

1 **Rapid screening of arsenic species in urine from exposed human by inductively**
2 **coupled plasma mass spectrometry with germanium as internal standard**

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10 **Abstract**

11 In the present work, internal standardization based on species-unspecific isotope dilution
12 analysis technique is proposed in order to overcome the matrix effects and signal drift originated in
13 the speciation of As in urine by HPLC-ICP-MS. To this end, ^{72}Ge has been selected as a pseudo-
14 isotope of As. The resulting mass flow chromatogram of the element allows the calculation of the
15 corrected overall species concentrations without requiring any methodological calibration,
16 providing high-throughput sample processing. The validation was carried out by analyzing a blank
17 human urine fortified at three concentration levels and an unspiked human urine sample containing
18 different species of arsenic. In all cases, recoveries ranging from 90 to 115% and RSD below 10%
19 were attained with this approach. Furthermore, the proposed method provided results in excellent
20 agreement with those obtained using standard additions and internal standard calibration, allowing a
21 fast way to assess human exposure to arsenic species.

22 **1. Introduction**

23 It is widely known that potential health risk to arsenic exposition depends on the chemical form
24 entering the human body, due to the different degree of toxicity of these compounds.¹⁻⁴ Urine is
25 regarded as an important biomarker of arsenic intake. In this regard, a speciation analysis of urine is
26 usually performed by HPLC-ICP-MS.⁵⁻¹⁰ However, signal drift and matrix effects are observed due
27 to urine matrix, hampering the quantification of such species.¹¹

28 The use of species-unspecific isotope dilution analysis allows the correction for those errors,
29 providing accurate and precise determinations of the sought element. In the case of arsenic, there is
30 only one isotope available to be measured (m/z 75); therefore, an internal standard of an element
31 close to the analyte mass has to be selected to follow the isotope dilution procedure.¹² This
32 approach, which we have called “pseudo-unspecific isotope dilution analysis”, could make possible
33 to obtain the accurate concentrations of the above mentioned species in a single run.

34 The developed method was applied for the analysis of human urine samples fortified at different
35 concentration levels and compared with internal calibration and the standard additions method.

36 2. Experimental

37 2.1. Instrumentation

38 The HPLC system consisted of an Agilent 1100 Series (Agilent, Waldbronn, Germany)
39 binary pump and auto injector with a programmable sample loop (100 μ L maximum). The
40 separations were performed on a PRP-X100 (Hamilton, Reno, NV, USA) anion-exchange
41 column (250 x 4.1 mm, 10 μ m). An additional Agilent 1100 Series binary pump was used to
42 continuously add the internal standard solution.

43 The outlet of the chromatographic column was connected through a T piece to a Meinhard
44 concentric nebulizer. An Agilent 7500cx inductively coupled plasma mass spectrometer
45 (Agilent Technologies, Tokyo, Japan) equipped with an octopole reaction cell using helium as
46 a reaction gas to reduce polyatomic interferences on arsenic was used in this work. For
47 HPLC-ICP-MS data acquisition, the “time resolved analysis” mode was used with 1 second of
48 integration time per mass.

49 For tuning of ICP-MS, a solution containing 10 μ g L⁻¹ of As made up in double deionized
50 water filtered through 0.45 μ m was monitored at m/z 75 and 72; the ion intensity, resolution
51 and mass axis were optimized.

52 2.2. Standards and reagents

53 Arsenite (As^{III}), arsenate (As^V), dimethylarsinic acid (DMA) and arsenobetaine form (AsB)
54 were delivered by Fluka (Buchs, Switzerland), while monomethylarsonic acid (MMA) was
55 from Carlo Erba (Milano, Italy). The stock solutions of arsenic species containing about 1000
56 mg L⁻¹ of As were prepared in water and maintained at 4 °C after standardization against an
57 atomic absorption arsenic standard solution (J.T. Baker, Phillipsburg, USA).¹³ Appropriate
58 dilutions of the stock solution were prepared daily, by weight, using double deionized water
59 to obtain the required concentration. Germanium internal standard and nitric acid was
60 purchased from Fluka.

61 A previously developed method⁵ based on HPLC-ICP-MS coupling system was optimized
62 in order to separate the five arsenic species in human urine. The mobile phase consisted of 4
63 mM ammonium phosphate (Merk, Darmstad, Germany), 4 mM ammonium hydrogen
64 carbonate (Fluka) and 4 mM ammonium sulfate (Sigma, St. Louis, MO, USA). The pH value
65 was adjusted to 8.9 by the addition of ammonium hydroxide (Trace Select, Fluka). These
66 solutions were filtered through a 0.45 µm membrane before use.

67 All the aqueous solutions were prepared with Milli Q Gradient A10 (Millipore, Molsheim,
68 France) water (18.2 MΩ cm).

69 *2.3. Analytical procedure*

70 Urine samples were diluted 5 fold with 0.1 % nitric acid before injection. The flow coming
71 from the column (0.95 mL min⁻¹) was mixed with the internal standard solution containing
72 around 15 ng g⁻¹ of Ge and 4 ng g⁻¹ of As (0.15 mL min⁻¹). The signals for m/z 75 and 72
73 were monitored over time. After smoothing of the data using moving average (n = 5) in order
74 to reduce noise level the isotope ratio ⁷⁵As/⁷²Ge was calculated. Then, the on-line pseudo-
75 isotope dilution equation was applied to each point of the chromatogram to obtain the mass
76 flow chromatogram. The amount of arsenic in each fraction was determined by integration of
77 the chromatographic peaks using the Origin 5.0 software (Microcal Software Inc.,
78 Northampton, MA, USA). Finally, the concentration of arsenic was computed by dividing the
79 As amount found by the injection volume.

80 3. Results and discussion

81 3.1. Selection of internal standard and development of the equation for on-line pseudo-isotope 82 dilution analysis

83 In order to appropriately correct for matrix-induced signal enhancement or suppression as
84 well as for drift instability of the instrument, the analyte and the internal standard should
85 undergo an equal relative signal intensity drift. To this end, mass-to-charge ratio and
86 ionization potential of both elements should be as close as possible, being especially critical
87 the first factor. In this regard, selenium seems the best candidate to use as internal standard for
88 arsenic speciation.^{12,14} However, this element is often present in urine samples, which can
89 produce errors in the normalization. By contrast, germanium is rarely present in urine samples
90 and has been satisfactory used for matrix effects correction.¹⁵ As a consequence, the isotope
91 ⁷²Ge was selected as internal standard.

92 The proposed procedure is based on post-column isotope dilution analysis.¹⁶ Briefly, this
93 technique consists in the on-line addition of an isotopically enriched solution of the sought
94 element after the chromatography separation to modify the original isotope abundances in the
95 sample. The resulting isotope ratio (in the mixture) of the most abundance isotope in the
96 sample and the spike permits to calculate the endogenous concentration contained in each
97 chromatographic peak. In the case of arsenic, a germanium internal standard is used instead of
98 a spike, owing to its monoisotopic character. Since both elements have different ionization
99 efficiencies, the experimental isotope ratio in the mixture R_m (⁷⁵As/⁷²Ge) will not provide the
100 As/Ge molar ratio. The instrumental response of Ge present in the mixture must be previously
101 normalized to As in order to correlate R_m with the analyte concentration. For this purpose, a
102 known amount of As was added within the internal standard solution and the corresponding
103 isotope ratio R_{IS} (⁷⁵As/⁷²Ge) was measured. Consequently, R_m and R_{IS} can be expressed as
104 follows:

105
$$R_m = \frac{N_s^{As} \cdot d_s \cdot f_s + N_{IS}^{As} \cdot d_{IS} \cdot f_{IS}}{N_{IS}^{Ge'} \cdot d_{IS} \cdot f_{IS}} \quad (1)$$

106
$$R_{IS} = \frac{N_{IS}^{As}}{N_{IS}^{Ge'}} \quad (2)$$

107 were N_s^{As} (mol g⁻¹) shows the amount of As in the sample with density d_s (g mL⁻¹) pumped at
 108 a flow rate f_s (mL min⁻¹), which is mixed with N_{IS}^{As} (mol g⁻¹) of As arising from the internal
 109 standard solution pumped at a flow rate f_{IS} (mL min⁻¹) and density d_{IS} (g mL⁻¹). The term
 110 $N_{IS}^{Ge'}$ (mol g⁻¹) is the concentration of Ge normalized to As, which must not be confused with
 111 the true amount of Ge. Indeed, $N_{IS}^{Ge'}$ would represent the mol g⁻¹ of ⁷²Ge contained in the
 112 internal standard solution if the ionization efficiency were the same as As. When we combine
 113 eqns. (1) and (2) the following expression is obtained:

114
$$R_m = \frac{N_s^{As} \cdot d_s \cdot f_s + N_{IS}^{As} \cdot d_{IS} \cdot f_{IS}}{(N_{IS}^{As} / R_{IS}) \cdot d_{IS} \cdot f_{IS}} \quad (3)$$

115 Please note that the true amount of Ge is not needed in the calculation. Rearranging eqn.
 116 (3) for N_s^{As} , we obtain:

117
$$N_s^{As} \cdot d_s \cdot f_s = N_{IS}^{As} \cdot d_{IS} \cdot f_{IS} \cdot \left(\frac{R_m}{R_{IS}} - 1 \right) \quad (4)$$

118 Concentrations in mol g⁻¹ can be expressed as concentrations in weight by taking into
 119 account the atomic weight of the element. Since the atomic weight of As in the sample
 120 (AW_s^{As}) and in the internal standard solution (AW_{IS}^{As}) are the same, eqn. (4) becomes:

121
$$C_s^{As} \cdot d_s \cdot f_s = C_{IS}^{As} \cdot d_{IS} \cdot f_{IS} \cdot \left(\frac{R_m}{R_{IS}} - 1 \right) \quad (5)$$

122 were C_s^{As} and C_{IS}^{As} are the mass concentrations (ng g⁻¹) of As in the sample and internal
 123 standard solution, respectively. $C_s^{As} \cdot d_s \cdot f_s$ has the units of ng min⁻¹ and it is the mass flow of

124 the sample eluting from the column, MF_s . Then, the final pseudo-isotope dilution equation has
125 the form:

$$126 \quad MF_s = C_{IS}^{As} \cdot d_{IS} \cdot f_{IS} \cdot \left(\frac{R_m}{R_{IS}} - 1 \right) \quad (6)$$

127 If the analyte concentration changes with time, e.g., during the chromatographic peak, MF_s
128 will also change with time. The integration of the chromatographic peak in the mass flow
129 chromatogram will give the amount of As in that fraction. The concentration is then easily
130 calculated knowing the sample volume injected. Eqn. (6) was thus used for calculations in the
131 present work.

132 3.2. Analytical results

133 In a preliminary study, the concentration of the As primary standard in the internal
134 standard solution was optimized. On the one hand, it has to be taken into account that a high
135 enough amount of exogenous As is required to minimize the m/z 75 background influence.
136 On the other hand, the higher amount of ^{75}As coming from the post-column solution the
137 higher baseline noise, leading to poorer detection limits of the endogenous species. Thereby, a
138 concentration of ca. 4 ng g^{-1} was selected as a compromise. The case of Ge internal standard
139 concentration is much less critical because it does not contribute to the signal of the analyte
140 eluting from the column and does not influence the final results. In addition, the
141 chromatographic and post-column flow rates were tested. The final values used for the mobile
142 phase and the internal standard solution (0.95 and 0.15 mL min^{-1} , respectively) allowed the
143 elution of all the species in a proper time without sacrificing the accuracy of the post-column
144 flow rate.

145 To a better understanding of the procedure, Fig. 1 illustrates the conversion from original
146 ICP-MS intensities to mass flow. The chromatograms corresponding to m/z 75 and 72 (a) are
147 first transformed into the isotope ratio chromatogram (b). It is worth stressing that ^{75}As and
148 ^{72}Ge background signal from mobile phase was negligible (data not shown), thus the baseline

149 of the isotope ratio chromatogram provides R_{IS} (see Fig. 1b). Next, equation (6) is applied to
150 the whole chromatogram. Finally, the mass flow peaks of Fig. 1c are integrated and divided
151 by the injection volume (50 μL). It should be remarked that the present strategy permits to
152 correct for errors derived from instrumental instabilities and matrix effects in the whole
153 chromatogram, since the Ge internal standard is continuously added to the effluent from the
154 column.

155 The proposed procedure was applied to the analysis of a blank urine sample spiked at 1, 5
156 and 10 $\mu\text{g As L}^{-1}$ (concentrations referred to the diluted urine injected). It is worth noting that
157 the blank urine used corresponds to an unexposed human and no As species were found when
158 it was analyzed by the conventional calibration method. Ten replicates for each of the three
159 fortification levels were carried out. Additionally, in order to check the suitability of ^{72}Ge as
160 internal standard the results were compared with those obtained using internal standard
161 calibration. To this end, calibration standards containing 0-20 $\mu\text{g L}^{-1}$ of As for each compound
162 were injected by triplicate within the post-column solution. Then, the isotope ratio
163 chromatogram was plotted (as exemplify in Fig. 1b). Satisfactory recoveries, between 90 to
164 105%, were obtained both for the medium and highest fortification levels when pseudo-
165 unspecific IDA was used. At the lowest fortification level, which was closed to the detection
166 limit, recoveries were in the range of 96-115% and coefficients of variation were below 10%
167 (Table 1). No significant differences were noticed between both methods, thus it seems that
168 calibration-free measurements based on eqn. (6) can be performed for the quantification of As
169 species in urine.

170 Intermediate precision ($n = 9$) was also estimated by analyzing replicates of the medium
171 fortification level on 3 different days. The coefficient of variation was found to be $<8\%$ in all
172 cases. Detection limits, defined as three times the signal-to-noise ratio in the mass flow
173 chromatogram were determined for the blank urine sample spiked at 1 $\mu\text{g L}^{-1}$ of each As

174 species. As can be seen in Table 1, LODs were $<0.7 \mu\text{g L}^{-1}$ in the diluted urine. In fact, the
175 continuous addition of arsenic post-column to normalize de germanium response increase
176 notably the detection limits. However, these values are satisfactory to evaluate the potential
177 risk of people exposed to inorganic arsenic. Actually, the American Conference of
178 Governmental Industrial Hygienists (ACGIH) and Deutsche Forschungsgemeinschaft (DFG)
179 set the BEI and BAT values for occupational arsenic exposure as $35 \mu\text{g As L}^{-1}$ and $50 \mu\text{g L}^{-1}$,
180 using the sum of inorganic arsenic, MMA and DMA.¹⁷

181 The validity of the proposed method for correcting instrument signal drift was tested with
182 the injection of a 15-h batch run. Fig. 2a compares the intensity chromatograms
183 corresponding to the first and last injections of the experiment. Appreciable signal drift was
184 observed between them, resulting in lower peak areas for the second injection. The
185 application of eqn. (6) lead to the mass flow chromatograms presented in Fig. 2b. As
186 expected, no significant differences were noticed between injections, which confirm that
187 appropriate correction of signal drift is achieved. It is worth mentioning the severe signal
188 suppression at the dead volume caused by the high salt content of urine sample (Fig. 2a). In
189 this case, instrumental instability was not totally overcome (Fig. 2b), most probably because
190 the Ge internal standard suffers more signal depression than As in the presence of high
191 concentration of Na. Anyway, such anomalous behavior does not affect any chromatographic
192 peak

193 Finally, the quantification of a human urine sample containing different species of arsenic
194 was performed by the present methodology, internal standard calibration and standard
195 additions (Table 2). The concentrations calculated by pseudo-unspecific IDA were in very
196 good agreement with those obtained using the other quantification strategies. These data
197 confirm the suitability of the developed procedure for arsenic speciation studies in human
198 urine.

199 **Conclusions**

200 A new procedure for the simultaneous determination of AsB, As^{III}, As^V, DMA and MMA
201 in human urine which does not require any methodological calibration graph and allows
202 correcting for instrumental instabilities has been developed. For this purpose, species
203 unspecific isotope dilution analysis has been adapted to As using ⁷²Ge as an additional isotope
204 of the sought element.

205 The proposed method has been successfully validated in spiked and unspiked human
206 urinesamples. In addition, the results were in excellent agreement with internal standard
207 calibration and standard additions.

208 The need for addition a known amount of As to normalized the Ge concentration increases
209 appreciably the LODs. This fact however, do not hampers the correct quantification of toxic
210 inorganic species of arsenic in urine of exposed humans

211 Therefore, the possibility to carry out the quantification of As species in a single run
212 provided by the developed procedure could be very useful to assess workplace, drinking water
213 or food exposure to inorganic arsenic.

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221

222 **References**

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Table 1. Analytical characteristics of the pseudo-unspecific IDA procedure obtained for a blank urine sample fortified at three different levels.

Species	1 $\mu\text{g As L}^{-1}$		5 $\mu\text{g As L}^{-1}$		10 $\mu\text{g As L}^{-1}$		Intermediate precision. 5 $\mu\text{g As L}^{-1}$ C.V. (%) (n=9)	LOD ^c ($\mu\text{g L}^{-1}$)
	Pseudo-IDA	IC ^b	Pseudo-IDA	IC	Pseudo-IDA	IC		
As ^B	96.8 (8.7) ^a	104.6 (10.0)	90.8 (1.8)	99.4 (1.5)	92.1 (2.2)	101.3 (0.9)	6.2	0.3
As ^{III}	99.5 (5.9)	111.0 (4.0)	100.7 (7.4)	106.5 (5.1)	101.1 (4.9)	104.8 (0.9)	7.4	0.6
DMA	102.0 (6.3)	106.5 (3.5)	97.5 (9.5)	94.3 (6.9)	100.0 (2.3)	94.4 (1.6)	3.5	0.3
MMA	114.6 (1.7)	115.6 (2.4)	101.1 (9.2)	101.8 (6.8)	102.6 (3.0)	101.6 (3.3)	5.5	0.4
As ^V	104.0 (3.6)	104.2 (5.4)	95.7 (6.6)	103.1 (6.8)	96.5 (1.1)	103.1 (0.4)	3.8	0.7

^a The uncertainty in the values corresponds to 1 s standard deviation of 10 independent HPLC-ICP-MS injections.

^b Recoveries calculated using internal standard calibration, for comparison.

^c Detection limits referred to diluted urine sample.

Table 2. Comparison of different methodologies to correct for matrix effects in the analysis of a human urine sample.

Method	AsB, $\mu\text{g L}^{-1}$	As ^{III} , $\mu\text{g L}^{-1}$	DMA, $\mu\text{g L}^{-1}$	MMA, $\mu\text{g L}^{-1}$	As ^V , $\mu\text{g L}^{-1}$	Sum of the species
Pseudo-unspecific IDA	15.5 ± 0.3	ND	3.2 ± 0.2	2.5 ± 0.5	3.8 ± 0.3	25.0 ± 1.3
Internal standard calibration	16.4 ± 0.3	ND	3.4 ± 0.2	2.1 ± 0.5	4.0 ± 0.5	25.9 ± 1.5
Standard additions	14.6 ± 0.1	ND	3.0 ± 0.1	2.4 ± 0.1	3.5 ± 0.1	23.5 ± 0.4

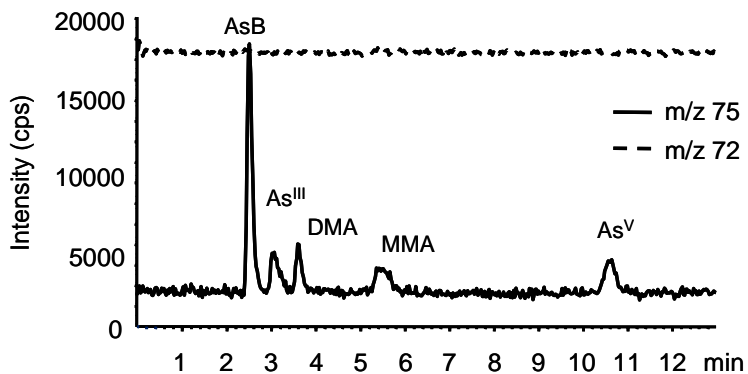
Figure captions

Figure 1.- Conversion process from intensities to mass flow using the pseudo-isotope dilution equation.

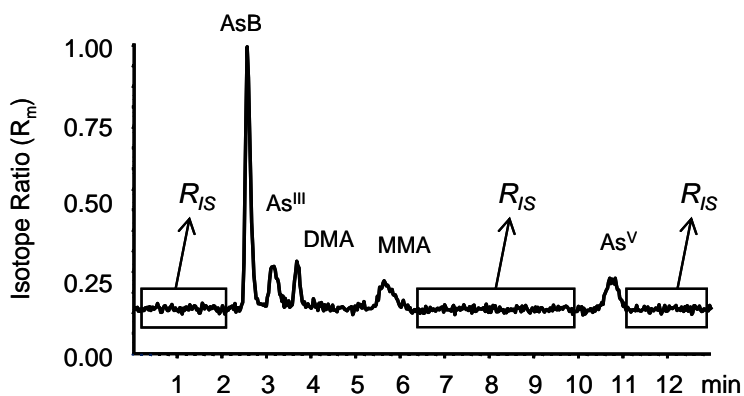
Figure 2.- Use of Ge as a pseudo-isotope of As to correct for instrumental signal drift observed during a 15-h batch run.

Figure 1

a) Intensity chromatogram



b) Isotope ratio chromatogram (⁷⁵As/⁷²Ge)



c) Mass flow chromatogram

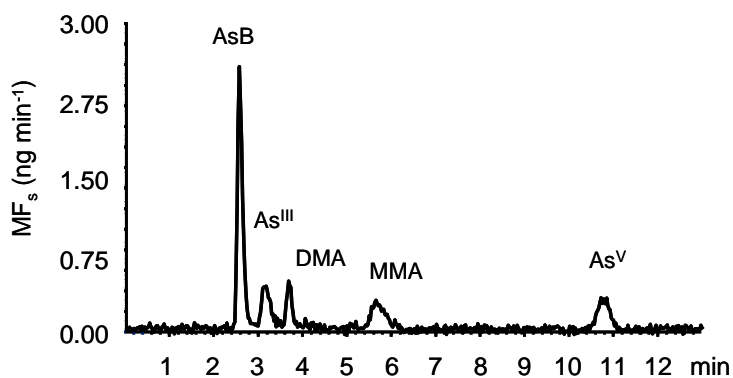
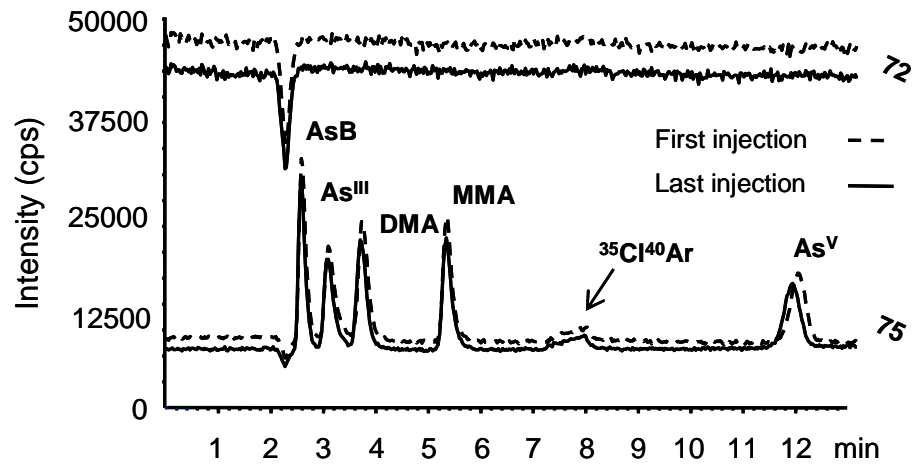


Figure 2

a) Intensity chromatograms



b) Mass flow chromatograms

