Journal of Engineering and Science Research 6 (2): 01-06, 2022
e-ISSN: 2289-7127

DOI: 10.26666/rmp.jesr.2022.2.1

Analysis of Heavy Metal and Microcystin Bioaccumulation in Local Bivalves

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Received: 13 January 2022; Revised: 29 February 2022; Accepted: 12 March 2022; Published: 30 April 2022

Abstract: Edible bivalves such as cockles, clams, mussels and oysters are considered as an important protein sources among local population. However, allergy to these bivalves has also been increasingly recognized. Production of allergen extracts with high quality, efficacy and safety are very important, as this influences the outcome of the diagnostic tests of allergy. Thus, the aim of this study was to analyse the heavy metals and microcystin concentrations in local bivalves in order to produce bivalve allergen extracts with the lowest/acceptable toxic contaminants. Five species of local bivalves (Malaysian cockle, Asian green mussel, Asian clam, carpet clam and tropical oyster) from several locations in Kuala Lumpur and Selangor were selected in this study. Analysis of heavy metals in the bivalve samples was conducted by Induces Coupled Plasma-Mass Spectrometry (ICP-MS), followed by analysis of microcystin concentrations by qualitative and quantitative tests using the Abraxis Microcystin Test Strip and Abraxis-Microcystin ELISA kit, respectively. Concentrations of heavy metals and microcystin in these bivalve samples were then compared to the permissible limits recommended by Malaysia Food Regulation (1985). The concentrations of Pb, Sn, Hg, Cd and Sb from all bivalve samples were lower than the limits. However, high level of As was found in the tropical oyster and Asian green mussel collected from Wangsa Maju, Kuala Lumpur. Meanwhile, the qualitative test indicated all bivalves samples were negative for the presence of microcystin (0 ppb). In contrary, quantitative analysis of microcystin concentrations in all bivalve samples indicated either none or lower microcystin concentrations when compared to the WHO's provisional tolerable daily intake (0.04 µg kg^{-1}). Hence, as a conclusion, based on the results of heavy metal and microcystin concentrations analysis, Malaysian cockle, carpet clam and tropical oyster from Rawang, Asiatic hard clam from Wangsa Maju and Asian green mussel from Ampang were suitable to be selected as the allergen sources for production of allergen extracts in future studies. In addition, these results also indicated that all bivalve samples are safe for human consumption.

Keywords: *Heavy Metal, Microcystin, Bivalve, Allergen Extract*

1. Introduction

Bivalves provide an essential part of the diet of Malaysian population [1]. The main edible bivalves include cockles, mussels, clams and oysters. Allergy to this type of molluscan shellfish among local populations has been recognized [1]. Despite the fact that uncertainty remains on the exact frequency and allergens involved in bivalve allergy locally, it is clear that this condition is common and is likely to have increased in frequency as it has to other shellfish. The main factor contributing to this uncertainty in

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frequency and allergens involved in local bivalve allergy is the scarcity of allergen extracts for these bivalves group [2].

Allergen extracts are widely used to diagnose and treat allergic diseases [3]. The limited availability of allergen extracts for the bivalve group shows a need for bivalve allergen extract production for use in *in vitro* and *in vivo* diagnostic tests [2]. Several factors influence the quality of the allergen extracts including the source and quality of the raw material used for preparation [3, 4]. The extract should contain the allergens derived from its source material and should not be contaminated with other source materials [3, 4]. This is a prerequisite for efficacy and safety of the extracts, as this influences the outcome of diagnostic tests either *in vivo* or *in vitro* [2, 5].

However, bivalves are filter feeders and feed on zooplanktons, algae and excreta of all aquatic organisms in the bottom sediment of water bodies and therefore accumulate toxic substances such as heavy metals and toxins [6-13]. Bivalves absorbed the toxic substances in the aquatic environment with food particles or through their gills in the process of respiration [12].

Heavy metals synonymously with trace metals are a class of non-degradable pollutants [9, 14]. The bioavailability of heavy metals in the aquatic is higher than in the terrestrial environment [9]. Common heavy metals found in aquatic habitats are arsenic (As), cadmium (Cd), mercury (Hg), lead (Pb), antimony (Sb) and tin (Sn). The discarding of waste, maritime transport and extensive run-off from urban and industrialised areas result in high levels of heavy metal pollution in marine environments [15, 16]. The elevated heavy metal concentrations in aquatic systems have harmful effects on animals living in these environments which include bivalves [9].

Cyanobacteria or previously known as 'blue-green algae' can form extensive blooms in aquatic ecosystems, therefore have a worldwide distribution. Some cyanobacteria produce toxins as secondary metabolites that might bioaccumulate in aquatic organisms including bivalves and are transferred through a food chain [7, 10]. Microcystins are hepatotoxins, the most hazardous toxins produced by cyanobacteria [6, 11]. In the liver, microcystins inhibit the key regulatory enzymes such asserine-threonine protein phosphatases that participate in the metabolism of carbohydrate and lipid, regulation of cell division and apoptosis rates [6]. Microcystins are produced commonly by strains of the genera *Microcystis, Planktothrix, Anabaena,* and *Nostoc* [17]. These toxins are very stable from most water treatment methods and therefore considered to have a high potential for bioaccumulation in aquatic organisms including bivalves [11, 18].

Selection of raw materials is the first step in preparing an allergen extract. The extract should only contain the allergen derived from its source material and should not contaminated with other source materials [3, 4, 19]. Therefore, this study was conducted to analyse the levels of heavy metal and microcystin in local bivalves in order to produce an effective and safe allergen extracts because these toxic contaminants may cause reactions that mimic allergy symptoms. Bivalves with the lowest contaminants or lower than the permissible limits will be selected as the allergen sources for allergen extract production to ensure that toxic contaminants were as low as possible in the extract.

2. Materials and Methods

Bivalve Samples

Five species of commonly consumed bivalves were selected in this study: *Anadara granosa* (Malaysian cockle), *Perna viridis* (Asian green mussel), *Corbicula fluminea* (Asian clam), *Paphia textile* (Carpet clam) and *Crassostrea belcheri* (Tropical oyster). All live bivalve samples were collected from local suppliers in Kuala Lumpur and Selangor as stated in Table 1. The bivalve samples were transported to the laboratory and stored at -20 °C freezer to reduce biological deterioration prior to analysis.

Table 1: Sampling of bivalve species

No.	Location	Species
1	Wangsa Maju, Kuala Lumpur	*Anadara granosa, Perna viridis, Corbicula fluminea, Paphia textile, Crassostrea belcheri*
2	Rawang, Selangor	*Anadara granosa Paphia textile Crassostrea belcheri*
3	Ampang, Kuala Lumpur	*Perna viridis*
4	Bangsar, Kuala Lumpur	*Corbicula fluminea*

Heavy Metal Analysis in Bivalve Samples

Sample Preparation

Before dissection, the bivalve samples were thawed at room temperature. The soft tissues from the bivalves were carefully dissected by removing the byssus, shell and hepatopancreas using a clean stainless steel scalpel. Whole tissues were rinsed with distilled water and subsequently subjected to freeze drying for 3 days. Dried tissues were then homogenized using an agate mortar and pestle and then stored in desiccators prior to analysis.

Sample Digestion

Samples for heavy metal analysis were digested in a Microwave System Multiwave Perkin-Elmer Model 3000. 0.5 g dried bivalves were weighed and placed into the

digestion vessels. 5.0 ml of concentrated analytical grade 65% v/v nitric acid (HNO_3) and 2.0 ml 30% v/v of hydrogen peroxide (H_2O_2) were added to each sample. The vessels were sealed and placed in the rotor for microwave digestion. The digestion of samples during the first phase was set at 600 W, followed by 5-min ramping and holding time. Then, at the second phase, the digestion power was increased to 1,400 W, followed by 5-min ramping and 10-min holding time. Finally, at the third phase, the power was set to zero with a holding time of 15 min. After completed, the digested samples were diluted with ultrapure water to 25 ml. The diluted final test solution samples were then filtered through a 0.45 µm acid resistant membrane. The analytical reagent blanks were also prepared using the same method as mentioned above minus the dried bivalve samples.

Inductively Coupled Plasma-Mass Spectrometer (ICP-MS)

Heavy metal concentrations were measured by ICP-MS. Procedural blanks and quality control samples made from the standard solutions for Arsenic (As), Lead (Pb), Tin (Sn), Mercury (Hg), Cadmium (Cd) and Antimony (Sb) were analysed every seven samples in order to check for sample accuracy. All standard solutions, control and samples were carried out in triplicate. The results were expressed on a dry-weight basis. In order to compare the results with Malaysian Food Regulation (1985)[20] guideline, the heavy metals concentrations in bivalve samples ia converted to wet values using the formula: Dry weight concentration = wet weight concentration x (100/100-moisture percentage). The calculations for the amount of moisture contents were determined based on the method described by other researchers [21].

Microcystin Analysis in Bivalve Samples

Microcystin Extraction

Microcystin extraction was performed following the procedure reported by Leao et al. [22]. 1 g of each dried bivalve sample was weighed. 15 ml of methanol was added to each sample, which was kept for 2 h on an orbital shaker at 80 rpm and then 15 h at 4 °C. Samples were then centrifuged at 5,000 rpm for 20 min. After centrifugation, the supernatants were removed and added to separation funnels and the pellets were re-extracted in 15 ml of methanol followed by centrifugation at 5,000 rpm for 20 min. The new supernatants were added to the previous funnel and 30 ml of hexan was added. After 5 min, the methanolic fraction was evaporated in a rotary evaporator at 55-60 °C while the hexanolic fraction was discharged. After the evaporation process, samples were re-suspended in 1 ml of ultrapure water followed by centrifugation at 14,000 rpm for 10 min. The final supernatants were then analysed for microcystin content.

Microcystin Analysis

Qualitative Test

The Abraxis Microcystin Test Strip was used. 200 µl of the sample was transferred to the conical test vial, shaken for 30 sec and incubated for 20 min. Test strips were then inserted into the conical vial. After 10 min, the test strips were removed and the results were read and interpreted according to the band intensity as described in the manufacturer's instruction.

Quantitative Test

A commercial Abraxis-Microcystin ELISA kit was used following the instructions of the manufacturer. 100 µl of the standard solutions, control and samples were pipetted into the wells of the plate and assayed in triplicate. 50 µl of the enzyme conjugate solutions were then added into each well. Subsequently 50 µl of the antibody solution was added and the wells were then covered with parafilm. The contents of the wells were mixed and incubated for 90 min at room temperature. After completed, the covering was removed and the contents of the wells were decanted. The plate was washed three times with 250 µl of wash buffer solution. The remaining buffer in the wells was removed by patting the plate dry on a stack of paper towels. 150 µl of substrate solution was then added into each well. The wells were then covered with parafilm and mix the contents of the wells. The plate was incubated for 20-30 min at room temperature in the absence of light before the addition of 100 µl of stop solution. The plate was then measured within 15 min using a microplate ELISA reader at 450 nm. All standard solutions, control and samples were carried out in triplicate. Microcystin concentrations in the bivalves were calculated as following:

$$[MC]\ \mu g\ g^{-1} = \frac{\left(\frac{[MC]\ \mu\ L^{-1}}{DHW\ (g)}\right)}{1000}$$

Where:

[MC] µg g^{-1} = Microcystin concentration in 1 g dry bivalve weight

[MC] µ L^{-1} = Microcystin concentration in 1 litre of the bivalve extract

DHW (g) = Dry bivalve weight

3. Results and Discussion

Concentration of Heavy Metals in the Bivalve Samples

Heavy metals present in high concentrations in aquatic

environments are bioaccumulated in the tissues of aquatic animals such as bivalves [9, 23-25]. The heavy metal concentration in bivalve samples studied are presented in Table 2. The concentration of heavy metals in these bivalve samples were compared to the permissible limits recommended by Malaysia Food Regulation (1985) [20]. In comparison with the permissible limits set by national standard for Pb, Sn, Hg, Cd and Sb, the concentrations from all bivalve samples were lower than the limits.

However, high accumulation of As was found in the samples of Tropical oyster (2.043 ppm) and Asian green mussel (1.575 ppm), collected from Wangsa Maju, Kuala Lumpur, which exceeded the limit set by Malaysia Food Regulation (1985) [20]. This probably due to anthropogenic activities in their habitats. Anthropogenic activities have directed to the release of heavy metals into the aquatic habitat [15, 16]. It should be noted that both Tropical oyster and Asian green mussel habitats maybe located along the western coastline of West Malaysia where 60% of the population are concentrated and most of the development activities that have led to an increase in the pollution levels of aquatic habitat [26].

In contrary, the As concentrations in other Tropical oyster and Asian green mussel samples were recorded less than the permissible limit (less than 1.0 ppm). Therefore, mussel and oyster samples from Ampang and Rawang were suitable to be selected for allergen extraction.

Table 2: Concentration of heavy metals in the bivalve samples

Species	Location	Heavy Metal Concentration (ppm)					
		Arsenic (As)	**Lead (Pb)**	**Tin (Sn)**	**Mercury (Hg)**	**Cadmium (Cd)**	**Antimony (Sb)**
Anadara granosa	Wangsa Maju, Kuala Lumpur	0.815 ± 0.007	0.798 ± 0.006	5.004 ± 0.011	0.051 ± 0.003	0.159 ± 0.002	0.004 ± 0.001
	Rawang, Selangor	0.861 ± 0.001	0.612 ± 0.008	4.588 ± 0.175	0.049 ± 0.003	0.153 ± 0.005	0.002 ± 0.001
Paphia textile	Wangsa Maju, Kuala Lumpur	0.937 ± 0.066	0.204 ± 0.011	3.789 ± 0.050	0.006 ± 0.001	0.120 ± 0.001	0.006 ± 0.004
	Rawang, Selangor	0.980 ± 0.021	0.665 ± 0.001	3.985 ± 0.036	0.005 ± 0.001	0.115 ± 0.011	0.003 ± 0.000
Crassostrea belcheri	Wangsa Maju, Kuala Lumpur	2.043 ± 0.076	0.229 ± 0.002	4.904 ± 0.010	ND	0.164 ± 0.010	0.002 ± 0.001
	Rawang, Selangor	0.945 ± 0.021	0.141 ± 0.006	5.010 ± 0.085	0.012 ± 0.002	0.172 ± 0.002	0.004 ± 0.002
Corbicula fluminea	Wangsa Maju, Kuala Lumpur,	0.603 ± 0.009	0.147 ± 0.003	4.254 ± 0.062	0.002 ± 0.001	0.050 ± 0.002	0.004 ± 0.001
	Bangsar, Kuala Lumpur	0.985 ± 0.018	0.097 ± 0.002	1.331 ± 0.075	0.002 ± 0.001	0.047 ± 0.005	0.003 ± 0.001
Perna viridis	Wangsa Maju, Kuala Lumpur,	1.575 ± 0.035	0.062 ± 0.001	3.620 ± 0.092	0.009 ± 0.001	0.030 ± 0.008	0.002 ± 0.001
	Ampang, Kuala Lumpur	0.829 ± 0.042	0.067 ± 0.002	4.099 ± 0.039	0.002 ± 0.001	0.028 ± 0.004	0.002 ± 0.001
Permissible limits (Malaysia 1985)		**1**	**2**	**40**	**0.5**	**1**	**1**

Malaysia Food Regulation (1985), Fourteenth Schedule (Regulation 38)
ND = Not detected

Concentration of Microcystin in the Bivalve Samples

It is known that microcystin accumulates in bivalves [6, 7, 27]. Microcystins are present in diverse organs and also in muscle tissues and other edible parts [17, 25]. Nevertheless, higher microcystin concentrations were accumulated in the hepatopancreas of bivalves indicating that this is the target organ for microcystins [27-30].

Table 3 shows the qualitative results of the microcystin analysis for the bivalve samples. It shows that all bivalve samples tested were negative for the presence of microcystin (0 ppb).

Table 4 indicates the quantitative analysis of microcystin concentrations in the bivalve samples. Lower microcystin concentrations were detected in all the bivalve samples when compared to the WHO's provisional tolerable daily intake (0.04 µg kg^{-1}). On the other hand, Asian clam collected from Bangsar, Kuala Lumpur did not contain any detecteable concentrations of microcystin. The reason for this result is unclear as there is no information about dominant phytoplankton species and the microcystin concentrations in the aquatic habitats where the bivalves were collected. A possible explanation may be that the hepatopancreas of each bivalve samples were removed before freeze drying.

Interstingly, these results indicate that all bivalves samples were considered have no microcystin contamination and therefore safe for consumption. These results also indicated that all bivalve samples were safe for preparation of allergen extracts.

Hence, based on the results of heavy metals and microcystin analysis, Malaysian cockle, carpet clam and tropical oyster from Rawang, Asiatic hard clam from Wangsa Maju and Asian green mussel from Ampang were selected as the allergen sources for production of allergen extracts.

Table 3: Qualitative results of the microcystin analysis for the bivalve samples

Species	Location	Results
Anadara granosa	Wangsa Maju, Kuala Lumpur	Negative (0 ppb)
	Rawang, Selangor	Negative (0 ppb)
Paphia textile	Wangsa Maju, Kuala Lumpur	Negative (0 ppb)
	Rawang, Selangor	Negative (0 ppb)
Crassostrea belcheri	Wangsa Maju, Kuala Lumpur	Negative (0 ppb)
	Rawang, Selangor	Negative (0 ppb)
Corbicula fluminea	Wangsa Maju, Kuala Lumpur,	Negative (0 ppb)
	Bangsar, Kuala Lumpur	Negative (0 ppb)
Perna viridis	Wangsa Maju, Kuala Lumpur,	Negative (0 ppb)
	Ampang, Kuala Lumpur	Negative (0 ppb)

Table 4: Concentration of microcystin in the bivalve samples

Species	Location	Microcystin concentrations (µg kg^{-1})
Anadara granosa	Wangsa Maju, Kuala Lumpur	0.03 ± 0.005
	Rawang, Selangor	0.02 ± 0.013
Paphia textile	Wangsa Maju, Kuala Lumpur	0.02 ± 0.008
	Rawang, Selangor	0.03 ± 0.001
Crassostrea belcheri	Wangsa Maju, Kuala Lumpur	0.03 ± 0.010
	Rawang, Selangor	0.03 ± 0.006
Corbicula fluminea	Wangsa Maju, Kuala Lumpur,	0.03 ± 0.005
	Bangsar, Kuala Lumpur	ND
Perna viridis	Wangsa Maju, Kuala Lumpur,	0.02 ± 0.011
	Ampang, Kuala Lumpur	0.01 ± 0.020
	Wangsa Maju, Kuala Lumpur	0.04 ± 0.010

ND = Not detected

4. Conclusion

In this study, the heavy metal and microcystin bioaccumulations in five species of local bivalves have been successfully determined. Bivalves that have lowest heavy metals and microcystins can be used as the sources of allergen extraction to be used in the *in vivo* and *in vitro* diagnostic tests in our future studies on allergen extraction and identification to ensure that toxic contaminats were as low as possible in the extracts.

5. Acknowledgements

The authors thank Universiti Pendidikan Sultan Idris (UPSI) for the fund award (UPSI 2015-0048-101-01).

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