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Research Note

Method for Speciation of Organoarsenic in Mussels by Liquid Chromatography Coupled to Electrospray Ionization and QTRAP Tandem Mass Spectrometry

F. P. SERPE,[1,2] R. RUSSO,[1] P. GALLO,[2] AND L. SEVERINO[1]*

[1]*Dipartimento di Patologia e Sanità Animale, Università degli Studi di Napoli Federico II, Via Delpino 1, Napoli, Italy; and* [2]*Dipartimento di Chimica, Istituto Zooprofilattico Sperimentale del Mezzogiorno, Via Salute 2, Portici, Italy*

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ABSTRACT

Arsenic toxicity to humans critically depends on the chemical form of the arsenic. The Expert Committee of the Food and Agriculture Organization and the World Health Organization defined a tolerable intake only for inorganic arsenic, although the toxicity of some organoarsenic compounds is known. Arsenobetaine (AsB), arsenocholine (AsC), dimethylarsinic acid (DMA), and monomethylarsonic acid (MMA) are abundant in shellfish. We present a fast and reliable method for identification of the type of organic arsenic in mussels by using liquid chromatography coupled to electrospray ionization tandem mass spectrometry on triple quadrupole with parallel determination of total arsenic by atomic absorption spectrophotometry. The method was validated by evaluating mean recoveries, repeatability, specificity, limits of quantification, and limits of detection that produced satisfactory results. The method was used to carry out the first survey of the concentrations of AsB, AsC, MMA, and DMA in seafood from southern Italy. Total As concentrations ranged from 1.38 to 12.79 mg/kg. AsB and DMA were detected in all samples (AsB: 0.72 to 10.36 mg/kg; DMA: 0.28 to 1.08 mg/kg), and concentrations of AsC and MMA ranged from 0.20 to 1.53 mg/kg. This method allowed us to rapidly and inexpensively identify arsenic types in fishery products and would be suitable for routine detection of organoarsenic compounds in molluscs.

Environmental pollutants such as metals can be found in fish and fishery products *(4, 13, 15–17)*. Arsenic (As) is a metal present in the environment in various oxidation states and often is combined in several organic compounds. As is present in seawater (average, 0.3 μg/liter) and in traces in almost all vegetables and animal tissues, deriving from rock erosion and human activities such as the electronics industry, production of pigments for glazes, and coal combustion *(8)*. As in seawater can undergo bioaccumulation and biomagnification in some marine animals and their derived foods, thus representing a risk to consumers. In particular, fish and shellfish are the source of most of the As ingested by humans (about 75%), although these foods usually represent a small percentage (2%) of the daily dietary intake *(18)*. As toxicity to humans critically depends on its chemical form and oxidation state *(5)*; inorganic As was declared a carcinogen for humans by the International Agency for Research on Cancer *(19)* and can induce adverse cardiovascular effects *(2)*. The metabolic biotransformation of As in marine animals through detoxification pathways can lead to a wide range of organoarsenic compounds. The Joint FAO/WHO Expert Committee *(19)* defined a provisional tolerable weekly intake of 0.015 mg/kg of body weight for total inorganic As; however, at least 32 forms of organic As are usually present in widely consumed foods such as fish *(1, 9)*. Arsenobetaine (AsB) and dimethylarsinic acid (DMA) are the most abundant As forms in shellfish *(4, 10)*. Monomethylarsonic acid (MMA) and arsenocholine (AsC) also are present in shellfish *(1, 10)*; these forms are traditionally considered less toxic than inorganic forms, although some recent studies indicate that DMA is a potential human carcinogen *(6, 20, 21)*.

Reliable test methods for identification of organic As compounds are necessary to set tolerable levels for both total inorganic and organic As in fishery products, to monitor their amounts in foods, and to support regulatory activity. Herein, we describe a simple method for identification of organic As in mussels by liquid chromatography coupled to electrospray ionization and tandem mass spectrometry on triple quadrupole (LC-ESI-MS/MS); method performances were evaluated in terms of mean recoveries of the analytes, precision, specificity, limits of quantification (LOQs), and limits of detection (LODs).

The method was applied to determination of organoarsenic compounds in field samples with the double aim of testing method suitability under routine conditions and carrying out the first investigation describing the concentrations of AsB, AsC, MMA, and DMA in seafood from southern Italy. Total As concentrations were also determined

* Author for correspondence. Tel: 0039 0812536272; Fax: 0039 0812536274; E-mail: lorella.severino@unina.it.

TABLE 1. *Multiple reaction monitoring MS/MS parameters*

Compound	Precursor ion (amu)	Product ion (amu)	Declustering potential (V)	Collision energy (V)
Arsenobetaine	178.9	105.2	40.00	35.00
	178.9	120.0	40.00	35.00
Arsenocholine	165.3	105.0	70.00	32.00
	165.3	121.3	70.00	32.00
Monomethylarsonic acid	152.8	90.8	−70.00	−25.00
Dimethylarsinic acid	136.8	106.8	−70.00	−35.00

by graphite furnace atomic absorption spectrophotometry (GF-AAS).

MATERIALS AND METHODS

Reagents. High purity water was produced in house with a Milli-Q deionizing system (Millipore, Bellerica, MA). High-performance LC (HPLC) grade acetonitrile, 70% (wt/vol) nitric acid, and 30% (vol/vol) hydrogen peroxide (Carlo Erba, Milan, Italy) were used for metal trace analysis. Dihydrogenammonium-phosphate and magnesium nitrate (Perkin Elmer, Milan, Italy) were used as matrix modifiers for GF-AAS. A standard stock solution of As at 995 ± 5 mg/liter was supplied by Merck (Milan, Italy). Samples were diluted with Milli-Q water. Laboratory glassware was washed before use with 10% (wt/vol) aqueous nitric acid, rinsed with Milli-Q water, and dried in a desiccator sheltered from atmospheric dust.

Reference standard solutions in water containing AsB at 0.518 ± 0.015 μmol/g (92.2 μg/g), AsC bromide at 0.374 ± 0.015 μmol/g (91.6 μg/g), MMA at 0.335 ± 0.015 μmol/g (46.2 μg/g), and DMA at 0.706 ± 0.024 μmol/g (97.4 μg/g) were purchased from the National Institute of Metrology (Beijing, China). Working standard stock solutions were obtained by weighing known volumes of each reference standard solution containing 10 μg of MMA and 20 μg each of AsB, AsC, and DMA. After drying under a gentle nitrogen stream, all standards stock solutions were dissolved in 200 μl of acetonitrile, giving final concentrations of 50.0 μg/ml for MMA and 100.0 μg/ml for AsB, AsC, and DMA. A mixed standard solution containing AsB, AsC, DMA, and MMA at 2,000 ng/ml was prepared daily by dilution in acetonitrile.

LC/ESI-QTRAP-MS/MS analysis. Instrumental determination of organoarsenic compounds was carried out with an Agilent 1200 HPLC system (Agilent Technologies, Santa Clara, CA) coupled to a 4000 QTRAP mass spectrometer interfaced with a Turboion electrospray source (Applied Biosystems, MDS Sciex, Foster City, CA). Chromatography was performed using a Kinetex PFP column (100 mm by 3.0 mm by 2.6 μm; Phenomenex, Torrence, CA) with a 0.3 ml/min flow rate at 25°C. Gradient elution was performed with Milli-Q water as mobile phase A and acetonitrile as mobile phase B using the following program: 90% A at time 0, up to 50% A at 8 min, down to 90% A at 13 min, and holding for 2 min to reequilibrate the column. The following ion source parameters were set: resolution Q1/Q3: unit; curtain gas temperature: 40°C; source temperature: 400°C; GS1/GS2: 20°C. AsB and AsC were analyzed in positive ionization at 4,500 V (IS), and MMA and DMA acids were analyzed in negative ionization at −4,500 V (IS).

MS/MS was conducted in the multiple reaction monitoring (MRM) mode. The entrance potential was set at 10 V and the cell exit potential was set at 42 V for all compounds. The declustering potential and collision energy were optimized for each compound and ion transition by infusion in LC flow conditions of standard solutions at 50.0 ng/ml (Table 1). Two product ions were detected for AsB and AsC, and one product ion was monitored for MMA and DMA. In Table 1, the fragmentation parameters and the precursor and diagnostic product ions for each compound are reported. For MMA, we observed a strong signal for the Na^+ adduct; therefore, the precursor ion $[M^{2-} + Na^+]^-$ was selected for MS/MS fragmentation. The other precursor ions selected were $[M + H]^+$ for AsB and AsC and $[M - H]^-$ for DMA (Fig. 1).

Quantitative analysis was performed with external standard calibration curves calculated by linear regression of mixed standard solutions in acetonitrile containing each compound at 50.0, 100.0, 200.0, 500.0, 1,000, and 2,000 ng/ml. The following product ions (base peaks) were chosen for quantitative analysis: *m/z* 120.0 (AsB), *m/z* 121.3 (AsC), *m/z* 106.8 (DMA), and *m/z* 90.8 (MMA).

GF-AAS analysis. Total As content was determined according to Serpe et al. *(15)* by GF-AAS with an Analyst 800 atomic absorption spectrophotometer equipped with a transverse heated graphite furnace atomizer and Zeeman effect for background correction (Perkin Elmer). Argon pressure was set at 3.5 bar, air pressure was 4.0 bar, resolution was 0.7 amu, and scanning time was 4 s. For quantitative determination of total As, external standardization calibration curves were calculated by linear regression using the standard solution at 5.00, 15.0, 30.0, and 60.0 ng/ml interpolated with the least squares approach. Spectrophotometer management, data acquisition, building of calibration curves, and linear regression were performed with the software WinLab 32 version 6.2 (Perkin Elmer).

Sample preparation. All mussel samples were collected by the local health authorities in the frame of the 2012 National Integrated Monitoring Plan *(11)* for environmental pollutants in foods. The samples were collected from mussel farms on the coast of Campania, Italy. Mussels were thawed, gutted, and homogenized in a blender. For each sample, 0.25 ± 0.01 and 0.75 ± 0.01 g were weighed and then lyophilized to be processed for LC/ESI-QTRAP-MS/MS and GF-AAS analyses, respectively.

Before LC/ESI-QTRAP-MS/MS analysis, the sample was weighed in a 10.0-ml glass tube, 2 ml of HPLC grade methanol was added, and the tube was placed in a sonicator for 15 min. After centrifugation at 3,000 × *g* for 10 min, the supernatant was separated, and the extraction was repeated with 2 ml of methanol. Both extracts were collected, filtered on a 0.22-μm-pore-size nylon filter, and evaporated to dryness at 50°C under a nitrogen stream. The residue was dissolved in 1.000 ± 0.002 ml of HPLC grade methanol, centrifuged at 9,500 × *g* for 2 min, and analyzed by LC/ESI-QTRAP-MS/MS.

Before GF-AAS analysis, the sample was placed in a teflon vessel with 5.0 ml of 70% nitric acid, 2.5 ml of 30% hydrogen peroxide, and 2.5 ml of Milli-Q water. The vessel was sealed and placed in an Ethos E microwave oven (Milestone, FKV, Milan). Microwave assisted digestion was performed with a mineralization

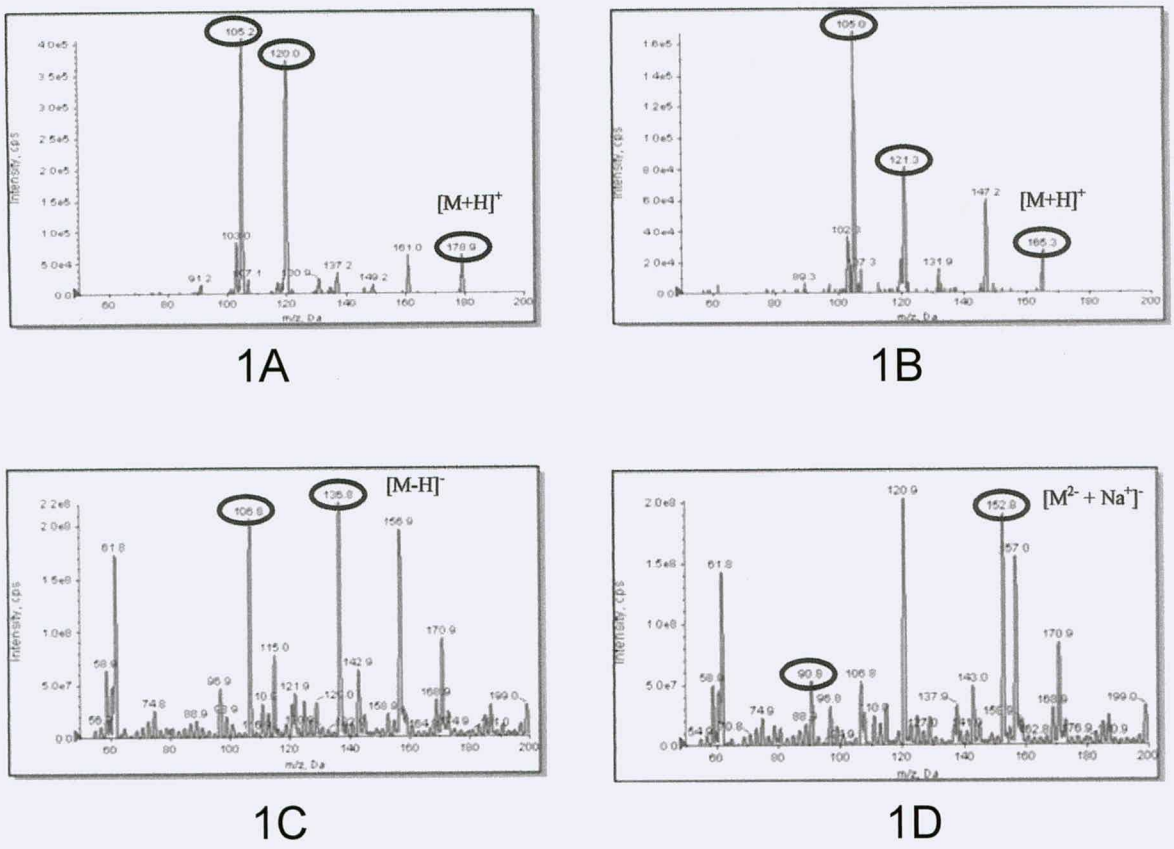

FIGURE 1. *Mass spectra of all transitions: (A) arsenobetaine Q1-Q3 (positive), (B) arsenocholine Q1-Q3 (positive), (C) dimethylarsinic acid Q1-Q3 (negative), and (D) monomethylarsonic acid Q1-Q3 (negative).*

program for 10 min at 190°C (constant temperature). The vessel was then cooled at room temperature, and the digestion mixture was transferred into a 50.00 ± 0.06-ml class A round-bottom flask and brought to volume with Milli-Q water.

Method performances. To evaluate the accuracy and precision of the LC/ESI-QTRAP-MS/MS method, aliquots of an As-free mussel sample were spiked at three concentrations by adding AsB, AsC, DMA, MMA at 500.0, 1,000, and 2,000 ng/ml. For total As determined by GF-AAS, As-free mussel samples were spiked at 100.0, 200.0, and 400.0 ng/g with a standard stock solution of As at 1,000 ng/ml.

As-free samples were obtained from the 2012 National Integrated Monitoring Plan *(11)* using the mussels containing the lowest total As concentrations.

Method accuracy was calculated in terms of mean recoveries at different spiking concentrations; method precision was evaluated in terms of the relative standard square deviation percentage (RSD%) within days. The linearity of the mass spectrometer detector response was assessed for AsB, AsC, DMA, and MMA by linear regression of calibration curves of standards mixed solutions at 50.00, 100.0, 200.0, 500.0, 1,000, and 2,000 ng/ml analyzed in triplicate. For total As, the linearity of the detector response was assessed by linear regression of calibration curves of standard mixed solutions at 5.00, 15.0, 30.0, and 60.0 ng/ml analyzed in triplicate. The LODs were calculated by analyzing five replicates of As-free mussel samples at concentrations corresponding to a 3:1 signal:noise (S:N) ratio; the LOQs were calculated as the concentrations corresponding to a 5:1 S:N ratio. The performance of the method for total As content was evaluated with a food analysis performance assessment scheme (FAPAS; Food and Environment Research Agency, Sand Hutton, UK) proficiency test.

Statistical analysis. Statistical parameters considered for the validation study of the LC/ESI-QTRAP-MS/MS method were RSD% and mean recovery for each organoarsenic compound. An analysis of variance single-tail test also was carried out for each organoarsenic compound on three groups of replicates spiked at the same concentration, with a maximum acceptable P value of 0.05. Calibration curve linearity was determined for six concentrations per compound, in triplicate, interpolated with the least squares approach.

Concentrations of total As, organic As (expressed as the sum of AsB, AsC, MMA, and DMA), and inorganic As for 13 representative samples of mussels from Campania were evaluated through calculation of median, mean, standard deviation, minimum and maximum values, and percentage of total As. An asymmetry test also was conducted to establish the trend and shape of the distribution of field data. Statistical analysis was carried out with Excel software, version 1997–2003 (Microsoft, Redmond, WA).

RESULTS

LC/ESI-QTRAP-MS/MS analysis. The experimental conditions for ionization and ESI-MS/MS fragmentation were optimized for all organocompounds studied; the corresponding MS/MS spectra are shown in Figure 1. AsB and AsC produced two significant product ions in positive ionization (Fig. 1A and 1B). MMA and DMA produced only one product ion in negative ionization (Fig. 1C and 1D). In Figure 2, the single MRM chromatograms of the organoarsenic compounds are shown; the Kinetex PFP column allowed for satisfactory separation and a short analysis time. No significant matrix interference were

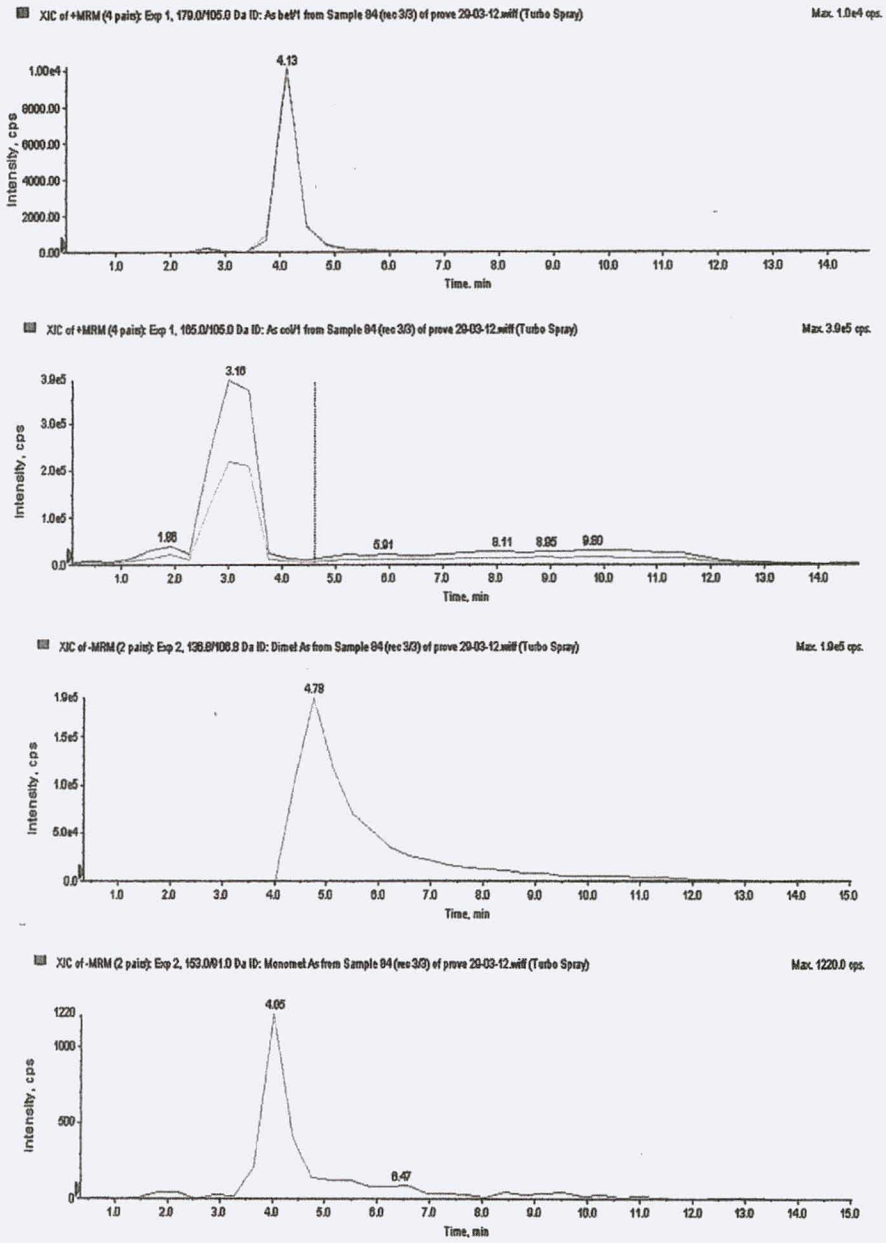

FIGURE 2. *LC-MS/MS elution profiles of arsenobetaine, arsenocholine, dimethylarsinic acid, and monomethylarsonic acid from a mussel sample spiked at 1,000 ng/ml.*

observed in As-free mussel samples, accounting for method specificity. The linearity of the detector response (Fig. 3) was satisfactory (R^2 = 0.9656 for AsB, R^2 = 0.9869 for AsC, R^2 = 0.9770 for MMA, R^2 = 0.9714 for DMA). Method accuracy in terms of mean recovery and repeatability (calculated as RSD%) at all spiking concentrations are reported in Table 2. Mean recoveries of 76 to 125% were measured for AsB, AsC, and MMA; DMA had a lower recovery (45 to 62%). RSD% of 2.5 to 27.5% accounted for method repeatability based on provisions of European Commission Decision 657/2002 *(3)* (Table 2).

The LODs, LOQs, and matrix interference were evaluated by analysis of five organoarsenic-free mussel samples. The concentrations corresponding to an S:N of 3:1 and 5:1 were calculated by extrapolation of the calibration curves for each compound at analyte retention times for AsB, AsC, DMA, and MMA (Table 2). No relevant matrix interference was observed.

Differences in recovery among the three replicates spiked at the same concentration were significant for all organoarsenic compounds because P values were always <0.05 among all groups.

GF-AAS analysis. The LOD (60 ng/g), LOQ (100 ng/g), and matrix interference were evaluated by analysis of five As-free mussel samples. No relevant matrix interference was observed. Detector response linearity was supported by an R^2 of 0.9976 (curve not shown). In Table 2, method accuracy and repeatability over three spiking concentrations are reported; mean recoveries of 95 to 104% and RSD% of $\leq$16% demonstrate method reliability *(3)* (Table 2). The proficiency test scheme from FAPAS was used for analyzing method performance, producing a z-score of -1.5.

Mussel survey. The methods developed were used to test 13 samples of mussels from the coast of Campania. In Table 3, the total organic As and inorganic As content and the relative content of each organoarsenic compound are

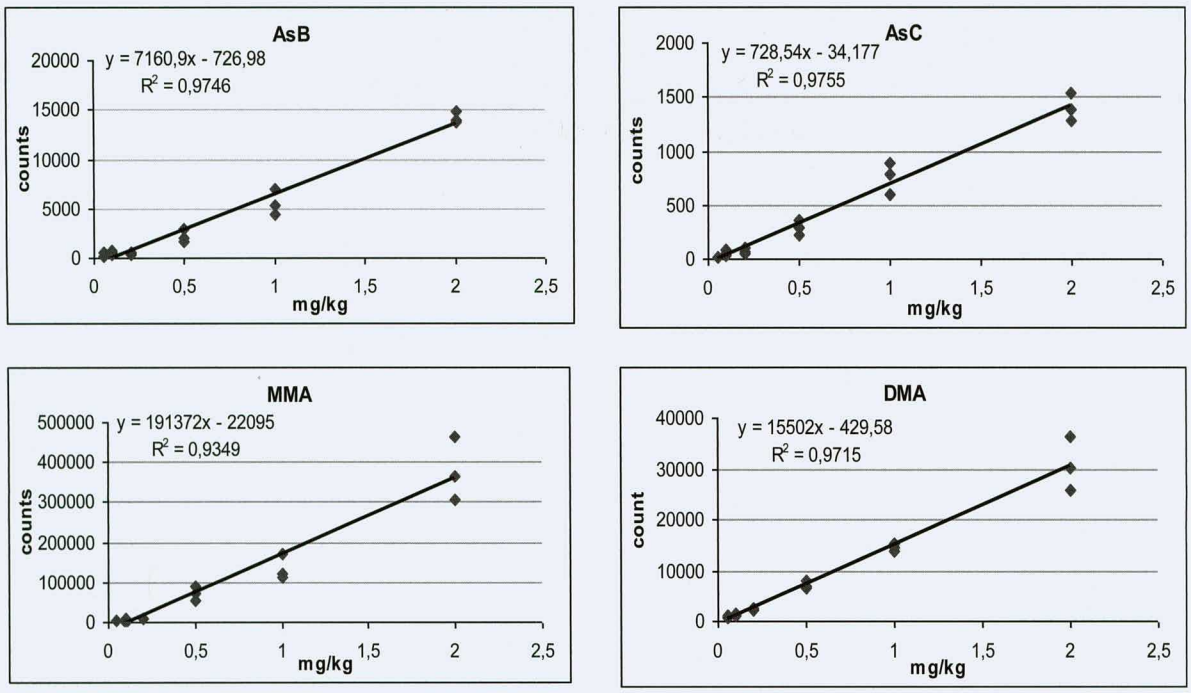

FIGURE 3. *Calibration curves obtained with three replicates of six spiking concentrations at 50.0, 100.0, 200.0, 500.0, 1,000, and 2,000 ng/ml interpolated with the least squares approach.*

shown for each sample. Total As concentrations ranged from 1.38 to 12.79 mg/kg. Most of the metal (from 89.9 to 100%) was in the organic compounds, usually AsB and DMA, detected in all samples of the survey. The concentrations of AsC and MMA were relatively low (0.20 to 1.53 mg/kg, representing 5 to 13% of total As); these compounds were detected in only 4 (30.8%) of the 13 samples tested.

All data grouped for analyte type were asymmetrically distributed, except DMA and inorganic As distribution in the 13 mussel samples, which were normally distributed with asymmetry coefficients of 0.58 and 0.43, respectively.

DISCUSSION

Current methods in use for identification of various forms of As are a complex and expensive combination of separation techniques such as HPLC and capillary zone electrophoresis and modes of detection such as atomic MS (e.g., inductively coupled plasma MS) and molecular (electrospray) MS in tandem mode for the characterization of molecular forms, as reported by McSheehy et al. *(9)*. Most laboratories dedicated to food control and food safety use a triple quadrupole system coupled with an HPLC separation unit, which is simpler and more versatile than the instrumentation listed above. In the present study, we used an LC/ESI-QTRAP-MS/MS analysis with a PFP chromatographic column as an alternative to the typical ion chromatography used for identification of various As forms, which can be complicated by the use of aqueous buffers and often two columns *(4, 7, 9, 12, 14)*. These characteristics combined with the extremely fast sample preparation and only 15 min for extraction made the method useful for a rapid identification of toxic inorganic As in a food sample.

For sample preparation, mussel samples were extracted with 100% methanol instead of water and mixed with dichloromethane to improve the chemical stability of the organoarsenic compounds and reduce the possible conversion of AsB in DMA, as described by Pizarro et al. *(12)*.

For in-house method validation, spiking concentrations were chosen on the basis of published data regarding the natural concentrations of organoarsenic compounds *(1, 7, 14)* and on the basis of a provisional weekly tolerance intake for inorganic As of 0.015 mg/kg *(16)* for a 70-kg individual eating 20 mussels of 500 mg every week *(8)*.

The survey carried out on mussels from southern Italy allowed for a comparison with similar data previously reported for *Mytilus galloprovincialis* and *Mytilus edulis*. Contamination in mussels from southern Italy was comparable to that in mussels from northern Italy (Venice lagoon), which was 0.14 to 0.50 mg/kg for AsC, 4.5 to 14.6 mg/kg for AsB, 0.25 to 1.6 mg/kg for DMA, and 11.9 to 39.3 mg/kg for total As *(1)*. Lower contamination levels were found in mussels from Belgium and in Chinese seafood; concentration ranges were 0.240 to 1.47 mg/kg for AsB, 0.010 to 0.059 mg/kg for DMA, and 0.001 to 0.036 mg/kg for MMA with a mean total As of 2.33 mg/kg in Belgium mussels *(14)* and 0.11 to 0.48 mg/kg for AsB and 0.18 to 0.58 mg/kg for total As in Chinese seafood *(7)*.

In conclusion, a simple and rapid method for identification of some organoarsenic compounds detectable in mussels by LC/ESI-MS/MS on a QTRAP mass spectrometer was developed and validated. The method was employed to perform the first survey describing the concentrations of AsB, AsC, MMA, and DMA in seafood from southern Italy. The concentrations of these compounds are comparable to those reported previously in mussels from other areas in Italy. These data could be used for a

TABLE 2. *Intralaboratory repeatability for LC/ESI-QTRAP-MS/MS analysis of arsenic forms*[a]

	Spiking concn:													
	100 ng/g		200 ng/g		400 ng/g		500 ng/g		1,000 ng/g		2,000 ng/g			
Arsenic form	Mean recovery (%)	RSD%	Mean recovery (%)	RSD%	Mean recovery (%)	RSD%	Mean recovery (%)	RSD%	Mean recovery (%)	RSD%	Mean recovery (%)	RSD%	LOD (ng/g)	LOQ (ng/g)
AsB							125	20.4	84	21.5	99	2.5	58	97
AsC							86	27.5	81	22.5	79	7.2	188	312
MMA							76	16.1	86	22.7	125	3.0	75	125
DMA							62	14.6	45	10.0	48	8.2	38	62
Total As	96	16	104	13	95	10							60	100

[a] Statistical parameters considered for method repeatability study were RSD% (relative standard deviation percent) and mean recovery for experiments conducted in triplicate. Method performances were evaluated by LOD (limit of detection) and LOQ (limit of quantification) assessment.

TABLE 3. *Concentrations of total As, organic As, and inorganic As for 13 representative samples of mussels bred in Campania, Italy*

		Concn: mg/kg (% of total As)[a]					
Sample no.	Total As (mg/kg)	AsB	AsC	MMA	DMA	Total organic As	Total inorganic As
1	2.18	1.29 (59)	ND	ND	0.74 (34)	2.03 (93.1)	0.15 (7)
2	4.41	3.13 (71)	0.57 (13)	ND	0.66 (15)	4.36 (98.9)	0.05 (1)
3	3.95	3.28 (83)	ND	ND	0.63 (16)	3.91 (99.0)	0.04 (1)
4	1.51	0.98 (65)	ND	ND	0.48 (32)	1.46 (96.7)	0.05 (3)
5	5.89	5.30 (90)	ND	ND	0.47 (8)	5.77 (98.0)	0.12 (2)
6	12.79	10.36 (81)	ND	1.53 (12)	0.90 (7)	12.79 (100)	ND
7	5.15	3.91 (76)	ND	ND	1.08 (21)	4.99 (96.9)	0.16 (3)
8	2.76	2.46 (89)	ND	ND	0.28 (10)	2.74 (99.3)	0.02 (1)
9	1.38	0.72 (52)	ND	ND	0.52 (38)	1.24 (89.9)	0.14 (10)
10	4.66	3.73 (80)	ND	ND	0.79 (17)	4.52 (97.0)	0.14 (3)
11	4.82	4.10 (85)	0.34 (7)	ND	0.39 (8)	4.83 (100)	ND
12	3.91	3.09 (79)	ND	0.20 (5)	0.55 (14)	3.84 (98.2)	0.07 (2)
13	8.06	7.33 (91)	ND	ND	0.73 (9)	8.06 (100)	ND
Median	4.41	3.28 (74)	ND	ND	0.63 (14)	4.36 (99)	0.05 (1)
Mean	4.73	3.82 (81)	0.07 (1)	0.13 (3)	0.63 (13)	4.66 (99)	0.07 (1)
SD	3.04	2.66	0.18	0.42	0.22	3.07	0.06
Minimum	1.38	0.72	ND	ND	0.28	1.24	ND
Maximum	12.79	10.36	0.57	1.53	1.08	12.79	0.16

[a] Total organic As is the sum of arsenobetaine (AsB), arsenocholine (AsC), monomethylarsonic acid (MMA), and dimethylarsinic acid (DMA). ND, not detected.

preliminary evaluation of possible risks to the consumers from consumption of mussels in Italy.

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