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ARTICLE

Chemical Speciation of Arsenic and Chromium in Seafood by LC-ICP-MS

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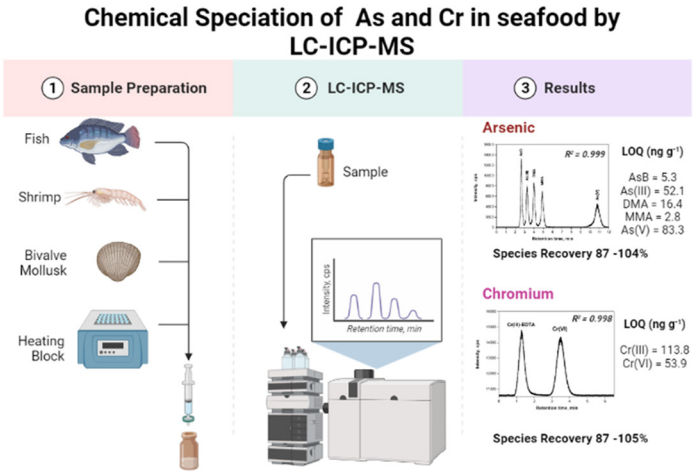

Two methods were developed for the As and Cr species determination in different kinds of seafood, exploring the LC-ICP-MS potential and chemometric approach to define the extraction conditions. Adequates separation and sensitivity data by LC-ICP-MS were obtained with 0.01 mol L^{-1} $(NH_4)_2HPO_4$ in 1% methanol (pH 8.0) for arsenic, and 0.015 mol L^{-1} Na_2SO_4, 0.005 mol L^{-1} EDTA, and 0.005 mol L^{-1} NaH_2PO_4 (pH 7.0) for chromium. The Doehlert matrix and Box-Behnken design defined the ideal extraction conditions for arsenic and chromium species. For As extraction, the optimized conditions employed 0.1 g of sample and 30 mmol L^{-1} HNO_3 at 90 °C for 45 min, and Cr, 0.1 g of sample and 0.045 mol L^{-1} [EDTA] at 90 °C for 40 min. Recoveries from 88 to 106% of arsenobetaine (AsB), arsenite [As(III)], dimethylarsinic acid (DMA), monomethylarsonic acid (MMA), arsenate [As(V)], Cr(III) and Cr(VI) were obtained for all evaluated samples. The limits of quantification provided by the proposed methods were 5.3; 52.1; 16.4; 2.8; 83.3; 113.8; 53.9 ng g^{-1} for AsB, As(III), DMA, MMA, As(V), Cr(III) and Cr(VI) respectively. The trueness was evaluated using certified reference materials and addition and recovery procedures. The sum of the species agreed with the total concentration of arsenic and chromium contents.

Keywords: LC-ICP-MS, seafood, chemical speciation analysis, Doehlert, Box-Behnken, sample preparation

INTRODUCTION

Arsenic (As) and chromium (Cr) are toxic elements naturally present in various chemical species with different toxicity levels. They are on the Priority List of the Agency for Toxic Substances and Disease Registry

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list (ATSDR)[1] and by the International Agency for Research on Cancer (IARC) as human carcinogens.[2] Different arsenic species are responsible for the toxicity and carcinogenicity of arsenic. Inorganic arsenite [As(III)] and arsenate [As(V)] are more toxic than methylated species such as monomethylarsonic acid (MMA) and dimethyarsenic acid (DMA).[3,4] Moreover, arsenobetaine (AsB), arsenocholine (AsC), and other arsenosugars are non-toxic[3]. Chromium(III) is considered essential for glucose metabolism, while Cr(VI) is carcinogenic.[1] European Food Safety Authority (EFSA) points to controversy regarding the intake of Cr(III) because, according to recent studies, no evidence of beneficial effects associated with chromium intake in healthy individuals has been found.[5]

Animal products such as seafood are recognized as an essential source of protein and rich in minerals.[6,7] Its consumption is associated with health benefits, as it helps in neurological development and the prevention of heart disease.[7,8] Despite the remarkable advantage of diet, seafood is able to absorb and metabolize potentially harmful elements in their tissues, including arsenic and chromium, posing risks to human health.[9] This contamination comes from several factors. Among others, the pesticide monosodium methyl arsenate and chromium residues from the leather tanning industry.[10,11]

Due to the different levels of arsenic and chromium species toxicity found in fish and the numerous forms of food contamination, the analysis of chemical speciation of As and Cr is increasingly necessary when it comes to food safety. Thus, several countries have established limits for these analytes in safe seafood consumption. Regulatory bodies have been reluctant to set a maximum limit for As due to the diversity of molecular forms found in seafood.[11,12] The Brazilian legislation defined 1.0 mg kg^{-1} of As in the total As content in fish as the maximum allowed.[13] But this limit, set for total arsenic, is inadequate because it is recognized that the predominant species of As in fish is AsB.[14] Maximum allowed limits of inorganic As species in fish are defined in European government legislation, and the limits vary according to country and the type of fish.[15,16] Brazilian legislation is already adapting to these needs. The maximum levels of inorganic As for baby food and pregnant women are already defined, and the same should occur with other types of food, mainly fish.[10,13,15,16] No legislation establishes the country's maximum limits for Cr.[13]

The choice of extraction method is one of the most critical steps during As and Cr chemical speciation since it is necessary to maintain the integrity of the species in all stages of analysis.[6,11,14,15,17–22]

Different methods have been proposed for sample preparation for As speciation in fish, the most common of which use diluted nitric acid solutions, methanol-water mixtures, or just water.[6,14] The use of ultrasound, microwave, and agitation are considered in these methods. Ultrasound extraction procedures can affect sample integrity if the parameters are not well adjusted.[23] On the other hand, microwave extraction methods with HNO_3 reduce sample preparation time and, consequently, the risk of species interconversion. Although different extracted solutions and heating temperatures have been proposed for As speciation in fish, establishing a single method for speciation analysis is not trivial, and evaluation for each type of fish is necessary. Furthermore, the accuracy of some procedures found in the literature did not present the expected rigor of analytical methods, leading to erroneous results.[21,24]

Several methods for extracting chromium species from food samples were found in the literature, however nonspecific to fish samples.[18] Alkaline salts are generally used to extract Cr(VI) in food samples. Ultrasound, microwave, stirring, and hot plate are used.[19,25-27] Extraction with EDTA at alkaline pH to stabilize Cr species (Cr(VI) through alkaline pH and Cr(III) through EDTA complexation) has been investigated.[28]

Hyphenated liquid chromatography with inductively coupled plasma mass spectrometry (LC-ICP-MS) is the most suitable combination for the speciation of As and Cr.[6,14,25] This coupling enables excellent and sensible As and Cr species separations using anionic or cationic chromatographic columns.[29]

Considering the discussion, LC-ICP-MS procedures for As and Cr speciation were explored to establish a single extraction and separation condition for the different seafood. Experimental design was applied to optimize the extraction conditions of the As(V), As(III), AsB, MMA, DMA, Cr(III), and Cr(VI) species in shrimp, fish, and bivalve mollusks.

MATERIALS AND METHODS

Instrumental

Speciation was performed in a liquid chromatograph (1260 Infinity II, Agilent Technologies, Tokyo, JHS, Japan) composed of a quaternary pump (G7111A, Agilent Technologies) and an autosampler with 100 positions (G129A, Agilent Technologies). Anion exchange columns (G3288-80000, 4.6 mm x 150 mm, and G3268-80001, 4.6 mm x 30 mm, Agilent Technologies) were used to separate arsenic and chromium species, respectively. For Cr species, a guard column (G3154-65002, 4.6 mm x 10 mm, Agilent Technologies) and a liquid chromatograph connection kit (G1833-65200, Agilent Technologies) were used. The elution procedure was achieved in isocratic mode with 1.0 mL min^{-1} (As) and 0.7 mL min^{-1} (Cr) as the flow of mobile phases. A PEEK capillary from the LC column outlet was coupled to the concentric nebulizer of the ICP-MS introduction system.[30] The integrated configuration method and the LC-ICP-MS sequential control were performed using the ICP-MS MassHunter (MH) software (Agilent Technologies).

The inductively coupled mass spectrometer (ICP-MS 7800, Agilent Technologies) was the used ICP-MS. It was fitted with a reaction and collision cell pressurized with high purity He (99.9999%) (White Martins-Praxair, Sertãozinho, SP, Brazil). Helium was used to avoid polyatomic interferences, $^{40}Ar^{35}Cl$ (samples with high chloride concentration, such as saltwater fish[29]), and $^{40}Ar^{12}C$ during ^{75}As and ^{52}Cr determination, respectively. High-purity argon(99.999%) (White Martins-Praxair) was used for plasma generation, nebulization, and auxiliary gas. The operational and instrumental parameters are presented in Table I.

Table I. Instrumental parameters of the LC-ICP-MS for chemical speciation analysis of As and Cr

Chemical speciation analysis of As (LC)	
Mobile phase	0.01 mol L^{-1} $(NH_4)_2HPO_4$ + 1% (v v^{-1}) CH_3OH pH 8.0
Mobile phase flow	1.0 mL min^{-1}
Elution mode	Isocratic
Injected volume	50 µL
Analysis time	720 s
Measurement	Time-resolved analysis
Chemical speciation analysis of Cr (LC)	
Mobile phase	0.015 mol L^{-1} Na_2SO_4, 0.005 mol EDTA, 0.005 mol NaH_2PO_4 pH 7.0
Mobile phase flow	0.7 mL min^{-1}
Elution mode	Isocratic
Injected volume	50 µL
Analysis time	360 s
Measurement	Time-resolved analysis
ICP-MS	
Plasma power	1550 W
Deep sampling	8 mm
Nebulizer gas flow rate	1.1 L min^{-1}
He gas flow	4.5 mL min^{-1}
Nebulizer	Micromist
Spray chamber	Scott type, double-pass
Isotopes	^{75}As, ^{52}Cr

The seafood samples were freeze-dried (Model EC, MicroModulyo, New York, NY), cryogenically ground (MA775, Marconi, Piracicaba, Brazil), and microwave oven digested (Multiwave Go Plus, Anton Paar, Graz, Austria) for total element acid determination. A pHmeter (W3b model, Bel Instrument, Monza MB, Italy) was used for pH measurements, and extractions of As and Cr for speciation analysis were performed with PFA (perfluoroalkoxy) flasks (Savillex, Eden Prairie, MN USA) in a digester block (MA4025, Marconi, Piracicaba, Brazil). The extract solution was dispersed in the samples using a vortex (Marconi, Piracicaba, Brazil). A centrifuge with a capacity for 15 and 50 mL tubes (Excelsa II 206 BL, FANEM, São Paulo, Brazil) was used to accelerate the particulate decantation.

Samples and reagents

Three samples of six fish species: amberjack (Seriola spp.), cobia (*Rachycentron canadum*), dogfish (*Carcharhinus plumbeus*), croaker (*Argyrosomus regius*), sole (*Paralichthys brasiliensis*) and sardines (*Sardinella brasiliensis*), were acquired in open markets and supermarkets of Aracaju, SE, Brazil. Three samples of bivalve tarioba mollusk (*Iphigenia brasiliensis*) from the estuary area of the city of Ilhéus, BA, Brazil, gray shrimp (*Litopenaeus vannamei*) and pink shrimp (*Farfantepenaeus paulensis*) acquired in open markets from two different cities of Sao Paulo State, Brazil, as well as their cephalothorax, legs, and shells, due to their use in the preparation of various Brazilian culinary dishes were also analyzed.

Seafood was refrigerated immediately after collection and kept on dry ice until arrival at the laboratory. Around 100-200 g of each laterodorsal muscle of the fish, the mollusks, and the parts of the shrimps were separated and lyophilized. After that, the samples were cryogenically ground and sieved through a 0.25 mm nylon mesh to samples homogenized.

Reference materials (fish protein (DORM-4, NRC, National Research Council of Canada), dogfish liver (DOLT-5, NRC), lobster hepatopancreas (TORT-3, NIST), oyster tissue (1566b, NIST), soil contaminated with hexavalent chromium (2701, NIST), and fish tissue reference material developed by the Institute for Energy and Nuclear Research (IPEN, São Paulo-SP, Brazil) were used to total and speciation methods validation.

All glass and plasticware were kept in 10% HNO_3 (v/v) for at least 24 h and washed with water before use. All solutions were prepared with analytical grade reagents and water with a resistivity of 18.2 MΩ cm, purified in a Milli-Q® system (Millipore, Bedford, MA, USA). Nitric acid obtained from a model distillacid BSB-939-IR acid purification apparatus (Berghof, Eningen, Germany) and hydrogen peroxide solution (30% wt., Sigma-Aldrich, Saint Louis, MO, USA) were used for sample preparation procedures.

Reference solutions for the determination of total As and Cr (0.1 to 20 μg L^{-1}) were prepared in 1% (v v^{-1}) HNO_3 from a standard solution for ICP-MS (Fluka, Buchs St. Gallen, Switzerland). Internal standard (IS) solution of 500 μg L^{-1} Ga was prepared in 1% (v/v) HNO_3 from a 1000 mg L^{-1} Ga IS solution (Fluka, Buchs St. Gallen, Switzerland).

For As analysis speciation, the mobile phase containing $(NH_4)_2HPO_4$ 0.01 mol L^{-1} (pH 8.0) was prepared daily by dissolving the salt $(NH_4)_2HPO_4$in water containing 1% (v v^{-1}) CH_3OH (methanol HPLC grade, Sigma-Aldrich). Mobile phase pH was adjusted with an NH_3 solution saturated in water. For Cr, the mobile phase 0.015 mol L^{-1} Na_2SO_4, 0.005 mol L^{-1} EDTA, 0.005 mol L^{-1} NaH_2PO_4 (pH 7.0) was prepared daily by dissolving the salts in water.

As(III), As(V), AsB, DMA, MMA, Cr(III), and Cr(VI) calibration solutions (0.1 to 20 μg L^{-1} as As or Cr) were prepared daily from the previously prepared 1000 mg L^{-1} stock solutions from the dissolution of the salts of $NaAsO_2$, KH_2AsO_4, $C_5H_{11}AsO_2$, $(CH_3)_2AsO_2Na{\cdot}3H_2O$, $CH_3AsO(ONa)_2{\cdot}6H_2O$, $Cr(NO_3)_3$, and K_2CrO_4 (Sigma-Aldrich) in water, respectively.

Sample preparation

Arsenic and chromium total determination

200 mg of each evaluated seafood sample was weighed, in triplicate, into PTFE-TFM microwave vials for sample digestion. Solutions of 6.0 mL of HNO_3 (7.0 mol L^{-1}) and 2.0 mL of H_2O_2 (30% m v^{-1}) were added to the samples, and the heating program (1) 180 ºC (20 min) and (2) 220 ºC (20 min) was performed.

After digestion, the solution was transferred to a volumetric flask, and the volume was set to 30 mL with water. ICP-MS was used for determination of total As and Cr.

Arsenic speciation

100 mg of seafood samples were weighed, in triplicate, into the PFA digestion vials. Then, 5 mL of 0.03 mol L^{-1} HNO_3 was added. The vials were closed with caps, mixed for 30 s, and heated in a digester block for 40 min at 90 °C. After cooling, the extracts were centrifuged at 3200 rpm for 5 min. A volume of 1 mL of the supernatant was 5-times diluted with the mobile phase. Finally, the extracted solution was filtered with a 0.22 µm PTFE syringe filter. The extracted solutions were stored in a refrigerator (4 ºC) for up to 24 h before performing the analysis, a condition where the species remained stable, according to previously published results by Schmidt et al.[14] The As speciation analysis was performed by LC-ICP-MS.

Chromium speciation

For Cr species extraction, 100 mg of seafood samples were weighed directly into the PFA digestion vials, and then 5 mL of EDTA 0.045 mol L^{-1} (pH 10.0) was added. The vials were closed with caps, mixed for 30 s, and heated in a digester block for 40 min at 90 °C. After cooling, the extract was centrifuged at 3200 rpm for 5 min. A volume of 1 mL of the supernatant was 10-times diluted with the mobile phase (pH 7.0). Finally, the extracted solution was filtered with a 0.22 µm PTFE syringe filter. The Cr speciation analysis was performed by LC-ICP-MS.

Optimization strategy

Arsenic species

The optimization of Ar species extraction was defined by using a Doehlert matrix. The factors and levels examined, amended from our previous studies, and based on the literature[6,31] were temperature (70, 80, and 90 °C) and time (15, 22.5, 30, 37.5, and 45 min). The data were analyzed with STATISTICA 12 software (with a 95% of confidence level).

Chromatographic Recovery (CR) was used as an analytical response to obtain a single experimental condition for all species. For this, Equation 1 was used.

$$\mathrm{CR}(\%) = \frac{\left(C_{As(III)} + C_{As(V)} + C_{MMA} + C_{DMA} + C_{AsB}\right)}{C_e} x100 \qquad \text{Equation 1}$$

where CR corresponds to the percentage of chromatographic recovery, C_e the total concentration of As in the extract, and $C_{As(III)}$, $C_{As(V)}$, C_{MMA}, C_{DMA}, and C_{AsB} the As species concentrations in the extract.

Chromium species

The optimization of Cr species extraction was defined by using Box-Behnken planning. The factors and levels examined, amended from our preliminary experiment[25] and based on the literature, were: temperature (50, 80, and 110 °C), time (10, 30, and 50 min), mass (50, 100, and 150 mg), and EDTA concentration (10, 40, and 70 mmol L^{-1}). The data were analyzed with STATISTICA 12 software (with a 95% of confidence level).

Chromatographic Recovery (CR) was used as an analytical response to obtain a single experimental condition for both chromium species. For this, Equation 2 was used.

$$\mathrm{CR}(\%) = \frac{\left(C_{Cr(III)} + C_{Cr(VI)}\right)}{C_e} x100 \qquad \text{Equation 2}$$

where CR corresponds to the chromatographic recovery percentage, *Ce* is the total chromium concentration in the extract, $C_{Cr(III)}$ the concentration of the trivalent chromium species in the extract, and $C_{Cr(VI)}$ the hexavalent species of chromium in the extract.

RESULTS AND DISCUSSION

As and Cr total determination

Recoveries from 87 to 99% were obtained by the proposed method and reference values of the certified reference materials (Table SI, Supplementary Material). No significant differences (*t*-test at 95%) were observed between the measured and reference values of the CRM. The t_{cal} (1.56) and t_{tab} (2.78) for As and t_{cal} (1.87) and t_{tab} (3.18) for Cr, confirm the method's trueness.

Optimization of As and Cr species chromatographic separation

Arsenic

To improve the separation of As species, $(NH_4)_2HPO_4$ 10 mmol L^{-1} mobile phase was studied with minor pH adjustments (8.0 to 8.65). In Figure 1, it is possible to observe the pH influence on the As species chromatographic separation. This parameter was studied in a univariate way using As reference solutions for each species (As(III), As(V), AsB, DMA, and MMA).

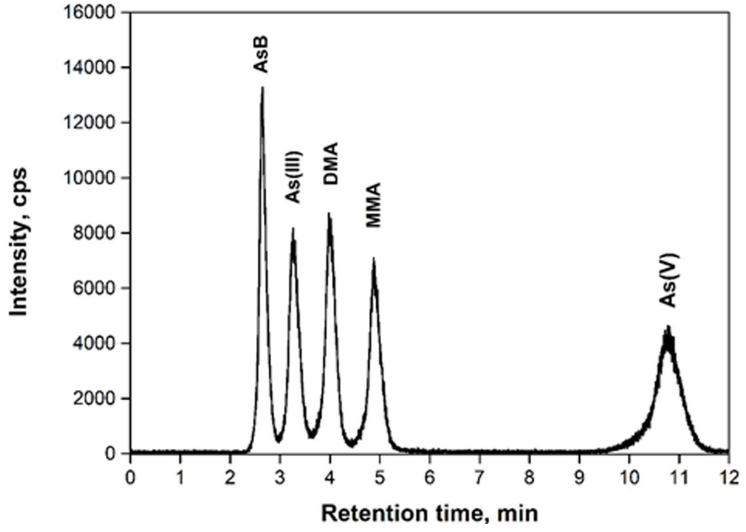

Figure 1. Separation chromatogram of As species using 0.01 mol L^{-1} $(NH_4)_2HPO_4$ in 1% (v/v) CH_3OH (pH 8.0) as mobile phase.

The pH 8.0 of mobile phase favored separation between As(III) and DMA. With the mobile phase at pH 8.65, the physicochemical characteristics of As(III) are altered to arsenous acid (pK_{a1} 9.2). Thus, its interaction with the stationary phase is reduced, and As(III) is eluted practically at the same time with the DMA species. This condition was observed at pH values above 8.25. On the other hand, at lower pH values, As(III) also interacts more with the stationary phase due to the greater equilibrium shift and the other species, making the peak wider, consequently raising the LOD. However, as an adequate resolution for As(III) and DMA at pH 8.0, this pH was defined for the mobile phase, even with the enlargement of the other species.

The solution 0.01 mol L^{-1} $(NH_4)_2HPO_4$ in 1% (v/v) CH_3OH (pH 8.0) was selected as the mobile phase. Even in isocratic mode, it allowed good separation and resolution in a reasonably short time.[23,32]

Doehlert experimental design to optimize the species extraction conditions used matrices containing nine experiments each, including temperature and time factors in seafood samples. Sample mass and extractor solution were adapted according to extraction procedures described in the literature.[11,14] Table II shows the Doehlert experimental design applied with real and coded values and the analytical response as a function of the chromatographic recovery.

Table II. Doehlert matrix design for factors optimizing involved in the seafood As species extraction

Experiment	Time (min)	Temperature (ºC)	CR(%)[a]
1	30.0 (0)	80 (0)	88.4[b]/85.1[c]
2	45.0 (1)	80 (0)	85.7[b]/83.2[c]
3	37.5 (0.5)	90 (0.866)	96.1[b]/92.3[c]
4	15.0 (-1)	80 (0)	65.4[b]/65.1[c]
5	22.5 (-0.5)	70 (-0.866)	75.7[b]/75.0[c]
6	37.5 (0.5)	70 (-0.866)	69.3[b]/69.4[c]
7	22.5 (-0.5)	90 (0.866)	80.3[b]/80.1[c]
8	30.0 (0)	80 (0)	89.2[b]/86.0[c]
9	30.0 (0)	80 (0)	88.5[b]/85.5[c]

[a]chromatographic recovery; [b]analytical response for gray shrimp; [c]analytical response for bivalve mollusk.

Analysis of variance (ANOVA table) was used to validate the developed statistical model. The F test was applied, and no lack of fit to the models worked was observed. The well-adjusted model was attested by the analysis of the variance table and the obtained graphs of predicted versus observed values and residuals versus predicted values. Equation 3 described the mathematical models for the gray shrimp sample and Equation 4 for the bivalve mollusk sample.

$$\text{Chromatographic recovery} = 88.76_{\pm 0.28} + 8.29_{\pm 0.24}(\text{Temperature}) - 6.17_{\pm 0.37}(\text{Temperature}^2) + 3.41_{\pm 0.17}(\text{Time}) - 2.84_{\pm 0.13}(\text{Time}^2) + 4.97_{\pm 0.22}(\text{Temperature x Time}) \quad \text{Equation 3}$$

$$\text{Chromatographic recovery} = 85.85_{\pm 0.28} + 7.35_{\pm 0.24}(\text{Temperature}) - 4.86_{\pm 0.37}(\text{Temperature}^2) + 2.73_{\pm 0.17}(\text{Time}) - 2.34_{\pm 0.13}(\text{Time}^2) + 3.79_{\pm 0.22}(\text{Temperature x Time}) \quad \text{Equation 4}$$

Through analysis of the variance of the model, it was verified that all terms were statistically significant. Additionally, the positive sign for the factors and their interaction indicates that there is a greater extraction of As species at moderately high temperatures and time. This condition was observed for the two models generated from the seafood analysis. A correlation coefficient greater than 0.97 (95% of confidence level) indicates an excellent agreement between predicted and observed values. The maximum response was determined by applying the Lagrange criteria to characterize the critical point as a maximum. The first derivative was adjusted concerning each factor, time or temperature, to obtain the optimal point coordinates. The best condition established was 40 min at 90 ºC for greater extraction of As species. These conditions are demonstrated in the response surfaces in Figure 2.

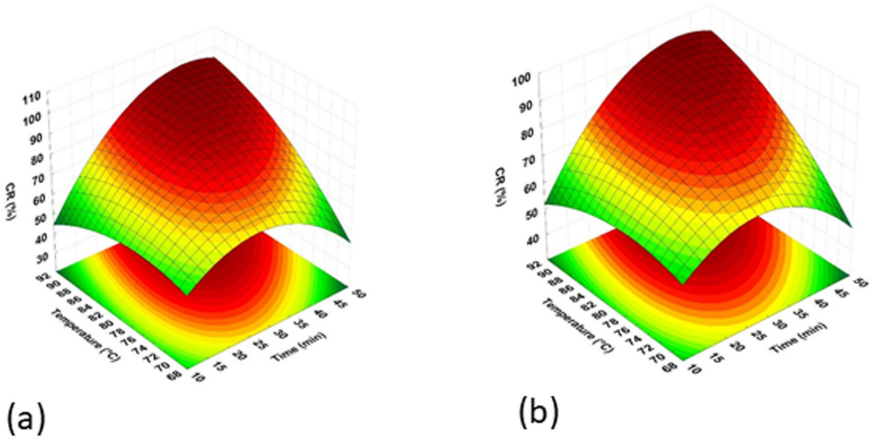

Figure 2. Response surfaces obtained from Doehlert experimental design for factors temperature versus time in (a) shrimp and (b) bivalve mollusk using chromatographic response as analytical response

Chromium

Mobile phases of 0.015 mol L^{-1} Na_2SO_4 + 0.005 mol L^{-1} EDTA + 0.005 mol L^{-1} NaH_2PO_4 (pH 7.0); 0.025 mol L^{-1} $(NH_4)_2SO_4$ + 0.005 mol L^{-1} EDTA + 0.001 mol L^{-1} NaOH (pH 8.0) and 0.005 mol L^{-1} EDTA (pH 10.0), were evaluated to improve the separation of Cr species. Figure 3 presents pH influence and mobile phase composition on chromatographic separation. These parameters were studied in a univariate way, using Cr reference solutions for Cr(III) and Cr(VI).

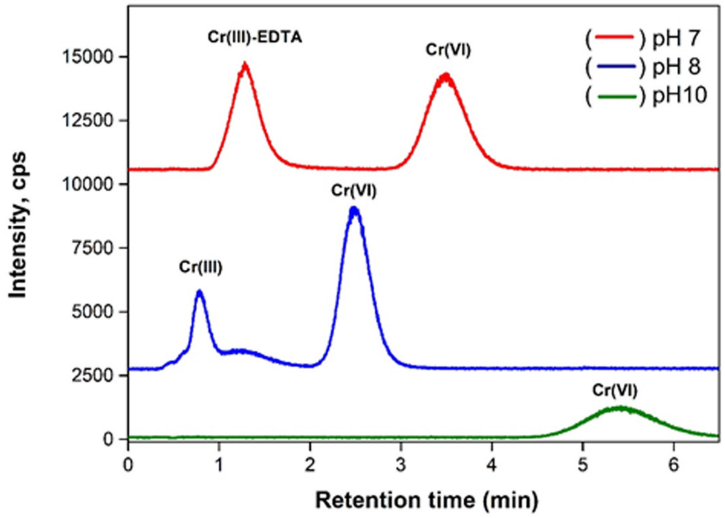

Figure 3. Chromium species separation. Injection of 0.05 mL solution containing Cr(III) and Cr(VI) using mobile phase composed of 0.015 mol L^{-1} Na_2SO_4 + 0.005 mol L^{-1} EDTA + 0.005 mol L^{-1} NaH_2PO_4 with (—) pH = 7.0; 0.025 mol L^{-1} $(NH_4)_2SO_4$ + 0.005 mol L^{-1} EDTA + 0.001 mol L^{-1} NaOH with (—) pH = 8.0, and 0.005 mol L^{-1} EDTA (—) with pH = 10.0

The mobile phase at pH 10.0 was unable to separate the Cr(III) species and had a longer elution time for Cr(VI), leading to a higher analysis cost due to the chromatographic run time and consequent higher analysis costs. At pH 8.0, Cr(III) and Cr(VI) were separated. However, the peak definition was not adequate for Cr(III), which is characterized by the low solubility in this medium. At pH 7.0 adequate resolution and

peak definition were observed since the complexation of Cr(III)-EDTA was effective, converting Cr(III) to its complexed form, facilitating the separation and determination of this analyte. Therefore, the mobile phase 15 mmol L^{-1} Na_2SO_4 + 5 mmol L^{-1} EDTA + 5 mmol L^{-1} NaH_2PO_4 (pH 7.0) was selected, considering the separation parameters, resolution, and time using the isocratic mode.

The Cr species extractions optimization was performed using a Box-Behnken experimental design. An experimental matrix containing 27 experiments was used to optimize the extraction conditions of chromium species. Factors such as temperature, EDTA concentration, time, and mass were studied. As Cr(VI) is usually absent in foods,[25] we added 0.01 mg L^{-1} Cr(VI) in all experiments. The Box-Behnken matrix, with the actual coded values and chromatographic recoveries, is shown in Table III.

Table III. Box-Behnken matrix design for factors optimizing involved in Cr species extraction

Exp[a]	Temperature (ºC)	Time (min)	Mass (g)	[EDTA] (mol L^{-1})	CR (%)[b]
1	50.0 (-1)	10.0 (-1)	100.0 (0)	40.0 (0)	64.6
2	110.0 (1)	10.0 (-1)	100.0 (0)	40.0 (0)	50.0
3	50.0 (-1)	50.0 (1)	100.0 (0)	40.0 (0)	61.8
4	110.0 (1)	50.0 (1)	100.0 (0)	40.0 (0)	86.4
5	80.0 (0)	30.0 (0)	50.0 (-1)	10.0 (-1)	48.0
6	80.0 (0)	30.0 (0)	150.0 (1)	10.0 (-1)	47.4
7	80.0 (0)	30.0 (0)	50.0 (-1)	70.0 (1)	58.2
8	80.0 (0)	30.0 (0)	150.0 (1)	70.0 (1)	60.9
9	80.0 (0)	30.0 (0)	100.0 (0)	40.0 (0)	81.8
10	50.0 (-1)	30.0 (0)	100.0 (0)	10.0 (-1)	49.1
11	110.0 (1)	30.0 (0)	100.0 (0)	10.0 (-1)	48.5
12	50.0 (-1)	30.0 (0)	100.0 (0)	70.0 (1)	59.1
13	110.0 (1)	30.0 (0)	100.0 (0)	70.0 (1)	59.1
14	80.0 (0)	10.0 (-1)	50.0 (-1)	40.0 (0)	49.1
15	80.0 (0)	50.0 (1)	50.0 (-1)	40.0 (0)	63.7
16	80.0 (0)	10.0 (-1)	150.0 (1)	40.0 (0)	65.5
17	80.0 (0)	50.0 (1)	150.0 (1)	40.0 (0)	65.5
18	80.0 (0)	30.0 (0)	100.0 (0)	40.0 (0)	84.6
19	50.0 (-1)	30.0 (0)	50.0 (-1)	40.0 (0)	53.7
20	110.0 (1)	30.0 (0)	50.0 (-1)	40.0 (0)	58.2
21	50.0 (-1)	30.0 (0)	150.0 (1)	40.0 (0)	51.8
22	110.0 (1)	30.0 (0)	150.0 (1)	40.0 (0)	61.8
23	80.0 (0)	10.0 (-1)	100.0 (0)	10.0 (-1)	49.7
24	80.0 (0)	50.0 (1)	100.0 (0)	10.0 (-1)	47.9

(continues on the next page)

Table III. Box-Behnken matrix design for factors optimizing involved in Cr species extraction (continuation)

Exp[a]	Temperature (ºC)	Time (min)	Mass (g)	[EDTA] (mol L^{-1})	CR (%)[b]
25	80.0 (0)	10.0 (-1)	100.0 (0)	70.0 (1)	59.1
26	80.0 (0)	50.0 (1)	100.0 (0)	70.0 (1)	68.2
27	80.0 (0)	30.0 (0)	100.0 (0)	40.0 (0)	82.8

[a]Experiment; [b]Chromatographic Recovery.

The model was validated using the abovementioned parameters. In this way, the mathematical model developed can be described by Equation 5.

$$\text{Chromatographic Recovery} = 49.39_{\pm 0.46} + 1.99_{\pm 0.40}(\text{Temperature}) + 5.65_{\pm 0.30}(\text{Temperature}^2) + 4.62_{\pm 0.40}\,\text{Time} + 3.95_{\pm 0.30}(\text{Time}^2) + 1.84_{\pm 0.40}(\text{Mass}) + 6.99_{\pm 0.30}(\text{Mass}^2) + 6.16_{\pm 0.40}([\text{EDTA}]) + 8.71_{\pm 0.30}([\text{EDTA}]^2) + 9.77_{\pm 0.69}(\text{Temperature x Time}) - 3.63_{\pm 0.69}(\text{Time x Mass}) \quad \text{Equation 5}$$

The model analysis verified that only all linear and quadratic terms and the interactions between temperature versus time and time versus mass were statistically significant. Additionally, the positive sign in the parameters and interactions indicates that these parameters should be applied at moderately high levels. The estimated correlation coefficient was 0.94 (95% of confidence level). Therefore, this indicates a satisfactory agreement between the values determined and predicted by the model. The Lagrange criterion allows characterizing the critical point as a maximum. The function of the first derived was adjusted with each variable, and a system of equations of the 1st degree was obtained to find the coordinates of the critical point. With the coordinates of the optimal point, the optimal conditions established are 100 mg of sample and 45 mmol L^{-1} of EDTA at pH 10.0 and 40 min heating at 90 ºC as the extracted solution for Cr species. These conditions are demonstrated in the response surfaces shown in Figure 4.

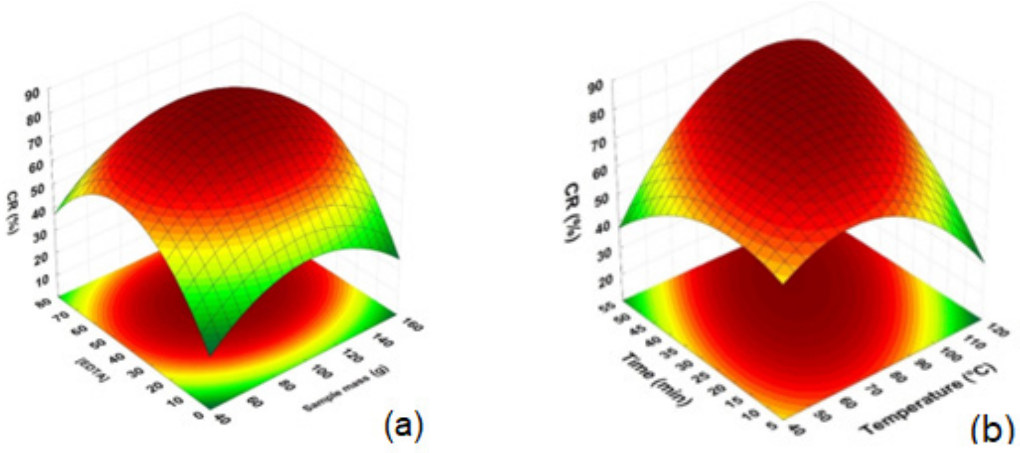

Figure 4. Response surfaces obtained from Box-Behnken experimental design for factors: (a) [EDTA] versus sample mass and (b) Time versus temperature using chromatographic response as analytical response.

Analytical parameters

The complete separation was obtained in 12 min and 6 min for As and Cr, respectively, with good resolution for both methods. Arsenic and Cr species were quantified by external calibration, ranging from 0.1 to 20 µg L^{-1} of As or Cr. Each species was determined by concentration correlation with the integration

area of the analytical signal. A good linear correlation (R^2 > 0.9998) for all As and Cr species was found. The LOD and LOQ were obtained from LD=3×SE/S and LQ=10×SE/S, respectively, where SE and S correspond to the standard error and slope of the linear regression, respectively.[33,34] Limits of quantification of 2.8; 5.3; 16; 52; and 83 ng g^{-1} (as As) were obtained for MMA, AsB, DMA, As(III), and As(V), respectively. For chromium, the LOQs were 114 ng g^{-1} (Cr(III)) and 54 ng g^{-1} (Cr(VI)).

Sample analysis and trueness

Arsenic

Different concentrations of As(III), As(V), AsB, DMA, and MMA were added directly to lyophilized seafood samples to evaluate the As species recoveries. The additions were carried out in the same order of magnitude as the original As concentration already present in each sample. When As species were initially ≤ LOQ, we added about 3 times the LOQ of the respective specie to the final solution (considering the extraction and dilution step). Recoveries from 87 to 99% were obtained (Table SII, Supplementary Material). The obtained values can be regarded as quantitative for all evaluated seafood, as recoveries between 70 and 120% are described as satisfactory in speciation analysis.[35]

Certified reference materials complementarily evaluated the method's trueness. The results agreed with the certified value of total certified As with a confidence level of 95% (Table IV). Chemical speciation in different kinds of fish, bivalve mollusks, and shrimp was performed with the use of the developed method (100 mg of sample, 0.03 mol L^{-1} HNO_3 as extracting solution, and 0.01 mol L^{-1} $(NH_4)_2HPO_4$ in 1% CH_3OH (pH 8.0) as mobile phase). The obtained results are presented in Table IV, and the chromatograms are in Figure S1 in Supplementary Material.

Different species of seafood present peculiarities in their composition, and the proposed extraction method was suitable for all analyzed samples. With the selected conditions, the As species sum agrees with the values obtained by the digestion method for all seafood samples with a confidence level of 95%.

All evaluated seafood are above 1.0 μg g^{-1}, the maximum allowed limit according to current Brazilian legislation.[13] In shrimp, the highest As values are presented by cephalothorax. This slice contains organs concentrating more arsenic in its structure. The AsB, a non-toxic As specie, is the predominant specie in all evaluated seafood. However, small amounts of DMA, As(III), and As(V) were also found in bivalve mollusks. The provisional weekly intake tolerance value of 15 μg kg^{-1} per body mass of inorganic As was proposed by the European Union. On the other hand, FAO and WHO concluded that this value presented a risk and considered it inappropriate. The provisional value of tolerance was revoked due to this controversy. Currently, there is no maximum limit of inorganic arsenic in seafood that would be tolerated for ingestion, and the establishment of specific legislation for these arsenic species is required.[16,36]

Table IV. Total As and As species in CRM, RM, and seafood, using the proposed method (mean ± standard deviation, mg kg^{-1}, n=3)

Sample	AsB	As(III)	DMA	MMA	As(V)	∑ As	Total As
IPEN[a]	5.15 ± 0.08	< 0.052	< 0.016	< 0.003	< 0.025	5.15 ± 0.08	5.80 ± 0.32
DOLT-5[b]	29.17 ± 1.31	< 0.052	0.031 ± 0.02	< 0.003	< 0.025	29.20 ± 1.33	31.02 ± 1.55
DORM-4[b]	5.37 ± 0.08	< 0.052	0.015 ± 0.01	< 0.003	< 0.025	5.38 ± 0.09	5.94 ± 0.14
1566b[b]	4.8 ± 0.28	0.056 ± 0.01	1.50 ± 0.02	< 0.003	< 0.025	6.36 ± 0.31	6.64 ± 0.19
TORT-3[b]	54.24 ± 0.78	< 0.052	0.022 ± 0.01	< 0.003	< 0.025	54.26 ± 0.79	59.78 ± 1.08
Shrimp muscle tissue[c]	11.81 ± 0.30	< 0.052	< 0.016	< 0.003	< 0.025	11.81 ± 0.30	12.55 ± 0.44
Shrimp carapace[c]	4.66 ± 0.07	< 0.052	< 0.016	< 0.003	< 0.025	4.66 ± 0.07	4.48 ± 0.02
Shrimp cephalothorax[c]	15.75 ± 0.55	< 0.052	< 0.016	< 0.003	< 0.025	15.75 ± 0.55	16.00 ± 0.40
Shrimp legs[c]	6.59 ± 0.30	< 0.052	< 0.016	< 0.003	< 0.025	6.59 ± 0.30	6.65 ± 0.37
Shrimp muscle tissue[d]	1.72 ± 0.09	< 0.052	< 0.016	< 0.003	< 0.025	1.72 ± 0.09	2.05 ± 0.10
Bivalve mollusk	4.25 ± 0.09	0.080 ± 0.004	0.090 ± 0.006	< 0.003	0.031 ± 0.003	4.45 ± 0.10	4.81 ± 0.11
Amberjack	5.41 ± 0.14	< 0.052	< 0.016	< 0.003	< 0.025	5.41 ± 0.14	5.62 ± 0.16
Cobia	4.875 ± 0.07	< 0.052	< 0.016	< 0.003	< 0.025	4.87 ± 0.07	5.22 ± 0.09
Sardine	56.75 ± 1.14	< 0.052	< 0.016	< 0.003	< 0.025	56.75 ± 1.14	60.1 ± 1.4
Croaker	10.1 ± 0.35	< 0.052	< 0.016	< 0.003	< 0.025	10.1 ± 0.35	9.52 ± 0.30
Dogfish	100.0 ± 3.1	< 0.052	< 0.016	< 0.003	< 0.025	100.0 ± 3.1	106.5 ± 2.88
Sole	5.24 ± 0.08	< 0.052	< 0.016	< 0.003	< 0.025	5.24 ± 0.08	5.49 ± 0.07

[a]Reference material; [b]certified reference material; [c]gray shrimp from supplier A; [d]pink shrimp from supplier B.
Certified values, mg kg^{-1}, IPEN (5.90 ± 0.27), DOLT-5 (34.6 ± 2.4), DORM-4 (6.80 ± 0.64), 1566b (7 .65 ± 0.65), TORT-3 (59.5 ± 3.8).

Cromium

Methods proposition that contemplates extraction of Cr(III) and Cr(VI) species without stability loss is a challenge because Cr(VI) is stable only in basic medium. Only soluble Cr(III) is extractable in this environment. An addition and recovery experiment of Cr(III) and Cr(VI) species in the seafood samples and the CRMs DORM- 4, DOLT-5, and TORT-3 was carried out. Different amounts of Cr species (Cr(III) and Cr(VI)) were added to the freeze-dried samples in the same order of magnitude as the original concentration of Cr already present in the samples. In Cr species originally below the LOQ, the amount corresponding to about 3 times the LOQ of the respective species was added to the determination solution (considering the extraction and dilution steps). Recoveries from 87 to 104% were obtained (Table SIII, Supplementary Material), and deemed quantitative to all samples. In speciation analysis, 70 to 120% recoveries can be considered satisfactory.[35]

Certified reference material soil contaminated with hexavalent chromium (NIST-2701) complementarily evaluated the trueness of the method. The result for hexavalent Cr, 535 ± 24.2 μg g^{-1}, agreed with the certified Cr(VI) value, with a recovery of 97% (at 95% of confidence level).

Chromium speciation in seafood samples was performed with the proposed method. It was used 100 mg of sample, 0.045 mol L^{-1} of EDTA (pH 10) as extracting solution, and 0.015 mol L^{-1} Na_2SO_4 + 0.005 mol L^{-1} EDTA + 0.005 mol L^{-1} NaH_2PO_4 (pH 7.0) as mobile phase. The results are presented in Table V, and the chromatograms are in Figure S2 in the Supplementary Material.

Table V. Total Cr and Cr species in CRM, RM, and seafood, using the proposed method (mean ± standard deviation, mg kg^{-1}, n=3)

Sample	Total Cr	Cr(III)	Cr(VI)	Soluble Cr(III) (%)
IPEN[a]	0.117 ± 0.003	0.015 ± 0.001	< 0.054	13
DOLT-5[b]	2.012 ± 0.030	0.449 ± 0.007	< 0.054	22
DORM-4[b]	1.847 ± 0.037	0.206 ± 0.004	< 0.054	11
1566b[b]	0.554 ± 0.036	0.141 ± 0.009	< 0.054	25
TORT-3[b]	1.782 ± 0.045	1.025 ± 0.026	< 0.054	57
Shrimp muscle tissue[c]	0.304 ± 0.008	0.093 ± 0.002	< 0.054	30
Shrimp carapace[c]	0.378 ± 0.013	0.120 ± 0.004	< 0.054	32
Shrimp cephalothorax[c]	0.828 ± 0.037	0.101 ± 0.005	< 0.054	12
Shrimp legs[c]	0.467 ± 0.016	0.187 ± 0.007	< 0.054	40
Shrimp muscle tissue[d]	2.034 ± 0.051	0.143 ± 0.004	< 0.054	7
Bivalve mollusk	10.95 ± 0.110	0.674 ± 0.007	< 0.054	6
Amberjack	0.324 ± 0.011	0.075 ± 0.003	< 0.054	23
Cobia	0.554 ± 0.028	0.073 ± 0.004	< 0.054	13
Sardine	0.485 ± 0.036	0.069 ± 0.005	< 0.054	14
Croaker	0.568 ± 0.020	0.072 ± 0.003	< 0.054	13
Dogfish	2.936 ± 0.029	0.337 ± 0.003	< 0.054	11
Sole	2.415 ± 0.181	0.246 ± 0.018	< 0.054	10

[a]Reference material; [b]certified reference material; [c]gray shrimp from supplier A; [d]pink shrimp from supplier B.
Certified values, mg kg^{-1}, DOLT-5 (2.35 ± 0.58), DORM-4 (1.87 ± 0.16), TORT-3 (1.95 ± 0.24), 1566b (-). IPEN (-). CRM 2701-contaminated soil with Cr(VI) presented recovery of 97%.

Chromium(III) is the predominant species in the seafood samples. As can be observed in Table V, the sum of Cr(III) and Cr(VI) values differs from the total Cr contents obtained after sample digestion. As previously stated, only a part of Cr(III) is soluble in the alkaline extracting solution. No legislation regulates the Cr content in seafood, even though it is carcinogenic and recently remarks low essentiality to the human organism, according to the evaluation of European Union studies.[5]

CONCLUSIONS

The method developed for As speciation in fish, shrimp, and bivalve mollusks was optimized for each matrix separately, considering its particularities and possible interferences during LC-ICP-MS determination. Otherwise, the established extracted and mobile phase conditions allowed the As species extraction and detection with adequate resolution and a short time. The proposed method presented suitable analytical parameters with excellent sensitivity, linearity, and selectivity. According to Brazilian legislation, all samples were above the maximum limit allowed for fish. However, as expected, AsB was the primary chemical specie present.

An additional method was developed for the speciation of soluble Cr(III) and Cr(VI) in fish, bivalve mollusks, and shrimp samples by LC-ICP-MS. The chemical species of Cr was extracted in an alkaline solution of 0.045 mol L^{-1} of EDTA at 90 ºC for 40 min. The species were determined with an adequate chromatographic resolution, excellent sensitivity, linearity, and good selectivity. Chromium(III) is the specie present in the samples, as expected in food samples.

Data Availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Conflict of interest

The authors declare that there is no conflict of interest regarding the publication of this article.

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Supplementary Material

Table SI. Total As and Cr trueness assessment in CRM by ICP-MS

Analyte	CRM[a]	Value (mg kg^{-1})		Recovery (%)
		Certified	Determined	
As	IPEN[b]	5.90 ± 0.27	5.80 ± 0.32	98
	DOLT-5	34.6 ± 2.4	31.02 ± 1.55	90
	DORM-4	6.80 ± 0.64	5.94 ± 0.14	87
	1566b	7.65 ± 0.65	6.64 ± 0.19	87
	TORT-3	59.5 ± 3.8	59.38 ± 1.08	99
Cr	IPEN[b]	-	0.117 ± 0.003	-
	DOLT-5	2.35 ± 0.58	2.012 ± 0.030	87
	DORM-4	1.87 ± 0.16	1.847 ± 0.037	98
	1566b	-	0.554 ± 0.036	-
	TORT-3	1.95 ± 0.24	1.782 ± 0.045	91
	2701	4.26 ± 0.12	4.24 ± 0.35	99

[a]certified reference material; [b]reference material.

Table SII. Recovery tests of As species (n = 3) in fish samples

Sample	AsB	As(III)	DMA	MMA	As(V)
Fish	97-101	99-103	90-94	96-99	99-104
Bivalve mollusk	92-104	88-93	93-97	95-98	97-100
Shrimp	97-103	92-96	91-95	87-91	91-95

Table SIII. Recovery tests of Cr species (n = 3) in fish samples and CRM

Sample	Cr(III) %	Cr(VI) %
Fish	92-104	88-93
Bivalve mollusk	97-101	90-96
Shrimp	92-103	91-99
DOLT-5	95-101	91-95
DORM-4	87-98	90-97
TORT-3	92-96	89-98

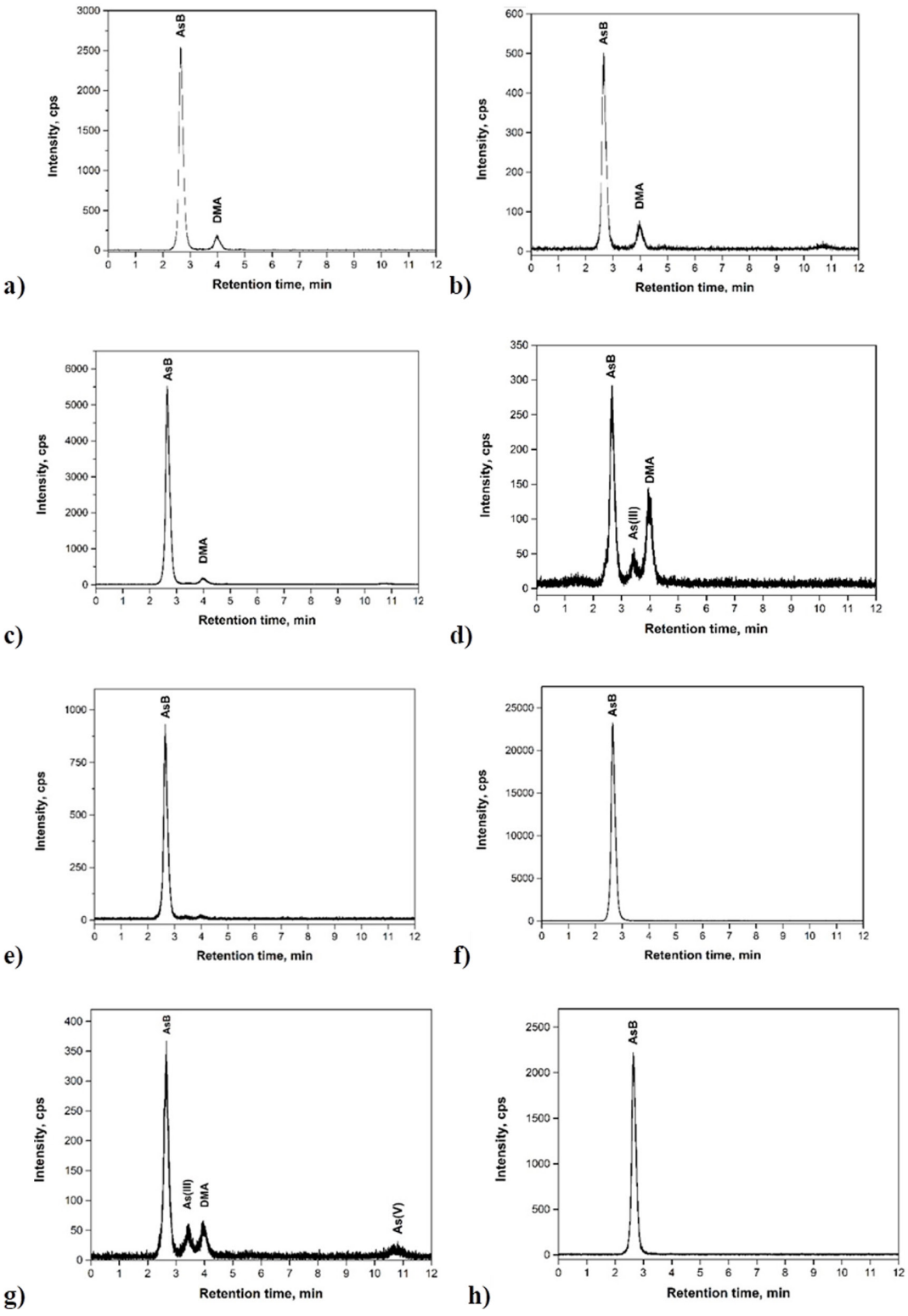

Figure S1. Chromatograms of As species in a) DOLT-5, b) DORM-4, c) TORT-3 d) 1566b, e) IPEN, f) dogfish, g) bivalve mollusk, and h) gray shrimp.

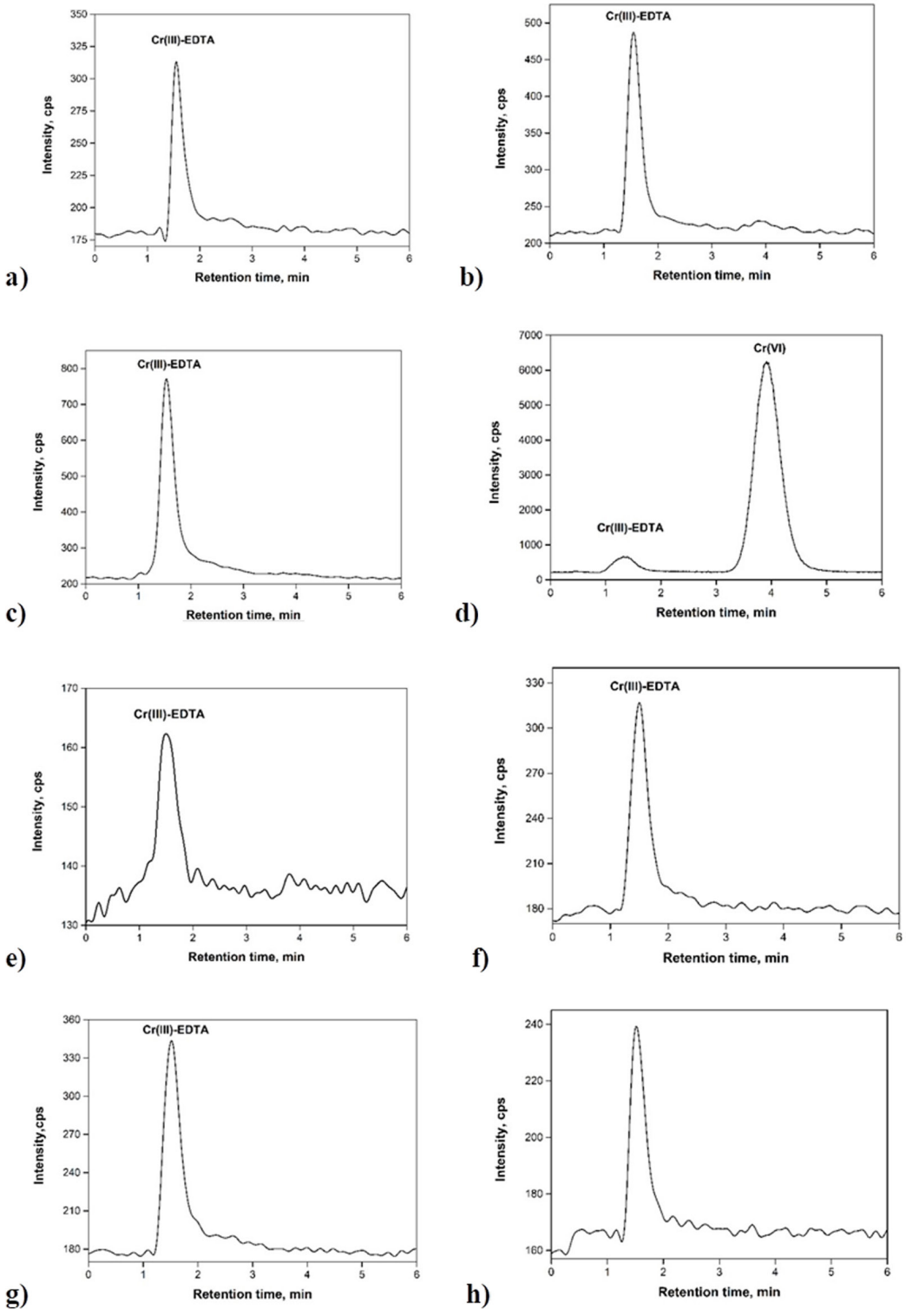

Figure S2. Chromatograms of Cr species in a) DOLT-5, b) DORM-4, c) TORT-3, d) NIST (2701), e) sardines, f) bivalve mollusk, g) gray shrimp, and h) pink shrimp.