Measurements of Methylmercury, Ethylmercury, and Inorganic Mercury Species in a Whole Blood Standard Reference Material: SRM 955c – Toxic Elements in Caprine Blood

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Abstract

Trace elemental speciation analysis is assuming more critical importance in nutritional and toxicological assessments, and despite the necessity to quantitatively differentiate elemental species in environmental and clinical samples, there are a limited number of reference materials suitable for this purpose. A whole blood certified reference material, SRM 955c Toxic Elements in Caprine Blood was developed for the determination of toxic metals in whole blood including mercury. The certification of methylmercury, ethylmercury, and inorganic mercury was carried out by triple spike speciated isotope dilution inductively coupled plasma mass spectrometry (TS-SID-GC/ICP-MS) and represents the first whole blood material certified for three mercury species. The certified values (± expanded uncertainty) of methylmercury, ethylmercury, inorganic mercury and total mercury in SRM 955c are 4.5 ± 1.0 µg L^{-1}, 5.1 ± 0.5 µg L^{-1}, 9.0 ± 1.3 µg L^{-1}, and 17.8 ± 1.6 µg L^{-1} respectively.

Key Words: ICP-MS, speciation, mercury, blood, methylmercury, ethylmercury, reference material

Introduction

Clinical measurements of biological samples such as blood, urine, and keratin tissue (i.e. hair and nails) have long been important tools in monitoring environmental and occupational exposure of contaminants and are used to determine whether adverse health effects are likely to occur. Mercury measurements in urine are commonly used to evaluate exposure to inorganic mercury, while whole blood is used to evaluate exposure to the highly toxic organomercury compounds (i.e. methylmercury and ethylmercury). Although methylmercury is the predominant form,[1, 2] inorganic mercury is also present[1] in blood. Ethylmercury has been of interest due to exposure from thimerosal (sodium ethylmercurithiosalicylate) which had been added (0.003 % - 0.01 %) to several vaccines for preservative and antimicrobial purposes.[3, 4]

Blood is a dynamic tissue, the composition of which is constantly changing throughout the body. Organomercury compounds may circulate in the blood for extended periods of time and therefore have an opportunity to accumulate in the central nervous system. The toxic effects of mercury are dependent on its chemical form and total mercury measurements do not necessarily provide sufficient information for toxicity and bioavailability assessments. For these reasons, the specific measurement of mercury species in whole blood is an ideal means to capture multiple exposure routes of both organic and inorganic forms of mercury.

While there have been several reports in the literature of the measurement of mercury species in blood,[2, 5-9] total mercury determinations in blood and urine are more common.[10-12] Simple and robust sample preparation and quantification methods, the relative cost of analysis, and the need to generate accurate results for clinical diagnostics,

are all critical considerations when laboratories are faced with increasing numbers of samples that require more detailed analysis reporting. In addition, the use of reference materials that are certified for elemental species of interest in clinical matrices like blood and urine are also of great importance in determining method accuracy and reproducibility as well as providing measurement quality control and assurance for unknown patient samples.

High accuracy quantification strategies such as standard addition with internal standards have been utilized for the determination of elemental species and total elemental content in a variety of matrices such as environmental and clinical samples.[13] Monperrus and co-workers demonstrated that external calibration is not as accurate a quantification technique when compared to speciated isotope dilution (SID) in the measurement of methylmercury and tributyltin in oyster tissue.[14] However, a recent example detailing the use of external calibration for the quantification of mercury species in blood provided good results for both methylmercury and inorganic mercury in SRM 966 Toxic Metals in Bovine Blood.[9] SID analysis has become a method of choice for the quantification of organometallic species when species-specific isotopically enriched spikes are available[14-18] and this approach has been utilized by National Metrology Institutes (NMI) for certification of organometallic species in reference materials.[19, 20]

With the growing need for accurate quantification methods for speciation measurements in clinical samples,[1] appropriate reference materials are needed in a variety of clinical matrices (e.g. blood and urine) with certified concentrations for a variety of elemental species. SRM 955c Toxic Elements in Caprine Blood is intended to replace SRM 966 Toxic Metals in Bovine Blood which currently has reference values for both

methylmercury and inorganic mercury at the occupational exposure level of concern (i.e. American Conference of Industrial Hygienists recommends mercury blood levels due to inorganic mercury exposure not exceed 15 μg L^{-1}). This work describes the determination of mercury species in whole blood as demonstrated through the certification analysis of SRM 955c Toxic Elements in Caprine Blood, including the first reference material certified for ethylmercury in whole blood.

Experimental

SRM 955c Toxic Elements in Caprine Blood

The collection and preparation of SRM 955c Toxic Elements in Caprine Blood (formally Lead in Caprine Blood) has been described previously.[21] Units of SRM 955c consist of four vials of frozen caprine blood at four concentration levels: a base level and three progressively elevated levels that contain endogenous lead and spiked inorganic arsenic, cadmium, ethylmercury inorganic mercury, and methylmercury. Each vial contains approximately 2 mL of whole blood.

Reagents and chemicals

Stock solutions of mercury compounds were prepared gravimetrically from methylmercury chloride (Sigma Aldrich, St. Louis, MO, USA) ethylmercury (Pfaltz & Bauer, Waterbury, CT, USA), and inorganic mercury (SRM 3133 Mercury Standard Solution, NIST, Gaithersburg, MD, USA), in Millipore 18.6 MΩ·cm water (Millipore, Bedford, MA, USA). Sodium tetrapropylborate (GALAB, Geesthacht, Germany) and ultra grade acetic acid (EMD Chemicals, Gibbstown, NJ, USA), were used in the derivatization of mercury species to volatile forms for analysis by gas chromatography inductively coupled mass spectrometry (GC/ICP-MS). Tetramethylammonium

hydroxide solution (TMAH 25 % in water) (Fluka, Switzerland) was used in the extraction of mercury from the blood matrix. HPLC grade hexane (Burdick and Jackson, MI, USA) dichloromethane (Burdick and Jackson), and alumina (Acros Organics, Geel, Belgium) were used in the solid phase extraction (SPE) sample cleanup process. Ultra high purity nitric acid (Fisher Scientific, Suwanee, GA, USA) was used to digest the blood, and tin (II) chloride (JT Baker, Phillipsburg, NJ, USA) and hydrochloric acid (JT Baker) were used in the reduction of inorganic mercury to Hg^0 for the total mercury analysis. Additionally, IRMM-670 ^{202}Hg enriched methylmercury (Institute for Reference Materials and Measurements, Geel, Belgium), ^{201}Hg enriched ethylmercury (AIT, Sunnyvale, CA, USA), ^{198}Hg enriched inorganic mercury (Trace Sciences International, Wilmington, DE, USA) were used as the species specific spikes while a ^{201}Hg enriched inorganic mercury spike derived from mercury (II) oxide (Oak Ridge National Laboratory, Oak Ridge, TN, USA) was used in the total mercury determinations.

Sample Preparation and Data Collection

Isotopically enriched spike preparation and calibration

A stock solution of inorganic isotopic spike standard solution used in the total mercury determinations was prepared by dissolving ^{201}HgO in nitric acid. The ^{201}Hg spike was then gravimetrically diluted and calibrated by reverse isotope dilution with SRM 3133 Mercury Standard Solution. The methylmercury isotopic spike was prepared by gravimetric dilution of IRMM-670 ^{202}Hg enriched methylmercury. A spike calibration of the IRMM-670 was accomplished by reverse speciated isotope dilution with a gravimetrically prepared solution of methylmercury chloride which had been previously calibrated for total mercury content with SRM 3133. The reverse isotope

dilution yielded a methylmercury concentration in good agreement with the certified value of IRMM-670; therefore, the certified concentration of the isotopic spike was used in all calculations. The ethylmercury isotopic spike was prepared by gravimetric dilution of ^{201}Hg enriched ethylmercury. A spike calibration of the ethylmercury was accomplished by reverse speciated isotope dilution with a gravimetrically prepared solution of ethylmercury chloride which had been previously calibrated for total mercury content with SRM 3133. The inorganic mercury isotopic spike standard solution was prepared by dissolving ^{198}Hg enriched metallic mercury in nitric acid. The inorganic mercury spike was calibrated by reverse isotope dilution using SRM 3133. Additionally, a solution of natural methylmercury, ethylmercury, and inorganic mercury was prepared from the stock solutions of natural methylmercury, ethylmercury, and SRM 3133 respectively in order to be used as a gravimetrically prepared control material and was also used for determining the mass bias correction.

Total mercury by cold vapor isotope dilution inductively coupled mass spectrometry (CV-ID-ICP-MS)

Vials of SRM 955c Level 3 and SRM 966 Level 2 (used as a control material) were removed from the -80 ºC freezer, allowed to warm to room temperature, and mixed by gently rolling the vials prior to sub-sampling. For the microwave dissolution procedure, 0.2 g test portions from five vials of SRM 955c Level 3 and 0.3 g of isotopically enriched ^{201}Hg were ~~added to~~accurately weighed into individual microwave vessels followed by the addition of 6 mL of concentrated HNO_3. The vessels were then sealed, and the contents digested to effect sample decomposition and spike equilibration in a high-pressure microwave oven (Multiwave, Anton PAAR, Graz, Austria). The

resulting digests were allowed to cool, the vessels pressure-vented, and the contents transferred to clean 50 mL polypropylene centrifuge tubes. The contents of the tubes were diluted with quartz-distilled water to 15 mL. The tubes were placed in a refrigerator at 4 °C overnight to allow degassing. The solutions were then further diluted to produce a concentration suitable for measurement by cold-vapor ICP-MS. All data were corrected for detector dead-time and instrument mass bias. The mercury concentrations were converted from a mass/mass to a mass/volume basis using the densities of the blood listed on the respective Certificate of Analysis.

Triple spike speciated isotope dilution gas chromatography inductively coupled plasma mass spectrometry (TS-SID-GC/ICP-MS)

Vials of SRM 955c Level 3 and SRM 966 Level 2 were removed from the -80 ºC freezer, allowed to warm to room temperature, and were then mixed by gently rolling the vials prior to sub-sampling. For the sample analysis, six aliquots of ≈ 0.5 g of SRM 955c Level 3 and three aliquots of ≈ 0.5 g of SRM 966 Level 2, three aliquots of the standard calibration mixture, and three method blanks (Milli-Q water) were accurately weighed into clean 10 mL borosilicate glass microwave vessels and spiked with a known mass of IRMM-670 ^{202}Hg enriched methylmercury CRM solution, ^{201}Hg enriched ethylmercury solution, and inorganic ^{198}Hg solution prepared in high-purity water. Approximately 3 mL of 25 % mass fraction TMAH ~~(Fluka, Switzerland)~~ in water was added to each sample vessel. The vessels were sealed, and the contents extracted in an Explorer focused microwave oven (CEM, Matthews, NC) at 35 W for 4.5 min. The resulting extracts were allowed to cool to room temperature and buffered with 0.550 mL of 1 M sodium acetate ~~(Sigma-Aldrich)~~ and 0.650 mL of UHP nitric acid ~~(J.T. Baker,~~

~~Phillipsburg, NJ)~~ to set the pH at 5. Approximately 100 μL of 20 % mass fraction sodium tetrapropylborate ~~(GALAB, Germany)~~ was added to the microwave extracts, and the samples were shaken for 5 min to ensure complete derivatization of the mercury species. Two mL of hexane were added to the derivatized solutions, and the samples were again vortexed to ensure complete extraction of the derivatized species into the organic layer. The tubes were centrifuged for 3 min at 1500 rpm to facilitate separation of the organic and aqueous phases, and the organic phase was then transferred to pre-cleaned autosampler vials. The samples then underwent a clean-up procedure involving SPE on 3 mL cartridges packed with 5 % (mass fraction) water-deactivated alumina. Before use, the alumina was baked overnight at 650 °C and deactivated prior to use, after cooling to room temperature in a desiccator. Using a SPE Rapidtrace workstation (Caliper Life Science, Hopkinton, MA), the SPE cartridges were first conditioned with 6 mL 1:1 (volume fraction) dichloromethane–hexane followed by 8 mL hexane, both at 1.2 mL min^{-1}. The sample was loaded on to the SPE column at a flow rate of 1 mL min^{-1}. The derivatized mercury species were collected in a 10 mL acid-cleaned glass culture tube by elution with 4 mL hexane delivered at a flow rate of 1 mL min^{-1}. Collected fractions were concentrated to ≈ 0.2 mL under a nitrogen stream with a Turbovap (Caliper Life Science) and analyzed by GC/ICP-MS. Quantification was achieved by speciated isotope dilution. The standard calibration mixtures and blanks followed the above procedure with the exception that they were not exposed to the microwave extraction in order to avoid significant decomposition of the organomercury spikes.

All data were corrected for detector dead-time and instrument mass bias determined from comparison of the isotope ratios of chromatographic peak areas from 50

ng g^{-1} standard solutions of methylmercury and inorganic mercury, with the natural isotope ratios from IUPAC. The individual mercury specie concentrations were converted from a mass/mass to a mass/volume basis using the densities of the blood listed on the respective Certificate of Analysis.

Instrumentation

CV-ID-ICP-MS

Total mercury was measured using ID-ICP-MS with a Thermo Elemental X7 (Winsford, UK) quadrupole ICP-MS instrument using cold vapor mercury generation with the reduction reaction in a commercial (CETAC, Omaha, NE, USA) glass reaction/separator cell and has been described previously.[22-24] Briefly, the generated Hg^0 was transferred to the ICP-MS instrument with 1/16" id Viton tubing, using an argon carrier gas. This gas stream was mixed with the plasma injector gas stream via a polyethylene T piece. The instrument operating conditions were optimized to yield maximum signal to background ratio under relatively dry plasma conditions. The raw counts were collected in Profile TRA mode monitoring mercury isotopes at m/z 201 and 202. The $^{201}Hg/^{202}Hg$ ratios were downloaded as CSV files to a Microsoft Excel spreadsheet for calculations of the total mercury concentration.

GC/ICP-MS

GC/ICP-MS analysis was performed with a Thermo Trace GC Ultra equipped with a 30 m DB-5MS+DG 250 μm i.d. (J & W Scientific, Folsom, CA, USA) capillary column coated with a 0.25 μm thick film of (5 %-phenyl)-methylpolysiloxane. The column temperature was held at an initial temperature of 80 °C for 3 min, ramped at 25 °C min^{-1} to 200 °C, then ramped at 70 °C min^{-1} to a final temperature of 280 °C and held

for 3 min. The programmable temperature vaporizing injector (PTV) maintained at 280 °C was used in the constant temperature (CT) splitless mode with helium used as the carrier gas flowing at a constant flow rate of 1.5 ml min^{-1}. The GC was coupled to a Thermo Elemental X7 quadrupole ICP-MS using a Thermo GC/ICP-MS commercial interface. The transfer column consisted of a 1 m MTX-5 280 μm i.d. stainless steel capillary (Restek, Bellefonte, PA) coated with a 0.1 μm thick film of (5 %-phenyl)-methylpolysiloxane heated at 275 °C. The chromatograms of the mercury species (Figure 1) were recorded in transient TRA mode monitoring mercury isotopes at m/z 198, 199, 200, 201 and 202 at dwell times of 50 ms each. For the speciated isotope dilution measurements, $^{200}Hg/^{198}Hg$, $^{200}Hg/^{201}Hg$, and $^{200}Hg/^{202}Hg$ ratios from the integrated peak areas of methylmercury, ethylmercury, and inorganic mercury were downloaded as CSV files to a Microsoft Excel spreadsheet for calculations of the individual mercury species concentrations.

WARNING: Methylmercury, ethylmercury, and derivatized forms of organomercury compounds are highly toxic and must be handled with appropriate personal protection. ~~*This requires the use of silver lined gloves, use of a fume hood, and approved breathing apparatus.*~~

Results and Discussion

TS-SID-GC/ICP-MS

Method-induced mercury transformation artifacts have been reported for sediments[25] and for biological materials[26, 27] but little work has been applied to clinical matrices. SID equations are designed to quantify reaction yields involved in possible mercury species transformations (e.g. methylation and demethylation) during sample preparation using different enriched labeled mercury spikes. This corrects the final

mercury species concentrations (methylmercury, ethylmercury, and inorganic) for method-induced transformation, and takes into account any isotopic species contamination from the species-specific spikes. There are numerous reports of the use of different SID approaches and equations for the quantification of elemental species[18, 20, 27-31] with comparisons of the different calculation equations/formulas providing similar results.[32] The TS-SID calculations used in the certification of 955c are based on those described by Rodriquez-Gonzalez *et al.*[33] with a modification described by Point *et al.*[26] which allows for the correction of species impurities in the spikes.

The results of the TS-SID concentration determinations for methylmercury, ethylmercury, and inorganic mercury in SRMs 955c, 966, and the gravimetrically prepared calibration solution are listed in Table 1, together with the expanded uncertainties at the 95 % confidence interval. The uncertainty in the certified value is calculated as an expanded uncertainty, $U = ku_c$, where u_c is the combined standard uncertainty calculated according to the ISO Guide and k is the coverage factor.[34] The sum of the mercury species for 955c Level 3 (18.6 µg L^{-1} ± 1.5 µg L^{-1}) is in good agreement with the total mercury value measured independently by CV-ID-ICP-MS (17.8 µg L^{-1} ± 1.6 µg L^{-1}). The measured values of methylmercury and inorganic mercury in SRM 966 which serve as the control material for both methylmercury and inorganic mercury are in good agreement with the reference values of SRM 966 from the NIST Certificate of Analysis. Also of note is the calculated value of ethylmercury in SRM 966 which, in fact, does not contain ethylmercury. The transformation yields calculated by the SID equations track the decomposition or formation reactions based solely on the isotopically enriched spikes. In this case, the isotopically enriched spike undergoes de-

ethylation and therefore the SID calculations apply the calculated de-ethylation correction of the spike to the sample. The calculated ethylmercury concentration values in the SRM 966 samples were highly variable resulting in the high uncertainty of the measurement. The decomposition of the ethylmercury spike does not necessarily mean that the ethylmercury is unstable in the SRM material; although, the stability of all the mercury species will be closely monitored especially due to a recent report that ethylmercury can be converted to inorganic mercury in blood at room temperature.[9]

Confirmation of ethylmercury quantification

Due to the lack of an available reference material certified for ethylmercury, a calibration mixture was prepared and analyzed by TS-SID. As mentioned previously, both the methylmercury chloride and ethylmercury chloride have been analyzed for total mercury content by ID-ICP-MS and are traceable to SRM 3133. This mixture served as a pseudo-control material for the ethylmercury determinations in SRM 955c. The measured values of standard calibration mixture of methylmercury, ethylmercury, and inorganic mercury prepared from methylmercury chloride, ethylmercury chloride, and SRM 3133, were in good agreement with the gravimetrically prepared solutions.

For confirmation of the ethylmercury TS-SID results, an additional set of samples of SRM 955c Level 1 containing no methylmercury or ethylmercury (total Hg 0.017 ± 0.011 μg L^{-1}) was spiked with various amounts of ethylmercury to produce a matrix-matched external calibration curve (Figure 2). Six vials of SRM 955c Level 1 were processed and analyzed as described above with ethyltin added as an internal standard to all of the samples. The results of the quantification of ethylmercury in SRM 955c Level 3 (4.74 μg L^{-1} ± 0.34 μg L^{-1}) with the matrix-matched external calibration curve built

from SRM 955c Level 1 were in good agreement with the TS-SID results. While only the measurement results of the TS-SID analysis were used in the calculation of the certified value of ethylmercury, the results from both the TS-SID analysis of the calibration mixture and the results of matrix-matched external calibration curve serve as confirmation of both the method and measurement accuracy.

It should be noted that ethylmercury exhibits decomposition to inorganic mercury during the sample preparation procedure. However, the amount of the decomposition of ethylmercury measured in the TS-SID measurements was consistent. The decomposition of the ethylmercury in the matrix-matched external calibration curve was also consistent despite the varying amounts of added ethylmercury. The primary reason that the matrix-matched external calibration curve was used as a confirmatory method using the spiked was that the blood matrix (SRM 955c Level 1) contained very little mercury and only ethylmercury was measured in the samples. This method would not be suitable for routine quantification of mercury species in clinical samples due to the decomposition of ethylmercury and possible transformation of other mercury species present in the samples.

The stability of the mercury species in SRM 955c Level 3 will continue to be monitored and the material has, to date, shown no instability when properly stored and handled. A full description of the collection and preparation of the SRM 955c material as well as information detailing all of the certified constituents and both the homogeneity and stability measurements in all four levels of SRM 955c will be detailed in a future publication.

Conclusions

NIST SRM 955c Toxic Elements in Caprine Blood Level 3 was certified for three mercury species and for total mercury. The certified values of methylmercury, ethylmercury, inorganic mercury, and total mercury are 4.5 μg L^{-1} ± 1.0 μg L^{-1}, 5.1 μg L^{-1} ± 0.5 μg L^{-1}, 9.0 μg L^{-1} ± 1.3 μg L^{-1}, and 17.8 μg L^{-1} ± 1.6 μg L^{-1} respectively. This SRM represents the first whole blood SRM material certified for ethylmercury. SRM 955c will prove useful in evaluating the accuracy of analytical methods and validation of measurement results for mercury speciation analysis in clinical blood samples, mercury exposure studies, and/or nutritional and toxicological assessments. Measurements of Levels 2 and 4, also spiked with the three mercury species, are planned to provide certified values for the three mercury species in all levels of SRM 955c.

Disclaimer

Certain commercial equipment, instruments, and materials are identified in this paper to adequately specify the experimental procedure. Such identification does not imply recommendation or endorsement by NIST nor does it imply that the equipment, instruments, or materials are necessarily the best available for the purpose.

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Figure Captions

Figure 1. GC/ICP-MS chromatogram of propylated mercury species in SRM 955c Level 3 (Peak identification; 1 - methylmercury, 2 - ethylmercury, and 3 - inorganic mercury).

Figure 2. Matrix matched standard addition plot for the determination of ethylmercury in 955c Level 3.

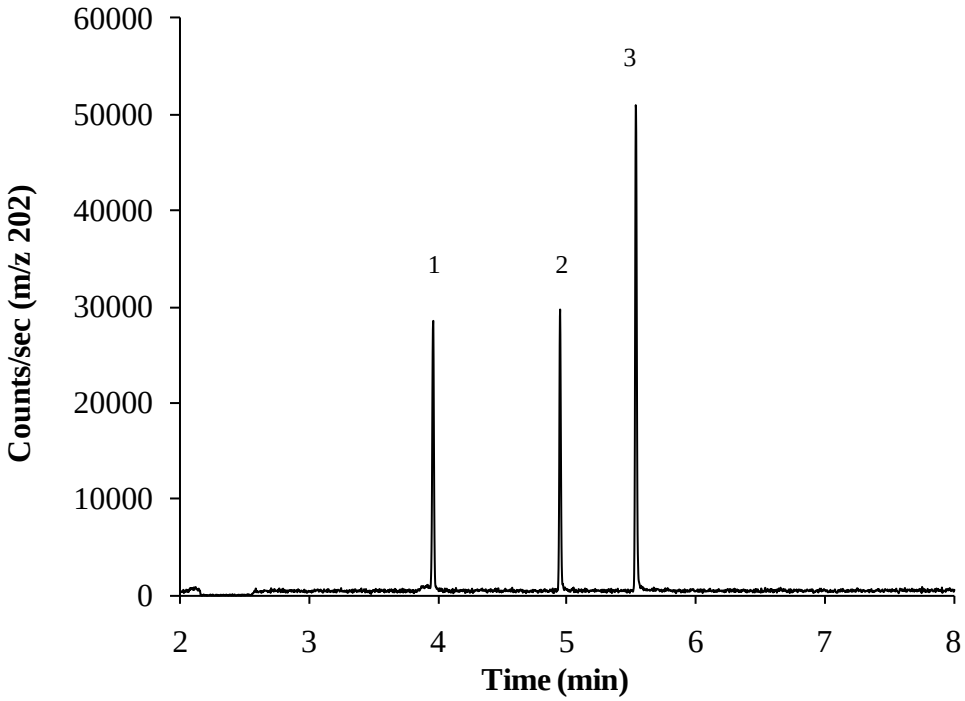
Table 1. Results of TS-SID quantitative results for SRM 966 Toxic Metals in Bovine Blood and SRM 955c Toxic Elements in Caprine Blood

$\mu g\ L^{-1}$ as Hg

	MeHg	EtHg	iHg	Total Hg
SRM 966 Level 2				
TS-SID	16.3 ± 0.8	1.0 ± 0.7	15.3 ± 1.0	32.0 ± 0.7*
Certified Value *(Reference Value)*	(16.4 ± 1.4)	--	(14.87 ± 0.93)	31.4 ±1.7
SRM 955c Level 3				
TS-SID	4.5 ± 1.0	5.06 ± 0.47	9.0 ± 1.3	17.8 ± 1.6*
Calibration Solution				
TS-SID	2.3 ± 0.7	4.7 ± 0.3	10.7 ± 0.9	--
Gravimetrically Prepared Value	2.2 ± 0.1	5.0 ± 0.1	9.7 ± 0.1	--

*CV-ID-ICP-MS result

The uncertainties associated with the measured values are expressed as expanded uncertainties, U, at the approximately 95 % level of confidence (k=2).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Time (min)

Figure 1.

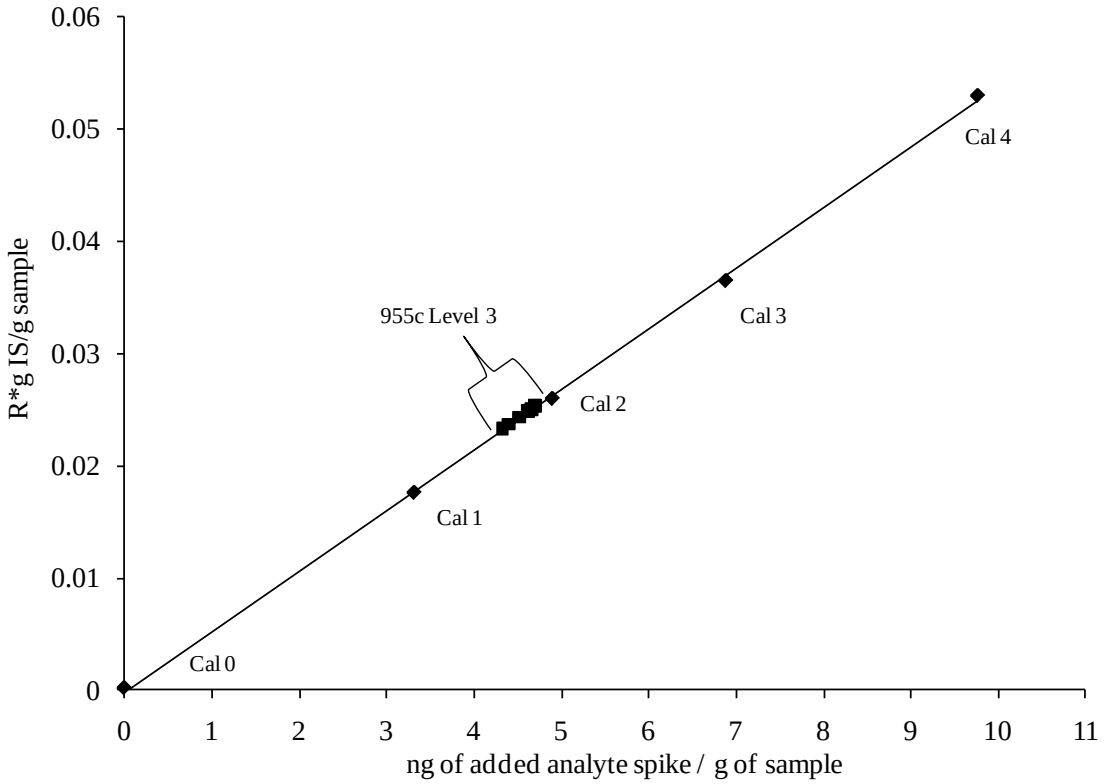
0.06
0.05
0.04
0.03
0.02
0.01
0
R*g IS/g sample
Cal 4
955c Level 3
Cal 3
Cal 2
Cal 1
Cal 0
0
1
2
3
4
5
6
7
8
9
10
11
ng of added analyte spike / g of sample

Figure 2.