Fast and Accurate Determination of Arsenobetaine (AsB) in Fish Tissues Using HPLC-ICP-MS

Application

Foods

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Abstract

A high performance liquid chromatography-inductively coupled plasma mass spectrometry method was developed for the fast and accurate analysis of arsenobetaine in fish samples extracted by accelerated solvent extraction. The combined extraction and analysis approach was validated using certified reference materials for arsenobetaine in fish and during a European intercomparison exercise with a blind sample. Up to six species of arsenic can be separated and quantified in the extracts within a 10-minisocratic elution. The method was optimized so as to minimize time-consuming sample preparation steps and to allow for automated extraction and analysis of large sample batches. A comparison of standard addition and external calibration showed no significant difference in the results obtained, which indicates that the liquid chromatography-inductively coupled plasma mass spectrometry method is not influenced by severe matrix effects. The extraction procedure could process up to 24 samples in an automated manner while the robustness of the developed high performance liquid chromatography-inductively coupled plasma mass spectrometry approach is highlighted by the capability to run more than 50 injections per sequence which equates to a total run-time of more than 12 hours. The method can therefore be used to rapidly and accurately assess the proportion of nontoxic arsenobetaine in fish samples with high total arsenic content during toxicological screening studies.

Introduction

The element Arsenic (As) has long been thought of as poisonous and highly toxic. However, it has since been shown that the toxicity of As is largely dependent on the form or “species” the arsenic is in. Arsenic is ubiquitous in the environment due to natural and anthropogenic sources, and the relative contribution of these factors is estimated as roughly 60% and 40% respectively. In the environment, As behaves in similar ways to the Group V elements nitrogen (N) and phosphorus (P). As a result of these similarities, arsenic gets taken into the biochemical pathways of N and P. This results in the formation of compounds such as arsenobetaine (AsB) in fish and arseno-sugars, which are found in marine algae. The toxicity of the inorganic As-species (such as arsenite, As(III) and arsenate, As(V)) is far greater than the organic forms, such as monomethylarsonic and dimethylarsinic acid (MMAA and DMA) and AsB. The International Agency for Research on Cancer (IARC) has classified inorganic arsenic as a human carcinogen, whereas AsB, the predominant form of As in most marine organisms [1], is considered nontoxic to humans. Although AsB is the major form of As in many marine organisms, it is not present in all fish species [2]; therefore, an evaluation of the proportion of AsB to the total As determined can give a useful and rapid estimate of the toxicological significance of a sample. In order to determine the toxicity of seafood, the determination of the total As alone is of limited value, and the different species of As have to be extracted, separated, and determined. Fast, reliable, and practical methods are therefore required that can provide speciation information for the screening of large sample batches.

Aims and Objectives

The aim of this study was to develop a semi-automated analytical method for the extraction and determination of As-species in fish tissues. Requirements for high sample throughput analysis were the automation of the extraction procedure as well as a fully automated separation and detection method capable of analyzing large sample batches (up to 50 injections per run) during overnight runs. In order to streamline the analytical procedure, an attempt was made to develop a method with a minimal number of sample preparation steps. It was intended that the method should be established using calibration by external calibration curves, rather than the lengthy alternative of standard additions. The use of an isocratic liquid chromatography (LC) elution can be favorable in terms of time-efficiency during the liquid chromatography-inductively coupled plasma mass spectrometry (LC-ICP-MS) analysis because it negates the need for column re-equilibration between injections.

Calibration Standards

The following standards were obtained from Fluka (Sigma-Aldrich, Gillingham, UK): di-sodium hydrogen arsenate heptahydrate ($AsHNa_2O_4.7H_2O$) ≥98.5%, sodium (meta)arsenite ($AsNaO_2$) ≥99.0%, and cacodylic acid (dimethylarsinic acid, DMA, $C_2H_2AsO_2$) ≥99.0%; monomethylarsonic acid disodium salt (MMAA, $CH_3AsNa_2O_3$) >98% was obtained from Argus Chemicals (Vernio, Italy). Arsenobetaine (AsB, $C_5H_{11}AsO_2$) was obtained from BCR (Brussels, Belgium) as a solution of AsB in water at 1031 ±6 (95% C.I.) mg/kg (BCR 626).

Extraction

Accelerated solvent extraction (ASE) has been used previously for As-speciation [3, 4] and was chosen as the sample preparation method because it allows for the automated extraction and online filtration of up to 24 samples. In addition, the extraction solution is collected in glass vials, which negates further sample preparation steps such as filtration or centrifuging.

The samples were extracted using a Dionex ASE 200 accelerated solvent extractor. Sample sizes from 0.1–0.3 g were weighed accurately into 11-mL stainless steel extraction cells fitted with filter papers and PTFE liners. The extraction program was set up as shown in Table 1.

Table 1. Extraction Conditions Used for ASE

Instrument	Dionex ASE 200
Preheat	2 min
Heat	5 min
Extraction steps	5 × 2 min
Temperature	100 °C
Pressure	1500 psi
Solvent	Methanol

HPLC-ICP-MS Methodology

The HPLC-ICP-MS instrumentation consisted of an Agilent Technologies 1100 HPLC system coupled to an Agilent Technologies 7500i ICP-MS fitted with a second roughing pump, which enhances sensitivity by increasing ion transmission across the interface. The HPLC system comprised a quaternary pump module, a vacuum degasser, a temperature controlled autosampler, and column compartment. The ICP-MS instrument was tuned for sensitivity, reduced oxides, and doubly charged species prior to connection to the liquid chromatograph by performing a standard instrument tune using a 10 ng/g solution of Li, Y, Ce, and Th in 1% HNO_3. The pulse to analog (P/A) factor was adjusted on a daily basis using a solution containing ~50 ng/g Li, Mg, Mn, Cu, As, Gd, Y, Cd, Pb, and Ba. After this optimization, a 50 ng/g solution of As in 1% HNO_3 was used to specifically optimize the sensitivity for arsenic. The ICP-MS nebulizer was then connected to the HPLC-column using a length of PEEK tubing (yellow, 1/16-inch od, 0.007-inch id). See Table 2 for the ICP-MS conditions used.

Table 2. ICP-MS Conditions Used for HPLC-ICP-MS Determination of As-Species

RF Power	1430–1550 W
RF Matching	1.89–1.92 V
Sampling depth	4.0–4.8 mm
Carrier gas flow	0.89–0.93 L/ min
Make up gas flow	0.10–0.14 L/ min
Optional gas	Oxygen at 5%
Spray-chamber temperature	0 °C
Cones	Platinum
Isotopes monitored	^{75}As ^{103}Rh ^{77}Se ($^{40}Ar^{37}Cl$) to monitor Cl interferences ^{53}Cr ($^{40}Ar^{13}C$) to monitor C interferences
Other parameters	Injector diameter: 2.4 mm Nebulizer 100 µL/min PFA, Two interface pumps used

In order to develop a rapid chromatographic separation of the main As-species in fish tissues, an anion exchange column (Hamilton PRP X-100) was chosen in combination with an isocratic elution profile. Several mobile phases were tested and the best separation of AsB and As(III) as well as DMA and MMAA was achieved within 10 min using 2.2-mM NH_4HCO_3/2.5-mM tartaric acid at pH 8.2 delivered at 1 mL/min isocratic flow. This evaluation was carried out initially using matrix-free calibration standards containing the species of interest and refined using an oyster tissue extract that contained arsenocholine (AsC), two arsenosugars (As-sug. B and As-sug. D), $TMAs^+$ and several unknown species in addition [5]. The injection volume for samples and standards was 50 µL.

In order to enhance the ionization of the As-species [6, 7], methanol was added to the mobile phase at concentrations ranging from 0.5% to 5% v/v. At concentrations above 1%, the chromatographic separation degraded significantly to the degree that base-line resolution between AsB and As(III) was no longer achieved. However, the addition of 1% MeOH to the mobile phase resulted in a significant improvement in the sensitivity (3–4-fold increase in peak height) for all analytes. A chromatogram for a 5-ng/g mixed calibration standard with the final chromatography conditions is shown in Figure 1.

Variations in Signal Response for Different As-Species

The chromatogram shows that the four species analyzed here have very different response factors with this method, even when made up to contain the same concentration of As in solution. This is further illustrated by the calibration curves and their respective slopes, as shown in Figure 2. Such differences in the analyte signal intensity were reported previously in the literature [7] and appear to be due to a combination of the ICP-MS hardware used and the plasma conditions, which are in turn affected by the mobile phase composition. This points to possible differences in the nebulization, transport and/or ionization of different species by such methods. In order to determine whether this effect could be attributed to the coupling of the ICP-MS with a liquid chromatograph, aqueous standards of AsB and As(III) were made up to equivalent concentrations as As and analyzed by direct aspiration without chromatography. This indicated that the signal response of AsB was ~10%–15% higher compared to the inorganic As standard and, therefore, the difference in signal response does not appear to be related to the coupling with a liquid chromatograph.

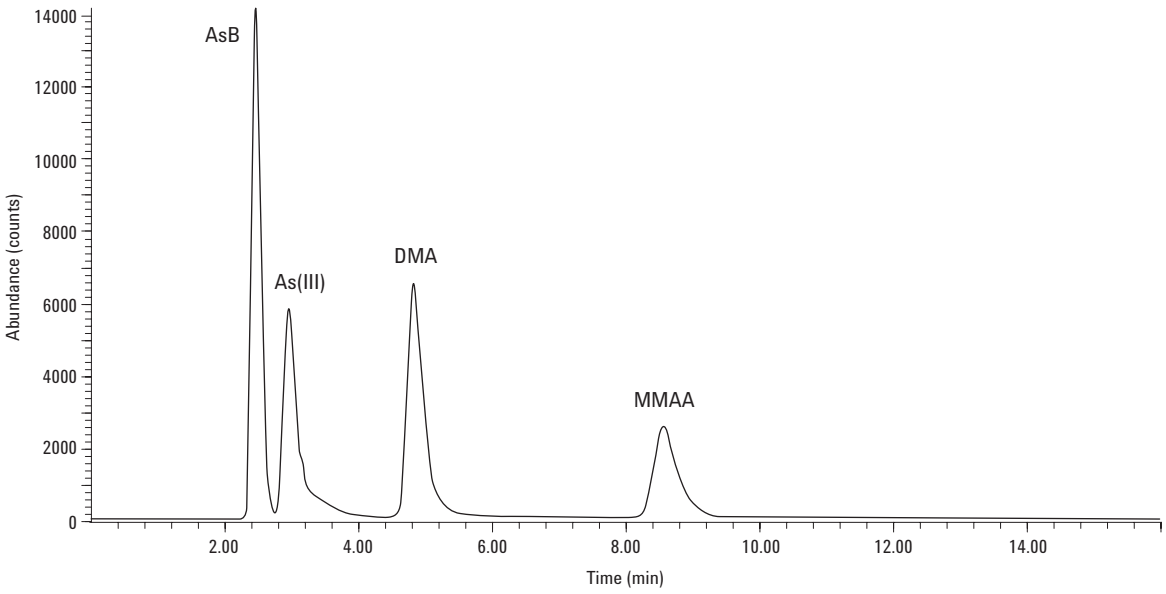

Figure 1. Chromatography A: 2.2-mM NH_4HCO_3 2.5-mM tartaric acid, 1% MeOH, pH 8.2, Hamilton PRP X-100 column. Concentration of standard ~ 5 ng/g as As.

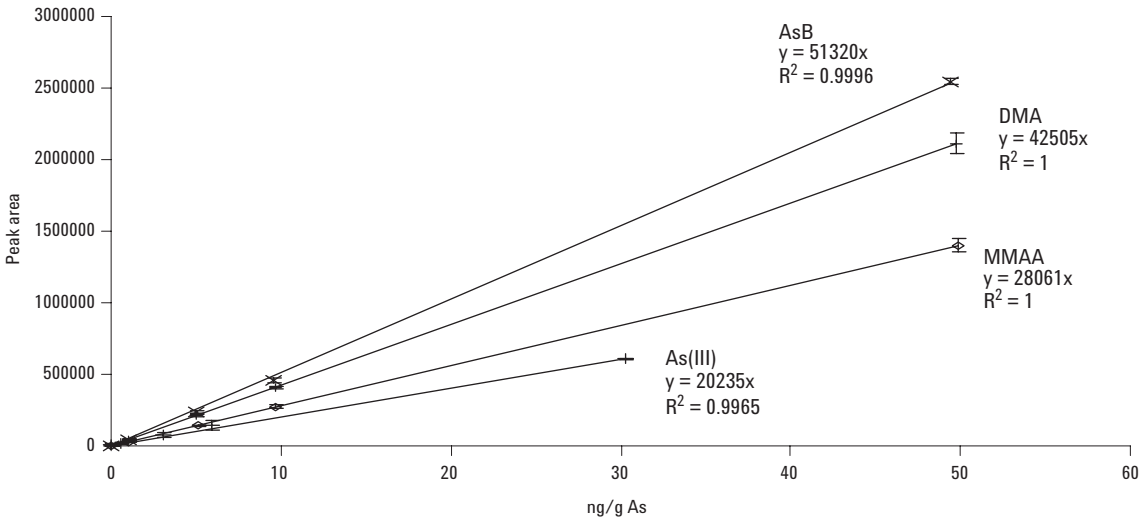

Figure 2. Calibration curves for AsB, DMA, MMAA and As(III) over a range of 0–50 ng/g as As.

In order to increase the signal intensity for species such as As(III) and MMAA by the approach described here, additional MeOH was added via a T-piece post-column so as not to impact on the chromatographic resolution. Although the relative volume of MeOH could be increased by 50%–70% in this way without deteriorating plasma stability, the relative signal responses of the four species were not influenced significantly. Because the relative signal response was stable on a day-to-day basis, no further attempts were made to equalize the signal responses.

The instrumental detection limit for AsB by this method was 0.04 ng/g as As. The linearity obtained, as indicated by the correlation coefficient of the calibration line, was 0.999–1.000 over a calibration range of 0–700 ng/g as As.

Plasma Disturbance Due to Elution of MeOH

During the analysis of fish samples, which had been extracted under the ASE conditions highlighted in Table 1, a disturbance of the plasma was observed between ~2.3 to 4.3 min after injection. This affected all of the isotopes monitored and the effect on ^{75}As and ^{103}Rh is highlighted in Figure 3. As can be seen from the chromatogram, the effect on these two isotopes is nonlinear. The ^{103}Rh signal decreases significantly during this time, whereas the ‘shoulder’ on the tailing side of the AsB peak indicates an increase in the ^{75}As signal.

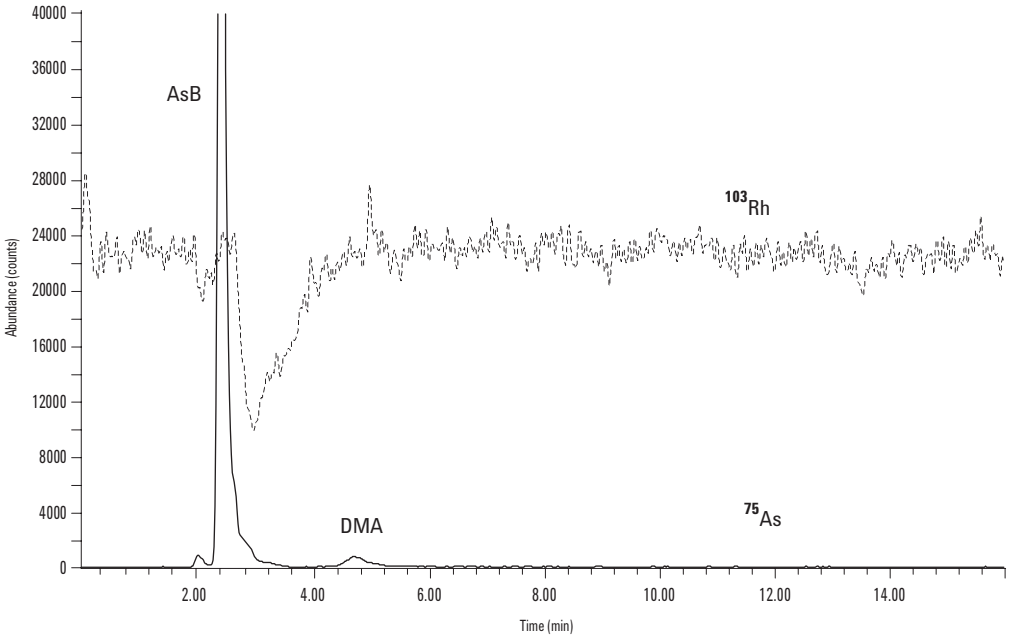

Figure 3. Signals for ^{103}Rh and ^{75}As in an undiluted fish extract. Notice the increase in the ^{75}As signal at the tailing side of the major peak (AsB) coinciding with the decrease in the ^{103}Rh signal.

The observed fluctuation in the signal intensities for the different isotopes coincides with the elution of the organic methanol fraction of the fish extracts from the analytical column. This effect could be reduced slightly by lowering the temperature of the spray-chamber from 5 °C to 0 °C, but the effect was not completely eliminated. During the injection of undiluted sample extracts, the volume of methanol that passes through the column and into the ICP-MS is ~10%. It has already been discussed that the addition of MeOH enhances the ^{75}As signal by increasing the ionization efficiency of this analyte; this effect is observed on a small scale here. Although there is no detectable As(III) in this fish material, the accurate quantitation of this compound (compared to aqueous calibration standards) could obviously lead to an overestimation if the signal of this analyte is enhanced due to the simultaneous elution of MeOH from the column. In this case, a standard addition calibration would represent a more accurate approach for quantitation. However, the spiking of each sample extract at different levels, which is necessary for this type of calibration, would make such an approach less suitable for a high sample-throughput application. In addition, the accurate integration of AsB is influenced by the signal increase on the tailing side of the peak.

In order to eliminate the effect of these signal variations on the accurate quantitation of the As-species in the methanolic extracts, the methanol fraction could either be reduced by evaporation or dilution with water. Dilution was chosen as the preferred option over evaporation in order to avoid possible analyte losses and because of time-efficiency. Whereas evaporation would either involve passing an inert gas over the solution or using rotary evaporation equipment, gravimetric dilutions were easily and quickly achieved by pipetting an aliquot of the extract into a sealed HPLC autosampler vial, weighing, and then adding the appropriate amount of water. In order to observe the effect of different dilution factors on the observed plasma disturbance, a fish extract was diluted 10-, 5-, and 2-fold in water and also injected undiluted. The effects of the different dilutions on the ^{103}Rh signal are shown by the chromatograms in Figure 4.

As demonstrated in Figure 4, a 10-fold dilution is sufficient to eliminate the plasma disturbance sufficiently; therefore, all extracts were diluted 1:10 in water prior to injection.

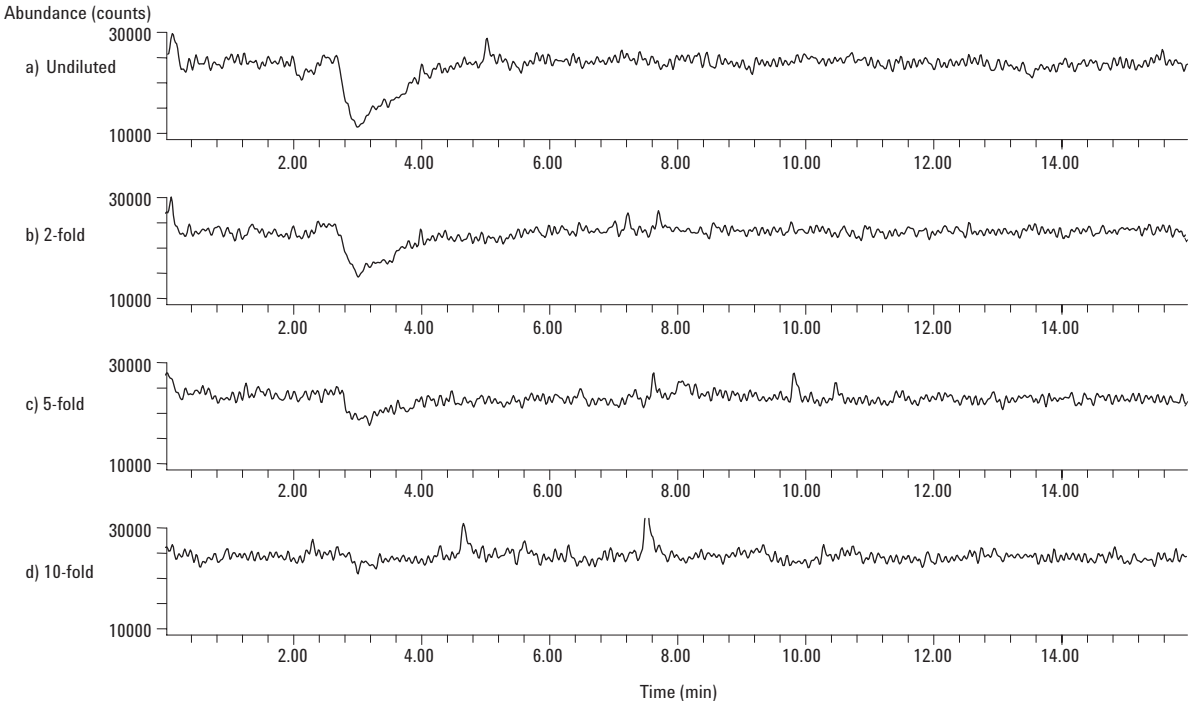

Figure 4. Signal of the internal standard ^{103}Rh, for fish sample extracts a) undiluted and diluted b) 2-fold, c) 5-fold and d) 10-fold.

Comparison of External Calibration and Standard Addition for the Quantitation of AsB in Fish Tissues

Due to the fact that arsenic is mono-isotopic, isotope dilution analysis cannot be used for the high-accuracy quantitation of this compound by LC-ICP-MS. In such circumstances, calibration by standard additions is often used in order to achieve matrix matching of standards and samples. It is also a useful technique in chromatographic applications where the possibility of retention time (RT) shifts of analytes due to matrix components exists. This can result in misidentification, and thus erroneous results. However, standard addition calibration can be very time- consuming because several aliquots of the sample require spiking with different levels of a calibration standard, and at least three levels of standard addition are needed for accurate quantitation of the same sample. External calibration by non-matrix matched standards can be used for applications where the difference in the matrix between samples and standards does not influence the accuracy of the result to a significant extent.

Standard addition calibration and non-matrix matched external calibration were compared for AsB in two certified reference materials (DORM-2, Dogfish muscle, NRC Canada and BCR 627, Tuna Fish, BCR EU) in order to assess whether the calibration technique used significantly influenced the accuracy or precision of the analytical result. The results showed that there was no significant difference in the mean results determined by the different calibration techniques with this method. The mean results for repeat analysis of both materials showed that the difference in the DORM-2 material was less than 1.4% and less than 4.5% for the BCR 627 material. When taking into account the standard deviations (SD) associated with the mean result obtained by each calibration technique, there was no statistically significant difference between the AsB results obtained by either approach in either of the fish tissue certified reference materials (CRMs).

Results of CRM Analysis

In order to test the accuracy of the developed ASE extraction and HPLC-ICP-MS method, a variety of certified and candidate reference materials of marine origin were extracted and analyzed. The samples included the certified fish reference materials DORM-2 and BCR 627, as well as an oyster tissue material (BCR 710)*, which is pending certification.

* The "MULSPOT" project has been financed by the SM&T Program (EU) (Contract SMT4-CT98-2232) and coordinated by ENEA (IT). The Project is at the certification stage and the material is not yet available on the market.

Table 3. Data Obtained for AsB in Two CRMs and a Candidate Reference Material

Expressed as mg/kg As unless otherwise stated	Measured value	Certified value
DORM-2 (Dogfish muscle)	16.3 ±0.9 (±1 SD)	16.4 ±1.1 (±95% C.I.)
BCR 627 (Tuna fish)	3.69 ±0.21 (±1 SD)	3.90 ±0.22 (±95% C.I.)
BCR 710 (Oyster tissue)† (Concentration as species)	31.8 ±1.1 (±1 SD)	32.7 ±5.1 (±1 SD)

† The data shown for this material is based on the consensus mean of the final certification round after the removal of statistical outliers.

Subsamples of the different materials (n = 4–6) were extracted, diluted in water, and analyzed as described above. The data for AsB determined in these samples is shown in Table 3. A chromatogram of the tuna fish material BCR 627 is shown in Figure 5.

The chromatogram indicates that the major species in this sample is AsB with two minor species, which were also extracted and detected. One peak was identified as DMA, and the peak labelled P1 is most likely to be AsC from RT matching. The data in Table 3 shows that the combined ASE/HPLC-ICP-MS methodology is capable of delivering accurate and reproducible results for AsB in these matrices. In addition, the extraction of other minor species, such as DMA and AsC, was achieved in the fish tissues; up to six species were extracted and separated in the oyster material, although none of these (apart from DMA) were quantified during this study. This DMA data for BCR 710 (730 ±30 ng/g DMA) showed a good agreement with the consensus mean value of the certification round (820 ±200 ng/g DMA).

Evaluation of Method Performance During a CRM Feasibility Study

The method performance was assessed in comparison to a number of European expert laboratories during the “SEAS” feasibility study organized by the The University of Plymouth Enterprise Limited and sponsored by the European Union (BCR, EU)‡. A fish material was prepared for this intercomparison by the University of Plymouth and distributed to participating laboratories. Participants were asked to determine AsB in a fish material from two different bottles using a methodology of their choice and making their determinations, at least, in duplicate on separate days.

The developed As-speciation method was used to extract and analyze the fish samples provided. A total of 12 subsamples from the two bottles were

‡The “SEAS” feasibility study was co-ordinated by The University of Plymouth Enterprise Limited (Plymouth, UK) under the EC contract: G6RD CT2001 00473 "SEAS" with the title: 'Feasibility Studies for Speciated CRMs For Arsenic in Chicken, Rice, Fish and Soil and Selenium in Yeast and Cereal'.

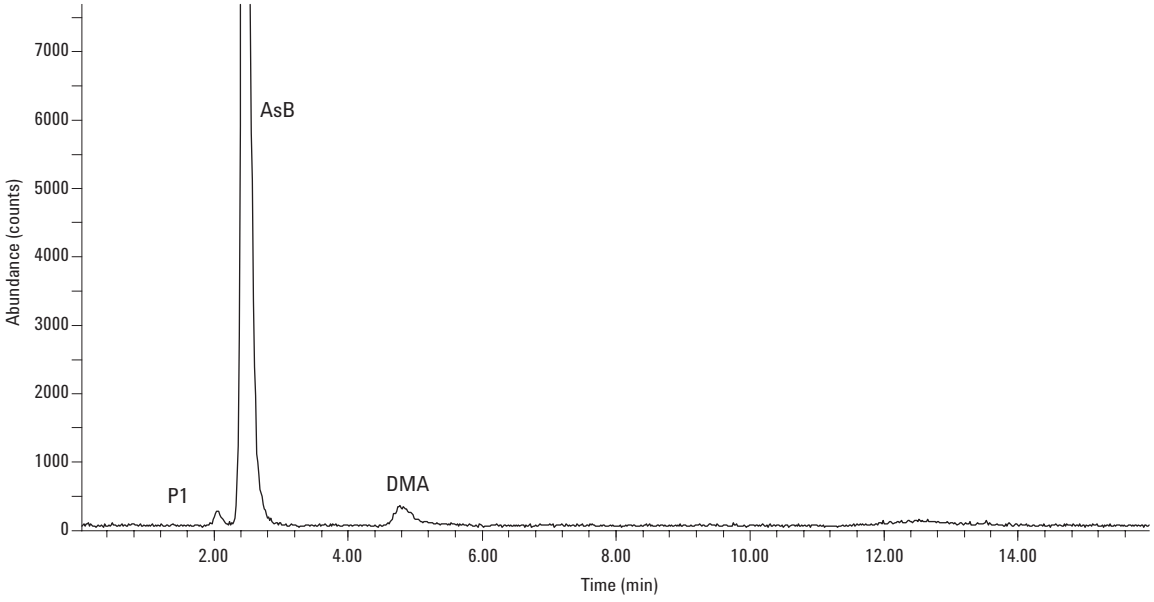

Figure 5. Chromatogram of a tuna fish extract (BCR 627) enlarged to show the detection of minor species in this material.

extracted and analyzed on 3 different days. The data were combined to provide the value labelled "LGC" in Figure 6 below. The error bars indicate the SD of the mean of individual results. The mean of all result (excluding a statistical outlier) together with 1 SD above and below the mean is indicated by the solid and dashed horizontal lines, respectively. The data provided by the combined ASE extraction and developed LC-ICP-MS methodology (94.92 ±3.95 mg/kg AsB) is in very good agreement with the mean result of all labs (95.72 ±7.79 mg/kg AsB, n = 11). The precision achieved was also satisfactory at 4.2% (RSD) for 12 subsamples from different bottles analyzed on 3 separate days. The performance of the method in this international intercomparison is highlighted by the good agreement with data provided by several European expert laboratories with longstanding expertise in As-speciation analysis. It should also be noted that the intercomparison was carried out with a blind sample of unknown concentration, rather than based on the analysis of a CRM with known certified values.

Conclusions

A robust and practical method has been developed based on accelerated solvent extraction and HPLC-ICP-MS analysis for the fast and accurate determination of AsB in fish samples. The benefits of the methods include automated extraction of up to 24 samples, minimal sample preparation steps (dilution only) after extraction, and rapid and automated analysis by HPLC-ICP-MS. The separation of four to six species of toxicological interest is achieved within 10 min using an isocratic elution. This increases the sample throughput by negating the column equilibration period needed with most gradient elution profiles.

The method was validated using commercially available CRMs and during a European intercomparison study with a fish sample of unknown concentration. The performance of the method was very satisfactory in terms of both accuracy and precision compared to several other expert laboratories.

This method can be used to rapidly determine the nontoxic proportion (AsB) in fish samples with high total As content and could therefore be used to determine whether a particular sample poses a toxicological risk in the food chain.

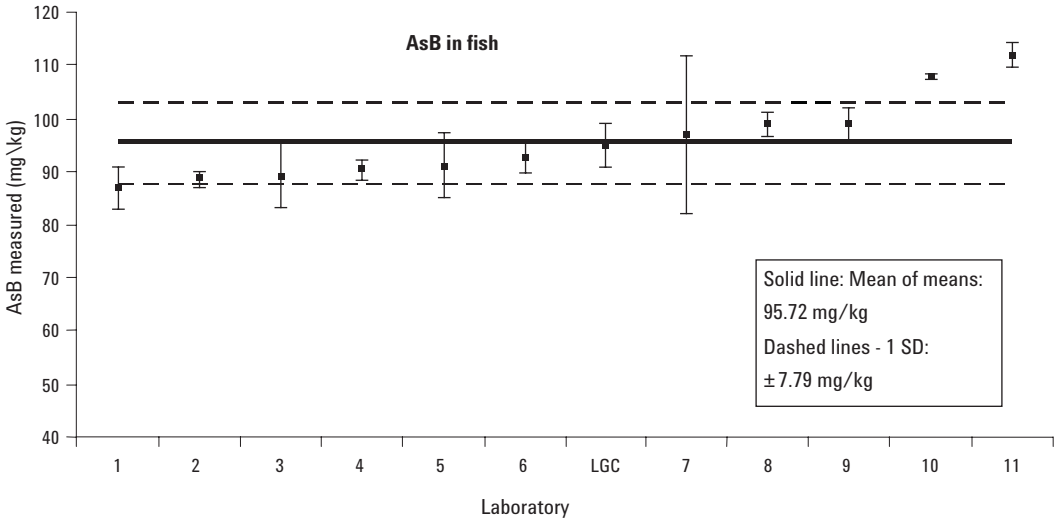

Figure 6. Comparison of data submitted by 12 participants for the determination of AsB in fish during the "SEAS" feasibility study. The error bars associated with the individual data points represent 1 SD of analysis of separate subsamples.

Acknowledgements

The work described in this application note was supported under contract with the Department of Trade and Industry (UK) as part of the National Measurement System Valid Analytical Measurement (VAM) program.

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Printed in the USA
October 14, 2003
5988-9893EN

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