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Comparison of *in vitro* digestion methods for determining bioaccessibility of Hg in rice of China

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ABSTRACT

Rice consumption can be a major pathway for mercury (Hg) exposure, which can cause health hazards to Chinese residents. To accurately assess the potential health risks, the bioaccessibility of Hg in rice samples collected at background and Hg-contamination sites was examined using four *in vitro* methods. The results showed that <50% of the total mercury (THg) was bioaccessible in the studied rice samples. The THg bioaccessibility in the rice samples collected at the Hg-contaminated site was higher than that observed at the background area. The bioaccessibility of arsenic (As) and cadmium (Cd) was also evaluated for comparison of the four *in vitro* methods used in this study. The Physiologically Based Extraction Test was found to be the most accurate method based on the consistency of the results compared to those reported in previous studies. The estimated daily intakes of THg *via* rice consumption using the bioaccessibility data were found to meet the recommendation value set by the JECFA and the WHO in both sites. However, the potential health risk was not negligible at the Hg-contaminated sites, due to the high THg concentration and bioaccessibility in the rice samples.

Introduction

Mercury (Hg) is a toxic heavy metal shown to cause adverse effects to human health. Consumption of fish and rice contaminated by Hg are the primary pathways of human exposure to Hg. The World Health Organization (WHO) estimated that the approximate daily intake of inorganic Hg to be 0.067 μg/kg body weight per day, which was at 3% of the estimated tolerable intake of 2 μg/kg body weight per day (WHO, 2003). The provisional tolerable weekly intake (PTWI) of the total mercury (THg) estimated by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) was 5 μg/kg body weight, *i.e.*, 0.71 μg/kg body weight per day (JECFA, 1972). Female fish consumers aged 15 to 45 were exposed to 0.33 μg/kg body weight per day, on average, as reported by the U.S. Environmental Protection Agency (USEPA, 1997). Compared with the residents of the western countries, Chinese residents do not consume the same amount of fish in their daily diets. In inland China, rice rather than fish is the major pathway for methyl-mercury (MeHg) to enter the human body (Feng and Qiu, 2008; Zhang et al., 2010).

To evaluate the potential health risk of Hg ingestion, *in vitro* experiment is a simple and effective method. The bioaccessibility of Hg in seafood obtained from *in vitro* experiment was

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Table 1 – Basic information of the sampling sites.

Sample no.	Sampling sites (city, province)	Group
HB1	Xiantao, Hubei	Background group
HB2	Enshi, Hubei	
JX1	Ganzhou, Jiangxi	
JX2	Ganzhou, Jiangxi	
GX	Beihai, Guangxi	
GD	Shaoguan, Guangdong	
HN	Changde, Hunan	
ZJ	Hangzhou, Zhejiang	
JS	Yangzhou, Jiangsu	
FJ	Jianou, Fujian	
SC	Nanchong, Sichuan	
GZ	Wangmo, Guizhou	
DB	Heilongjiang	
XJ	Wensu, Xinjiang	
WS1	Wanshan, Guizhou	Mining group
WS2		
WS3		
WS4		
WS5		

applied in many studies gathered from different countries or regions. Various levels ranging from 7.45 to 25.2% and from 10.9 to 33% of total Hg (THg) in gastric and intestinal phases had been reported for fish in Hong Kong (Wang et al., 2013). And the bioaccessibility of THg in 10 fish and seafood species in Spain was reported from 17% ± 6% to 77% ± 6% (Cano-Sancho et al., 2015). Otherwise, the MeHg bioaccessibility of 10 seafood species in North America (Montreal, Canada) ranged from 50.1%± 19.2% to 100% (Siedlikowski et al., 2016); and 42%± 5% to 57% ± 2% in tuna and swordfish in Spain (Cano-Sancho et al., 2015) was reported using *in vitro* experiments. In the aforementioned studies, THg bioaccessibility data were used to calculate the estimated daily intake (EDI) and compared with the recommended limit to accomplish the risk/benefit assessment accurately. Hg ingestion *via* rice consumption should be the dominant health risk for Chinese residents, but under the vacancy of the bioaccessibility data, the assessment of the health risk simply use the THg concentration in rice will not be accurate.

Several *in vitro* methods, including static methods of *In Vitro* Gastrointestinal (IVG), Solubility Bioaccessibility Research Consortium (SBRC), Physiologically Based Extraction Test (PEBT), Deutshe Institut für Normung (DIN) and dynamic model of Simulator of the Human Intestinal Microbial Ecosystem (SHIME) are available for bioaccessibility tests. Each method uses different constituents of simulated gastro-intestinal juice and extraction time. The bioaccessibility of cadmium (Cd) and arsenic (As) in rice was reported in this study and the *in vitro* method was adopted from Zhuang et al. (2016). The U.S. Food and Drug Administration (USFDA) evaluated the health risk of As in rice based on bioaccessibility and bioavailability data derived from *in vitro* and *in vivo* studies (USFDA, 2016). In this study, four static methods (IVG, SBRC, PBET, DIN) were applied for an accurate Hg health risk assessment since the methods have not been applied for Hg in rice. Verification of different methodologies was accomplished using available bioaccessibility data of As and Cd.

In this research, we aimed to (1) ascertain the optimal *in vitro* method for evaluating Hg bioaccessibility in rice using the reported As and Cd bioaccessibility data, and (2) assess the health risk of Chinese exposed in Hg in rice.

1. Materials and methods

1.1. Samples

The rice samples in this study were collected in different provinces of China and separated into the background group (14 samples) and the mining group (5 samples). The sampling sites are listed in Table 1, and the locations of sampling sites were showed in Appendix A Fig. S1 (WS1-5 and JX1-2 were in the map scale, thus only WS and JX were showed in Appendix A Fig.

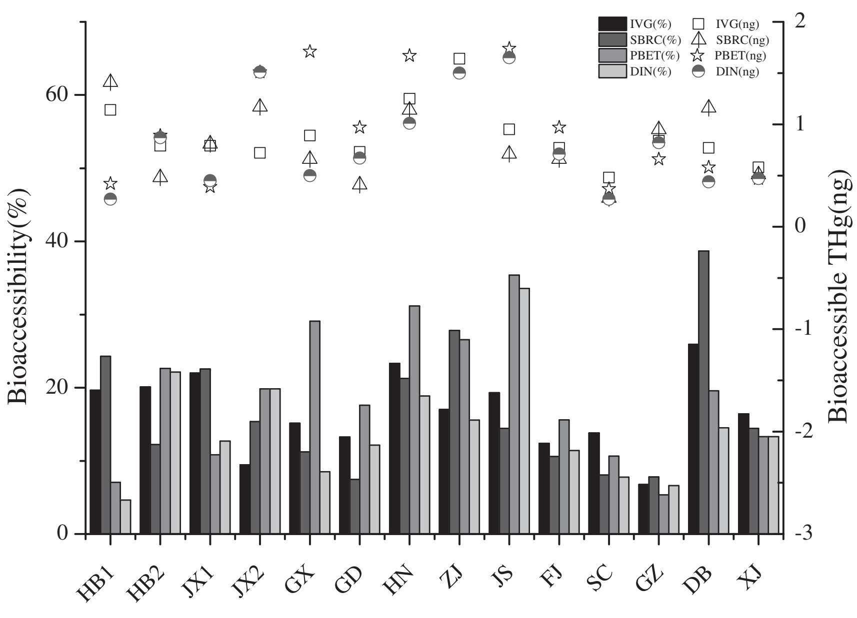

Fig. 1 – THg bioaccessibility and bioaccessible fraction of gastric phase in the background group.

S1). The mining group was collected in the Wanshan Hg mining area, Guizhou, China.

1.2. *Sample preparation and Hg, As and Cd analysis*

Whole rice plants were collected in all sampling sites and only rice seeds were used in this study. Rice seeds were separated into husk and brown rice with a huller and then further polished into white rice. All the white rice samples were ground into 100 mesh and preserved in sealed polyethylene bags. For each sample, 0.5 g of samples of background group and 0.2 g of samples of mining group were weighed into 15 mL centrifuge tubes. Deionized water was added to make up 1 mL volume which was heated in the water bath at 100°C for 20 min to simulate the cooking process, due to the fact that the rice people consumed were normally cooked. For *in vitro* experiment, all samples were weighed 1 g into 50 mL centrifuge tubes and added water to 5 mL, then water bathed in 100°C for 30 min.

Five milliliters of HNO_3 was added to each cooked sample at 95°C for 3 hr to digest the sample for THg analysis and 5 mL of KOH (25% *W/V*) was added to each cooked sample at 75°C for 4 hr to MeHg measurement. After water bath, deionized water was added to 15 mL. Determination procedure were followed the USEPA method 1630 (USEPA, 1998) and 1631 (USEPA, 1996), respectively, with a cold vapor atomic fluorescence system (CVAFS, Model III detector, Brooks Rand Instruments). The analysis of the Cd and As concentrations was followed by the procedure outlined by Zhang et al. (2015) using inductively coupled plasma mass spectrometry (ICP-MS, NexION 300X, PerkinElmer).

1.3. *In vitro bioaccessibility experiments*

Four *in vitro* methods (IVG, SBRC, PBET and DIN) were utilized in this study. Simulated gastric juice was added to the cooked samples to make up a total 50 mL of solution. The mixtures were then shaken at 120 r/min and 37°C for duration as shown in Table 2. After that, each sample was centrifuged for 20 min at 3000 r/min. The supernatant was transferred into another 50 mL centrifuge tube. To the residue, we added simulated intestinal juice to 50 mL, then shaken at 120 r/min at 37°C, and then centrifuged at 3000 r/min for 20 min before the supernatant was collected. The supernatant collected from two extractions were frozen at −18°C until analysis. The ingredients, pH and extraction time of each method are listed in Table 2 (Ng et al., 2013). All chemicals were of reagent grade and obtained from Sigma-Aldrich.

1.4. *Calculation method*

The bioaccessibilities (%) of Hg, Cd and As were calculated by Eq. (1) according to USEPA (2012):

$$\text{Bioaccessibility} = \frac{C_{\text{ext}} \cdot V_{\text{ext}}}{C_{\text{sample}} \cdot M_{\text{sample}}} \cdot 100\% \quad (1)$$

where, C_{ext} (ng/mL) is heavy metals concentration in extraction solution of *in vitro* experiment, V_{ext} (mL) is volume of extraction solution, C_{sample} (ng/g) is heavy metals concentration in samples, M_{sample} (g) is mass of samples.

Table 2 – Ingredients and parameters of four *in vitro* methods.

Method	Ingredients (per L)	pH	Extraction time (hr)
IVG			
Gastric	10 g pepsin, 8.77 g NaCl	1.8	1
Intestinal	3.5 g bile, 0.35 g pancreatin	5.5	1
SBRC			
Gastric	30.03 g glycine	1.5	1
Intestinal	1.75 g bile, 0.5 g pancreatin	7.0	4
PBET			
Gastric	1.25 g pepsin, 0.5 g sodium malate, 0.5 g sodium citrate, 420 μL lactic acid, 500 μL acetic acid	1.5	1
Intestinal	1.75 g bile, 0.5 g pancreatin	7.0	4
DIN			
Gastric	1 g pepsin, 3 g mucin, 2.9 g NaCl, 0.7 g KCl, 0.27 g KH_2PO_4	2.0	2
Intestinal	9 g bile, 9 g pancreatin, 0.3 g trypsin, 0.3 g urea, 0.3 g KCl, 0.5 g $CaCl_2$, 0.2 g $MgCl_2$	7.5	6

The pH was adjusted by HCl (12 M) and NaOH (saturated solution). IVG: *in vitro* gastrointestinal; SBRC: solubility bioaccessibility research consortium; PEBT: physiologically based extraction test; DIN: Deutshe Institut für Normung; SHIME: dynamic model of Simulator of the Human Intestinal Microbial Ecosystem.

After calculation of the bioaccessibility data, the health risks caused by rice consumption were assessed. The estimated daily intake (EDI) (USEPA, 2000) was used for the potential risk assessment. The equation of the EDI was calculated by Eq. (2).

$$\text{EDI} = \frac{\text{C} \cdot \text{IR}}{\text{BW}} \quad (2)$$

where, EDI (ng/(kg·BW·day)) is estimated daily intake, C (ng/g) is the contaminant concentration in rice, IR (g) is the ingestion rate of rice, BW (kg) is body weight.

1.5. QA/QC

The limits of detection (LODs) of THg and MeHg were 0.03 ng/L and 0.02 ng/L, respectively, as determined using the USEPA method 1630 (1998) and 1631 (1996). All data in this study represented duplicate with the relative standard deviation (RSD) ranging from 2.3% to 7.8%.

A certified reference material (CRM), the National Research Center of Certified Reference Material tangerine leaf (GBW-10020), was used to calculate the recovery of the THg, Cd and As. The recoveries were at range of 98.6%–105.7%, 102.8–104.6% and 96.4%–106.7% for THg, Cd and As, respectively.

2. Results

2.1. *Hg, As and Cd concentrations in rice samples*

The concentrations of the THg and As (Table 3) in the background group were all lower than the Chinese National

Table 3 – THg, MeHg, As and Cd concentration in studied samples (ng/g).

Sample ID	As	Cd	THg	MeHg	MeHg/THg (%)
HB1	205.63	0.67	5.75	3.42	59
HB2	33.96	93.56	3.93	2.62	67
JX1	88.24	18.24	3.54	0.96	27
JX2	107.33	758.73	7.61	5.47	72
GX	107.69	229.58	5.88	4.45	76
GD	65.58	18.98	5.51	4.21	76
HN	90.68	11.34	5.36	2.17	40
ZJ	62.17	0.83	9.63	5.73	59
JS	76.99	214.81	4.92	3.67	75
FJ	124.68	23.54	6.22	5.04	81
SC	59.83	55.32	3.47	1.10	32
GZ	127.02	50.66	12.06	10.31	85
DB	106.10	3.38	2.93	1.98	68
XJ	98.47	252.99	3.53	1.70	48
WS1	228.36	13.74	143.45	30.73	21
WS2	184.59	16.33	173.03	40.89	24
WS3	118.17	21.10	144.56	14.77	10
WS4	96.29	195.60	126.02	20.38	16
WS5	108.66	19.86	117.32	10.70	9

Standard of 20 ng/g and 500 ng/g, respectively (NHFPC, 2012). The mining group all exceeded the THg limit, whereas the As concentration were lower than the standard. The concentration of Cd at JX2, GX, JS and XJ exceeded the national standard of a maximum of 200 ng/g.

2.2. Hg, As and Cd bioaccessibility

For background group, the THg bioaccessibility of the IVG, SBRC, PBET and DIN methods were at range of 6.8%–25.9%, 7.4%–38.7%, 5.4%–35.4% and 4.6%–33.6%, respectively. The total quantities of THg which dissolved in the simulated gastro-intestinal fluid were expressed as bioaccessible fraction. The bioaccessible fraction of the THg in gastric phase mostly was below 2 ng due to the low THg concentration in the rice of the background area (shown in Fig. 1). In contrast, the THg bioaccessibility of the gastric phase of the Hg mining area rice was elevated, except the values obtained from the SBRC method (Fig. 2). The bioaccessibility of the PBET and the DIN methods were significantly elevated with a percentage of 28.0%–40.7% and 26.4%–41.9%, respectively. Due to the high THg concentration in the mining group, the bioaccessible fraction was higher than that of the background area, which was over 30 ng in the gastric supernatant of the IVG, PBET and DIN methods.

After the second extraction (intestinal phase), the bioaccessibilities of the THg in background group by the four methods were elevated to 10.3%–47.3%, 11.4%–44.9%, 6.5%–47.0% and 7.5%–36.7% using four *in vitro* methods, respectively, though the bioaccessible fraction of the THg was mostly below 1 ng (Fig. 3). For the mining group (Fig. 4), the THg bioaccessibility estimated by the PBET method were consistently higher than 40%, whereas the values by the IVG and DIN methods were at range of 28.2%–37.8% and 29.8%–45.3%, respectively.

Compared to the THg, the bioaccessible extraction percentage of As and Cd was much higher (Figs. 5 and 6). For each method, the percentage varied significantly. The extraction efficiency of the PBET method was more consistent than that of other three methods in which the bioaccessible percentage of As and Cd was steadily over 65%. The DIN method had the lowest bioaccessible percentage of As. For Cd, the lowest efficiencies were by the IVG and SBRC methods, respectively. The different ingredients and pH values that were used in each of the four *in vitro* methods likely impact the extraction efficiency of different metals.

3. Discussion

3.1. Comparison of Hg bioaccessibility of rice and fish

With four *in vitro* methods, the THg bioaccessibility of background group was all below 50%. The low THg bioaccessible percentages (<50%) were also reported in some fish species (Laird et al., 2009; Cano-Sancho et al., 2015; Wang

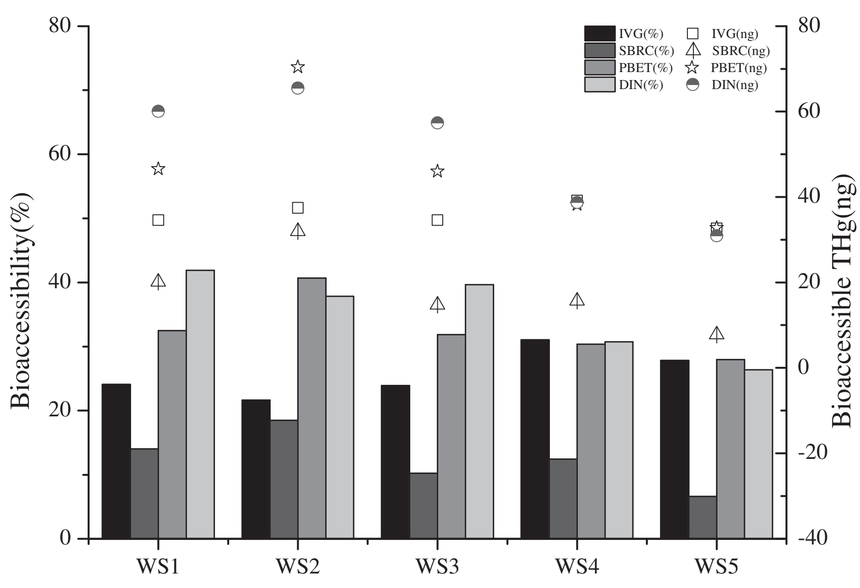

Fig. 2 – THg bioaccessibility and bioaccessible fraction of gastric phase in the mining group.

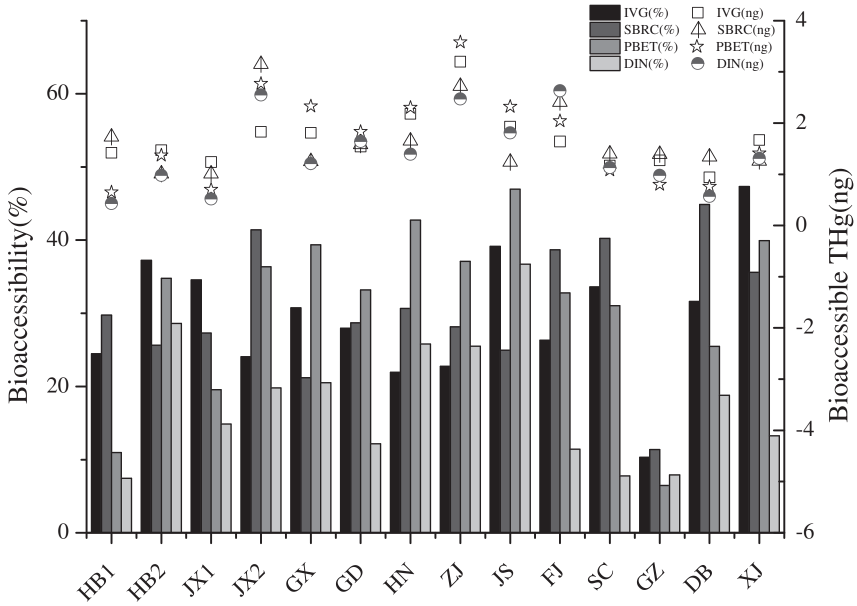

Fig. 3 – THg bioaccessibility of gastric and intestinal phase and bioaccessible fraction in the background group in intestinal phase.

et al., 2013; Calatayud et al., 2012). Similarly, the THg bioaccessibility in rice samples of the background group showed large variation, and this pattern was also observed in across fish species data (Table 4), which indicated that the effects of different species on THg bioaccessibility exists in both food matrices.

Compared with the background group, rice samples in the mining group were same species (Indica rice). Hence, for comparison with single species of rice and fish, the samples of the mining group were chosen. In fish species containing high THg concentration such as the meager (boiled, 220 ± 10 ng/g), tuna (raw, 910 ± 1060 ng/g), blue shark (raw, 2250 ± 660 ng/g), the THg bioaccessibility were reported of 91% ± 5.1%, 78%± 6% and 94%± 3% (Afonso et al., 2015a, 2015b; Matos et al., 2015). Similarly with these fish species, the THg concentrations in the mining group were much higher than the background group. But the THg bioaccessibility in the mining group were below 60%, which were much lower than the values of fish. It was speculated that the inorganic mercury (IHg) in rice grains primarily binds to cysteine (Meng et al., 2014), which was different from the dominance of MeHg cysteine complexes in muscle tissue of fishes (Kuwabara et al., 2007). Consequently, in these two food matrices, the different combination mode of Hg resulted in the difference of THg bioaccessibility.

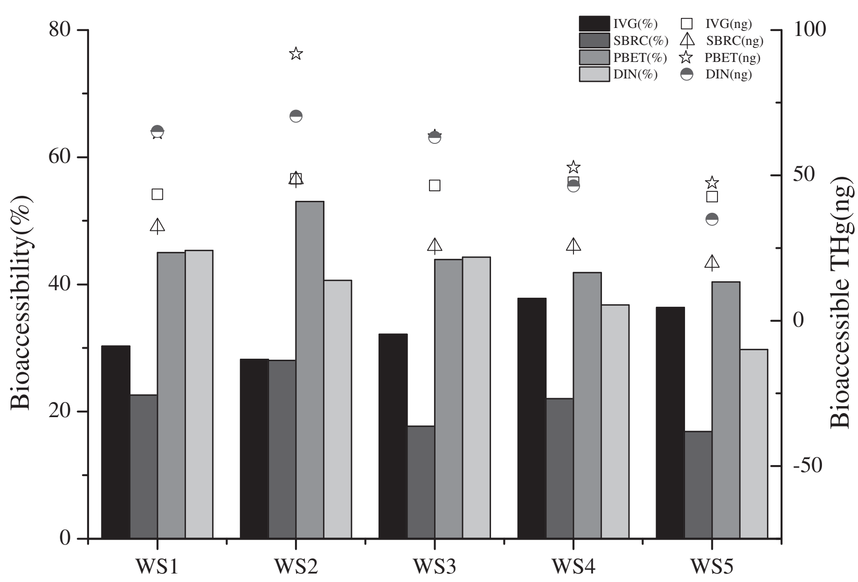

Fig. 4 – THg bioaccessibility of gastric and intestinal phase and bioaccessible fraction in the mining group in intestinal phase.

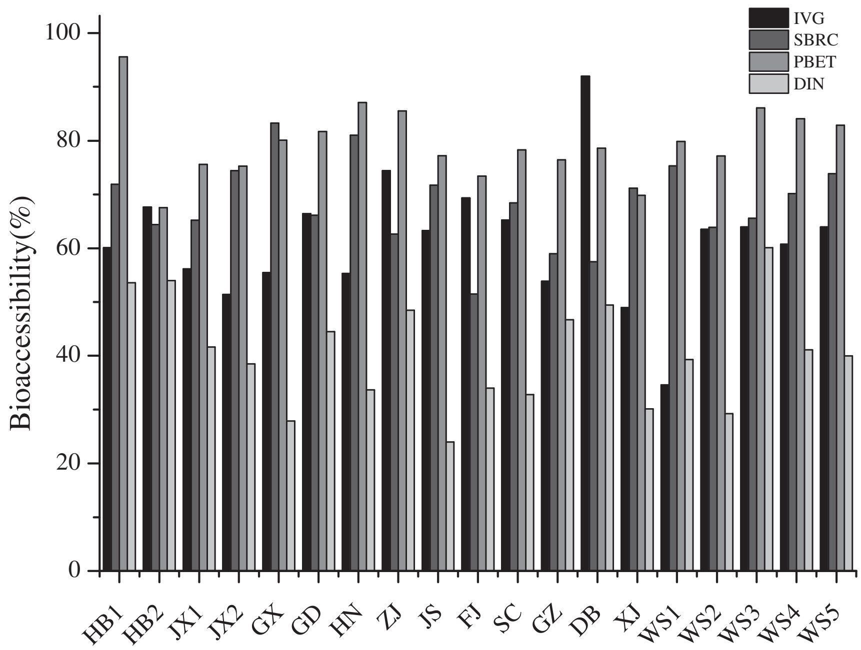

Fig. 5 – The bioaccessibility of As in rice samples by four *in vitro* methods.

3.2. Comparison of THg bioaccessibility between two groups

It is evident that the bioaccessibility percentages of THg in mining group were significantly higher than that of the background group. Two possible explanations are: 1) the impacts from THg and MeHg concentrations; 2) the influences of different rice cultivars. Mean THg and MeHg concentrations in rice samples of the mining group were 141 ± 21.4 and 23.5 ± 12.3 ng/g respectively, about 25 and 6 times higher than those found in the background group.

For the species influences on Hg bioaccessibility, the data from fish studies have been verified in Table 4, which showed large variations of the THg bioaccessibility in different fish species. Similarly, the THg bioaccessibility of the background group showed a large range of variations, besides the MeHg/THg percentage of background group was 27%–85%, similar to the variation of THg bioaccessibility of background group. This may be a result of different rice species used in this study. The rice samples from Northeast China and Xinjiang Uygur Autonomous Region were Japonica rice, while the samples from other

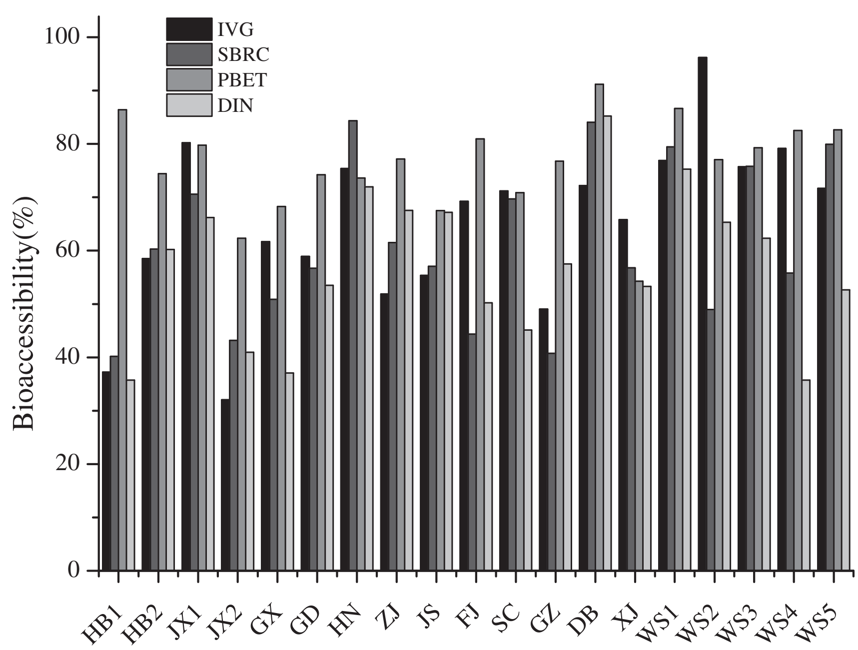

Fig. 6 – The bioaccessibility of Cd in rice samples by four *in vitro* methods.

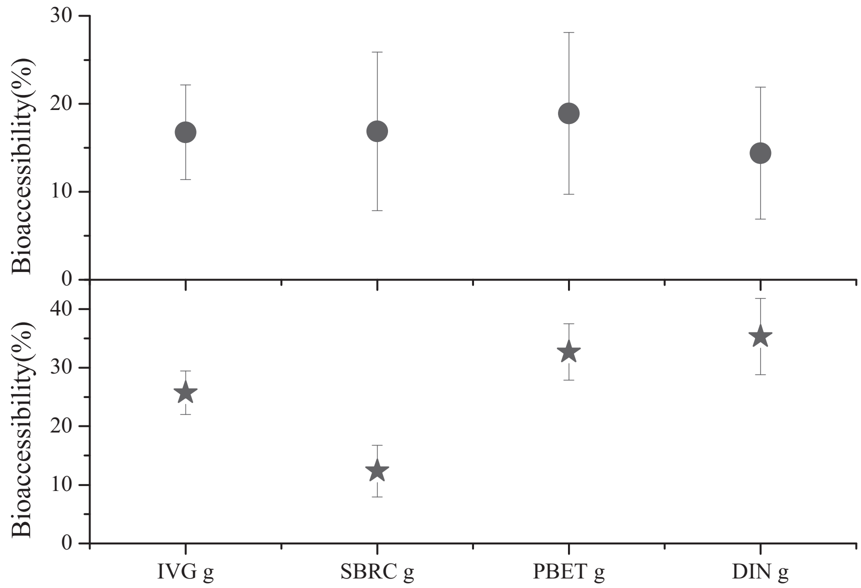

Fig. 7 – Comparison of THg bioaccessibility in gastric phase by four *in vitro* methods. *●—Bioaccessibility data of rice samples in background area; ★—Bioaccessibility data of rice samples in mining area.

provinces were the Indica rice samples. The rice planted in the mining area was Indica rice, thus the bioaccessibilities of mining group was relatively more consistent. The THg bioaccessibility difference caused by rice cultivars needs to be further investigated. The higher THg bioaccessibility of mining group can be explained by: 1) the difference of THg concentration between two groups; 2) the large variation of THg bioaccessibility in background group; 3) the range of THg and MeHg bioaccessibility.

3.3. Evaluation of four *in vitro* methods

The four *in vitro* methods were evaluated for obtaining representative bioaccessible fractions of the THg extraction in the gastric and gastrointestinal phases (Figs. 7 and 8). As seen in Figs. 6 and 7, the PBET method generally showed a higher extraction efficiency of the THg. Moreover, in gastrointestinal phase of the mining group, the PBET had the most consistent extraction percentage among the four methods. In addition, the USFDA (2016) reported a 70%–90% bioaccessibility of inorganic As based on a number of *in vitro* and *in vivo* studies. Caster et al. (2016) reported a 54%–96% of As bioaccessibility in rice. The results in the two studies were comparable with the results obtained in this study using PBET method. Thus, to assess the Hg bioaccessibility in rice, the PBET method should be relatively more accurate.

Table 4 – The bioaccessibility of THg in fish of former researches.

Food matrix	THg bioaccessibility(%)	Reference
Tuna	39 ± 9–78 ± 6	Afonso et al. (2015b)
Salmon	46–49	Laird and Chan (2013)
Meagre	54 ± 14–91 ± 5.1	Afonso et al. (2015a)
Salmon	32.2 ± 9.4–89.8 ± 0.1	Costa et al. (2015)
Freshwater fish (10 species)	32.7–48.7	Wang et al. (2013)
Marine fish (10 species)	21.4–51.7	
Blue shark	52 ± 5–94 ± 3	Matos et al. (2015)
Marine fish (13 species)	35–106	Calatayud et al. (2012)
Marine fish (10 species)	17 ± 6–77 ± 6	Cano-Sancho et al. (2015)

3.4. Potential health risk assessment

The EDI values (Table 5) were estimated based on the bioaccessibility data of the two groups. The body weight (BW) was 66.2 kg and 57.3 kg for males and females, respectively, as reported by the National Health and Family Planning Commission of the People's Republic of China (2015). The rice ingestion rate (IR) was 238.3 g/day for the national average value (Jin, 2008). The mean THg concentration was 6.0 ng/g and 140.9 ng/g for the background and mining group, respectively. The C value was calculated by the average THg concentration multiplied by the average bioaccessibility.

Compared with the PTWI of 0.71 μg/kg body weight per day recommended by the JECFA and the tolerable intake (TI) value of 2 μg/kg body weight per day suggested by the WHO (2007), all the EDI values in this study fell below the reference values. Compared with the MeHg ingestion caused by a dietary intake of 0.04 μg/kg body weight per day in the United States and 0.09 μg/kg body weight per day in France reported by the WHO (2004), the health risk caused by rice consumption for residents in the background area of China was also low. However, for residents in the mining area, the EDI values all exceeded 120 ng/kg body weight per day, higher than that in the background area. The health risk of residents in the mining area was especially notable because rice was staple food of the local residents.

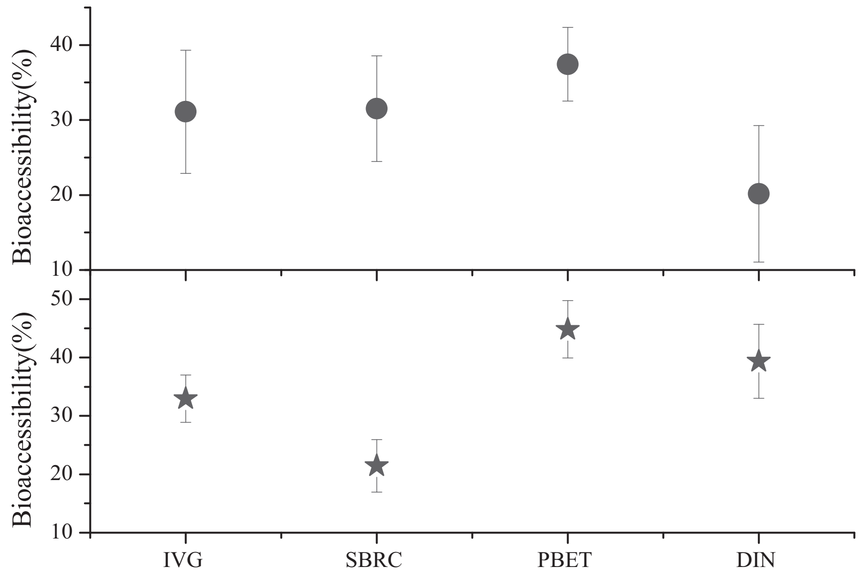

Fig. 8 – Comparison of THg bioaccessibility in gastrointestinal phase by four *in vitro* methods. *●—Bioaccessibility data of rice samples in background area; ★—Bioaccessibility data of rice samples in mining area.

4. Conclusion

This study characterized the THg bioaccessibility of 14 rice samples at the background sites and 5 samples at Hg mining area using four *in vitro* methods. The bioaccessibility of Cd and As were also assessed to evaluate the accuracy of the bioaccessibility estimates. The results showed that the PBET method had relatively higher consistency. The THg bioaccessibility of all samples was <50%, lower than most fish data reported. For residents in the background area, the health risk caused by rice consumption was low, due to the low THg and MeHg concentration in rice. More attention should be paid to residents in the mining area, because the relatively higher THg and MeHg concentration in rice. Thus even the EDI of the THg was below the PTWI and TI values but still much higher than that in the background area. They were confronted chronic exposure of THg and MeHg on rice consumption.

Table 5 – The average bioaccessibility(AVB) and EDI (ng/kg body weight per day) of four *in vitro* methods.

	IVG	SBRC	PBET	DIN
AVB_B (%)	29.43	30.61	31.20	17.90
AVB_M (%)	32.96	21.44	44.84	39.36
Male				
EDI_B	7.59	7.90	8.05	4.62
EDI_M	198.70	129.26	270.31	237.32
Female				
EDI_B	8.77	9.12	9.30	5.34
EDI_M	229.56	149.33	312.29	274.18

Footnote "B" means background group, "M" means mining group. IVG: *in vitro* gastrointestinal; SBRC: solubility bioaccessibility research consortium; PEBT: physiologically based extraction test; DIN: Deutshe Institut für Normung; EDI: estimated daily intake.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jes.2017.10.008.

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