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Sequential injection analysis implementing multiple standard additions for As speciation by liquid chromatography and atomic fluorescence spectrometry (SIA-HPLC-AFS)

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ABSTRACT

An analytical procedure for multiple standard additions of arsenic species using sequential injection analysis (SIA) is proposed for their quantification in seafood extracts. SIA presented flexibility for generating multiple specie standards at the ng mL^{-1} concentration level by adding different volumes of As(III), As(V), monomethylarsonic (MMA) and dimethylarsinic (DMA) to the sample. The mixed sample plus standard solutions were delivered from SIA to fill the HPLC injection loop. Subsequently, As species were separated by HPLC and analyzed by atomic fluorescence spectrometry (AFS). The proposed system comprised two independently controlled modules, with the HPLC loop acting as the intermediary device. The analytical frequency was enhanced by combining the actions of both modules. While the added sample was flowing through the chromatographic column towards the detection system, the SIA program started performing the standard additions to another sample. The proposed method was applied to spoiled seafood extracts. Detection limits based on 3σ for As(III), As(V), MMA and DMA were 0.023, 0.39, 0.45 and 1.0 ng mL^{-1}, respectively.

1. Introduction

Speciation of arsenic has been accomplished by different hyphenated schemes, usually involving high-performance liquid chromatography (HPLC) or capillary electrophoresis (CE) columns coupled to atomic spectrometers. These separation techniques have been frequently coupled to inductively coupled plasma mass spectrometer (ICP-MS), due to its wide applicability, and seldom to atomic fluorescence spectrometry (AFS), which is limited to species forming volatile compounds. However, analytical characteristics of As speciation by hydride generation atomic fluorescence spectrometry (HG-AFS), namely detection limit, sensitivity, repeatability, and reproducibility were found comparable to those of HPLC-ICP-MS [1].

HPLC and HG-AFS have been combined to determine As species, in urine [2] and seafood [3] samples. The biotransformation of inorganic arsenic included the production of species named arsenite (As(III)) $As(OH)_3$, arsenate (As(V)) $AsO(OH)_3$, monomethylarsonic acid (MMA) $(CH_3)AsO(OH)_2$ and dimethylarsinic acid (DMA) $(CH_3)_2AsO(OH)$. Also, the less stable monomethylarsonous acid $(CH_3)As(OH)_2$ and dimethylarsinous $(CH_3)_2As(OH)$ were identified [2] by HPLC-HG-AFS using a reverse phase C18 column. The anion exchange chromatographic column Hamilton PRP-X100 is frequently used for separation of those arsenic species. In a recent review on speciation analysis by HPLC-HG-AFS, 89 citations corresponded to arsenic speciation by HPLC-AFS, 54 of which used the PRP-X100 column for As(III), As(V), MMA, and DMA [4].

Flow systems have usually been described for in-line extraction of As species from soil samples placed in a column and determination by HG-AFS [5]. The arsenic extracted fractions were in-line oxidized to As(V) previously to quantification involving the standard addition method (SAM). This procedure was needed for overcoming sample matrix effects.

The management of solutions by a sequential injection analyzer (SIA) has been exploited for automation of SAM [6]. Sequential injection standard addition was applied for quantification of Hg in river water samples. In that approach, in-line digestion carried out by a BrCl solution and UV-irradiation promoted the oxidation of Hg species to Hg^{2+}, allowing the sequential reduction of Hg in the sample with further detection by cold vapor AAS [6].

SAM was previously implemented in a SIA system [7] using a mono-segmented scheme to promote different dilutions of a single standard solution for the determination of Fe(II) in an anti-anemic medicine [8]. The mono-segmented approach is based on defining a constant total volume partitioned among the sample, reagent, and variable volumes of the standard solution. The mono-segmented sequential injection approach constituted the basis of a calibration method using SIA complementary dilutions [9]. The sample, stan-

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dards and diluents solutions were combined and interacted in the SIA holding coil at various volumetric proportions under computer control.

The potentialities of SIA for chemical speciation have been reviewed elsewhere [10]. Its versatility for speciation is related to the combination of flow diagrams combining a 10-port selection valve and 1 or 2 detection systems. In a previous paper of our group, it was demonstrated that the management of arsenic solutions by SIA resulted in a pronounced reduction of the formed hazardous waste residues [11], so that SIA ranks among the environmentally friendly approaches towards chemical analysis.

In this paper, an instrumental configuration using SIA coupled to HPLC-HG-AFS is proposed for implementing SAM aiming at simultaneous quantification of four As species in each sample. The reason for coupling SIA to the HG-AFS was the high sensitivity of the combination to detect As(III), As(V), MMA and DMA which is comparable with that displayed by ICP-MS, in addition to the low cost of the equipment and operation. The proposed procedure was applied to the analysis of spoiled seafood samples to allow the joint quantification of the most toxic arsenic species avoiding interconversions.

2. Experimental

2.1. Instrumentation

The components of the SIA-HPLC-AFS system are presented in Fig. 1.

The SIA system (FIA-Lab, Fiatron, Seattle, USA) with an 8-port selecting valve, a 1 mL syringe pump, and a 0.8 mL holding coil was used to perform standard additions to the sample. Solutions from the holding loop were delivered through port number 8 to fill a loop 150 cm long made of peek tubing 0.5 mm id, installed in the chromatographic injector (mod 7125, Rheodine, Berkeley, USA). The isocratic HPLC pump (Shimadzu, LC-10AD, Kyoto, Japan) was connected to a selector valve of the mobile phase solutions C or E. The chromatographic guard pre-column and column PRP-X100 (250 mm × 4.1 mm, 10 μm packed particles) (Hamilton, Reno, USA) were used. The parts of the equipment used for atomic fluorescence (Excalibur, PS Analytical, Orpington, UK) were a detector, a hydride generator separator, a gas liquid separator (GLS), and an atomization source. An 8-channel peristaltic pump (Ismatec, Glattbrug, Swiss) was used for pumping the sodium tetraborohydride and HCl reagents. An auxiliary argon flow was introduced just before the GLS in order to accelerate the transport of the evolved gases towards the atomizer.

Sample extractions were performed using an ultra-sonic bath (40 kHz, Thornton, Piracicaba, Braz.). Total As was determined by ICP OES (Optima 3000, Perkin Elmer, Norwalk, USA).

2.1.1. The SIA program

The SIA system was programmed according to the steps presented in Table 1. The analytical sequence started by introducing defined volumes of water, sample, As species, and water into the holding coil (HC). Solutions inside the HC were mixed by reversing the flow direction. After mixing, the syringe was filled with a defined volume of water by the lateral inlet used to pump the HC solutions through port 8 to fill the sample loop of the chromatographic injector.

2.1.2. SIA-HPLC-HG-AFS system

The systems scheme in Fig. 1, left side, shows the SIA components used to perform standard additions to the sample. The volume pumped by the syringe pump was calculated so as exactly fill the loop in the HPLC injector port. This was followed by operation of the injector, leaving the sample solution inside the loop to be carried by the isocratic pump through the PRP-X100 column. In this case, the less concentrated phosphate buffer E was pumped. Thereafter, the HPLC pump was operated and the buffer solution in C was sent through the HPLC column. Species leaving the HPLC column were carried to a hydride generation unit, receiving the reagents pumped by a peristaltic pump (PP). This was followed by introductions of Ar plus H_2 into a gas/liquid separation chamber (GLS), so as to efficiently carry the gases to the atomic fluoresce spectrometer (AFS).

2.2. Chemicals

All solutions were prepared with deionized water (>18.2 MΩ cm) from a Milli-Q system (Millipore, Bedford, USA). Standard solutions for arsenite and arsenate were prepared by dissolving $AsNaO_2$ or $Na_2HAsO_4{\cdot}7H_2O$ (Merck, Darmstadt, Germany) in phosphoric acid. The monomethylarsonic (MMA) and dimethylarsinic (DMA) acids were from Supelco (Sigma–Aldrich, Munich, Germany). Working standard solutions of 100.0 ng mL^{-1} of each As specie were used for performing the standard additions.

Mono and dibasic potassium phosphate solutions at pH 6.2 in concentrations of 12 and 24 mmol L^{-1} were used as mobile phase solutions for the chromatographic separations [12]. Chromatographic grade methanol and nitric acid from Merck (Darmstadt, Germany) were used for column regeneration. Sodium tetrahydroborate 1.3% $NaBH_4$ (Nuclear, São Paulo, Brazil) was prepared with 0.1 mol L^{-1} NaOH. Hydrochloric acid 1.5 mol L^{-1} was used for post-column hydride generation of As species.

2.3. Sample treatment

About 0.25 g of lyophilized shark (*Pleonacea glauca*) muscular tissue was extracted with 20.0 mL of water in an ultrasonic bath for 1 h. This solution was stored in a refrigerator for 4 months. Also, the liquid from a can of natural bivalves (*Mesodesma donacium*), whose expiration date had occurred 3 years prior to the analysis, was filtered through a 0.2 μm pore cellulose acetate filter just before analysis. Accordingly to the manufacturer, the sample contained water, salt, and the preservatives INS 451i (a stabilizer) and INS 385 (an antioxidant). A mass of 0.25 g of dogfish muscle (DORM-2) CRM from the National Research Council of Canada was extracted with 10 mL of water. Extracts were centrifuged at 3500 rpm and the filtrated supernatant made up to 10 mL final volume.

2.4. The in-line standard additions of multiple As species

The SIA system in Fig. 1 was programmed to perform standard additions of three concentrations of the four As species to the sample. Samples were analyzed in a first run to observe the main As species occurring and the dilution degree required to support additions. This was attained by programming steps 1, 2, 7 and 8 in Table 2, which control the volumes of water and sample introduced into HC. Depending on the number of species to be determined and their concentrations, the volumes of sample, standards and water were then reprogrammed according to the steps 3–6 sequence in Table 2.

The system was dimensioned to perform the addition of four As species standard solutions to the sample in less than 1 min by performing the actions represented in Table 2. Taking in consideration the different concentration ranges of sample species, the experimental sequence of additions presented in Table 2 was followed. To perform the additions, a variable plug of water was inserted through valve 6, in both extremes of the sample and standard solutions, in order to keep constant the total volume. This water amount added in each run was programmed following the equation in Table 2.

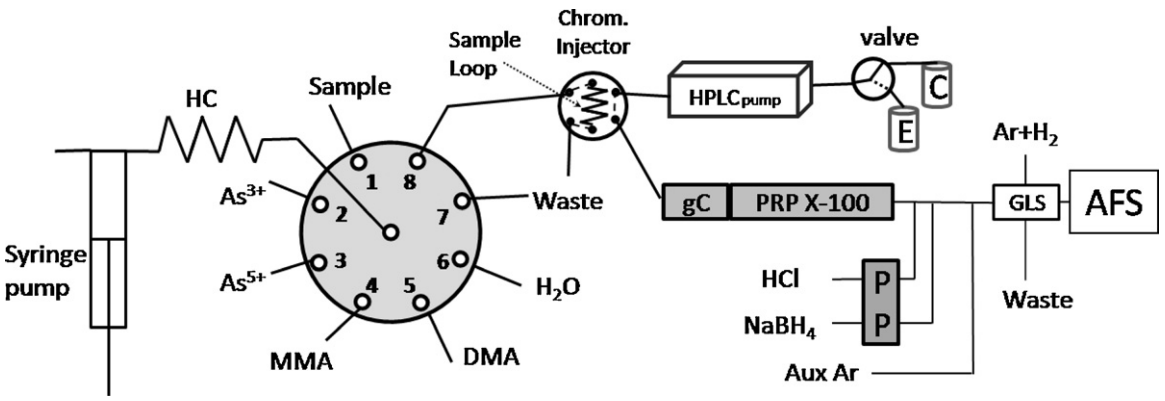

Fig. 1. Scheme of the SIA-HPLC-AFS system. The modules from left to right are the SIA instrument with syringe pump, HC holding coil, the 8 valves, the chromatographic injector, the HPLC pump to carry solutions E or C through the guard and chromatographic column. Solutions to generate hydrides are pumped by PP, receiving an auxiliary Ar before rising the gas liquid separation device, where a stream of Ar plus H_2 is introduced to carry the analytes to the AFS atomic fluorescence spectrometer.

Table 1
Sequential additions of 4 As species to the sample.

Step	Action	SIA port	V (μL)	Flow rate (μL s^{-1})	Action
HC filling					
1	Aspirate water into HC	6	V_6	50	Aspirate
2	Collect sample into HC	1	V_1	50	Aspirate
3	Introduce As(III) standard	2	V_2	50	Aspirate
4	Introduce As(V) standard	3	V_3	50	Aspirate
5	Introduce MMA standard	4	V_4	50	Aspirate
6	Introduce DMA standard	5	V_5	50	Aspirate
7	Collect sample into HC	1	V_1	50	Aspirate
8	Aspirate water into HC	6	V_6	50	Aspirate
Solution mixing inside HC					
9	Alternate flow direction	8	300	200	Aspirate
10	(six repeated cycles)	8	300	200	Dispense
Solution delivering to the HPLC injection loop					
11	Fill syringe with water	8	320	100	Aspirate
12	Transfer solution to the LC sample loop	8	–	100	Empty

V_i = volume of each solution introduced by port i; HC = holding coil; V_i = variable volumes.

The dilution factor for the samples was adjusted to the full scale of the AFS equipment. The sample was introduced in the front and rear of the standards sequence to improve mixing inside HC. Mixing was also improved by programming the syringe pump to alter by six turns the aspirate/dispense actions of 300 μL at high flow-rate (200 μL s^{-1}). After this cycle, the valve on the top of the syringe changed its position to fill the syringe with 320 μL of water from the lateral inlet. Afterwards, this volume drained completely the HC by transferring the sample plus standards to the chromatographic loop. The sample prepared by SIA (500 μL) was transferred from the HC to fill the sample loop in the chromatographic injector (300 μL).

Table 2
SIA program to calculate the sample plus four As species volumes to fill HC.

Function	Sampling position	Valve port	Volume (μL)
Composition of a solution (total = 500 μL)	Water	6	$250 - \frac{1}{2}\sum_{n=1}^{n=5} V_n$
	Sample	1	1/2 V_1
	As(III)	2	V_2
	As(V)	3	V_3
	MMA	4	V_4
	DMA	5	V_5
	Sample	1	1/2 V_1
	Water	6	$250 - \frac{1}{2}\sum_{n=1}^{n=5} V_n$

HC = holding coil; V_i = variable volumes of each solution introduced into HC-holding coil. The total volume aspirated through the valves was 500 μL.

2.5. Evaluation of the system performance

The system performance was evaluated by generating analytical curves in triplicate by inserting water instead of sample in the sequence of Table 1. The limits of detection (LOD) for each As specie was calculated using the slope of analytical curves and 3σ of the blanks. A noteworthy feature of the proposal is the feasibility of adjusting the sample dilution factor required to perform the SAM.

3. Results and discussion

The SIA configuration in Fig. 1 and the program to control the management of solutions enabled the performance of a reliable routine analysis of As species in solutions applying quantification by SAM. The SIA configuration using a mono-segmented flow scheme described earlier [7,8] was tried. However, small bubbles probably remained in the system, provoking disturbances on the HPLC isocratic pumping. In order to prevent such a possibility from occurring, a two-step cleaning was performed in between analysis of samples.

Mixed standard solutions containing four As species in concentrations range from 0.0 to 7.5 ng mL^{-1} were efficiently generated by SIA from the corresponding standard solutions (100 ng mL^{-1}) using the proposed innovation. The scans of the four species are shown in Fig. 2a, as well as the calibration curves in terms of peak area obtained in triplicate (Fig. 2b).

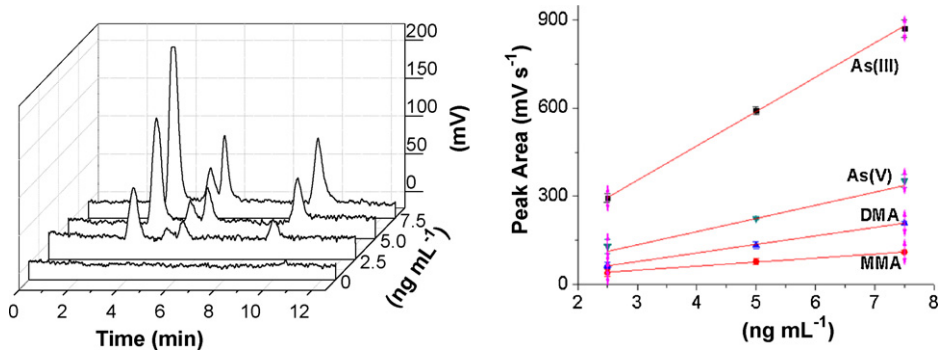

Fig. 2. SIA-HPLC-AFS chromatograms generated by the system for standards of As species in concentrations from 0.0 to 7.5 ng mL^{-1}. The peaks corresponding to As(III), DMA, MMA and As(V) are shown from left to right.

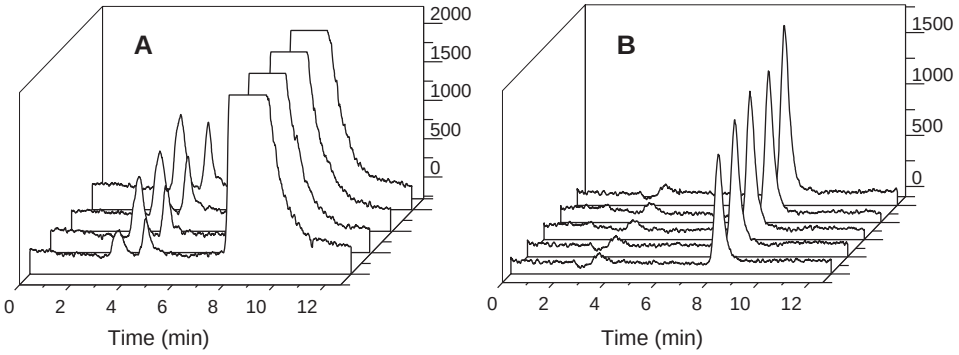

Fig. 3. Chromatograms generated by applying SAM using SIA-HPLC-AFS to aqueous extracts of shark mussel. In (A), from left to right, peaks corresponding to As(III), DMA, MMA and As(V) species are shown after 1:10 dilution. Scans to the back correspond to additions of 2.5, 5.0 and 7.5 μg L^{-1} As(III) and 4.0, 6.0 and 8.0 μg L^{-1} DMA. In (B), the same sample after 1:100 dilution and additions of 4.0, 6.0, 8.0 and 12 μg L^{-1} of As(V).

The regression lines of As species presented in Table 3 denoted sensitivities related to their efficiencies in producing arsine (AsH_3). The limits of detection (LOD) were calculated by considering 3σ of the base line and the respective sensitivity. LOD for all four As species were comparable to those obtained by HPLC coupled to ICP-MS [4]. The good linearity and correlation factors of the analytical curves demonstrate that no inter-conversion among species during the addition procedure occurred.

The system performance with respect to generation of standards in different concentrations can be evaluated by looking at the correlation graph obtained in triplicate (Fig. 2b). The regression equation and correlation factors relying on peak area demonstrate the feasibility of the proposed instrumental setup to perform the standard additions method. The accurate production of four standards of As species at different concentrations is a positive factor to proceed applying SAM for quantification in marine samples.

The high sensitivity of the proposed instrumental arrangement allowed detection of low analyte concentrations; even when the application of SAM requires sample dilution. A SIA sample dilution step previously to the standard addition procedure was implemented, so as to attain the detection range. However, it should be pointed out that during the multi-species standard additions the sample dilution factor remained constant. However, the programmed multiple addition of As species by SIA was attained by adjusting the volumetric dilution of the added species.

Table 3
Analytical curves and LOD's for As(III), As(V), MMA and DMA.

	Regression equation	R^2	LOD (μg L^{-1})
As(III)	$y = 115.4x + 8.44$	0.9997	0.023
As(V)	$y = 44.53x + 12.5$	0.9921	0.39
MMA	$y = 28.83x - 8.18$	0.9991	0.45
DMA	$y = 13.96x + 6.60$	0.9976	1.00

Number of data points = XX; number of replications = YY.

The multi-species analysis of shark extract indicated the presence of three As species (Fig. 3). The proposed method was able to precisely determine these species by applying two different dilution factors. The scans presented in Fig. 3a were obtained after dilution of 1/10 and in Fig. 3b the dilution was 1/100 (v/v). The dilution degree of 1/10 (v/v) was considered appropriate for performing additions of As(III) and DMA, as can be observed in Fig. 3a. So, the additions of 0.0, 2.0, 4.0 and 6.0 μg L^{-1} As(III) and 0.0, 4.0, 8.0 and 12.0 μg L^{-1} DMA were programmed. For application of SAM to quantify As(V), a dilution factor of 1/100 (v/v) was required. Scans shown in Fig. 3b correspond to the 1/100 diluted shark sample (shown in the front) followed by scans (shown in the back) obtained after additions of 4.0, 8.0, 12.0 and 16.0 μg L^{-1} As(V).

It is worthwhile mentioning that the high content of As(V) was due to an oxidation process that probably occurred during the sample storage at 4 °C. The analytical curves of As species after additions on samples extracts and the extrapolation to the concentration axis are presented in Fig. 4. Arsenic species in DORM-2 extracts as determined in triplicate using the proposed approach presented concentrations in mg kg^{-1} of 0.037 ± 0.02 for As(III), 0.048 ± 0.010 MMA and 0.456 ± 0.040 DMA. The values are in agreement to those reported recently by Leufroy et al. [13], although As(V) was not detected.

Liquid from canned bivalves were found to contain As(III) (as shown in Fig. 4), which is the most toxic As species. The sample was processed after a 20% (v/v) dilution. The scattering of data was expected, since this sample was slightly turbid, even after filtration.

The corresponding regression curves for each As specie (Fig. 4), the dilution factors and the calculated concentrations of As species in the shark sample solution as well as the corresponding total

Table 4
Concentration of As species in shark extracts, in dry basis calculated from the SAM regression and total As (df = volumetric dilutions).

Species	SAM regression	Species concentrations		As (mg kg^{-1})
		Solution (μg mL^{-1})	Sample (μg g^{-1})	
As(III)	$Y = 41.97X + 191.7$ df = 1:10	0.46	0.037	21.8
As(V)	$Y = 15.32X + 383.8$ df = 1:100	2.50	0.20	105.6
MMA	<LOD			
DMA	$Y = 9.73X + 178.8$ df = 1:10	0.184	0.015	8.0

Total As from the inorganic and methylated arsenic species 135.4 mg kg^{-1}.

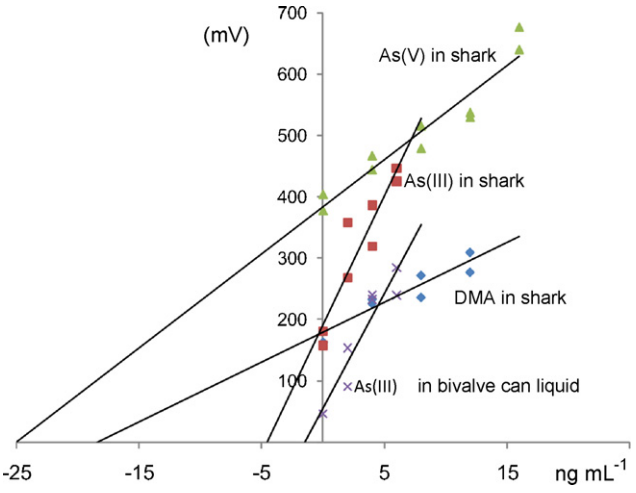

Fig. 4. Correlation functions after SAM for the samples, shark extract and liquid in canned seafood. Species in shark As(V), As(III) and DMA and As(III) in liquid in can.

arsenic contribution from each species are summarized in Table 4. Values in the 3rd column are the concentrations of species in solution as determined by SAM, followed by the total species content in the raw sample.

The liquid from the canned bivalve presented As(III) of 2.1 ng mL^{-1} calculated from the regression curve in Fig. 4 ($Y = 37.34X + 55.65$). The SAM calibration curves for As(III) presented slopes of 37.34 and 41.97 for the samples. Both values were different from the slope of 51.48 obtained for standard solutions, indicating the occurrence of matrix effects which were avoided by applying the standard addition method. From Tables 3 and 4 is possible to notice that matrix effects also affect sensitivity of As(V) and DMA.

Results of total As in 20 mL of shark extract by ICP OES presented concentration of 1.7 mg L^{-1}, or 34 μg of As from 0.25 g of sample which corresponds to 136 mg kg^{-1} in the shark muscle. However, from Table 3 species presented a total of 134.84 mg kg^{-1} As. The small difference could be attributed to high oxidation degree of the original organic As species, such as Arsenobetain and arsenosugars highly present in sea products [3]. The found results are of the same order to those reported earlier from 60 ± 8 to 114 ± 20 mg kg^{-1} for muscle tissue from three species of dog shark [14]. However, those results indicate that most of the sample As was oxidized to As(V).

4. Conclusions

The determination of As(III), As(V), MMA and DMA in seafood samples by coupling SIA with the HPLC-HG-AFS to perform the standard addition method was demonstrated. Quantification of As species in seafood extracts are affected by matrix effects which were overcome by the standard addition method (SAM). SIA presented flexibility for generating additions of different concentrations for each As species, demonstrating that either standard additions or dilutions can be easily performed, as routinely required in the analytical laboratory. Multiple species standards prepared by SIA at ng mL^{-1} concentration were accurately added to the sample. When large dilution was required, a two-step SAM can be applied. The in situ multi-species addition avoided inter-species conversions prior to analysis and reduced the consumption of As standards, which qualifies the method as a clean chemistry procedure.

The feasibility of this approach can be applied to other matrices while maintaining simplicity, ruggedness and analytical sensitivity.

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