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Change of Arsenic Speciation in Shellfish after Cooking and Gastrointestinal Digestion

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ABSTRACT: Shellfish is a common part of indigenous cuisines throughout the world and one of the major sources of human exposure to arsenic (As). We evaluated As speciation in shellfish after cooking and gastrointestinal digestion in this study. Results showed that washing and cooking (boiling and steaming) can reduce As exposures from shellfish. The use of spices during cooking processes also helped to reduce the bioaccessibility of total As. Through mass balance calculations, we verified the transformation of methylated As compounds into inorganic As in shellfish takes place during cooking and that As demethylation can occur during simulated gastrointestinal digestion. *In vivo* demethylation of As after gastrointestinal digestion was also demonstrated in laboratory mice. This increase in inorganic As during digestion suggests that risks of As toxicity from shellfish consumption are being underestimated. Further studies on the mechanisms of As speciation transformation in food are necessary for more thorough risk assessments.

KEYWORDS: *shellfish, As, cooking, speciation, bioaccessibility*

1. INTRODUCTION

Shellfish is a common part of indigenous cuisines throughout the world, and it plays an important role in helping people to reach a healthy balance of omega-3 and omega-6 fats in their diets.[1] However, some shellfish may contain high levels of arsenic.[2,3] Arsenic (As) is ubiquitous in the environment in water, rocks, soil, and air, and it is considered to be a class 1 nonthreshold carcinogen.[4,5] Sources of As to the environment include both natural processes and anthropogenic activities. The toxicity and carcinogenicity of As varies in accordance with its different speciations. To date, toxicological assessments of human exposure to As have focused on inorganic As (iAs).[6,7] Research in recent years has demonstrated that organic As compounds like arsenolipids and arsenosugars, which are considered to be far less toxic than inorganic As and even non toxic, can be metabolized by humans.[8] The As in shellfish is present primarily in the form of organic compounds (more than 90% of the total As).[4] Arsenobetaine (AsB) is the major As species in most shellfish.[9] Besides, methylated As compounds including dimethylarsinic acid (DMA^V) and monomethylarsonic acid (MMA^V) are also present in shellfish; MMA^V and DMA^V are classified as possibly carcinogenic to humans (Group 2B in agents classified by the IARC Monographs).[6,10]

Human exposure to As from shellfish occurs via ingestion. Thus, some authors have investigated the bioaccessibility of As after shellfish intake.[11,12] Initial studies pointed out that As is highly bioaccessibile (up to 100%) in some food (mainly rice, algae, and fish).[13−15] However, each species of As in food can have various levels of bioaccessibility in the gastric environment or intestinal tract.[16,17] For example, Juhasz et al.[13] reported that the 89% of bioavailable As was in the form of iAs, while the bioavailability of DMA and MMA was found to be much lower. In recent research, demethylation of methylated As compounds from rice and seaweed was found to occur during simulated in vitro digestion, and this led to an increasing amount of iAs.[18] Increases of iAs during gastrointestinal digestion could boost As toxicity after the intake of food containing As. However, the contribution rates and mechanisms of demethylation of DMA or MMA in food passing through the gastrointestinal tract remain uncertain. In addition, to the best of our knowledge, the extent of in vivo As demethylation in the gastrointestinal tract is not known.

Processing and cooking have an obvious effect on the As contents and bioaccessibility of As in food.[19,20] Dahl et al.[21] reported that freezing and storage could cause decreases in AsB and total As in some food. Washing has been shown to release a portion of the As from food (e.g., rice and hijiki).[22] Cheyns et al.[23] analyzed the concentrations of total As and As species in different foodstuffs (including molluscs) and showed that there was a decrease in the levels of total As and the types of As species analyzed after common kitchen practices (e.g., boiling, steaming, and frying). In addition, heating can induce As

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speciation changes, e.g., one study found that AsB will decompose into trimethylarsine oxide (TMAO) at 150 °C (like during grilling and roasting) or TMAO and trimethylarsonium (TMA^+) at 160 °C or above.[24] However, only a few studies have investigated the effects of cooking on As speciation changes in food. It has been reported that when temperatures between 150 and 190 °C are used, portions of the AsB in fish will decompose.[25] Cheyns et al.[23] found that total As was reduced during cooking and that iAs was released most easily followed by the organic species, whereby the small organic molecules were released more easily than the large ones. Nonetheless, the amount of As released during cooking can vary between different preparation processes and foodstuffs. Use of dietary additives [e.g., Fe(II), Fe(III), aluminum, titanium, and tannic acid] has been claimed to reduce the solubility of iAs and DMA.[12] Spices (e.g., cooking oils, salt, pepper) are widely used in boiling, baking, and other types of cooking methods, and these spices include numerous chemicals that may also have an impact on the release of As or the speciation changes.[26] However, to the best of our knowledge, the influence of those spices on the release of As or the speciation changes during cooking is still uncertain.

The objective of the present study was to evaluate the presence of total As and As species in three widely consumed types of shellfish, and the effects of various cooking processes and spices on As speciation changes in shellfish were considered. Additionally, through physiologically based extraction *in vitro* tests, the distribution of various As species in the bioaccessible fraction was determined, and through *in vivo* experiments with actual animals (mice), demethylation reactions during gastrointestinal digestion were ascertained.

2. MATERIALS AND METHODS

2.1. Samples. Scallop (*Argopecten irradias*), clam (*Mactra quadrangularis Deshayes*), and oyster (*Ostrea gigas Thunberg*) samples were purchased from Guangzhou City and Qingdao City in China. In each treatment, samples were studied in triplicate. Samples were prepared by first removing the shell. The tissues were then washed with the same mass of ultrapure water three times, and the As contents in the discarded washing water were determined. Stainless steel tools were used to cut and homogenize the tissues. Because of the higher As concentrations detected in shellfish from Guangzhou, we chose these samples for the cooking experiments. The chosen samples were boiled, steamed, or baked. Due to the higher moisture content in oysters, the homogenized tissues were difficult to boil or steam, and thus we only subjected these tissues to baking.

On the basis of the cooking habits of people, we selected several spices for analysis including the spices of oil (peanut oil, Lu Hua, China), salt (pure NaCl), pepper (white pepper ground, McCormick, China), and lemon juice (squeezed from fresh lemon) during the cooking experiments in our study. As was not detected in these spices. The amounts of shellfish spices used were based on practical cooking practices and the literature;[27] the water to shellfish ratio was 1:1, and the oil, salt, pepper, and lemon juice were added in amounts of 7, 2, 0.5, and 4 g per 100 g of shellfish, respectively. The water remaining after cooking was discarded.

Boiling was performed in a Teflon sauce pot filled with ultrapure water. Shellfish tissues were dipped into the boiling water for 5 min at 100 °C, and three boiling treatments were used (Boiled 1#: boiled with only water; Boiled 2#: boiled with water containing salts; Boiled 3#: boiled with water containing salts and lemon juice). Steaming was done in a steamer at 100 °C for 10 min, and three steamed treatments were used (Steamed 1#: steamed with nothing; Steamed 2#: steamed with salts; Steamed 3#: steamed with salts and lemon juice). Baking was done in a baking oven for 10 min at 120 °C, and five baking treatments were used (Baked 1#: baked with nothing; Baked 2#: baked with oil; Baked 3#: baked with oil and salts; Baked 4#: baked with oil, salts, and pepper; and Baked 5#: baked with oil, salts, lemon juice, and pepper).

The raw and cooked samples were separated into two subsamples; one was frozen and stored at −80 °C and the other one was freeze-dried (frozen for 48 h at −45 °C under low pressure) and afterward stored at −80 °C until further analysis.

2.2. Reagents. All solutions were prepared by using high-purity water with a resistivity of 18.2 mΩ·cm obtained from a water purification system (Milli-Q Element, Millipore, U.S.A.). Porcine pepsin, pancreatin, and sodium cholate were purchased from Sigma-Aldrich Chemical. Sodium citrate, malic acid, glacial acetic acid, lactic acid, nitric acid, hydrochloric acid, sodium hydroxide, sodium bicarbonate, and ammonia were of guaranteed reagent grade.

2.3. *In Vitro* Digestion. *In vitro*digestive juices were prepared following the method used by Ruby et al. (1996) with modifications.[28] We adjusted 1 L of ultrapure water to pH 2.0 with 12 N HCl and added 1.25 g of porcine pepsin, 0.5 g of malic acid, 0.5 mL of glacial acetic acid, and 420 μL of lactic acid. Intestinal digestive materials were prepared by adding porcine bile and pancreatin into the test meals at a mass ratio of food matrices to bile and to pancreatin of 1:0.175 and 1:0.05, respectively. The blank digestion solution was also analyzed in each batch of samples.

During the digestion, 500 mL of gastric solution was added to 5 g of homogenized freeze-dried shellfish sample. Each test was done in triplicate, and all aliquots were incubated at 37 °C in a shaking incubator at 150 rpm for 1 h; then, a 30 mL aliquot of gastric digestion was sampled during shaking. For intestinal digestion, the pH was adjusted to 5.3 with saturated sodium bicarbonate and porcine bile and pancreatin were added; the pH then was adjusted to 7.0 with 1 M NaOH, and the mix was incubated at 37 °C in a shaking incubator at 150 rpm. After 2 h of intestinal digestion, a 30 mL aliquot of gastrointestinal digestion was sampled during shaking.

The bioaccessible fraction was pretreated as follows: the solution was centrifuged at 5000 × *g* for 20 min to separate the aqueous phase from residual materials, and the supernatant was filtered through a 0.22-μm cellulose acetate disk filter. The residue pellet was freeze-dried and afterward stored at −80 °C until further analysis.

2.4. *In Vivo*Testing. The *in vivo* experiments were performed in compliance with the Guide for the Care and Use of Laboratory Animals (8th edition).[29] The experiments were conducted by the Guangdong Laboratory Animals Monitoring Institute in China (animal experiment certification: No. 00191797; animal quality certification: No. 44007200050564). Laboratory mice were randomly divided into four groups (26−30 g per mouse, six mice per group, half male and half female). The mice were not fed for 1 d before As exposures to empty the gut and maximize their digestion of the solution containing As species (DMA^V, MMA^V, and inorganic As^V). The four groups were exposed to As species for 15, 30, 60, and 120 min, respectively. In addition, one group was set as the blank control group (four mice, half male and half female) and was not exposed to any As species. Mice were exposed to As species by gavage (concentration: 50 ppb; volume: 1.5 mL). The stomach and the intestine were sampled after exposure. Whole stomachs or intestines were first minced and then extracted with 10 mL of 0.01 mol·L^{-1} nitric acid by microwave extraction for 1 h. The obtained solutions were centrifuged at 5000 × *g* for 20 min, and the supernatants were filtered through a 0.22-μm cellulose acetate disk filter.

2.5. Determination of Total As (tAs) Contents. The total As (tAs) in shellfish and residue pellets after in vitro tests was extracted from samples following a previously described method.[30] Briefly, 0.2 g samples were transferred into Teflon reactors and 4 mL of 14 mol·L^{-1} HNO_3 were added; this mixture was predigested for 4 h, and then, 1 mL H_2O_2 (30% v/v) was added. Next, the samples in the Teflon reactors were incubated in a microwave system for 30 min (1600 W, 180 °C).

The concentrations of tAs in shellfish, bioaccessible fractions, and residue pellets were determined by using a quadrupole inductively coupled plasma mass spectrometry unit (ICP-MS; Agilent 7700x, Agilent, U.S.A.).[31] The ICP-MS instrument was operated with helium

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Table 1. Contents of As and Its Chemical Forms ($\mu g \cdot g^{-1}$ dw) in the Collected Shellfish[a,b,c,d]

samples		moisture (%)	total As	AsB	DMA^V	MMA^V	iAs
Clam (Q)	Raw	85.2	4.1 ± 1.0	2.4 ± 0.4	1.0 ± 0.1	0.3 ± 0.01	0.03 ± 0.005
Clam (G)	Raw	78.9	4.9 ± 0.7	2.0 ± 0.7	1.5 ± 0.1	0.9 ± 0.09	0.1 ± 0.02
	Boiled 1#	75.0	2.2 ± 0.3	1.0 ± 0.1	0.6 ± 0.09	0.5 ± 0.09	0.08 ± 0.01
	Boiled 2#	71.7	3.0 ± 0.8	1.4 ± 0.3	0.9 ± 0.1	0.5 ± 0.09	0.09 ± 0.07
	Boiled 3#	71.3	3.3 ± 0.3	1.5 ± 0.2	1.0 ± 0.1	0.5 ± 0.08	0.08 ± 0.01
	Baked 1#	66.8	4.7 ± 0.8	2.1 ± 0.3	1.8 ± 0.06	0.8 ± 0.02	0.1 ± 0.05
	Baked 2#	62.6	4.6 ± 0.1	2.0 ± 0.03	1.7 ± 0.1	0.9 ± 0.03	0.1 ± 0.01
	Baked 3#	63.3	4.7 ± 0.9	2.0 ± 0.2	1.7 ± 0.09	0.9 ± 0.05	0.1 ± 0.06
	Baked 4#	65.2	4.9 ± 0.04	2.2 ± 0.1	1.6 ± 0.08	0.8 ± 0.07	0.1 ± 0.04
	Baked 5#	63.0	4.7 ± 0.7	2.1 ± 0.1	1.5 ± 0.06	0.9 ± 0.05	0.1 ± 0.1
Scallop (Q)	Raw	84.2	6.2 ± 1.0	4.5 ± 0.2	1.4 ± 0.1	0.01 ± 0.007	0.1 ± 0.08
Scallop (G)	Raw	82.3	8.6 ± 1.0	5.7 ± 0.4	2.1 ± 0.09	0.2 ± 0.02	0.1 ± 0.02
	Steamed 1#	75.0	6.8 ± 1.0	4.0 ± 0.1	1.7 ± 0.2	0.1 ± 0.09	0.06 ± 0.01
	Steamed 2#	75.7	7.4 ± 0.5	4.3 ± 0.2	1.7 ± 0.3	0.1 ± 0.05	0.06 ± 0.02
	Steamed 3#	76.3	7.6 ± 1.1	4.6 ± 0.9	1.9 ± 0.2	0.1 ± 0.03	0.06 ± 0.01
	Baked 1#	61.8	8.4 ± 1.2	5.9 ± 0.3	2.2 ± 0.1	0.2 ± 0.02	0.09 ± 0.03
	Baked 2#	60.6	8.2 ± 1.0	5.6 ± 0.3	2.2 ± 0.3	0.2 ± 0.03	0.1 ± 0.02
	Baked 3#	60.3	8.7 ± 1.0	5.6 ± 0.3	2.1 ± 0.07	0.2 ± 0.03	0.09 ± 0.03
	Baked 4#	61.2	8.7 ± 1.0	5.3 ± 0.3	2.2 ± 0.2	0.2 ± 0.02	0.08 ± 0.04
	Baked 5#	60.0	8.9 ± 1.0	5.7 ± 0.3	2.3 ± 0.1	0.2 ± 0.09	0.09 ± 0.02
Oyster (Q)	Raw	86.3	4.5 ± 0.3	3.3 ± 0.1	0.9 ± 0.04	<DL[e]	0.2 ± 0.04
Oyster (G)	Raw	85.1	6.3 ± 0.9	4.0 ± 0.1	1.3 ± 0.3	<DL	0.4 ± 0.09
	Baked 2#	68.3	6.1 ± 0.3	4.0 ± 0.2	1.0 ± 0.1	<DL	0.5 ± 0.01

[a]Triplicate analyses of total As and As species were carried out (means ± standard deviation). [b]Boiled 1#: Boiled with water; Boiled 2#: Boiled with water containing salts; Boiled 3#: Boiled with water containing salts and lemon juice. [c]Steamed 1#: Steamed with water; Steamed 2#: Steamed with water containing salts; Steamed 3#: Steamed with water containing salts and lemon juice. [d]Baked 1#: Baked with nothing; Baked 2#: Baked with oil; Baked 3#: Baked with oil and pepper; Baked 4#: Baked with oil, pepper and lemon juice; Baked 5#: Baked with oil, pepper, lemon juice and salts. [e]DL detected level.

(5 $mL \cdot min^{-1}$) as the collision cell gas for removing polyatomic interferences from argon chloride ($^{40}Ar^{35}Cl$). The detection limit for As was 0.0022 $\mu g \cdot L^{-1}$.

^{72}Ge (10 $\mu g \cdot L^{-1}$) was chosen as the internal standard element. A multielemental solution (1 $\mu g \cdot L^{-1}$ in 2% HNO_3) (Agilent, U.S.A.) was used as the tuning solution for the ICP-MS work. The As in the certified reference materials [GBW 10024 (GSB-15 chemical composition of scallop), GBW 10050 (GSB-28 chemical composition of shrimp), and GBW 10068 (elements and organotins in oyster tissue), China; DORM-4, fish protein, Canada] was extracted and determined in the same manner as the samples. The recovery rate ranged from 82% to 111%.

2.6. As Speciation Analyses. The As species in the initial shellfish matrices, in vitro bioaccessible fractions, residue fractions, and mice gastric/intestinal fractions were separated by an Agilent 7700 ICP-MS connected to a high-pressure liquid chromatography unit (Prin-cen Elspe-2 HPLC, Prin-cen Scientific Ltd., China). A Prin-cen Specia Fast Column (4.6 × 100 mm^2, Prin-cen Scientific Ltd., China) and dual mobile phases of ammonium nitrate solution (phase A: NH_4NO_3 8 $mmol \cdot L^{-1}$; phase B: NH_4NO_3 20 $mmol \cdot L^{-1}$; flow rate = 1.2 $mL \cdot min^{-1}$; injection volume = 30 μL; room temperature) were used for the identification of As species. Extraction of individual species of As from raw and cooked shellfish was based on the GB 5009.11−2014 method with modifications.[32] Briefly, 0.5 g of homogenized shellfish sample was extracted with 20 mL of 0.01 $mol \cdot L^{-1}$ nitric acid solution by microwave extraction for 1 h, and then, samples were centrifuged at 8000 × g for 15 min. Next, the samples were filtered through a 0.22-μm cellulose acetate disk filter. The same procedure was used to prepare and extract the standard reference material (SRM 1568b, rice flour, U.S.A.; DORM-4, fish protein, Canada) and blank solutions.

Arsenious acid solution (iAs^{III}, GBW 08666), arsenic acid solution (iAs^V, GBW 08667), monomethylarsonic acid solution (MMA^V, GBW 08668), dimethylarsinic acid solution (DMA^V, GBW 08669), and arsenobetaine solution (AsB, GBW 08670) were used to prepare the calibration standards for the individual species of As. Detection limits for the individual species on the ICP-MS instrument were 0.0083, 0.0052, 0.0123, 0.0145, and 0.0103 $\mu g \cdot L^{-1}$ for iAs^{III}, iAs^V, MMA^V, DMA^V, and AsB, respectively. The concentration of iAs represents the sum of iAs^{III} and iAs^V. SRM 1568b and DORM-4 were included in every sample batch for evaluations of accuracy. The recovery rates for DMA, MMA, and iAs in SRM 1568b were 81−122%, 91−124%, and 80−115%, respectively. The recovery rates of AsB in DORM-4 were 94−119%. The column recoveries (sum of species/total As in the extract) ranged from 79% to 122% (an average of 95%). In addition, the quality control blank spikes of 5 ppb run in triplicate with each batch during the analysis experiments produced average AsB, DMA, MMA, iAs^{III}, and iAs^V concentrations of 5.09, 4.96, 5.16, 4.57, and 4.78 ppb.

The bioaccessibility (%) and residue percentages (%) of As after in vitro digestion were calculated by using the following two equations:[12,28]

$$\text{bioaccessibility (\%)} = \frac{\text{bioaccessible concentration}}{\text{total concentration in shellfish}} \times 100\% \quad (1)$$

$$\text{residue (\%)} = \frac{\text{residue concentration}}{\text{total concentration in shellfish}} \times 100\% \quad (2)$$

The bioaccessible concentration was obtained from the in vitro digestion extraction data, and the residue concentration was obtained from the data for the residue pellets.

2.7. Statistical Analyses. Statistical analyses were performed by using SPSS 21.0 (SPSS, Chicago, IL, U.S.A.) and OriginLab 9.0 (OriginLab, U.S.A.). Differences in values among treated groups were tested by using the one-way analysis of variance (ANOVA) procedure followed by a least significant difference (LSD) test. All concentrations in this paper were based on the dry weight (dw). All data were expressed as the means or the means ± the standard deviation (SD). Means were considered significantly different if $p < 0.05$.

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Table 2. Average Converted As Contents C_i ($\mu g \cdot g^{-1}$ dw[a]) in Discarded Washing Water and Boiling Soup and Percents of Origin Concentration in Shellfish (%), Triplicate Analyses of Total As and As Species Were Carried Out

samples		tAs		AsB		DMA^V		MMA^V		iAs	
		C_i	%	C_i	%	C_i	%	C_i	%	C_i	%
Washing Water	Clam (G)	2.1	30.4	1.9	48.2	0.2	9.9	<DL[b]	ns[c]	0.009	7.3
	Clam (Q)	3.3	34.5	3.0	45.3	0.3	13.9	<DL	ns	0.007	14.9
	Scallop (G)	3.4	24.3	3.2	31.5	0.2	5.8	<DL	ns	0.01	12.6
	Scallop (Q)	2.9	31.7	2.6	36.9	0.1	7.2	<DL	ns	0.03	16.2
	Oyster (G)	3.6	36.0	3.5	46.2	0.3	16.3	<DL	ns	0.01	2.3
	Oyster (Q)	3.6	35.2	3.3	43.5	0.1	10.4	<DL	ns	0.02	8.0
Clam (G) Soup	Boiled 1#	2.5	52.2	1.1	53.8	0.6	41.0	0.2	24.1	0.5	408.0
	Boiled 2#	1.9	38.1	0.9	45.3	0.4	25.4	0.1	13.7	0.4	333.1
	Boiled 3#	1.4	28.0	0.6	29.9	0.3	20.1	0.1	11.1	0.4	346.7

[a]The contents of As in the discarded washing water and soup ($\mu g \cdot L^{-1}$) were converted as $\mu g \cdot g^{-1}$ dw raw shellfish. [b]DL detected level. [c]ns, not significant.

3. RESULTS AND DISCUSSION

3.1. As in Raw Shellfish. The contents of tAs and various species of As in the three kinds of raw shellfish that were analyzed are listed in Table 1. The average tAs concentrations in the shellfish that originated from Qingdao City (4.117, 6.211, and 4.476 $\mu g \cdot g^{-1}$ in clams, scallops, and oysters, respectively) were lower than those from Guangzhou City (4.878, 8.609, and 6.347 $\mu g \cdot g^{-1}$ in clams, scallops, and oysters, respectively).

As speciation was determined by using HPLC-ICP-MS. The results showed that AsB was the predominant species of As in all of the shellfish samples analyzed in this study; this species accounted for 49.5%, 69.0%, and 69.2% of the tAs in clams, scallops, and oysters, respectively (Table 1), which is similar to the findings from other previous studies of marine samples.[9] In addition, it was also found that AsB contents were correlated with the tAs levels, and the correlation coefficient (r) was equal to 0.83 for the three types of shellfish ($p < 0.05$). Methylated As compounds were also present in the shellfish samples, and the toxicity of these species represents another major concern just after that of iAs.[6] On average, DMA^V was the most predominant methylated As compound, and it accounted for 27.7%, 23.7%, and 20.4% of the tAs in clams, scallops, and oysters, respectively. The average MMA^V content in raw shellfish samples was 0.260 $\mu g \cdot g^{-1}$, and the highest value was detected in clams that originated from Guangzhou City (0.892 $\mu g \cdot g^{-1}$), while the lowest value was detected in oysters that originated from Qingdao City (0.014 $\mu g \cdot g^{-1}$); on average, this species accounted for 7.5% of the tAs. Inorganic As was detected in all shellfish samples in this study, and it was mainly in the form of iAs^V in raw samples. However, iAs contributed far less to the tAs in shellfish samples (1.1−6.9%) than the organic As species. These values are in accordance with those reported by other studies on shellfish, which correspond to 0.2−8.9%.[3]

According to the results presented in Table 2, washing released about 32.4%, 28.0%, and 35.6% of the original tAs from clams, scallops, and oysters, respectively. To date, and to the best of our knowledge, no specific data on As being released from shellfish into washing water have been reported in the literature, and thus, the results obtained in our study cannot be compared. However, our results are in agreement with As studies on the washing of other foodstuffs (e.g., rice, hijiki). For example, washing can reduce the tAs content in dry rice samples up to 24%.[22] After soaking hijiki in water for 15 min, one study reported the release of 28% of the tAs from dry samples after the water was removed.[23] The average percent decrease of tAs in shellfish following washing was a little higher than that reported in studies on other foods. Variations in the initial As concentration in the food as well as the washing water quantity may account for these differences. In this study, we washed the shellfish three times, and the total sum of the water quantity was three times the shellfish mass. Therefore, to reduce As concentrations in food, use of large volumes of washing water would likely be the most effective approach. As shown in Table 2, As released from shellfish into washing water was mainly in the form of AsB. The average percentages of the decrease in the initial AsB content in shellfish were calculated to be 46.8%, 34.2%, and 44.9% for clams, scallops, and oysters, respectively. Meanwhile, the washing procedure reduced DMA^V and iAs on average by 10.6% and 10.2%, respectively. Moreover, MMA^V was not detected in the washing water, and the change in the contents of MMA^V in shellfish before and after washing was not significant in this study ($p > 0.05$). Cheyns et al.[23] reported that soaking could reduce only 7% of the AsB and/or other cationic and uncharged species in dry hijiki samples, while it decreased DMA^V and iAs by 70% and 40%, respectively.[23] The differences between that study and the present one may be accounted for by the fact that the As species distributions in the food matrices were different.

3.2. As in Shellfish after Cooking. The contents of tAs and species of As in the three kinds of shellfish after cooking are shown in Table 3. The tAs decreased markedly after boiling and steaming based on the dry weight, and the content did not change significantly after baking ($p > 0.05$). The concentration of tAs in shellfish decreased by 33.0−54.1% and 12.3−20.6% after boiling and steaming, respectively. Results in this study are similar to those of Cheyns et al.[23] who reported that steaming reduced the tAs by 12−24% in mussels based on the dry weight. In relation to the use of spices (oil, salt, lemon juice, and pepper) during cooking processes, the results showed that salt and lemon juice had an adverse effect on the decrease of As during boiling and steaming, while the As concentrations in shellfish baked with and without spices did not differ significantly ($p > 0.05$) (Tables 2 and 3). A possible explanation for the differences in the As release behavior under different cooking procedures is as follows: soluble As dissolved into the cooking water during boiling and steaming, but As was hardly volatile during the heat treatments (<130 °C) applied in this study (for instance, the boiling point of $AsCl_3$ is 130 °C and the boiling point of As_2O_3 is 456 °C).[8,23,33,34] In addition,

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Table 3. As Bioaccessibility (%) and Residue Percents (%) after Gastrointestinal *In Vitro* Test of the Collected Shellfish[b,c,d,e]

			Total As	AsB	DMA^V	MMA^V	iAs
Clam (Q)	Raw	Bioaccessibility	79.2±2.1	82.2±1.5	59.5±2.1	56.2±2.9	119.0±2.6
		Residue percents	17.3±3.0	21.0±4.3	20.5±3.4	17.6±4.9	75.4±19.3
Clam (G)	Raw	Bioaccessibility	76.1±0.9	82.0±8.2	56.7±3.1	50.9±2.9	126.0±8.8
		Residue percents	18.9±4.3	13.0±6.2	33.3±3.0	34.1±7.5	46.0±18.3
	Boiled 1#	Bioaccessibility	66.7±3.4	66.0±1.7	45.1±1.3	38.8±1.8	188.2±3.8
		Residue percents	28.3±2.4	29.0±2.8	44.9±3.9	46.2±3.7	88.2±9.7
	Boiled 2#	Bioaccessibility	62.5±2.5	63.9±3.5	48.8±2.1	30.9±1.8	152.5±7.5
		Residue percents	32.5±1.9	31.1±2.1	41.2±3.1	54.1±4.3	52.5±10.1
	Boiled 3#	Bioaccessibility	60.3±4.2	60.9±1.6	44.3±3.1	23.8±1.0	153.5±7.5
		Residue percents	34.7±2.1	34.1±3.9	45.7±3.9	61.2±3.1	53.5±9.3
	Baked 1#	Bioaccessibility	70.0±2.8	73.5±1.5	22.2±1.9	32.3±1.2	219.7±4.2
		Residue percents	25.0±3.1	21.5±1.9	67.8±5.9	52.7±0.9	73.7±8.1
	Baked 2#	Bioaccessibility	64.0±4.2	68.8±5.6	18.8±0.9	31.4±2.3	247.8±6.4
		Residue percents	31.0±3.9	26.2±1.5	71.2±2.9	53.6±1.8	97.8±10.4
	Baked 3#	Bioaccessibility	60.6±6.7	65.2±1.2	18.7±0.9	24.6±1.0	211.4±4.3
		Residue percents	34.4±2.5	29.8±1.1	71.3±1.8	60.4±1.7	61.4±12.1
	Baked 4#	Bioaccessibility	58.5±1.4	65.1±0.8	18.3±1.1	23.6±1.2	167.2±6.5
		Residue percents	36.5±1.8	29.9±2.1	71.7±0.9	61.4±2.4	67.2±8.7
	Baked 5#	Bioaccessibility	61.1±1.3	68.4±3.0	22.7±1.9	28.3±2.5	169.1±8.8
		Residue percents	33.9±2.1	26.6±0.8	67.3±4.1	56.7±4.9	69.1±9.2
Scallop (Q)	Raw	Bioaccessibility	90.4±3.5	92.2±1.9	45.7±2.5	<DL[a]	99.9±6.8
		Residue percents	11.2±3.5	6.8±4.4	26.1±2.4	<DL	62.5±19.8
Scallop (G)	Raw	Bioaccessibility	83.6±7.1	86.8±2.7	58.3±1.9	19.8±2.2	111.6±2.7
		Residue percents	12.0±2.1	8.2±1.7	38.7±0.9	48.1±7.8	11.6±13.3
	Steamed 1#	Bioaccessibility	77.5±5.8	80.6±3.6	52.9±1.9	12.1±1.0	140.4±1.8
		Residue percents	18.5±3.3	14.4±2.1	45.1±2.4	52.7±3.1	40.4±8.5
	Steamed 2#	Bioaccessibility	70.0±5.2	77.3±1.2	45.7±1.5	18.5±0.5	136.2±8.6
		Residue percents	26.0±3.9	17.7±3.9	58.4±2.8	48.9±1.9	36.2±7.9
	Steamed 3#	Bioaccessibility	69.2±8.7	76.4±2.5	36.7±0.9	12.6±0.9	132.7±2.3
		Residue percents	26.8±4.3	18.6±3.1	66.4±1.1	52.4±3.1	32.7±7.1
	Baked 1#	Bioaccessibility	73.9±4.5	76.6±1.5	31.3±0.9	17.2±2.6	133.3±2.3
		Residue percents	22.1±5.9	18.4±1.9	66.6±1.9	49.7±3.9	33.3±6.9
	Baked 2#	Bioaccessibility	70.7±8.7	71.6±3.2	30.7±1.2	10.9±2.5	150.3±5.2
		Residue percents	25.3±2.9	23.4±4.1	64.6±2.5	53.5±2.5	50.3±5.9
	Baked 3#	Bioaccessibility	65.9±7.8	69.7±1.9	16.7±2.2	5.0±0.8	155.7±4.7
		Residue percents	30.1±5.1	25.3±4.1	77.0±3.2	57.0±2.7	55.7±10.2
	Baked 4#	Bioaccessibility	74.9±8.9	69.8±2.7	21.3±1.5	3.4±0.5	165.2±3.7
		Residue percents	21.1±3.9	25.2±3.9	76.6±1.9	58.0±0.9	65.2±8.6
	Baked 5#	Bioaccessibility	67.8±7.2	70.6±3.1	22.8±0.7	6.6±0.8	161.9±4.3
		Residue percents	28.2±5.3	24.4±2.8	74.2±0.8	56.0±1.6	61.9±8.5
Oyster (Q)	Raw	Bioaccessibility	90.2±3.6	89.3±4.2	54.2±2.6	<DL	109.2±4.8
		Residue percents	9.4±3.2	11.1±6.3	13.8±1.6	<DL	47.8±16.6
Oyster (G)	Raw	Bioaccessibility	88.4±2.5	87.2±3.1	39.0±1.5	<DL	114.0±5.0
		Residue percents	10.0±1.9	7.8±4.1	26.1±2.3	<DL	48.5±11.7
	Baked 2#	Bioaccessibility	69.1±2.1	70.2±1.8	23.6±2.1	<DL	134.3±2.5
		Residue percents	26.9±2.9	24.8±4.4	25.9±1.5	<DL	34.3±8.3

[a]DL detected level. [b]Triplicate analyses of total As and As species were carried out (means ± standard deviation) (in red). [c]Boiled 1#: Boiled with water; Boiled 2#: Boiled with water containing salts; Boiled 3#: Boiled with water containing salts and lemon juice. [d]Steamed 1#: Steamed with water; Steamed 2#: Steamed with water containing salts; Steamed 3#: Steamed with water containing salts and lemon juice. [e]Baked 1#: Baked with nothing; Baked 2#: Baked with oil; Baked 3#: Baked with oil and pepper; Baked 4#: Baked with oil, pepper and lemon juice; Baked 5#: Baked with oil, pepper, lemon juice and salts.

lemon juice and salt may bind with As in shellfish and alter the dissociation constants of soluble As.[12]

As a consequence, the distribution of chemical forms of As in cooked shellfish varied with that in raw samples. In general, contents of iAs, AsB, DMA^V, and MMA^V in shellfish decreased after cooking. It was hard to collect the entire contents of residual water after steaming, and thus, only the discarded boiling soup was investigated in this study (Table 2). Results showed that about 34.30% of the tAs was released from clams into the soup after boiling without spices. As mentioned above, salt and lemon juice had an adverse effect on the As release from the shellfish into the soup, and the percentages corresponded to 21.85−27.60%. Besides, we found that iAs (mainly iAs^V) as well as AsB and DMA^V were the main chemical forms of As in the boiling soup. Obviously, the contents of iAs in soup were all far more than the initial values in raw clams (~four times higher), which means that it is more dangerous for us to drink this kind of soup[35]. With respect to the effects of cooking practices on As in foods, the results found in the scientific literature were focused mainly on the cooked food matrices and generally ignored the cooking water. In our study, As concentrations were calculated based on the dry weight and we excluded the factor of moisture loss. Ultrapure water was used in this study, and ultrapure water samples with/without the five species of As were heated in the same cooking pots as well. We determined the arsenic contents in the water after cooking and found that the recovery of all individual As species ranged from 79% to 122% with an average of 95%, which means that the cooking water had no effect on the changes of As in shellfish after boiling. Besides, after mass balance accounting [recovery rate = 100% * (contents in boiled shellfish + contents in boiling soup)/

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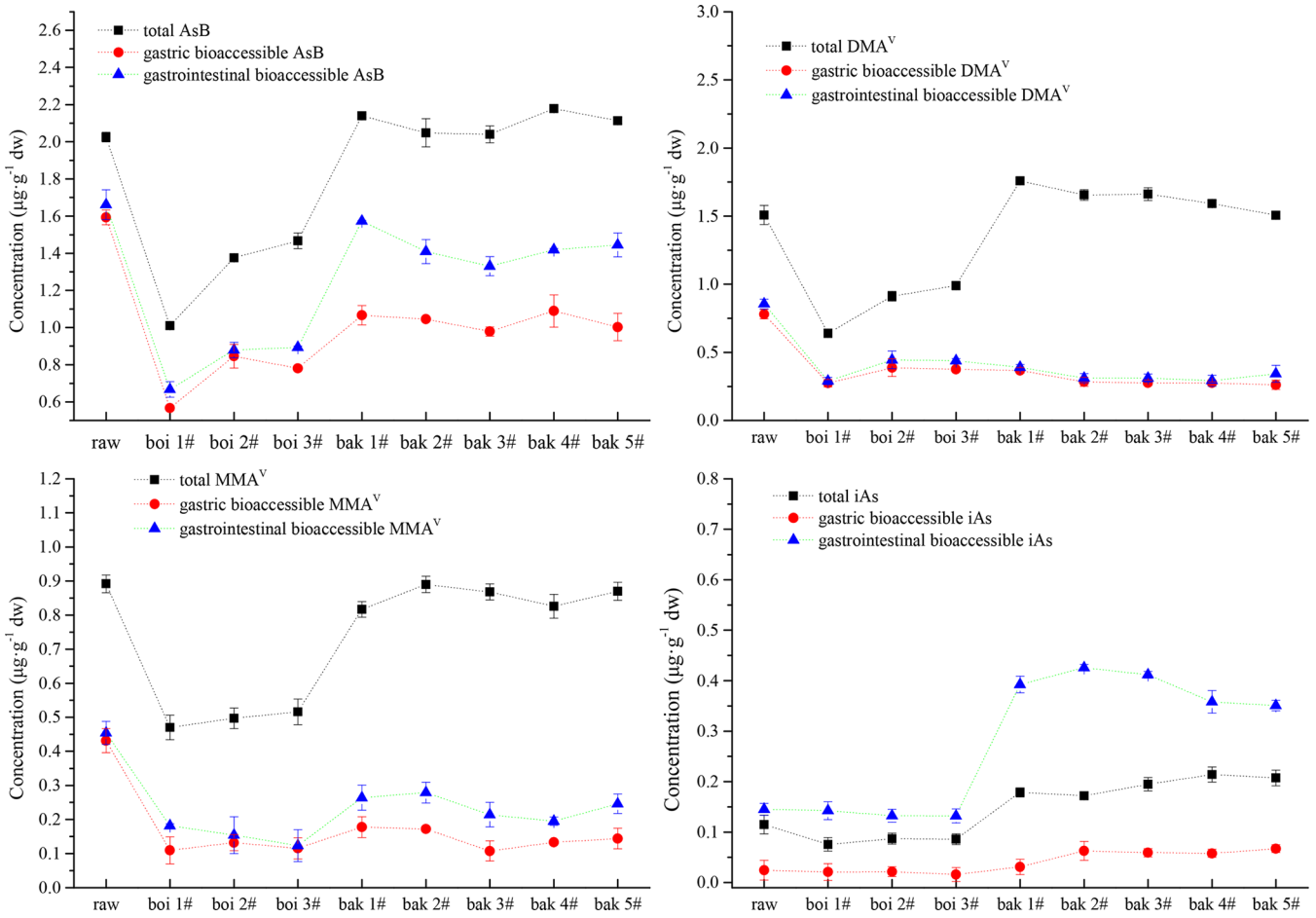

Figure 1. Bioaccessibility of AsB, DMA^V, MMA^V, and iAs in raw and cooked clam (boi: boiled clam; bak: baked clam). Boiled 1#: Boiled with water; Boiled 2#: Boiled with water containing salts; Boiled 3#: Boiled with water containing salts and lemon juice. Baked 1#: Baked with nothing; Baked 2#: Baked with oil; Baked 3#: Baked with oil and pepper; Baked 4#: Baked with oil, pepper and lemon juice; Baked 5#: Baked with oil, pepper, lemon juice, and salts. Total quantity was the concentration in raw and cooked clam samples.

contents in raw shellfish], the data showed that the recovery rates of AsB, DMA^V, MMA^V, and iAs were 108.1%, 78.4%, 71.7%, and 434.6%, respectively. In addition, during boiling, the loss of DMA and MMA (0.4804 $\mu g \cdot g^{-1}$) was slightly larger than the increase of iAs (0.3851 $\mu g \cdot g^{-1}$). Therefore, we assumed that the transformation of DMA^V and MMA^V into iAs^V may have taken place during cooking. Yet more research is needed to gain insight into the true transformation activities.

3.3. Bioaccessible As from Raw and Cooked Shellfish. This section pertains to the bioaccessible As from shellfish obtained through the physiologically based extraction test (PBET) in this study. Generally speaking, high proportions of tAs in the raw shellfish samples were bioaccessible (Table 3), and the percentages ranged from 76.1% to 93.6%, which is similar to the high bioaccessibility of As in seafood reported previously.[15] The bioaccessibility of As is related to the solubility of arsenical compounds in the food matrices in the gastrointestinal tract.[7] As given in Tables 1 and 3, the distribution percentages of chemical forms of As contained in the three types of shellfish were different, and therefore, the values of As bioaccessibility were different as a result.

The bioaccessibility values for various As species are also listed in Tables 1 and 3. The bioaccessibility of AsB was consistent with that of tAs in this study, and these values were much higher than those of DMA^V and MMA^V. Meanwhile, we found that gastric bioaccessible iAs was very low (Figures 1 and 2); however, gastrointestinal bioaccessible percentages of iAs ascended sharply and were apparently 99.9−126.0% (Table 1). The gastric bioaccessible iAs was less than the bioaccessible iAs after gastric plus intestinal digestion steps; one reason for this could have been the presence of pancreatic lipases that favored the solubilization of iAs, and another reason might have been the existence of bile that can form aqueous suspensions of micelles that jointly promote the release of protein-bound iAs and thus increase the iAs bioaccessibility from shellfish after gastrointestinal digestion.[11] The bioaccessibility of iAs in the gastrointestinal phase reached up to 126% in our study, which was in good agreement with the reports of Leufroy et al.,[16] who showed that iAs in scallops is highly bioaccessible following consecutive leaching by digestive reagents (188%). However, they did not discuss the reason. Chávez-Capilla et al.[17] detected As^V in gastrointestinal extracts with rice control samples (no As^V was detected in the original rice samples) and suggested that the detected As^V might have come from the demethylation of MMA and DMA during the gastrointestinal digestion. Zhao et al.[18] also reported that demethylation of DMA and MMA into iAs occurs during the gastrointestinal digestion of seaweed. In our study, through calculations of contents of all chemical forms of As in the gastrointestinal digestion solutions and residues (Table 1), it was demonstrated that there was no significant loss of AsB ($p > 0.05$), while it was observed that about 20.0−47.1% and 24.6−

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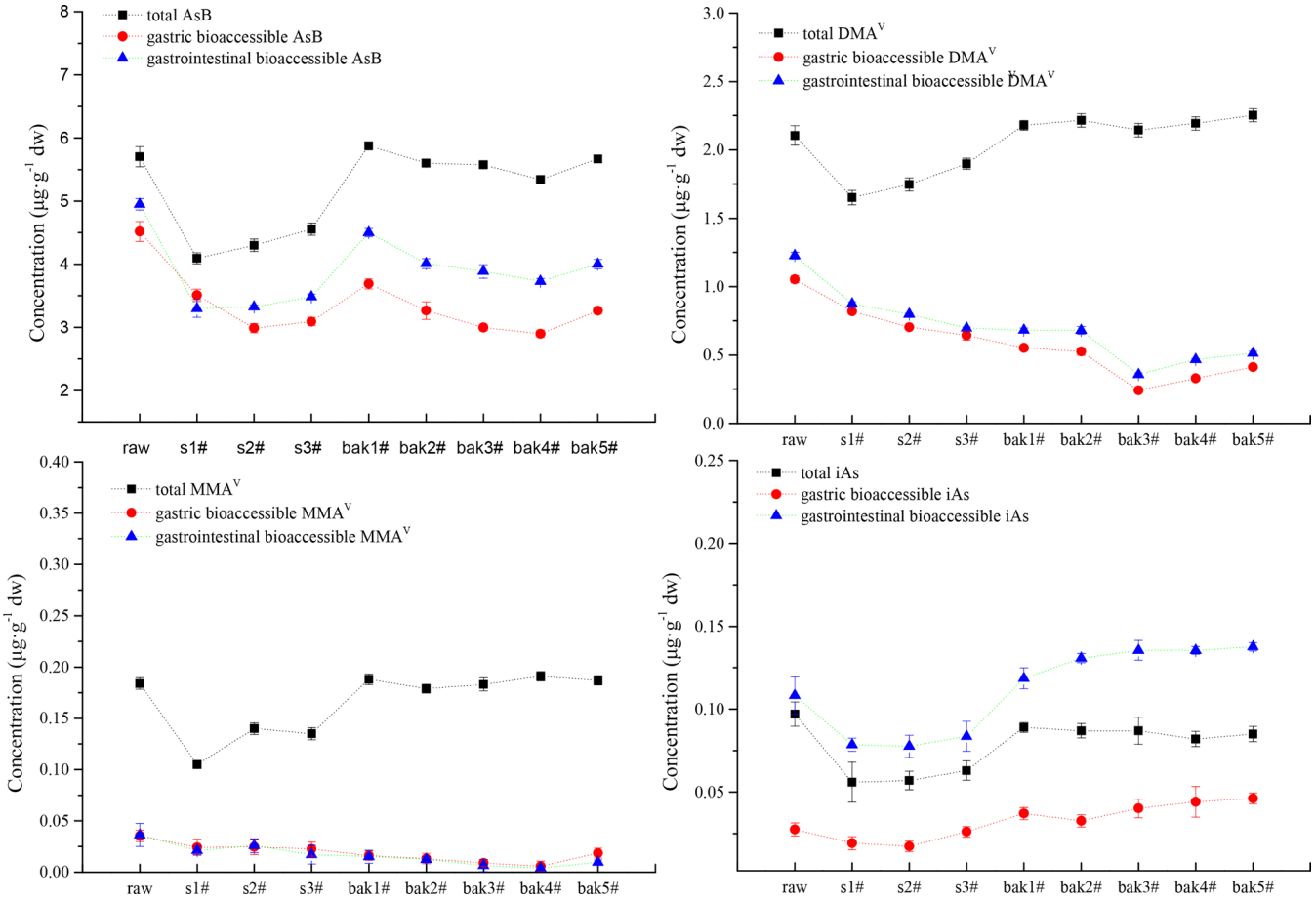

Figure 2. Bioaccessibility of AsB, DMA^V, MMA^V, and iAs in raw and cooked scallop (s: steamed scallop; bak: baked scallop). Steamed 1#: Steamed with water; Steamed 2#: Steamed with water containing salts; Steamed 3#: Steamed with water containing salts and lemon juice. Baked 1#: Baked with nothing; Baked 2#: Baked with oil; Baked 3#: Baked with oil and pepper; Baked 4#: Baked with oil, pepper, and lemon juice; Baked 5#: Baked with oil, pepper, lemon juice, and salts.Total quantity was the concentration in raw and cooked scallop samples.

Table 4. Percents of Inorganic As (iAs^V) to the Total As in Gastric/Intestinal Juice (Means ± Standard Deviation)

Digestion method	Exposed As species	Percents of inorganic As (iAs^V) to the total As (%)*		
		Gastric	Intestinal	Gastrointestinal
In vitro	DMA^V	19.0±2.1	11.5±1.5	23.3±2.8
	MMA^V	3.1±1.5	5.8±1.9	8.4±1.2
	iAs^V	99.8±1.3	99.6±2.1	99.0±1.1
In vivo	DMA^V	12.7±8.5	-	29.3±14.8
	MMA^V	6.8±3.9	-	6.4±3.4
	iAs^V	89.1±9.4	-	78.3±4.1

*The percents of iAs^V to the total As (%) is (iAs^V contents/measured total As contents) × 100%

100% of the DMA^V and MMA^V was lost, respectively. Besides, the losses of DMA and MMA (clams: 0.2466 $\mu g \cdot g^{-1}$; scallops: 0.1179 $\mu g \cdot g^{-1}$; oysters: 0.4830 $\mu g \cdot g^{-1}$) were also higher than the increases of iAs (clams: 0.1453 $\mu g \cdot g^{-1}$; scallops: 0.0684 $\mu g \cdot g^{-1}$; oysters: 0.2921 $\mu g \cdot g^{-1}$) after in vitro tests. Each test was repeated in triplicate, and the average results were used; the average error was less than 10%. Therefore, data in this study indicate that is possible that portions of the bioaccessible methylated As in shellfish are demethylated during intestinal digestion.

Cooking procedures (boiling, steaming, and baking in this study) seemed to be important factors that affected the bioaccessibility of As. As shown in Table 3, the bioaccessibility of tAs from shellfish decreased by 18.5−19.2%, 8.2−26.1%, and 5.4−31.7% after boiling, steaming, and baking, respectively. Notably, As has a high affinity for sulfhydryl groups in peptides and proteins, and heat processing denatures proteins and accelerates the breaking of bonds between As and shellfish proteins, which facilitates its solubilization.[4,23] Thus, the most important process influencing As bioaccessibility in cooked shellfish is the release of soluble As into water, which decreases the As bioaccessibility. In relation to the As speciation in the bioaccessible fraction, cooking lowered the bioaccessibility of AsB from shellfish, and the average percent decrease was 17.0%

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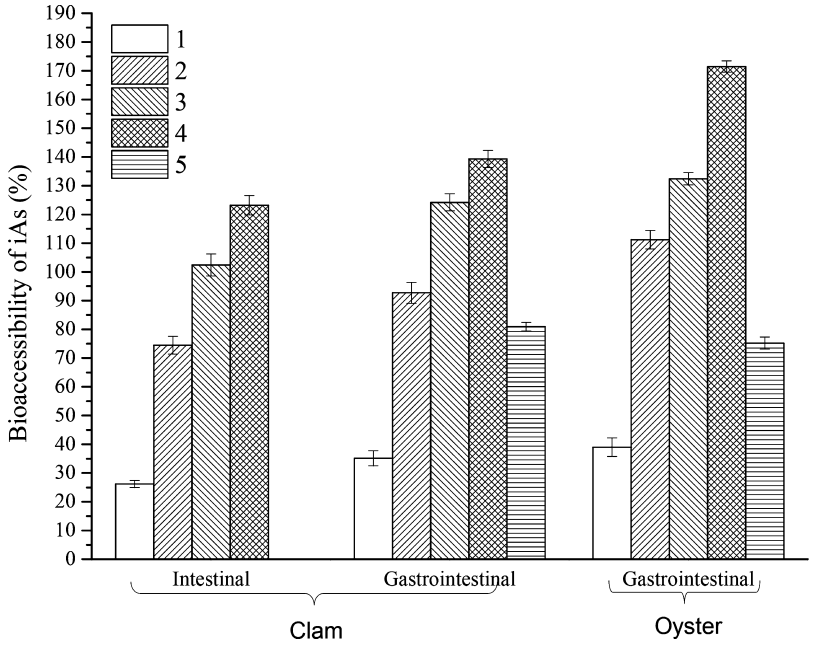

Figure 3. Intestinal/gastrointestinal bioaccessibility of iAs in shellfish (1. nothing added in the intestinal/gastrointestinal digestion, pH = 7.0; 2. adding sodium cholate, pH = 7.0; 3. adding pancreatin, pH = 7.0; 4. adding sodium cholate and pancreatin, pH = 7.0; and 5. adding sodium cholate and pancreatin, pH = 2.0).

($p < 0.05$). Conversely, the bioaccessibility of iAs in shellfish after cooking increased by 17.8−96.7%. However, the contents of iAs in cooked shellfish still accounted for a tiny fraction (Table 3). The demethylation of DMA^V and MMA^V during simulated gastrointestinal digestion could have led to the increase in the iAs bioaccessibility during the cooking of shellfish.[17]

The copresence of other components and shellfish matrices can potentially affect the bioaccessibility of As in the gastrointestinal tract.[7] In this study, we found that the use of spices (oil, salt, and lemon juice) during the cooking processes (boiling, steaming, and baking) helped to reduce the bioaccessibility of tAs ($p < 0.05$). Nonetheless, the reduction effect was not obvious when pepper was added. Consequently, the bioaccessibility of AsB, DMA^V, and MMA^V in cooked shellfish differed from that in raw matrices. Therefore, we have concluded that some spices employed during cooking may reduce the solubility of organic As from shellfish.

3.4. As Demethylation Validation. To verify the internal demethylation of DMA and MMA, *in vitro* and *in vivo* tests of aqueous solutions containing DMA and MMA were also conducted here. As shown in Table 4, the results revealed that the demethylation of DMA^V occurred more readily than that of MMA^V. The difference in the acid dissociation constants between MMA^V ($pK_{a1} = 4.2$) and DMA^V ($pK_{a1} = 6.22$) could explain these results.[34] When DMA^V solutions were used in the *in vitro* tests, the average percentages of iAs^V in relation to the tAs were 19.0%, 11.5%, and 23.3% in the gastric, intestinal, and gastrointestinal digestion solutions, respectively. In regard to the exposures of DMA^V in laboratory mice, the percentages of iAs^V were in the range of 5.1% to 22.8% and 15.3% to 62.3% in the gastric and intestinal tract, respectively. In effect, As methylation could also have occurred during gastrointestinal digestion (Table 4). When the mice were exposed to iAs^V for 2 h, the methylation percentage was on the average of 11% and 22% after gastric and gastrointestinal digestion, respectively. The methylation reaction was not significant after *in vitro* tests, which may have been due to the fact that As methylation under abiotic conditions is more complicated than its demethylation process. As mentioned above, iAs contributed far less to the tAs in shellfish samples (1.1−6.9%). Thus, As demethylation in shellfish during gastrointestinal digestion was an important process observed during our study.

Our findings strongly suggest that the gastrointestinal tract played an important role in As demethylation during As exposures from shellfish. During the digestion of shellfish, the acid environment in a simulated stomach (pH 2.0) will denature shellfish proteins, which can lead to the more efficient hydrolysis of peptide bonds and the release of As during the gastric phase.[11] In this study, the demethylation of DMA and MMA during gastric digestion was not obvious (Figures 1 and 2). As shown in Figure 3, when pancreatin and sodium cholate were both excluded, the average intestinal bioaccessibility of iAs in clams was 26.2%. When pancreatin and sodium cholate were added independently, the intestinal bioaccessible percentages of iAs in clams reached to 74.51% and 102.41%, respectively. When both pancreatin and sodium cholate were included, the value reached up to 123.19%. This tendency was in accordance with the gastrointestinal bioaccessibility of iAs in clams and oysters under different conditions (Figure 3). Besides, acidity and alkalinity can also affect the iAs intestinal bioaccessibility, and neutral or weakly alkaline conditions may suitable for changes in bioaccessibility to occur. Here, we suggest that sodium cholate may have been the determining factor that promoted the demethylation reactions during intestinal digestion and that pancreatin facilitated it in the neutral or weakly alkaline environment. Sodium cholate is a mixture of the bile salts glycocholate and taurocholate, while pancreatin is obtained from secretions of the pancreas and includes a number of enzymes.[36] Thus, more research is needed to gain insight into the mechanisms of the demethylation reactions of As in food during intestinal digestion and the specific chemicals and enzymes involved.

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Given that the results indicate that iAs concentrations in cooked shellfish after simulated gastrointestinal digestion were much higher (up to 247.8%) than those in raw shellfish, this paper presents important information suggesting that risks of As toxicity from shellfish consumption are being underestimated.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS USED

tAs, total arsenic; iAs^{III}, arsenious acid; iAs^{V}, arsenic acid; MMA^{V}, monomethylarsonic acid; DMA^{V}, dimethylarsinic acid; AsB, arsenobetaine; LSD, least significant difference; SD, standard deviation; ND, not detected; DL, detected level; SRM, standard reference material; HPLC, high-pressure liquid chromatography; ICP, inductively coupled plasma; MS, mass spectrometry; dw, dry weight

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