring data for these chemicals that are representative for European children, teenagers and adults. In order to collect biomonitoring data with EU wide coverage, studies conducted in northern, southern, western and eastern Europe have been aligned to HBM4EU. RMA is one of the aligned studies on teenagers from northern Europe. Except for data on benzophenones and arsenic in urine, we have earlier reported serum concentrations of per- and polyfluorinated compounds and urine concentrations of phthalate- and DiNCH-metabolites to HBM4EU. Within HBM4EU, data from all aligned studies will be evaluated together to e.g. investigate if there are geographical differences in exposure.

Benzophenones are UV-filters that are mainly used in sunscreens and other cosmetic and personal care products. They are also used in other consumer products to protect the products against UV radiation, i.e. paints, varnishes, adhesives, sealants and food packaging (Frederiksen et al., 2017). Benzophenone-3 (BP-3) is one of the most widely used benzophenones and it has been used as sunscreen agent for over 40 years (Kim & Choi, 2014). Because of the widespread use, UV-filters are spread to the environment, and benzophenones can for example be found in lakes, rivers, sediment and fish (Kim & Choi, 2014). Benzophenones have shown endocrine disrupting effects in animal studies and are suspected to be associated with birth outcomes and involved in reproductive disorders in humans (Kim & Choi, 2014). Oral and dermal exposure are the main routes of human exposure to UV-filters.

Arsenic (As) is a metalloid naturally present in the environment and can also be emitted as a result of industrial activities. The toxicity of As depends on chemical structure. The inorganic forms (arsenite (AsIII) and arsenate (AsV)), are highly toxic, while organic forms (e.g. arsenosugars and arsenobetain

(AsB)) are assumed to be of no toxicological concern. Adverse effects associated with long-term ingestion of inorganic As in humans are e.g. skin lesions, cancer, neurotoxicity, developmental toxicity and cardiovascular disease (EFSA, 2009). Humans are mainly exposed to As from food and drinking water. Fish and seafood contributes most to the dietary total As exposure. However, it is mostly the organic forms that are found in fish and seafood, while the main contributors to inorganic As are cereals and cereal products including rice (EFSA, 2009, Kollander et al. 2019). Contamination of drinking water with inorganic As is a serious problem in some regions, and for the worldwide public health, exposure from drinking water is most important (Hughes, 2006). Inorganic As is metabolised to monomethylarsonic acid (MMA) in the human body, and MMA is further methylated to dimethylarsinic acid (DMA) and eliminated with urine together with unmetabolized inorganic As (Hughes, 2006).

MATERIALS AND METHODS

Recruitment and sampling

The study population was a biomonitoring subgroup of Riksmaten Adolescents 2016-17, a nationally representative cross-sectional school-based dietary survey conducted by the SFA. Details of the recruitment process and study design are described elsewhere (Moraeus et al., 2018). Briefly, students in school grade 5 (12-year-olds), grade 8 (15-year-olds), and 2nd year of high school (18-year-olds) were recruited between September 2016 and May 2017. Nationally representative schools were invited to participate in the study and one or two school classes from each participating school was included. In the biomonitoring subgroup, 2377 students from 62 schools from all parts of Sweden were invited. 1305 students (55%) participated and donated a spot urine sample. Ethical approval for the survey was obtained from the Regional Ethical Review Board in Uppsala (No. 2015/190). Written informed consent was obtained from all participants and from the legal guardians if younger than 16 years.

Within HBM4EU, 300 individuals from each aligned study are selected for analysis of prioritized chemicals. The selection of 300 individuals from RMA was as far as possible conducted according to the approach suggested by the HBM4EU statistical working group. In brief, participants should be 12-17 years old, not be hospitalized/institutionalized, and have appropriate sampling matrix, sample volume and necessary questionnaire/sample information available. However, the inclusion criteria "participants should have lived at least 5 years in the catchment area" could not be fulfilled, because that information was not available. Among participants fulfilling the inclusion criteria, participants were randomly selected to be evenly distributed with respect to sex, degree of urbanization, maternal education level and sampling season.

Analysis

300 spot urine samples were sent to HBM4EU-qualified laboratories for analysis. The laboratory selected for analysis of UV-filters (benzophenones) was Department of Growth and Reproduction, Rigshospitalet, Region Hovedstaden, Copenhagen, Denmark (RegionH). The laboratory selected for analysis of arsenic, arsenic species and creatinine was Institute and Outpatient Clinic of Occupational, Social and Environmental Medicine, Friedrich-Alexander Universität, Erlangen-Nürnberg, Germany (IPASUM).

The UV-filters benzophenone-1 (BP-1, 2,4-dihydroxybenzophenone, cas no 131-56-6), benzophenone-2 (BP-2, 2,2',4,4'-tetrahydroxybenzophenone, cas no 131-55-5), benzophenone-3 (BP-3, 2-hydroxy-4-methoxybenzophenone, cas no 131-57-7), and benzophenone-7 (BP-7, 5-chloro-2-hydroxybenzophenone, cas no 85-19-8) were analyzed by a method developed for UV filters. In short, isotope dilution TurboFlow-LC-MS/MS was used with preceding enzymatic de-conjugation, where the benzophenones were deconjugated by a mixture of ß-glucuronidase (Escherichia coli K12) and sulfatase from Aerobacter Aerogenes. A detailed description of the method including standards and other materials and equipment, sample preparation, method validation, limits of detections (LOD) and quality characteristics of the method hereunder linear range, matrix effects, intra-day and inter-day accuracy and precision has previously been published (Frederiksen et al., 2017). The samples were measured in six batches each including 50 samples, calibration standards, three blanks and three urine pool controls spiked with a mixture of the four benzophenones at low and high concentration levels, where the recovery for all benzophenones in both the low and high spike level were >85%, while the relative standard deviation in both the low and high spike level were <10%, except for BP-2_{low} (14%), BP-7_{low} (16%) and BP-7_{high} (20%). LOD for BP-1, BP-2, BP-3 and BP-7 was 0.01, 0.08, 0.03 and 0.05 ng/mL, respectively. The laboratory is a selected expert laboratory in HBM4EU and has previously successfully participated in the quality programs, ICI/EQUAS.

Total arsenic (As total), arsenite (AsIII), arsenate (AsV), dimethylarsinic acid (DMA), monomethylarsonic acid (MMA) and arsenobetaine (AsB), were analyzed according to a method described in detail in Appendix 1 (Standard Operation Procedure provided by IPASUM). Briefly, arsenic species were separated by anion-exchange high-performance liquid chromatography (anion-exchange HPLC) and detected by inductively coupled plasma mass spectrometry (ICP-MS). LOQ was 0.05 µg/L for As total and 0.10 µg/L for AsIII, AsV, DMA, MMA and AsB.

Statistics

Statistical analyses were performed using the software package STATA version 14.2. Urine concentrations below LOD (benzophenones) or LOQ (arsenic species) were replaced by LOD/√2 or LOQ/√2. The concentrations of analytes are reported unadjusted (raw data) and adjusted for urine

creatinine. Adjustment for creatinine was not performed for BP-2 and BP-7, since these analytes had concentrations below LOD in 96 and 92% of the samples, respectively.

Analysis of variance (ANOVA), followed by Tukey's multiple comparison test, was conducted to determine if there were differences in exposure (creatinine adjusted contaminant concentrations) between girls and boys or between school grades. Gender and school grade was included in the ANOVA-models. The contaminant concentrations were log-transformed (base e) to approximately conform to normality before the tests.

RESULTS AND DISCUSSION

Characteristics of the 300 teenagers from RMA who were selected for analyses of benzophenones and arsenic are shown in table 1. Urine concentrations of benzophenones and arsenic species are presented in table 2 and 4. Creatinine-adjusted concentrations of benzophenones and arsenic species per gender and school grade are presented in table 3 and 5.

Table 1. *Population characteristics (N=300).*

Variable		**mean ± sd**	**min-max**
Age (years)		15.3 ± 1.7	12.0-17.9
Urine creatinine (g/L)		1.46 ± 0.70	0.21-6.2
		N	**%**
Gender	Boys	150	50
	Girls	150	50
School grade	5th grade (12-year-olds)	25	8
	8th grade (15-year-olds)	176	59
	2nd year of high school (18-year-olds)	99	33

sd, standard deviation

Benzophenones

Almost all participants had concentrations of BP-1 and BP-3 above LOD, while concentrations of BP-2 and BP-7 were below LOD in more than 90% of the samples (Table 2). BP-3 was the UV-filter with highest concentrations (mean 15 ng/mL, median 1.2 ng/mL). BP-3 was earlier analysed by Lund university in urine samples from the whole RMA study population (N=1104), with similar results (density adjusted mean 12 ng/mL, median 0.8 ng/mL) (Livsmedelsverket & Naturvårdsverket, 2020). The observed BP-3 levels are similar or somewhat lower than those reported in Swedish young adults (22-year-olds, mean 17 ng/mL, median 2.2 ng/mL) (Zettergren et al., 2020), Swedish first-time mothers (geometric mean 2.3 ng/mL, median 1.8 ng/mL) (Bjermo et al., 2019), Danish children (6-11 yrs, mean 17 ng/mL, median 1.8 ng/mL) (Frederiksen et al., 2013), Spanish 4-year-olds (median 1.9 ng/mL) (Casas et al., 2011) and Belgian adolescents (12-19 yrs, median 3.6 ng/mL) (Dewalque et al., 2014). In

Danish children and adolescents (6-21 yrs), the BP-1 and BP-3-concentrations varied depending on type of urine sample (24-h, 1st morning or 2nd morning urine), but the median concentrations of BP-1 (0.54-0.97 ng/mL) and BP-3 (0.92-2.04 ng/mL) were similar to the medians in the present study (Frederiksen et al., 2017).

Girls had significantly higher concentrations of BP-1 and BP-3 in urine than boys (Table 3). Higher exposure to BP-1 and BP-3 in women than in men has also been observed in Swedish young adults and Danish adolescents (Zettergren et al., 2020, Frederiksen et al., 2017), probably due to the presence of benzophenones in personal care products and cosmetics (Kim and Choi, 2014) and a higher use of such products in women than in men.

There was a tendency of higher exposure to BP-1 and BP-3 in older participants, although not statistically significant (Table 3). In the earlier analyses of BP-3 in urine samples from the whole RMA study population (N=1104), participants in 2nd year of high school had significantly higher BP-3 concentrations than participants in grade 5 (Livsmedelsverket & Naturvårdsverket, 2020). Similarly, Danish adolescents (14-20 yrs) had significantly higher urine concentrations of BP-3 than children (5-13 yrs) (Frederiksen et al., 2014).

Table 2. *Urine concentrations of benzophenones in teenagers participating in Riksmaten adolescents 2016-17 (N=300). Both unadjusted (raw, ng/mL) and creatinine-adjusted (adj crea, µg/g creatinine) concentrations are presented. Because of the large number of samples <LOD, creatinine adjustment of BP-2 and BP-7 concentrations was not performed.*

Biomarker	LOD	N (%) >LOD	AM	sd	GM	5th percentile	median	95th percentile	min-max
BP-1									
raw	0.01	292 (97)	4.8	18	0.74	0.05	0.67	20	<LOD-203
adj crea			3.7	15	0.57	0.05	0.49	13	<LOD-202
BP-2									
raw	0.08	12 (4)	<LOD	0.05	<LOD	<LOD	<LOD	<LOD	<LOD-0.75
BP-3									
raw	0.03	283 (94)	15	83	1.3	<LOD	1.2	43	<LOD-1042
adj crea			15	118	1.0	0.05	0.92	33	<LOD-1928
BP-7									
raw	0.05	23 (8)	0.05	0.08	<LOD	<LOD	<LOD	0.20	<LOD-0.85

LOD, limit of detection; AM, arithmetic mean; sd, standard deviation; GM, geometric mean
Concentrations below LOD were replaced by LOD/√2 before adjustment for creatinine and in the calculations of mean, sd and percentiles.

Table 3. *Back-transformed least square means (µg/g creatinine) with 95% confidence intervals of benzophenones in urine from Swedish teenagers per gender and school grade.*

	BP-1	BP-3
Gender		
Boys	0.31 (0.22-0.39)	0.65 (0.45-0.84)
Girls	1.1 (0.81-1.4)	1.7 (1.2-2.2)
p-value	<0.0001	<0.0001
School grade		
Grade 5	0.48 (0.16-0.80)	0.69 (0.18-1.2)
Grade 8	0.66 (0.48-0.84)	1.2 (0.81-1.5)
2nd year high school	0.84 (0.56-1.1)	1.3 (0.84-1.8)
p-value	0.25	0.28

Differences between the means were estimated by ANOVA with gender and school grade included in the model.

Arsenic

Almost all participants (91-100%) had concentrations of total arsenic and arsenic species above LOQ (Table 4). Organic AsB showed the highest concentrations, followed by DMA and MMA.

Table 4. *Urine concentrations of total arsenic and arsenic species in teenagers participating in Riksmaten adolescents 2016-17 (N=300). Both unadjusted (raw, ng/mL) and creatinine-adjusted (adj crea, µg/g creatinine) concentrations are presented.*

Biomarker	LOQ	N (%) >LOQ	AM	sd	GM	5th percentile	median	95th percentile	min-max
As total									
raw	0.05	300 (100)	54	112	24	4.6	21	187	1.8-1114
adj crea			40	75	19	3.7	16	141	2.1-634
AsIII									
raw	0.10	296 (99)	0.33	0.29	0.27	0.12	0.26	0.78	<LOQ-3.8
adj crea			0.26	0.21	0.21	0.08	0.20	0.60	<LOQ-2.3
AsV									
raw	0.10	273 (91)	0.26	0.23	0.21	<LOQ	0.20	0.69	<LOQ-1.7
adj crea			0.21	0.17	0.16	<LOQ	0.15	0.56	<LOQ-1.1
MMA									
raw	0.10	300 (100)	1.4	0.62	1.3	0.50	1.4	2.5	0.12-4.1
adj crea			1.2	0.61	1.0	0.34	1.1	2.3	0.08-3.7
DMA									
raw	0.10	300 (100)	4.3	3.9	3.3	0.95	3.4	9.6	0.18-35
adj crea			3.2	2.7	2.5	0.97	2.4	7.4	0.11-17
sum iAs (sum of AsIII, AsV, MMA and DMA)									
raw			6.3	4.2	5.4	2.2	5.4	12	1.0-36
adj crea			4.8	3.0	4.2	1.9	4.0	10	1.0-18
AsB									
raw	0.10	296 (99)	38	89	8.4	0.22	11	140	<LOQ-836
adj crea			28	60	6.5	0.18	8.5	105	<LOQ-554

LOQ, limit of quantification; AM, arithmetic mean; sd, standard deviation; GM, geometric mean.
Concentrations below LOQ were replaced by LOQ/√2 before adjustment for creatinine and in the calculations of sum iAs, mean, sd and percentiles.

Arsenic and arsenic species were earlier analysed by the SFA in 123 urine samples from participants in RMA (Livsmedelsverket & Naturvårdsverket, 2020). However, those results were reported in µg/kg (equal to density-adjustment) which makes them difficult to compare with the results from the present study. Nevertheless, comparing raw data from the present study (in µg/L) with the SFA results (in µg/kg) shows that the analyses performed by the SFA resulted in lower concentrations of total As (median 13 µg/kg), MMA (0,5 µg/kg), DMA (2,4 µg/kg) and AsB (8.7 µg/kg) than the present study. It should be noted that only a few samples that were analysed by the SFA and in the present study were sampled from the same individuals, and that the distribution between the age groups differed in the two studies.

The total As concentration in the present study (geometric mean 19 µg/g creatinine) was higher than in Flemish adolescents (14-15 yrs) (geometric mean 9.3 µg/g creatinine) (Baeyens et al., 2014) and also higher than in U.S. adolescents (12-19 yrs) participating in the NHANES-study in 2013-14 (geometric mean 5.2 µg/g creatinine) (CDC, 2017). The reasons for these differences are unknown. However, the sum of inorganic As and major metabolites (AsIII, AsV, MMA and DMA) was similar in the present study (geometric mean 4.2 µg/g creatinine) as in the Flemish and U.S. studies (geometric mean 3.6 and 4.6 µg/g creatinine, respectively). Thus, it does not seem like the exposure to toxic inorganic As is higher in the present study. In a Swedish study of children (8-10 yrs) living in a contaminated glasswork area in Lessebo municipality, the geometric mean concentration of the sum of AsIII, AsV, MMA and DMA was 6.9 µg/g creatinine (Mattisson et al,. 2020). The higher concentrations in the Lessebo study were mainly due to higher concentrations of DMA and AsV.

Table 5*. Back-transformed least square means (µg/g creatinine) with 95% confidence intervals of arsenic species in urine from Swedish teenagers per gender and school grade.*

	As total	AsIII	AsV	MMA	DMA	AsB
Gender						
Boys	21 (17-25)	0.21 (0.19-0.23)	0.15 (0.13-0.17)	0.99 (0.90-1.1)	2.6 (2.3-2.9)	8.4 (5.7-11)
Girls	17 (14-20)	0.21 (0.19-0.23)	0.17 (0.15-0.19)	1.0 (0.91-1.1)	2.5 (2.2-2.7)	5.3 (3.6-7.0)
p-value	0.17	0.58	0.13	0.82	0.55	0.04
School grade						
Grade 5	17 (9.6-25)	0.20 (0.15-0.25)	0.19 (0.13-0.24)	1.1[ab] (0.86-1.4)	3.1 (2.3-3.9)	5.2 (1.2-9.3)
Grade 8	21 (18-25)	0.22 (0.20-0.24)	0.17 (0.15-0.18)	1.1[b] (0.96-1.1)	2.6 (2.3-2.8)	7.9 (5.6-10)
2nd year high school	15 (12-19)	0.20 (0.18-0.22)	0.14 (0.12-0.16)	0.87[a] (0.77-0.97)	2.4 (2.1-2.7)	5.3 (3.2-7.4)
p-value	0.08	0.45	0.17	0.02	0.18	0.23

Differences between the means were estimated by ANOVA with gender and school grade included in the model. Different letters indicate significant differences between grades ($p<0.05$) according to Tukey's multiple comparison test.

Concentrations of AsB were higher in boys than in girls (Table 5). This may be due to a higher consumption of fish in boys in RMA (Livsmedelsverket, 2018a). There were no clear differences in exposure to arsenic between school grades, but MMA was higher in participants in grade 8 than in participants in 2nd grade of high school (Table 5).

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APPENDIX 1

Arsenic and arsenic compounds – Determination of arsenic species (As(III), As(V), monomethylarsonic acid, dimethylarsinic acid and arsenobetaine) in urine by HPLC-ICP-MS

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1. General principles

The analytical method described hereinafter permits the simple and specific determination of the arsenic species arsenic(III) (As^{3+}), arsenic(V) (As^{5+}), monomethylarsonic acid (MMA), dimethylarsinic acid (DMA) and arsenobetaine (AsB) in urine. The limits of quantitation allow the quantification of the above-mentioned arsenic species in both occupational and environmental medicine.

To this end, the arsenic species are separated by anion-exchange high-performance liquid chromatography (anion-exchange HPLC) and detected by inductively coupled plasma mass spectrometry (ICP-MS). The samples are prepared by diluting the urine

with mobile phase B. Calibration is performed using mixed standards of the different arsenic species.

2. Equipment, chemicals and solutions

2.1 Equipment

HPLC: Two-channel gradient pump and autosampler (Agilent)
Analytical column: PRP-X100 (250 x 4.1 mm) (Hamilton)
ICP mass spectrometer: NexION 350D (Perkin Elmer) coupled to the HPLC by a Meinhard nebulizer and cyclone spray chamber
pH-meter: pHenomenal pH 1000 (VWR)
Piston-stroke pipettes with variably adjustable volume between 10–100 µL, 100–1000 µL and 1–5 mL with suitable pipette tips (e.g. Eppendorf)
Various volumetric flasks and beakers made of glass (e.g. Schott)
Various polypropylene containers with cups (e.g. Sarstedt)
1.5 mL polypropylene vials for the autosampler (e.g. Sarstedt)

2.2 Chemicals

Ammonium dihydrogen phosphate (e.g. Merck No. 1.01126.0500)
Sodium nitrate (e.g. Sigma-Aldrich No. S5506-250)
Sodium acetate (e.g. Merck No. 1.06268.1000)
Sodium hydroxide (e.g. Merck No. 1.06466.0500)
Ethanol (e.g. Merck 1.00983.1000)
Deionised water, Millipore Direct 8 (e.g. Milli-Q)
Argon 4.6 (e.g. Air Liquide)

2.3 Solutions

Table 1: Overview of the weighing for the different solutions

Solution	Weighing/mL deionised water
Ammonium dihydrogen phosphate (1 M)	11.5 g/100 mL H_2O
Sodium nitrate (1 M)	8.499 g/100 mL H_2O
Sodium acetate (1 M)	8.203 g/100 mL H_2O
Sodium hydroxide (1 M)	5.8 g/100 mL H_2O

Table 2: Overview of the composition of the two mobile phases

Reagent	Mobile phase A (1 L)	Mobile phase B (1 L)
1 M Ammonium dihydrogen phosphate	0.5 mL (0.5 mM)	15 mL (15 mM)
1 M Sodium nitrate	2.4 mL (2.4 mM)	2.4 mL (2.4 mM)
1 M Sodium acetate	8 mL (8 mM)	8 mL (8 mM)
Ethanol	10 mL	10 mL
pH-value (adjust with 1M NaOH)	7.5	9.0

2.4 Calibration standards

Working solution I (WS I) (1 mg/L):

Each species is diluted at a ratio of 1:1000 by transferring 100 µL each of MMA, DMA, AsB, As(V) and As(III) into 100 mL volumetric flasks. After that, each flask is made up to the mark with deionised water

Working solution II (WS II) (100 µg/L):

100 µL of each WS I is pipetted into a 1.5 mL polypropylene container. The container is filled with 500 µL mobile phase A and the solution is thoroughly mixed.

Calibration standards:

Calibration standards are prepared in the concentration range up to 25 µg/L by diluting the two working solutions according to the scheme given in Table 3.

The calibration standards must be freshly prepared every day.

Table 3: Pipetting scheme for the preparation of calibration standards

Calibration standard	Concentration of the calibration standard	Volume of WSI / WSII	Volume of mobile phase A	Final volume
	[µg/L]	[µL]	[mL]	[mL]
0	0	0	10	10
1	0.1	10 WS II	9.99	10
2	0.25	25 WS II	9.975	10
3	0.5	50 WS II	9.95	10
4	2	200 WS II	9.8	10
5	10	100 of each WS I	9.5	10
6	25	250 of each WS I	8.75	10

3. Sample preparation

The urine samples are brought to room temperature and mixed thoroughly. Afterwards, the urine samples are diluted 1:1 with the mobile phase B. The final volume should not be <100 µL to avoid partial injections. The diluted samples are filled into vials, homogenized and transferred to the autosampler.

4. Operational parameters

Analysis was performed using HPLC with an anion exchange column coupled with ICP-MS detection.

4.1 High performance liquid chromatography

Separation column:	PRP-X100
Length:	250 mm
Inner diameter:	4.1 mm
Particle size:	10 µm
Separation principle:	Anion exchange
Pre-column:	PRP-X100, 10 µm
Mobile phase A and B:	See Table 2
Flow rate:	1.2 mL/min
Injection volume:	50 µL

The gradient program is given in Table 4.

Table 4: Gradient program for the determination of arsenic species in urine

Time [min]	Eluent A [%]	Eluent B [%]
0	100	0
2.5	100	0
5.5	0	100
13	0	100
13.5	100	0
21.5	100	0

The HPLC is directly connected to the Meinhard nebulizer with a cyclone spray chamber of ICP-MS by Teflon tubing.

4.2 Inductively coupled plasma mass spectrometry

The settings described in Table 5 are intended as a rough guide only. These parameters must be optimized individually for each system. Additional setting and

parameter optimization may be required when using spectrometers from other manufacturers.

Table 5: Overview of the different system parameters

Plasma power	1500 W
Plasma gas	15 L/min argon
Auxiliary gas	0.6 L/min argon
Nebulizer gas	0.95 – 1.05 L/min argon
Nebulizer	Meinhard
Nebulizer chamber	Cyclone type (18 – 25 °C, ambient temperature)
Analysed mass traces	75
Cone material	platinum
Reaction gas	none

5. Analytical determination

50 µL each of the samples prepared as described in Section 3 are injected into the HPLC-ICP-MS system. It is recommended to determine each sample in duplicate. Identification of the arsenic species is based on the retention times compiled in Table 6. The retention times given are intended as a rough guide only. Users of the method must ensure proper separation performance of the analytical column used influencing the resulting retention behaviour of the analytes. A reagent blank (mobile phase A instead of the urine sample) is included in each analytical run.

Table 6: Retention times of the analysed arsenic species

Species	Retention time [min]
AsB	2
As(III)	3
DMA	5
MMA	9
As(V)	11

Figure 1 shows an example of a typical chromatographic separation.

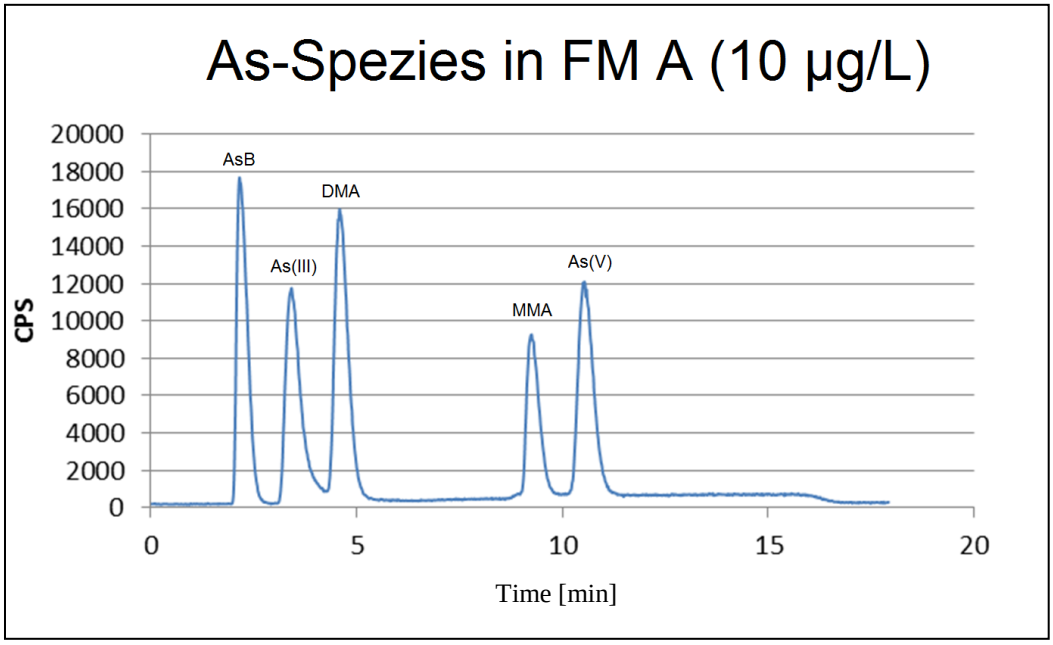

Figure 1: Example of a chromatogram of arsenic species standard number 5

6. Calibration

The calibration solutions prepared as described in Section 2 are randomly placed in the sample list to compensate for sensitivity variations. After ten samples, a new set of calibration is measured and is used for quantification. The calibration graph is obtained by plotting the concentration of the calibration solution against the peak area of the ^{75}As mass peak. Under the described conditions, the calibration graphs are linear for all analytes in a range between the detection limit and 25 µg As/L. Calibration should be performed anew for each day of analysis.

7. Calculation of the analytical results

To calculate the analyte concentration in a sample, the peak area of the ^{75}As mass peak of the analytes determined in the analysed urine sample is inserted into the corresponding calibration graph. The arsenic concentration is obtained in µg per litre urine. Any reagent blank values, which may be present, are accounted for by subtraction.

8. Quality control

To check precision, two quality control samples with known and constant analyte concentrations are analysed within each analytical run. One analytical run consists of the calibration and ten urine samples. For quality control, samples from G-EQUAS are used. When stored at -18 °C, aliquots of these solutions can be used for several years for quality control. The nominal value and the tolerance ranges of the quality control material are determined in the regular program of G-EQUAS. The measured values of the control samples analysed within each analytical run should each be within the determined tolerance ranges.

9. Evaluation of the method

9.1 Precision

To determine within-day precision, urine samples with a known concentration (G-EQUAS) of each individual arsenic species are processed several times in parallel and then analysed. The obtained within-day precision data are given in Table 7.

Table 7: Within-day precision for the determination of arsenic species in urine (n=8)

Analyte	Concentration [µg As/L]	Standard deviation (rel.) [%]	Prognostic range [%]
As(III)	0.39	10.9	25.8
As(V)	0.40	12.8	30.3
MMA	1.35	7.5	12.0
DMA	5.22	6.6	15.6
Arsenobetaine	69.5	2.0	5.6

Day-to-day precision was determined using urine samples (G-EQUAS) with a known concentration of each individual arsenic species. Therefore, the urine samples were processed on different days and analysed. The results are summarized in Table 8-9.

Table 8: Day-to-day precision for the determination of arsenic species in urine (Q_{low}, n=8

Analyte	Mean value [µg As/L]	Standard deviation (rel.) [%]	Prognostic range [%]
As(III)	0.39	9.1	21.6
As(V)	0.40	13.9	32.9
MMA	1.35	5.6	13.4
DMA	5.22	4.5	10.7
Arsenobetaine	69.5	2.4	5.5

Table 9: Day-to-day precision for the determination of arsenic species in urine (Q_{high}, n=8)

Analyte	Mean value [µg As/L]	Standard deviation (rel.) [%]	Prognostic range [%]
As(III)	1.6	4.9	11.7
As(V)	4.1	3.5	8.4
MMA	2.3	6.3	14.9
DMA	9.3	1.6	3.7
Arsenobetaine	126.94	1.9	4.6

9.2 Accuracy

The relative recovery was calculated on the basis of the determined concentrations of the day-to-day precision data. The results are summarized in Table 10.

Table 10: Relative recovery rates for the determination of arsenic species in urine (Q_{low})

Analyte	Concentration [µg As/L]	Mean value [µg As/L]	Mean relative recovery [%]
As(III)	0.39	0.40	113
As(V)	0.40	0.38	94
MMA	1.35	1.27	94
DMA	5.22	5.30	101
Arsenobetaine	69.5	70.3	101

9.3 Limits of detection and limits of quantitation

The detection and quantitation limits were determined according to DIN 32645 [2008]. For this purpose, an equidistant 10-point calibration was carried out, whereby the samples in urine were analysed three times in a concentration range of 0.1-1 µg/L. The detection and quantification limits can be calculated from the standard deviation of the calibration function obtained in accordance with DIN 32645. The results are presented in Table 11.

Table 11: Detection limits and quantitation limits of the arsenic species analysed

Analyte	Detection limit [µg As/L]	Quantitation limit [µg As/L]
As(III)	0.03	0.1
As(V)	0.02	0.1
MMA	0.02	0.1
DMA	0.03	0.1
Arsenobetaine	0.04	0.1