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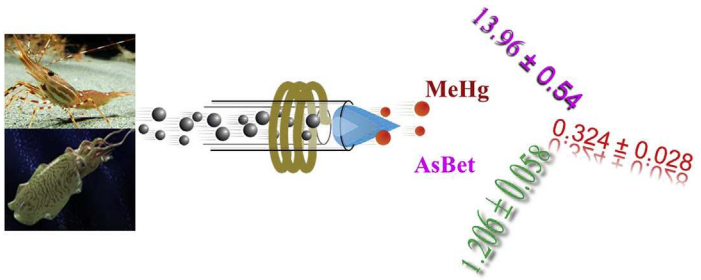
HIGHLIGHTS

- The accurate and SI traceable determination of arsenobetaine (AsBet) and methylmercury (MeHg) in prawn and squid tissues.
- Primary standards of AsBet and MeHg characterized by ^{1}H-NMR ensure the final results traceable to SI.
- First report of SI traceable measurements of AsBet and MeHg in prawn and cuttlefish.

GRAPHICAL ABSTRACT



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ABSTRACT

Methods based on species specific isotope dilution were developed for the accurate and SI traceable determination of arsenobetaine (AsBet) and methylmercury (MeHg) in prawn and cuttlefish tissues by LC-MS/MS and SPME GC-ICPMS. Quantitation of AsBet and MeHg were achieved by using a ^{13}C-enriched AsBet spike (NRC CRM CBET-1) and an enriched spike of Me^{198}Hg (NRC CRM EMMS-1), respectively, wherein analyte mass fractions in enriched spikes were determined by reverse isotope dilution using natural abundance AsBet and MeHg primary standards. Purity of these primary standards were characterized by quantitative ^{1}H-NMR with the use of NIST SRM 350b benzoic acid as a primary calibrator, ensuring the final measurement results traceable to SI. Validation of employed methods of ID LC-MS/MS and ID SPME GC-ICPMS was demonstrated by analysis of several biological CRMs (DORM-4, TORT-3, DOLT-5, BCR-627 and BCR-463) with satisfying results.

The developed methods were applied for the determination of AsBet and MeHg in two new certified reference materials (CRMs) prawn (PRON-1) and cuttlefish (SQID-1) produced jointly by Thailand Institute of Scientific and Technological Research (TISTR) and National Research Council Canada (NRC). With additional measurements of AsBet using LC-ICPMS with standard additions calibration and external calibration at NRC and TISTR, respectively, certified values of 1.206 ± 0.058 and 13.96 ± 0.54 mg kg^{-1} for AsBet as As (expanded uncertainty, $k = 2$) were obtained for the new CRMs PRON-1 and SQID-1, respectively. The reference value of 0.324 ± 0.028 mg kg^{-1} as Hg (expanded uncertainty, $k = 2$) for MeHg was obtained for the SQID-1 based on the results obtained by ID SPME GC-ICPMS method only,

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whereas MeHg in PRON-1 was found to be < 0.015 mg kg^{-1}. It was found that AsBet comprised 69.7% and 99.0% of total As in the prawn and cuttlefish, respectively, whereas MeHg comprised 94.5% of total Hg in cuttlefish.

1. Introduction

Arsenic and mercury are toxic trace elements in the environment and the toxicity of arsenic and mercury depends on their chemical forms (speciation) [1,2]. For arsenic, inorganic As(III) and As(V) are considerably more toxic than the organic species such as monomethylarsonic acid (MMA) or dimethylarsinic acid (DMA) [1,3] whereas arsenobetaine (AsBet) and arsenocholine (AsC) are believed to be nontoxic [4], although this view is not held universally [5]. In contrast to arsenic, organic methylmercury is more toxic than inorganic Hg(II) [2]. Marine organisms are known to accumulate and biotransform arsenic and mercury, and this could provide a major route of exposure for humans to arsenic and mercury through consumption of marine food products [6–11]. As a result, many countries have set guidelines for seafood consumption to safeguard human health, and efforts have been devoted to the development of sensitive, accurate and rapid analytical methods for the speciation of As^{4-} [5,7,8,12–20] and Hg [10,11,21–26] in seafood products.

In general, quantitative speciation of arsenic and mercury in biological samples requires efficient extraction, good chromatographic separation and detection. Compromise in any of these steps can contribute to the difficulty of the analysis and degrading the accuracy of the results. More importantly, the measurement results are required to be traceable to the International System of Units (SI) [27], as this is the basis to achieve comparable measurement results from different laboratories to ensure fair trade globally. As defined in Vocabulary in Metrology [28], measurement traceability is the property of the result of a measurement or the value of a standard whereby it can be related to stated references, usually national or international standards, through an unbroken chain of comparisons, all having stated uncertainties. Therefore, SI traceable primary pure standard with known purity and associated uncertainty, as well as matrix-matched certified reference materials (CRMs) for method validation are required to establish the measurement traceability. Currently, the choice of SI traceable primary standards and matrix CRMs for speciation analysis of AsBet and MeHg (two major species in fish, prawn and cuttlefish) are limited. As shown in Table 1, only a few CRMs which have certified values for AsBet and/or MeHg from National Research Council Canada (NRC, Ottawa, ON, Canada), Institute for Reference Materials and Measurements (IRMM, European Commission, Geel, Belgium), National Institute of Standards & Technology (NIST, Gaithersburg, MD, USA) and National Metrology Institute of Japan (NMIJ, Tsukuba, Japan), are available on the market.

Table 1
Current available CRMs certified for MeHg and/or AsBet.

Name of CRM	Matrix	Analyte certified	Source
DORM-4	Fish	AsBet and MeHg	NRC, Canada
TORT-3	Lobster	AsBet and MeHg	NRC, Canada
DOLT-5	Dogfish liver	AsBet and MeHg	NRC, Canada
BCR-627	Fish	AsBet	IRMM, Belgium
ERM-CE464	Fish	MeHg	IRMM, Belgium
BCR463	Fish	MeHg	IRMM, Belgium
SRM1946	Fish	MeHg	NIST, USA
SRM2976	Mussel	MeHg	NIST, USA
CRM7402a	Fish	AsBet and MeHg	NMIJ, Japan
CRM7403a	Fish	AsBet and MeHg	NMIJ, Japan

In terms of primary (calibrant) standards for AsBet and MeHg, only BCR-626 (IRMM, Geel, Belgium) and NMIJ-7901a (NMIJ, Tsukuba, Japan) are available. Assessment of purities of these organometallic standards is far from trivial. As exemplified in the 2007 CCQM-P96 international intercomparison study [29] under the auspices of the International Committee of Weights and Measures (CIPM), it was discovered that the certified value of AsBet in both reference materials BCR-626 (IRMM, Geel, Belgium) and NMIJ-7901a (NMIJ, Tsukuba, Japan) which were used as primary calibrators for the determination of AsBet in fish, were biased and both of which shared a common traceability link through BCR-626. As a result of the CCQM-P96 study, the certified value for NMIJ-7901a was revised by 20% in 2009 [30] whereas only the expanded uncertainty was subsequently revised for the BCR-626 in 2009 from 0.6% to 7%.

To address these needs, this paper describes protocols for the accurate and SI traceable determination of AsBet and MeHg in two new prawn and cuttlefish reference materials, named PRON-1 and SQID-1, respectively. Isotope dilution (ID) calibration was applied for the determination of AsBet and MeHg by LC-MS/MS and SPME GC-ICPMS, respectively, with use of a ^{13}C-enriched arsenobetaine spike (NRC CRM CBET-1) and ^{198}Hg-enriched methylmercury (NRC CRM EMMS-1) spike, both synthesized at NRC. Purities of primary standards of the natural abundance arsenobetaine bromide (NRC CRM ABET-1) and methylmercury chloride (purchased from Sigma-Aldrich) were both characterized by quantitative ^1H-NMR with the use of NIST SRM 350b benzoic acid as a primary calibrator. This is first time reporting the use of quantitative ^1H-NMR for the determination of purity of MeHgCl primary standard, ensuring the MeHg results in cuttlefish and prawn CRMs traceable to SI. Two secondary methods of standard addition calibration by LC-ICPMS and an external calibration LC-ICPMS were used for the determination of AsBet at NRC and TISTR, respectively. Validation of the above employed methods at NRC was demonstrated by analysis of several biological Certified Reference Materials (DORM-4, TORT-3, DOLT-5, BCR-627 and BCR-463) with satisfying results.

2. Experimental

2.1. Instrumentation

A 5975C GC-MS and 7500 ICPMS from Agilent Technologies (Mississauga, ON, Canada), were used for the determination of MeHg. A DB-5 column (5% diphenyl, 95% polydimethylsiloxane, 30 m × 0.28 mm × 0.5 μm) was used for the separation of mercury species. A commercial heated GC transfer line for splitting the GC eluent to ICPMS and MS detectors was custom designed and made by Agilent Technologies as described in the previous work [26]. A manual SPME device, equipped with a fused silica fiber coated with a 100 μm film of polydimethylsiloxane (Supelco, Bellefonte, PA, USA), was used for the sampling of propylated MeHg from the headspace above its aqueous solutions. For convenience, SPME sampling was conducted in a regular fumehood. ICPMS

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optimization was carried out by monitoring the signal of 1% xenon in argon sample gas for tuning. Typical operational conditions for GC-MS and ICPMS are outlined in Table 1S (Supplementary Information). Analyses were performed in SIM mode for GC-MS and ions of $m/z = 256$ and 260 from propylated MeHg fragment. Mercury isotopes ^{202}Hg and ^{198}Hg were monitored for GC-ICPMS.

An Agilent 8800 ICPMS and an Agilent HPLC 1200 Series (Agilent Technologies Canada Inc., Mississauga, Ontario, Canada) with a cation exchange column, Supelcosil™ LC-SCX (250 mm × 2.1 mm × 5 μm) and a Supelguard SCX Guard Column from Supelco (Bellefonte, PA, USA) were used for the determination of AsBet using standard addition calibration LC-ICPMS approach. A 6 mM ammonium formate solution adjusted to pH 3.0 with formic acid in 5% acetonitrile solution was used as mobile phase. Helium mode was used to minimize $ArCl^+$ interferences on $^{75}As^+$. Optimization of the Agilent 8800 ICPMS was performed as recommended by the manufacturer; typical operating conditions of ICPMS and LC are summarized in Table 2S (Supplementary Information).

An Agilent 7700 ICPMS and an Agilent HPLC 1200 Series (Agilent Technologies Thailand Ltd., Bangkok, Thailand) with a ODS column (250 mm × 2.1 mm × 5 μm) were used for the determination of AsBet using external addition calibration LC-ICPMS approach. A 10 mM sodium 1-butanesulfonate, 4 mM malonic acid, 4 mM tetramethylammonium hydroxide and 0.05% methanol (pH 3.0) solution was used as mobile phase at a flow 0.75 mL min^{-1}. Helium mode was used to minimize $ArCl^+$ interferences on $^{75}As^+$. Optimization of the Agilent 7700 ICPMS was performed as recommended by the manufacturer; typical operating conditions of ICPMS and LC are summarized in Table 3S (Supplementary Information).

A Thermo TSQ Quantiva triple quadrupole mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA) was used for all measurements for AsBet with ID LC-MS/MS approach. A Zorbax HILIC Plus-RR, (4.6 mm × 100 mm × 3.5 μm) column was used for ID LC-MS/MS measurements. A 5 mM ammonium formate (pH 3.5) solution was used as mobile phase A and acetonitrile was used as mobile phase B. 35% A and 65% B were used for isocratic elution. MS/MS mode was used ($m/z = 179 \rightarrow 120$ and $m/z = 180 \rightarrow 120$). Optimization of the TSQ-MS was performed as recommended by the manufacturer by using a 50 ng g^{-1} AsBet standard solution in LC buffer and tuning for $m/z = 179$; typical operating conditions are summarized in Table 4S (Supplementary Information). The coupling of LC to ICPMS or TSQ-MS was accomplished by directing the eluent from the column to the nebulizer of the ICPMS or to the TSQ-MS electrospray inlet through a 1 m and 0.3 m length PEEK tubings (0.13 mm id, 1.59 mm od), respectively.

A Branson 3510 sonication system (Branson, Danbury, CT, USA) was used for extraction of arsenic species from samples. A Thermo IEC Centra CL3 (Thermo Fisher Scientific, San Jose, CA, USA) was used for centrifuging samples.

A 400 MHz NMR spectrometer (Varian Inova, Santa Clara, CA, USA) was used for the determination of the purity of methylmercury chloride primary standard.

2.2. Reagents and solutions

A methanolic solution of potassium hydroxide (0.25 g mL^{-1}) was prepared by dissolving KOH (Fisher Scientific, Nepean, Canada) in methanol. Hydrochloric acids were purified in-house prior to use by sub-boiling distillation of reagent grade feedstock in a quartz still. High purity glacial acetic acid was obtained from GFS Chemicals Inc. (Powell, OH, USA). OmniSolv® methanol (glass-distilled) was purchased from EM Science (Gibbstown, NJ, USA). High purity de-ionized water (DIW) was obtained from a NanoPure mixed bed ion exchange system fed with reverse osmosis domestic feed water (Barnstead/Thermolyne Corp, Dubuque, IA, USA). A 0.05 g/mL solution of sodium tetrapropylborate was prepared by dissolving $NaBPr_4$ (GALAB, Geesthacht, Germany) in DIW. A 0.5 M ammonium acetate buffer at pH 5 was prepared by dissolving appropriate amounts of NH_4OAc (Fisher Scientific, Nepean, Ontario, Canada) in water and adjusting the pH with glacial acetic acid.

High purity methylmercury chloride purchased from Sigma Aldrich Canada Ltd. (Oakville, ON, Canada) was characterized for its purity by quantitative ^{1}H-NMR (see below). MeHg individual stock solution of 3300 μg g^{-1} as Hg was prepared in methanol and kept refrigerated until use. Natural abundance MeHg working standard solution of 0.58 μg g^{-1} was prepared by serial diluting the stock solution with 50% methanol in DIW. ^{198}Hg enriched Me^{198}Hg spike solution at nominal concentration of 0.12 μg g^{-1} in 50% methanol was prepared from an isotopically enriched $CH_3^{198}HgCl$ (Me^{198}Hg) stock synthesized in our laboratory (available at NRC as CRM EMMS-1) from a commercially available inorganic ^{198}Hg (abundance of mercury-198, $x(^{198}Hg) = 96.5\%$) [31]. To determine the mass fraction of the enriched Me^{198}Hg spike and final analyte mass fraction in the samples, double isotope dilution method was applied.

HPLC grade acetonitrile was purchased from EMD Chemicals INC (Darmstadt, Germany). High purity formic acid (88%) was obtained from GFS Chemicals Inc. (Powell, OH, USA). A 6 mM solution of ammonium formate used for LC-ICPMS approach was prepared by quantitative dissolution of solid ammonium formate (Certified, Thermo Fish Scientific, Ottawa, ON, Canada) in DIW and adjusted pH to 3.0 with proper amount of formic acid. A 5 mM ammonium formate, pH 3.5 and acetonitrile were prepared and used as mobile phase A and B for the LC-MS/MS approach.

Natural abundance arsenobetaine bromide was synthesized in-house [19] and its purity of 0.9930 ± 0.0025 g/g (expanded uncertainty, $k = 2$) was established by quantitative ^{1}H NMR with traceability to NIST SRM 350b benzoic acid [32]. This natural isotopic composition AsBet is available as CRM ABET-1 from NRC. An AsBet stock solution of 1000 mg kg^{-1} (as As) was gravimetrically prepared in DIW and kept refrigerated. An AsBet standard solution of 10.94 mg kg^{-1} (as As) was prepared by the appropriate dilution of the AsBet stock. Similarly, ^{13}C-enriched AsBet was also synthesized in house [19] and was used for the determination of AsBet in biological tissues by species specific isotope dilution. ^{13}C enriched AsBet are available as CRMs BBET-1 and CBET-1 from NRC. A stock solution of ^{13}C-enriched AsBet CBET-1 at 1000 mg kg^{-1} (as As) was prepared by dissolution of this material in DIW. A working spike solution, approximately 1.5 mg kg^{-1}, was prepared by gravimetric dilution of the enriched AsBet stock. The concentration of ^{13}C-enriched AsBet spike was verified by reverse spike isotope dilution using the natural abundance AsBet standard. The relative isotopic abundances of the protonated natural abundance and ^{13}C enriched AsBet molecular ions ($(CH_3)_3AsCH_2COOH^+$ and $(CH_3)_3AsCH_2{}^{13}COOH^+$) were calculated based on the IUPAC recommended isotopic abundance of C, H, and As and enriched C using the isotopic distribution calculator in Analyst software 1.4.1 (MDS Sciex, Toronto, ON, Canada) as detailed in the previous work [19].

Biological tissue CRMs of DORM-4 and TORT-3 from National Research Council Canada (NRC, Ottawa, ON, Canada), and BCR-463 and BCR-627 from Institute for Reference Materials and Measurements (IRMM, European Commission, Geel, Belgium), were used for the validation of methods used for the certification of PRON-1 and SQID-1.

2.3. Preparation of new CRMs PRON-1 and SQID-1

Two new reference materials of PRON-1 (giant river prawn,

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Macrobrachium rosenbergii) and SQID-1 (Pharaoh cuttlefish, *Sepia pharaonis*), 10 kg of each, were prepared from edible grade prawn and cuttlefish, collected in the area of Bangprakong River (Thailand) and Andaman Sea (Myanmar), respectively. For prawn, the head, shell, tail and vein along the back of prawn were removed and only the flesh was kept. Similarly, for cuttlefish, the head, skin, transparent cartilage spine were removed and only flesh was used. The harvested meat materials were thoroughly washed with deionized water, homogenized, freeze-dried, defatted with hexane (for cuttlefish) and vacuum-dried. The resulting powder was bottled at Thailand Institute of Scientific and Technological Research (TISTR). The materials were later irradiated with gamma rays at 18.1 kGy at Gem Irradiation Center of Thailand Institute of Nuclear Technology before shipping to NRC Canada.

2.4. Sample preparation for the determination of purity of natural abundance MeHgCl primary standard

The purity of natural abundance arsenobetaine bromide primary standard was determined by quantitative ^{1}H NMR as detailed in a recent study [32]. Similarly, the purity of natural isotopic abundance methyl mercury chloride (MeHgCl), purchased from Sigma-Aldrich Canada Ltd. (Oakville, ON, Canada), was determined using the same technique. In brief, three replicate samples of methylmercury chloride (12–20 mg) and benzoic acid (NIST SRM 350b, 7–10 mg) as an internal calibrator were weighed in separate clean aluminium foil boats on an analytical micro-balance (UMT-2, Mettler-Toledo) and co-dissolved in 4 mL CD_3OD (Cambridge Isotope Laboratories, Andover, MA, USA) in a 20 mL vial. The solutions were thoroughly vortex-mixed and 0.7 mL of each sample solution was transferred into clean, dry, argon-flushed 5 mm NMR tube (Wilmad pp535 tubes, LabGlass, Buena, NJ, USA). The NMR tubes were flame sealed prior to measurements.

Proton NMR spectra were acquired using the 400 MHz NMR spectrometer at 23 °C. Free induction decays were acquired with 32 scans and 44915 points, apodized with a decreasing exponential, Fourier transformed, and manually phased and baseline corrected. The integrals at 8.01 ppm (2H, benzoic acid including ^{13}C satellite signals) and 0.92 ppm (3H, methyl mercury including ^{13}C and ^{199}Hg satellite signals) were used for quantitation.

2.5. Sample preparation for the determination of MeHg

Sample preparation for the determination of MeHg followed previous reported procedures [11,33,34]. In brief, 250 mg subsamples were spiked with an appropriate mass of the enriched $Me^{198}Hg$ spike solution (0.12 μg g^{-1}). 20 mL of 25% methanolic KOH solution was added to each vial, and vails were mechanically shaken for 2 h and then stored at 4 °C until analysis. Three procedural blanks (spiked with 20% of the amount of enriched spike used for the samples) were processed along with the samples. Four reverse spike isotope dilution calibration solutions were prepared to determine the mass fraction of the enriched $Me^{198}Hg$ spike. A 110 mg of the enriched $Me^{198}Hg$ spike solution (0.12 μg g^{-1}) and 120 mg of 0.5849 μg g^{-1} natural abundance MeHg solution were accurately weighted into a vial and diluted with 20 mL of 25% methanolic KOH.

For SPME headspace sampling, a 4.0 mL volume of digest or reverse spike isotope dilution calibration solution was transferred to a 22 mL glass vial. After 10 mL of 0.5 M NH_4OAc buffer, 1.75 mL of HCl and 1 mL of 5% $NaBPr_4$ were added, and the vial was capped with a PTFE coated silicone rubber septum. The SPME needle was inserted through the septum and headspace sampling was performed for 10 min under vigorous stirring. The collected analytes were then desorbed from the SPME fiber onto the GC column. Intensities of ^{202}Hg and ^{198}Hg were monitored with ICPMS detection and peak areas were used to derive the $^{202}Hg/^{198}Hg$ isotope ratios which were then used to calculate the analyte mass fractions in the samples.

2.6. Sample preparation for the determination of AsBet by standard addition LC-ICPMS at NRC

For standard addition calibration, 250 mg subsamples were accurately weighted into individual pre-cleaned 50 mL PE plastic tubes. A suitable mass of natural AsBet standard was added to each of the spiked samples, resulting in a spiked concentration of 1.5–2.0-fold higher than that of the endogenous AsBet concentration in the samples. Similarly, for procedural blanks, spiked and unspiked solutions were prepared as above. Into each tube, 25.0 g of DIW was then added. Tubes were then capped and sonicated for a total of 45 min. After centrifugation at 2500 rpm for 10 min, an aliquot of the supernatant (10 mL) was transferred and filtered through a 0.45 μm filter into a clean 15 mL PE plastic tube prior to LC-ICPMS measurements. Samples were injected in a sequence of spiked sample-unspiked sample-unspiked sample-spiked sample. Response at $^{75}As^{+}$ was monitored. At the end of the chromatographic run, the acquired data were processed via Chromatographic Data Analysis software on the ICPMS instrument to yield peak areas, which were used to calculate the analyte mass fraction in the CRMs.

2.7. Sample preparation for the determination of AsBet by isotope dilution LC-MS/MS at NRC

The extraction procedure for AsBet used for the ID LC-MS/MS approach was the same as described above. In brief, 250 mg subsamples were accurately weighed into individual pre-cleaned 50 mL PE plastic tubes. A suitable mass of the ^{13}C enriched AsBet spike was then added to each vessel to achieve a near one-to-one ratio for reference ion and spike ion (protonated ions of $(CH_3)_3As^{+}CH_2COOH$) at *m/z* 179 and 180. Three procedural blanks (spiked with 50% of the amount of enriched AsBet used for the samples) were run along with the samples. The contents of each tube were then diluted with 25 g DIW prior to sonication. The rest of preparation was the same as above. The extracts were diluted 10–100-fold with 90% acetonitrile solution prior to LC-MS measurements. Calibration of the 1.5 mg kg^{-1} ^{13}C enriched AsBet spike was achieved by reverse spike isotope dilution. Four replicate reverse ID solutions were prepared by accurately weighing 2.0 g of 1.5 mg kg^{-1} ^{13}C-enriched AsBet spike solution into pre-cleaned 50 mL PE plastic tubes, 0.33 g aliquots of 10.94 mg kg^{-1} natural abundance AsBet standard solution were added, the content of each tube was then diluted with 25 g DIW and was then 10-fold diluted in 90% acetonitrile solution prior to LC-MS/MS measurements. Similarly, a mass bias correction solution was prepared by gravimetrically mixing 0.3311 g of 10.94 mg kg^{-1} natural abundance AsBet standard solution and 2.0169 g of 1.5014 mg kg^{-1} ^{13}C enriched AsBet spike solution, resulting in a ratio of 1:1 for *m/z* 179 and 180.

Sample extracts and four reverse ID calibration solutions were analyzed by LC-MS/MS on the same day. Mass bias calibration solution was repeatedly injected between samples to monitor the mass bias draft and to obtain mass bias correction factor as described in an earlier study [19]. MS/MS mode was used and the integrated peak areas for *m/z* of 120 from 179 to 120 from 180 were used to obtain the reference/spike ion ratios which were then used to calculate the analyte mass fractions in the samples.

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2.8. Sample preparation for the speciation of As using LC-ICPMS at TISTR

Subsequent determination of AsBet in SQID-1 was performed at TISTR using LC-ICPMS with an external calibration. 500 mg subsamples were accurately weighed into individual pre-cleaned 40 mL glass tubes, 2 mL of 0.15 M HNO_3 was then added. Tubes were then capped and heated to 100 °C in a heating block for 2 h. After centrifugation at 4000 rpm for 5 min, DIW was added into an aliquot of the supernatant to result in a final volume of 10 mL. The supernatants were transferred and filtered through a 0.45 μm filter syringe into 2 mL glass vials prior to LC-ICPMS measurements. AsBet (NMIJ-7901a) was purchased from NIMJ and a concentration of 1 mg L^{-1} (as As(V)) AsBet solution was prepared in DIW. Eight external calibration standards in a range of 0.2–15 ng g^{-1} were prepared from the 1 mg L^{-1} (as As(V)) AsBet solution.

Following injection of the sample (20 μL) to the LC-ICPMS, data acquisition was automatically triggered by the Agilent 7700 Series ICPMS control software on ICPMS instrument to yield peak areas, which were used to calculate the analyte mass fractions in the CRMs.

3. Results and discussion

3.1. Determination of purity of natural abundance MeHgCl primary standard

To ensure measurement results are traceable to the SI, the purity for the primary standard used must be traceable to the SI with known purity and its associated uncertainty. Thus, efforts were made for the determination of purity of MeHgCl by ^{1}H-NMR, similar to the determination of purity of AsBet in a previous study [32]. The determination of purity of the MeHgCl primary standard using ^{1}H-NMR is based on NIST SRM 350b benzoic acid as a calibrator, which is co-dissolved with MeHg in a known mass ratio. The integrated signal area per proton per mole for the analyte MeHgCl at 0.92 ppm (3H) including both ^{13}C and ^{199}Hg satellites and calibrator at 8.01 ppm (2H) were used to calculate the purity, shown in Fig. 1. A purity value of 0.9794 ± 0.0044 g/g (expanded uncertainty, $k = 2$) was obtained for the MeHgCl primary standard based on the following equation:

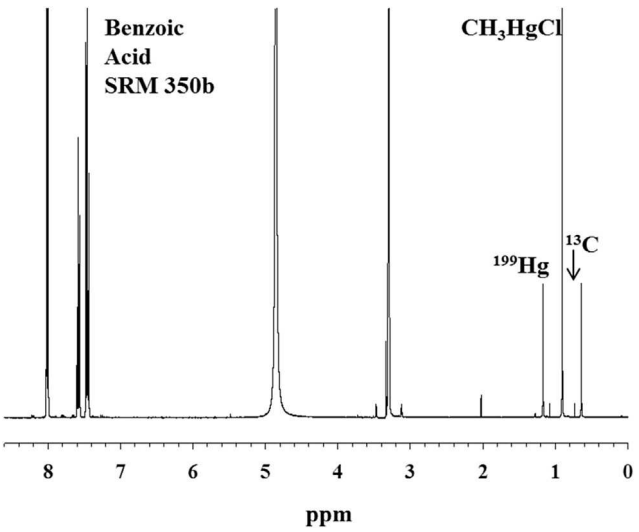

Fig. 1. Quantitative ^{1}H-NMR spectrum of CH_3HgCl admixed with the NIST benzoic acid SRM 350b.

$$w = \frac{I_{an}}{I_{cal}} \cdot \frac{N_{cal}}{N_{an}} \cdot \frac{M_{an}}{M_{cal}} \cdot \frac{m_{cal}}{m_{an}} \cdot Pcal \quad (1)$$

where: w is mass fraction of CH_3HgCl (g/g), P_{cal} is purity (g/g) of the primary calibrator, I is the integrated signal area, N is number of protons integrated, M is molar mass (g/mol), m is weighed mass (g), and subscripts (an) and (cal) refer to analyte and calibrator respectively.

3.2. Determination of MeHg in PRON-1 and SQID-1

Initially, tandem GC-MS and GC-ICPMS mode which allows simultaneous detection of MeHg in two systems was attempted for PRON-1 prawn and SQID-1 cuttlefish extracts. It was found that signals from the propylated MeHg fragment ions of m/z 256 and 260 in PRON-1 extract were too low, only twice the signals obtained from sample blanks. Thus, for the final determination of MeHg in both CRMs was performed on GC-ICPMS alone. The following equation [26,34] was used to calculate MeHg as Hg mass fraction in all CRMs:

$$w_x = w_z \cdot \frac{m_y}{D \cdot m_x} \cdot \frac{m_z}{m'_y} \cdot \frac{A_y - B_y \cdot R_n}{B_{xz} \cdot R_n - A_{xz}} \cdot \frac{B_{xz} \cdot R'_n - A_{xz}}{A_y - B_y \cdot R'_n} - f_b \cdot w_b \quad (2)$$

where w_x is the mass fraction of the analyte in the sample expressed on a dry mass basis, w_z is the mass fraction of the natural abundance standard (mg kg^{-1}), w_b is the mass fraction of analyte in the blank normalized for the sample weight (mg kg^{-1}), m'_y is the mass of the enriched spike used to prepare the blend solution of both enriched and natural abundance standard (g), m_y is the mass of enriched spike used to prepare the blend solution of enriched standard and sample (g), m_x is the mass of sample used (g), D is the correction factor for moisture content in the sample obtained by using the dry-mass of the sample which was held in a vacuum oven for three weeks divided by its initial mass of sample, A_y is the abundance of the reference isotope (^{202}Hg) in the enriched spike, B_y is the abundance of the spike isotope (^{198}Hg) in the enriched spike (Hg isotope abundances in the enriched $Me^{198}Hg$ were determined in house using MC-ICPMS and traceable to NRC NIMS-1 Hg isotopic standard), A_{xz} is the abundance of the reference isotope (^{202}Hg) in the sample or in the natural abundance standard, B_{xz} is the abundance of the spike isotope (^{198}Hg) in the sample or in the natural abundance standard, R_n is the measured and mass bias corrected $^{202}Hg/^{198}Hg$ ratio in the blend solution of the spike and the sample, R'_n is the measured and mass bias corrected reference/spike isotopic ratio in the blend solution of the spike and the natural abundance standard, and f_b is a blank correction factor. When only blank contributes to the ID process, $f_b = 1$ and when blank contributes to both ID and reverse ID process then [30]:

$$f_b = \left(1 - \frac{m_y}{m'_y} \cdot \frac{A_y - B_y \cdot K \cdot r}{B_{xz} \cdot K \cdot r - A_{xz}} \cdot \frac{B_{xz} \cdot K \cdot r' - A_{xz}}{A_y - B_y \cdot K \cdot r'}\right) \quad (3)$$

Fig. 2 shows a chromatogram obtained by SPME GC-ICPMS and Hg peaks are well separated. Results obtained using SPME ID GC-ICPMS for MeHg are summarized in Table 2. It is evident, results obtained for MeHg in CRMs of DORM-4, TORT-3 and BCR-463 are in agreement with certified values, respectively, confirming the accuracy of the SPME GC-ICPMS method.

3.3. Quantitation of AsBet in PRON-1 and SQID-1 at NRC using LC-MS/MS and LC-ICPMS

IDMS technique has been recognized as a primary method for

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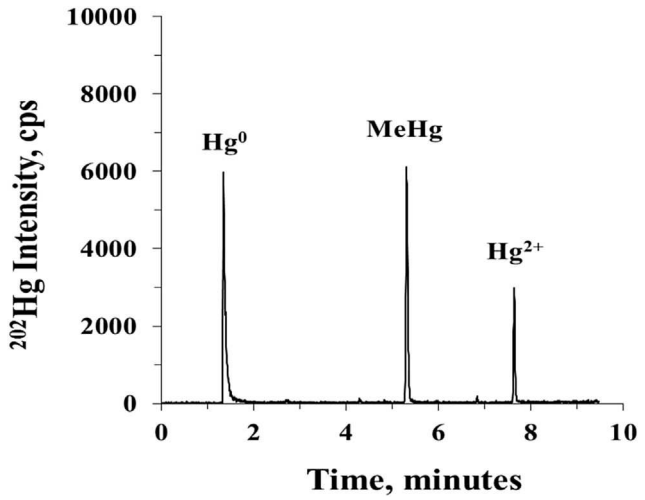

Fig. 2. A chromatogram obtained by SPME GC-ICPMS from SQID-1 extract for MeHg determination, ^{202}Hg isotope monitored. The dead volume is at 0.5 min.

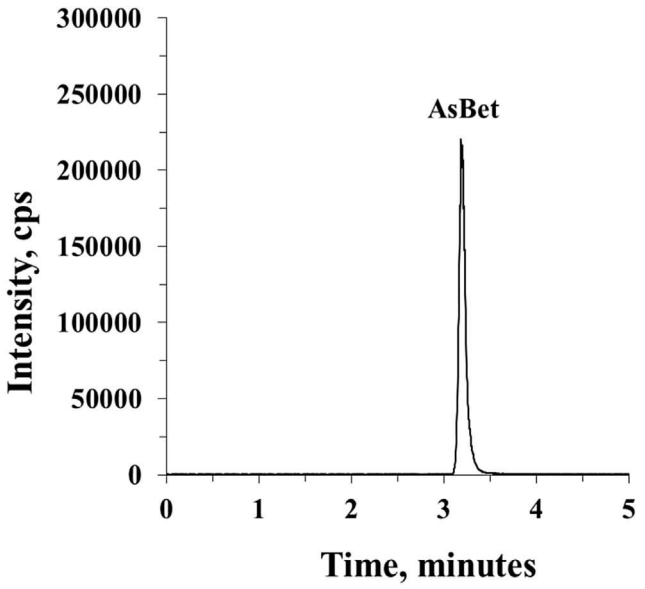

Fig. 3. Spectrum obtained by LC-MS/MS from diluted SQID-1 extract on Zorbax HILIC Plus-RR column, m/z 120 from 179 monitored. The dead volume is at 1 min.

the determination of trace metals. To achieve accurate and precise results, isotope dilution calibration using LC-MS/MS was thus developed for the determination AsBet in the two new CRMs PRON-1 and SQID-1 using Eq. (1). MS/MS mode was selected on the TSQ-MS instrument for the quantitation of AsBet. As shown in Fig. 3, AsBet peak is eluted out at 3.2 min in SQID-1 extract. Ions at m/z 179 to 120 and.180 to 120 were selected as reference and spike ions for ID analysis using a ^{13}C enriched AsBet to generate the final results. Results are summarized in Table 3, note that replicate measurements are from different extracts.

For comparison purpose, subsequent experiments using standard addition calibration LC-ICPMS were performed. Helium mode was used as a reaction cell gas to minimize any possible polyatomic interferences such as $^{40}Ar^{35}Cl^+$ and $^{38}Ar^{37}Cl^+$ on $^{75}As^+$ for the LC-ICPMS approach. Two-point standard addition calibration was used with the LC-ICPMS and analyte mass fraction was obtained using the following equation [19]:

$$w_x = \frac{I_{un} \cdot w_{std} \cdot m_{sp}}{\left(I_{sp} \cdot \frac{m_{f(sp)} \cdot m_{x(un)}}{m_{f(un)}} - I_{un} \cdot m_{x(sp)}\right) \cdot D} - w_{blank} \quad (4)$$

where w_x is the blank corrected mass fraction of AsBet in the sample (mg kg^{-1}); w_{std} is the analyte mass fraction in the natural abundance AsBet standard solution (mg kg^{-1}); m_{sp} is the mass of natural abundance AsBet standard solution added to the spiked sample (g); $m_{f(sp)}$ is the sum of the mass of m_{sp} (g) and DIW; $m_{x(sp)}$ is the mass of sample used to prepare the spiked sample (g); $m_{x(un)}$ is the mass of sample used to prepare the unspiked sample (g); $m_{f(un)}$ is the final mass of the unspiked sample (g); $m_{f(sp)}$ is the final mass of the spiked sample (g); D is the correction factor for moisture content in the sample, obtained from the ratio of sample masses after and before drying, respectively; I_{un} is the measured peak area of the analyte in the unspiked sample (counts); I_{sp} is the measured peak area of the analyte in the spiked sample (counts); w_{blank} is the analyte mass fraction in the procedural blank normalized to the sample mass (mg kg^{-1}).

Results are summarized in Table 3. Student T test was performed on two data sets obtained by LC-ICPMS and ID LC-MS/MS at NRC, and the obtained t-values were all smaller than the t-critical values, confirming no significant difference between results obtained by LC-ICPMS and ID LC-MS/MS for both new reference materials of PRON-1 and SQID-1, respectively. Importantly, results obtained for two CRMs of DORM-4 and TORT-3 by both methods are in agreement with certified values, respectively, confirming the accuracy of employed methods. However, about 11% difference is evident between the NRC measured AsBet value and the certified value in BCR-627 fish, possibly due to the biased certified value of AsBet in BCR-626[29–30] which was used as calibrator during the certification of BCR-627 fish.

3.3.1. *Quantitation of AsBet at TISTR using LC-ICPMS*

As shown in Fig. 4, As species in a mixed standard solution and SQID-1 extract were well separated using ODS column obtained by LC-ICPMS at TISTR. External calibration was applied for the determination of AsBet, As(III), As(V) and DMA in SQID-1. Values of 13.09 ± 0.37 mg kg^{-1} (SD, $n = 8$), 0.016 ± 0.006 mg kg^{-1} (SD, $n = 4$), 0.024 ± 0.009 mg kg^{-1} (SD, $n = 4$) and 0.023 ± 0.007 mg kg^{-1} (SD, $n = 5$) were obtained for AsBet, As(III), As(V) and DMA, respectively. Note that replicates are from different extracts. A value of

Table 2
Results for MeHg (as Hg) by SPME ID GC-ICPMS.

Name of sample	w(MeHg), mg kg^{-1} (as Hg)	Number of replicate extracts	Certified value, mg kg^{-1} (as Hg)
PRON-1	<0.015	6	
SQID-1	0.324 ± 0.008[a]	6	
DORM-4	0.342 ± 0.016	3	0.354 ± 0.031[b]
TORT-3	0.139 ± 0.006	3	0.137 ± 0.012
BCR-463	2.83 ± 0.14	3	2.83 ± 0.15

[a] Standard deviation.
[b] Expanded uncertainty ($k = 2$).

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Table 3
Results for AsBet content (as As) in SQID-1, PRON-1 and related CRMs.

Material	Method of analysis	Measured value mg kg^{-1} (as As)	Replicates	Certified value, mg kg^{-1} (as As)
PRON-1	LC-ICPMS	1.210 ± 0.065[a]	9	
	ID LC-MS/MS	1.204 ± 0.022	6	
SQUID-1	LC-ICPMS	13.96 ± 0.15	9	
	ID LC-MS/MS	13.97 ± 0.13	6	
	LC-ICPMS (TISTR)	13.09 ± 0.37	8	
DORM-4	LC-ICPMS	3.92 ± 0.14	6	3.95 ± 0.36[b]
	ID LC-MS/MS	3.94 ± 0.05	3	
BCR627	LC-ICPMS	3.54 ± 0.04	6	3.90 ± 0.22
	ID LC-MS/MS	3.48 ± 0.03	3	
TORT-3	LC-ICPMS	53.41 ± 0.78	3	54.9 ± 2.5
	ID LC-MS/MS	52.85 ± 0.84	3	
NMIJ-7402a	LC-ICPMS (TISTR)	32.94 ± 2.5	4	35.50 ± 1.8

[a] Standard deviation.
[b] Expanded uncertainty ($k = 2$).

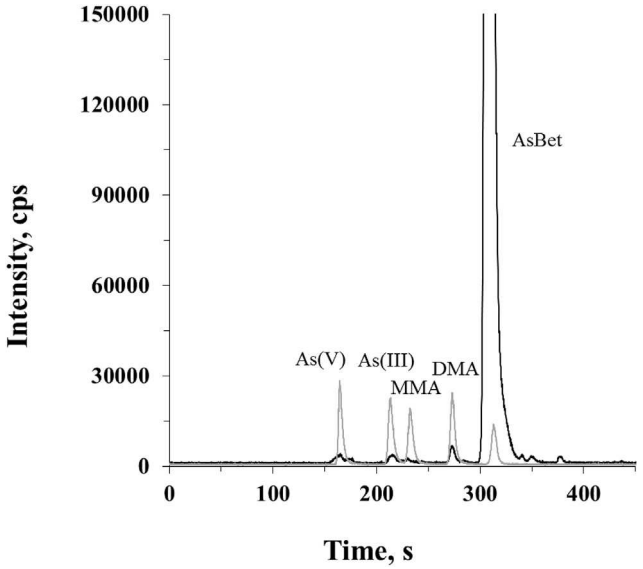

Fig. 4. Chromatograms obtained by LC-ICPMS from a mix arsenic standard solution of 5 ng mL^{-1} (grey line) and SQID-1 extract (black line) on ODS column. The dead volume is at 50 s.

32.94 ± 2.5 mg kg^{-1} (SD, $n = 4$) for AsBet was obtained in NMIJ-7402a, in agreement with the certified value of 35.50 ± 1.8 ($k = 2$) mg kg^{-1}. Note that t-values of 6.4 and 6.5 (>t-critical values of 2.2 and 2.3) were obtained from Student-T test by comparing data obtained by LC-ICPMS at TISTR to data obtained by ID LC-MS/MS and LC-ICPMS at NRC, respectively, confirming a significant difference between the data obtained at TISTR and data obtained at NRC. Nevertheless, final assigned certified value was based on weighted mean and the slightly lower value obtained at TISTR has little influence on the final results (see below section).

3.4. Certified/reference values in PRON-1 and SQID-1

Certified values based on dry weight for AsBet in PRON-1 and SQID-1 were obtained using weighted mean of three sets of data. Since only one set of data of MeHg in PRON-1 and SQID-1 is available, thus reference values were assigned for MeHg in the two new CRMs. The expanded uncertainty (U_{CRM}) in the certified or reference value is equal to $U = ku_c$ where u_c is the combined standard uncertainty calculated according to the JCGM Guide [35] and k is the coverage factor. A coverage factor of two (2) was applied for all measurands. The U_{CRM} accounts all sources of uncertainties from batch characterization (u_{char}), between-bottle variation or homogeneity (u_{hom}), and uncertainty related to inconsistency between the various measurement methods (u_{method}). Uncertainty for batch characterization is obtained using the following equation based on ID, standard addition or external calibrations for the mass fraction of analyte in the sample:

$$u^2(y) = \sum_{i=1}^{N} \left(\frac{\partial f}{\partial x_i}\right)^2 u^2(x_i) + 2 \cdot \sum_{i=1}^{N-1} \times \sum_{j=i+1}^{N} \left(\frac{\partial f}{\partial x_i}\right) \cdot \left(\frac{\partial f}{\partial x_j}\right) \cdot u(x_i) \cdot u(x_j) \cdot r_{(i,j)} \quad (5)$$

where $y = f(x_1, x_2, \ldots, x_N)$, the partial derivatives $\partial f/\partial x_i$ are often referred to as sensitivity coefficients, $u(x_i)$, $u(x_j)$ is the standard uncertainty associated with the input variables x_i and x_j, and $r(_{i,j})$ is the correlation coefficient. The material was tested for homogeneity at NRC. Results from nine sub-samples were evaluated using the DerSimonian-Laird random effects model [36]:

$$u_{hom}^2 = \frac{Q - (N-1)}{\sum u_i^{-2} - \sum u_i^{-4} \Big/ \sum u_i^{-2}} \quad (6)$$

where u_i are the uncertainty estimates of the individual measurements, individual N is the number of sub-samples, $Q = \sum u_i^{-2}(x_i - \mu)^2$, x_i are the results of each sub-sample, and μ is their weighted average. Uncertainties related to inconsistency between the various measurement methods (u_{method}) was estimated from the average results of independent methods using, again, the DerSimonian-Laird random effects model. Combined uncertainty, u_c, is then calculated using the following equation:

$$u_c^2 = u_{char}^2 + u_{hom}^2 + u_{method}^2 \quad (7)$$

Certified or reference values and uncertainties for MeHg and AsBet in two new CRMs are listed in Table 4. Results obtained are traceable to the SI through gravimetrically prepared natural abundance primary AsBet and MeHg standards whose purities were established by ^{1}H-NMR with use of NIST SRM 350b benzoic acid as calibrator. To the best of our knowledge, this is first report of SI traceable determination of AsBet and MeHg in prawn and cuttlefish.

As shown in Table 5, AsBet values obtained in prawn and cuttlefish samples are within the reported ranges in previous studies for prawn (shrimp) [37–42] and cuttlefish [42,43], respectively. AsBet comprised 69.7% and 99.0% of total As in the prawn and cuttlefish CRMs, respectively, which are within the range obtained

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Table 4
Certified or reference values and uncertainty components for PRON-1 and SQID-1.

Parameter	PRON-1 AsBet (as As), mg kg^{-1}	SQID-1 AsBet (as As), mg kg^{-1}	SQID-1 MeHg (as Hg), mg kg^{-1}
Certified or reference value	1.206	13.96	0.324
u_{char}	0.029	0.22	0.014
u_{hom}	0.000	0.15	0.000
u_{method}	0.000	0.00	0.000
u_c	0.029	0.27	0.014
U_{CRM} ($k = 2$)	0.058	0.54	0.028

Table 5
Comparison of total As, AsBet, total Hg and MeHg (mg kg-1, dry weight) in prawn and cuttlefish with values in the literature.

Sample	AsBet (as As)	Total As	AsBet/Total As	Reference
PRON-1 Prawn	1.206 ± 0.058[a]	1.73 ± 0.12[a]	69.7%	This study
Prawn	26.4–95.7	27.4–98.8	96.4–99.6%	[40]
Prawn	1.26–5.93	1.95–7.06	64.5–84.0%	[38]
Prawn	NA	NA	42%	[37]
Prawn	0.11–11	1.7–11	4.3–100%	[41]
Prawn	5.0	6.6	84.5%	[39]
Prawn	34	27	79.4%	[42]
SQID-1, Cuttlefish	13.96 ± 0.54[a]	14.1 ± 2.2[a]	99.0%	This study
Cuttlefish	61–114	93–134	82-86%	[42]
Cuttlefish	63.2 ± 3.3[b]	63.7 ± 0.9[b]	99.2%	[43]
Sample	**MeHg (as Hg)**	**Total Hg**	**MeHg/Total Hg**	**Reference**
SQID-1, Cuttlefish	0.324 ± 0.028[a]	0.342 ± 0.054[a]	94.7%	This study
Cuttlefish	0.15–2.9[c]	0.25–4.8[c]	60%	[44]
Cuttlefish	0.26–1.6	0.30–1.8	86.6–88.8%	[45]

[a] Expanded uncertainty, k = 2.
[b] Standard deviation.
[c] Calculated from wet weight.

in previous studies, as shown in Table 5. Currently, there are only a limited number of discussions on MeHg content in cuttlefish [44,45]. It is evident that MeHg value obtained in the new cuttlefish CRM SQID-1 is within the range obtained in these studies. Based on the total Hg content in the cuttlefish SQID-1, MeHg comprised 94.5% of total Hg, similar to these observed in previous studies [44,45]. These results confirm that the two new CRMs PRON-1 and SQID-1 are suitable for their use for the validation of methods for AsBet and MeHg measurements in such matrices.

4. Conclusions

Two methods based on species specific isotope dilution by LC-MS/MS and SPME GC-ICPMS which provide accurate and the SI traceable determination of AsBet and MeHg in biological tissues were developed. The purities of primary standards of arsenobetaine bromide and methylmercury chloride were characterized by ^{1}H-NMR with use of NIST SRM 350b benzoic acid as calibrator, ensuring values assigned in two new prawn and cuttlefish CRMs are traceable to SI. AsBet and MeHg values obtained in prawn PRON-1 and cuttlefish SQID-1 are within the range obtained in previous studies [37–45], suitable for their use for the validation of methods for such measurements. To the best of our knowledge, this is first report of SI traceable measurements of AsBet and MeHg in prawn and cuttlefish.

Author contributions

L. Yang has performed LC-ICPMS analysis at NRC and drafted this manuscript. B. Thiensong and S. Deawtong performed LC-ICPMS experiments at TISTR. P. Kumkrong has coordinated the physical production of the CRM and the measurement input of TISTR. G. McRae and P. Maxwell performed experiments for the ID LC-MS and GC-MS, respectively, at NRC. J. Meija calculated the final certified values and uncertainties for the two CRMs. P. Mai Le and Anthony Windust carried out the quantitative ^{1}H-NMR based purity evaluation. Z Mester has conceived this project and coordinated the execution of this research.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.aca.2016.09.031.

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