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Synthesis, characterization and application of a two-fold ^{13}C-labeled calibration standard for the analysis of arsenobetaine using HPLC–ESI-MS/MS without high resolution mass spectrometry

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ABSTRACT

A procedure has been developed for the determination of arsenobetaine in fish matrix by HPLC–ESI-MS/MS. Hereby (trimethylarsonium)-1,2-^{13}C-acetate (arsenobetaine) is used as internal calibration standard. Arsenobetaine was determined in a fish material (*Sea Bass*) with an expanded uncertainty of 3.8%.

1. Introduction

There is an immense interest on the analysis of arsenic species due to the variety of these arsenic compounds and their differences in toxicity and mobility. The high toxicological potential of inorganic arsenic species is known to cause adverse health effects. Methylated arsenic species like monomethylarsonic acid and dimethylarsinic acid possess lower toxic effects but are carcinogenic. Arsenobetaine and arsenocholine are the major arsenicals in seafood and were supposed to be nontoxic, but new studies indicate that they are transformed or metabolized in vivo to more toxicological relevant species [1,2].

Essential for risk assessment is the need to understand and to elucidate their pathways and long-term effects in vivo. The applied methods of analysis for arsenic species are generally based on separation techniques such as CE, HPLC or GC coupled with detection systems like HG-AAS, HG-AFS and ICP [3–5]. HPLC–ICP-MS has been established as reference for the analysis of arsenic compounds [6,7]. However, the increasing numbers of publications on real samples show the shortfall and limitations of this technique. These include a risk of co-eluting species, retention time irreproducibility, the presence of unidentified compounds, the impossibility of identifying them if reference standards are unavailable and a risk of misidentification of analytes solely based on the retention time matching with reference standards. Additionally, the ICP-MS is not as prevalent as other detection techniques because of its higher-than-average expenses for purchase and operation. Moreover, for monoisotopic arsenic isotopic dilution mass spectrometry (IDMS) with ICP-MS is not applicable.

In the late 1980s molecular detection became available with commercial mass spectrometry detectors [8]. Possible ionization methods are desorption, fast atom bombardment (FAB), atmospheric pressure (AP) and electrospray (ES) ionization. To fulfill the need for quality assurance and quality control for speciation analysis and in addition to focus on the use of certified reference materials, the validation and evaluation of state-of-the-art analytical techniques with isotope labeled calibration standards for arsenic species is obligatory. In contrast to HPLC–ICP-MS, deuterated or ^{13}C-labeled analytes can be used as internal standards for HPLC–ESI-MS. Hydrogen labels like ^{2}H or ^{3}H are notorious for their isotope effects and exchange reactions [9]. Hence, stable isotope tracers of choice are the ^{13}C-labeled species [10,11].

Our aim was to develop, characterize and evaluate a new approach based on a two-fold ^{13}C-labeled arsenobetaine ((trimethylarsonium)-1,2-^{13}C-acetate, Fig. 1) calibration standard. For the first time this standard was used in real samples. We were able to demonstrate that this standard is a powerful tool to assist

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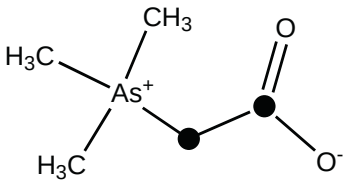

Fig. 1. (Trimethylarsonium)-1,2-^{13}C-acetate (arsenobetaine), the dots indicate the positions of the ^{13}C labels.

the determination, quantification and monitoring of arsenic species in complex matrices.

2. Material and methods

2.1. Synthesis of two-fold ^{13}C-labeled arsenobetaine

The synthesis for the unlabeled compound has been described by Lagarde et al. [12] (Fig. 2). A two-necked 25 mL round-bottom flask equipped with septum, a condenser and a gas inlet was twice evacuated and flushed with argon as a protection against oxidation (i.e. the formation of trimethyl arsenoxide). The flask was charged with ethyl bromoacetate-$^{13}C_2$ (1.22 g, 7.2 mmol, Sigma–Aldrich, Schnelldorf, Germany, 99%) and cooled externally to −35 °C. To this liquid neat trimethylarsane (0.77 mL, 7.2 mmol, Sigma–Aldrich, Schnelldorf, Germany, 99,9%) was added slowly *via canula*. After completion of the addition the reaction mixture was allowed to warm to room temperature and was stirred for an additional 20 h, during which time a solidification to a white solid was observed. Argon was used to flush out excess trimethylarsane, which was collected in a cooling trap. The resulting white solid was dried under vacuum (3×10^{-2} mbar) and identified as $[Me_3As^{13}CH_2{}^{13}CO_2Et]Br$ by ^{1}H and ^{13}C NMR spectroscopy in CD_3OD.

The ion exchange resin Amberlite® IRA OH 400 (45 g, Sigma–Aldrich, Schnelldorf, Germany) was suspended in 1 M aqueous NaOH (150 mL) and transferred into a chromatography column (approx. 2.5 cm diameter and 35 cm length). The resin was washed with deionized water until neutrality. $[Me_3As^{13}CH_2{}^{13}CO_2Et]Br$ was dissolved in 20 mL deionized water and carefully transferred onto the top of the resin. The material was eluted with approx. 500 mL water. The eluate obtained was evaporated to dryness to yield a colorless solid, which was dried under vacuum. The crude product was dissolved in methanol and precipitated with acetone. The crystallized colorless prisms were isolated on a glass frit (porosity 4) and dried under vacuum (3×10^{-2} mbar) for several days. They were identified as (trimethylarsonium)-1,2-^{13}C-acetate (arsenobetaine) by ^{1}H and ^{13}C NMR spectroscopy in CD_3OD. Yield: 1.14 g, 88%.

m.p. 203 ° C ^{1}H NMR (500 MHz, CD_3OD) δ = 3.37 (dd, $^1J^{13}C^1H$ = 136.9, $^2J^{13}C^1H$ = 5.5 Hz, 2H, $^{13}CH_2$), 1.89 (d, $^3J^{13}C^1H$ = 1.4 Hz, 9H, CH_3). ^{13}C NMR (125 MHz, CD_3OD) δ = 171.15 (d, $^1J^{13}C^{13}C$ = 49.8 Hz, C= O), 35.43 (d, $^1J^{13}C^{13}C$ = 49.8 Hz, CH_2), 8.24 (CH_3).

2.2. Purity assessment

Purity determination was performed by using HPLC–ESI-MS/MS, qNMR and coulometric Karl-Fischer. The used calibration standard was a commercial available solid arsenobetaine (Fluka, Seelze, Germany). The purity of this dried material was determined at BAM by qNMR and ICP-OES (total As concentration). The content in the standard solution was corrected for purity (purity: qNMR in methanol against benzoic acid NIST 350b (NIST, Gaithersburg, America): 89.34 ± 0.2%, ICP-OES: 90.3 ± 0.6%). The moisture content of the *Sea Bass* was determined at 5.64 ± 0.24% [1], by taking a portion of 0.3 g fish material and drying it in an oven at 105 ° C to constant weight. The result was verified by coulometric Karl-Fischer (5.43 ± 0.06% [2]).

Table 1
MS/MS-Operating conditions.

HPLC 1100			
Inj. vol. [μL]	5		
Flow rate [μL]	300		
Gradient	0–1 min		100% A
	1–6 min		0–25% B
	6.1–16 min		100% A
4000™ QTRAP™			
CUR [psi]	20		
CAD [arbitrary units]	6		
IS [V]	2500		
TEM [°C]	450		
GS1 [psi]	40		
GS2 [psi]	50		
Mode	ESI positive		
MRM [*m* /*z*]	Precursor ion	Quantifier	Qualifier
	179	105	120
	181	105	120

2.3. Analysis of arsenobetaine in a fish sample (Sea Bass)

The moisture assessment was carried out simultaneously with the analysis. Arsenobetaine was determined by HPLC–ESI-MS/MS with two-fold ^{13}C-labeled arsenobetaine as internal standard. The used calibrants were the NMIJ CRM 7901-a and a calibration solution prepared from solid arsenobetaine (Fluka, Seelze, Germany) dissolved in ultra pure water. To validate our analysis the BCR-627 tuna fish (IRMM, Geel, Belgium) was used. The applied equipments were a pressurized liquid extraction system ASE 200™ (Dionex Corporation, Sunnyvale, USA) and a HPLC 1100 (Agilent Technologies, Waldbronn, Germany) coupled to an ESI-MS API 4000™ QTRAP™ LC/MS/MS (AB SCIEX, Darmstadt, Germany). After cleaning all glassware and the ASE-frits with nitric acid overnight, it was washed with ultra pure water and dried. A 0.3 g sample was weighted into a 11 mL ASE cell. Two of five samples were spiked with 1.8 μg native arsenobetaine. The ASE extraction was performed with two cycles at 20 mL with 50 vol.% MeOH under pressure. The operating parameters were 0 min preheat, 5 min heat, 2 min static time, 60 vol.% flush, 60 s purge, 80 bar and 60 ° C for 5 cycles. The resulting extract was diluted to 100 g with ultra pure water. A 5 mL aliquote was evaporated to near dryness at 40 ° C with nitrogen, sonnicated in an ultrasonic bath for 60 min and cleaned up using commercial C_{18}-SPE cartridges (6 mL, 500 mg, J.T. Baker, Griesheim, Germany). The extract was diluted to 50 g with ultra pure water, 1 mL was given into PP vials (CHROMOPHOR, Fuessen, Germany) and assessed three times with HPLC–ESI-MS/MS. The parameters are given in Table 1. The applied column was a Hamilton PRP X-110, 2, 1 × 100 mm, 7 μm particle size. The eluents used were (A) 5% acetonitrile in ultra pure water and (B) 100 mM acetate buffer pH (5.8) with 5% acetonitrile in ultra pure water. A chromatogram is presented in Fig. 3 for a fish sample with 1,7 μg unlabeled arsenobetaine and 1,1 μg two-fold labeled arsenobetaine diluted in one liter ultra pure water after SPE clean up.

[1] Combined uncertainty.
[2] Combined uncertainty.

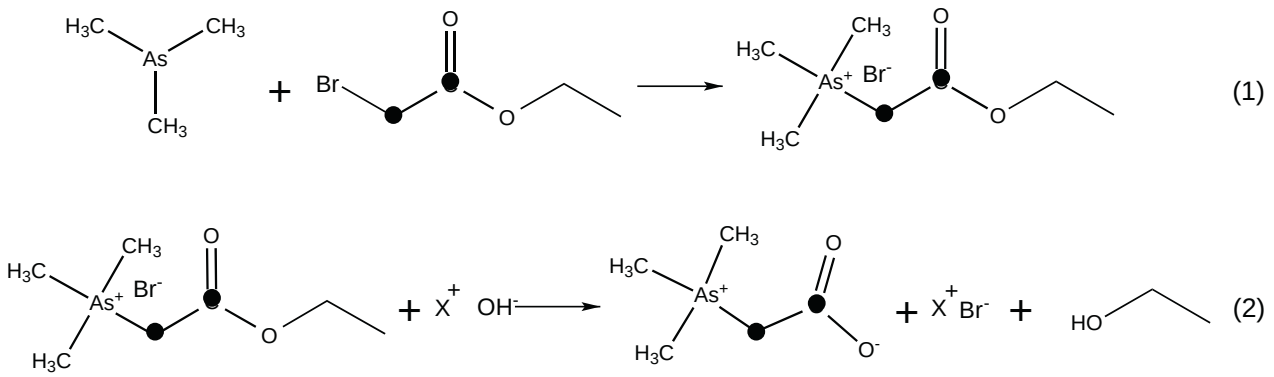

Fig. 2. Two step synthesis of (trimethylarsonium)-1,2-^{13}C-acetate from trimethylarsane and ethyl-1,2-^{13}C-bromoacetate; X^+OH^- symbolizes an anion exchange resin.

3. Results

All analytical results were calculated on a dry-mass basis. The calibration data sets for the NMIJ CRM 7901-a and the in-house calibrants are illustrated separately in Fig. 4.

To validate our analysis of the marine fish material (*Sea Bass*) the BCR-627 tuna fish (IRMM, Geel, Belgium) was used as a control sample: found with C_{18}-SPE clean-up 51 ± 1 μmol/kg (cert. 52 ± 3 μmol/kg). For calibration purposes our own arsenobetaine and the CRM solution was used. The combined calibration data is illustrated in Fig. 5. The average arsenobetaine content in the marine fish material was determined at 5.76 mg/kg with an expanded uncertainty of 3.82%.

During this study the results after clean-up were compared to the values determined without C_{18}-SPE clean-up. By measuring the raw extract we found 50.0 ± 0.5 μmol/kg for the BCR-627 tuna fish. The mean results for the *Sea Bass* after clean-up were statistically the same as those without C_{18}-SPE purification. Although we achieved slightly better results after clean-up the benefit of an C_{18}-SPE clean-up could not be proven by our results. Nevertheless, we recommend to remove nonpolar components to protect the analytical system and to avoid the effect of ion suppression for samples of lower arsenobetaine content. Additionally, the influence of a different extraction pressure and temperature was investigated. The results are shown in relative arsenobetaine content versus the extraction conditions in Fig. 6. Temperature and pressure have no significant influence on the ASE-extraction of arsenobetaine.

It was assumed that the major contributions to the combined uncertainty of the mean arise from the precision of the method including possible sample inhomogeneity, the concentration of the

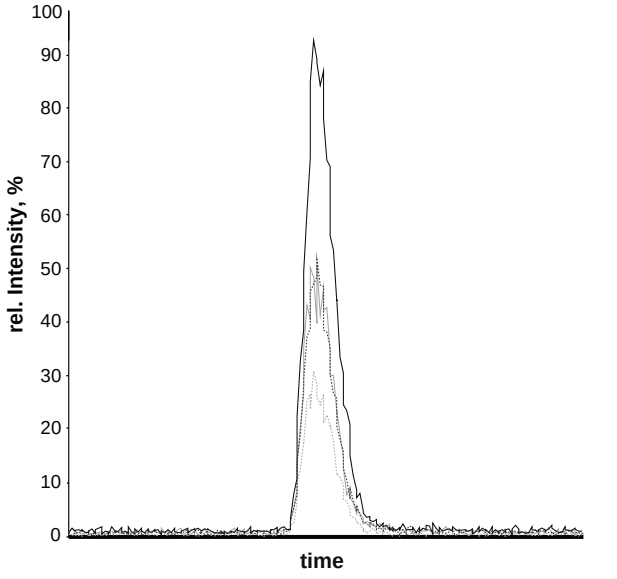

Fig. 3. Chromatogram of *Sea Bass* sample with 1,7 μg native arsenobetaine and 1,1 μg two-fold labeled arsenobetaine diluted in one liter ultra pure water after SPE clean up; MRM 179 → 120 in black, MRM 179 → 105 dotted in black, MRM 181 → 120 in gray, MRM 181 → 105 dotted in gray.

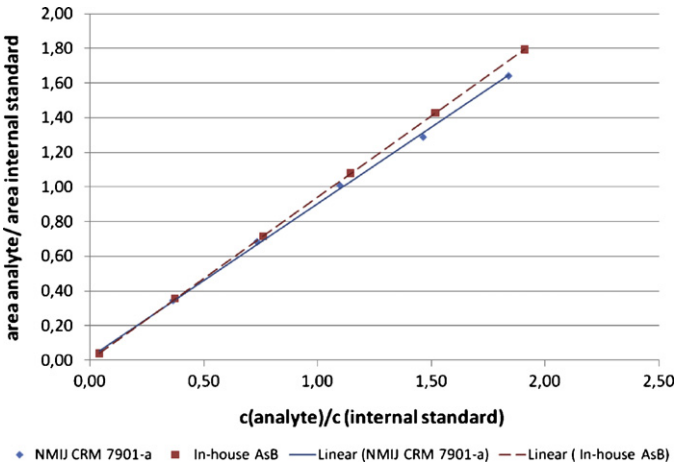

Fig. 4. Calibration data, complete specification of the measurement equations are: $y = 0.93796x + 3.92E^{-03}$, $R^2 = 0.99999$ (for calibration with own AsB) and $y = 0.8823x + 0.0213E^{-02}$, $R^2 = 0.9991$ (linear calibration with NMIJ CRM 7901-a).

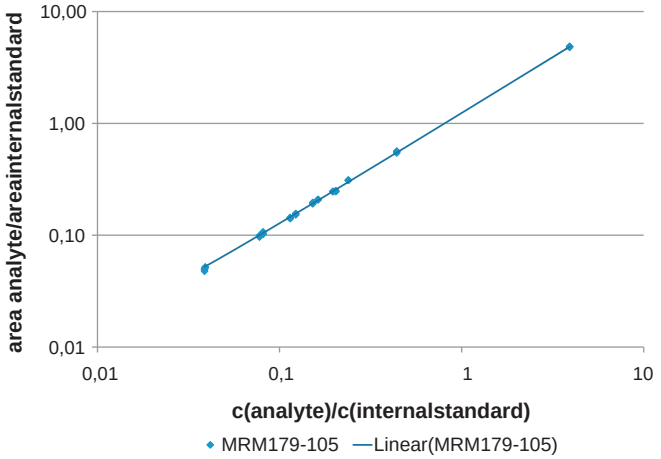

Fig. 5. Calibration data for the measurement of the *Sea Bass* and the BCR-627 tuna fish material, complete specification of the measurement equation: $y = 1.2344x + 0.0043$, $R^2 = 0.9999$.

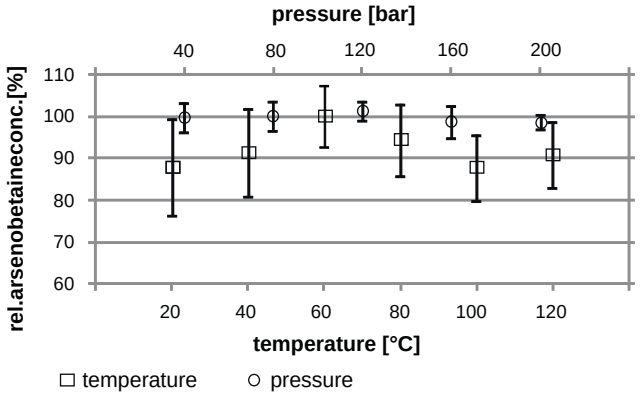

Fig. 6. Influence of temperature and pressure during extraction; results are shown in relative arsenobetaine concentrations.

calibration standard NMIJ CRM 7901-a and the dry mass correction. The standard deviation of the mean of the replicate measurements was taken as an estimate of method precision. It covers not only the precision associated with the measurement but also the precision of weighing out the sample, spiking it with the internal standard, extraction, clean up, calibration, etc. as these operations were repeated during the course of the experiment. A separate estimate of their individual uncertainties was therefore not carried out. The concentration of the calibrant solution and its uncertainty were taken from the certificate. The uncertainty of the dry mass correction factor was determined as the standard deviation of the mean of the replicate dry mass measurements.

4. Discussion

Because of the multitude of arsenic species and their differences in toxicity and mobility the species analysis of arsenic compounds is of special interest. In case of inorganic arsenic compounds a high toxicological potential is known to give rise to adverse health effects. Arsenobetaine and arsenocholine are the major arsenicals in seafood and were believed to be nontoxic but at this moment more and more hints appear that they are transformed or metabolized to more toxicological relevant species in vivo.

A procedure has been developed for the accurate determination of arsenobetaine in fish matrix by HPLC–ESI-MS/MS. The HPLC–ESI-MS/MS is a prevalent detection system and has lower expenses for purchase and operation than the HPLC–ICP-MS. Moreover, for monoisotopic arsenic the IDMS with ICP-MS is not applicable. The method uses for the first time ((trimethylarsonium)-1,2-^{13}C-acetate (two-fold ^{13}C-labeled arsenobetaine) as internal standard. With this standard no high resolution mass spectrometric system is needed. The simple ASE-extraction allows the parallel handling of many samples. The precision of our method is comparable to and even better than HPLC–ICP-MS-methods. Arsenobetaine was determined in marine fish material (*Sea Bass*) with an expanded uncertainty of 3.8%. Similar methods applying HPLC–ICP-MS have an expanded uncertainty in the same range for the determination of arsenobetaine in BCR-627 [13]. Therefore, the precision of our method is comparable or even better than standard protocols if using IDMS.

At the moment this method is limited to samples containing arsenobetaine as main component. Therefore it is well suited for the determination of arsenobetaine in seafood. Other matrices have to be evaluated. Although toxicity could not yet be proven for arsenobetaine, the correct content of this species is important for reassuring that the budget of arsenic species is in reasonable correlation to the total arsenic content. Our aim is to similarly analyze MMA and DMA as arsenic analytes with lower responses by HPLC–ESI-MS/MS. We are confident that the ongoing improvement of the sensitivity of ESI-MS/MS-systems will allow the detection of all organic arsenic compounds with ^{13}C-labeled standards. Furthermore, possible degradation products of arsenobetaine can be identified during stability tests by tracking the isotopic label in the corresponding metabolites. The origin of arsenobetaine or its transformation products can be determined by using the labeled compound. In addition, the standards will help to establish an HPLC–ESI-MS/MS method as an economic alternative for the species analysis of arsenic compounds. Finally by ESI-MS/MS species can be quantified in a reliable way by using a defined multiple reaction monitoring (MRM), a standard technique for quantitative HPLC–ESI-MS/MS.

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