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Seasonal Variations of Bromophenols in Brown Algae (*Padina arborescens*, *Sargassum siliquastrum*, and *Lobophora variegata*) Collected in Hong Kong

HAU YIN CHUNG,*[,†,‡,§] WING CHI JOYCE MA,[†] PUT O. ANG, JR.,[†,#] JOO-SHIN KIM,[⊥] AND FENG CHEN[||]

Department of Biology, Environmental Science Programme, Food and Nutritional Sciences Programme, and Food Science Laboratory, The Chinese University of Hong Kong, Shatin, New Territories, Hong Kong, China; Pulmuone, Research and Development Center, Pulmuone Company Ltd., Seoul 120−600, South Korea; and Department of Food Science and Human Nutrition, Clemson University, 224 Poole Agriculture Center, Clemson, South Carolina 29634

Distributions and seasonal variations of the key seafood flavor compounds including 2-bromophenol, 4-bromophenol, 2,4-dibromophenol, 2,6-dibromophenol, and 2,4,6-tribromophenol in three species of brown algae (*Padina arborescens*, *Sargassum siliquastrum*, and *Lobophora variegata*) found in Hong Kong waters were investigated. Bromophenols were extracted by simultaneous steam distillation and solvent extraction apparatus and analyzed by gas chromatography−mass spectrometry. On a dried weight basis, the total bromophenol content (TBC) determined varied widely with seasons (from 40.9 to 7030 ng/g). The TBCs detected were higher in winter and lower in summer. Except for 2-bromophenol, the rest of the bromophenols were detected in all of the algal samples. The TBC of *L. variegata* was generally the highest among all of the algae collected. Relatively high concentrations of bromophenols in algae supported the fact that marine algae were major producers of bromophenols in the marine environment.

KEYWORDS: Bromophenol; brown algae; flavor; Asia

INTRODUCTION

The occurrence of bromophenols, a group of key flavor compounds in seafood, has been extensively investigated (*1*−*3*). Bromophenols (**Figure 1**), including 2-bromophenol (compound **1**), 4-bromophenol (compound **2**), 2,4-dibromophenol (compound **3**), 2,6-dibromophenol (compound **4**), and 2,4,6-tribromophenol (compound **5**), were detected in many kinds of seafood. They were important flavor compounds responsible for the sealike and brinelike flavor in marine animals (*1*, *4*). The flavor threshold values of compounds **1**, **2**, **3**, **4**, and **5** in water were 3×10^{-2}, 23, 4, 5×10^{-4}, and 6×10^{-1} ng/g, respectively (*5*). Studies have strongly suggested that the bromophenols present in seafood were obtained from nature (*1*, *5*). Australian marine algae were shown to contain a significant amount of bromophenols contributing to the flavor of marine fish (*3*, *6*). The presence of bromophenols in algae is due to their possession of innate bromoperoxidases, which are a group of enzymes responsible for brominating organic substrates in the presence of bromide ion and hydrogen peroxide (*7*, *8*).

Situated in southeastern China, Hong Kong provides a suitable environment for some marine algae to thrive. These algae are a major dietary source for many marine organisms (*9*). Several species of brown algae such as *Padina* spp., *Sargassum* spp., and *Lobophora* spp. grow profusely in the waters of Hong Kong. These algae are members of the marine benthic communities commonly growing on rocks in subtropical waters (*10*). They are readily available and were collected throughout the year of this study except during the dieback period. These algae are not commonly consumed by humans. However, bromophenols synthesized by the algae could be important flavor components in the organisms used as seafood dwelling in local waters. Information on the levels of bromophenols among the various local algae and their seasonal variations was not available. Therefore, in the current study, the bromophenol contents of selected marine algae commonly found in Hong Kong were investigated, and their seasonal variations were monitored.

MATERIALS AND METHODS

Sample Collection and Preparation. Three species of marine algae, *Padina arborescens*, *Sargassum siliquastrum*, and *Lobophora variegata*, were freshly collected from Tung Ping Chau, a remote island in the

* Corresponding author (telephone + 852-2609-6149; fax +852-2603-5745; e-mail anthonychung@cuhk.edu.hk).
[†] Department of Biology, The Chinese University of Hong Kong.
[‡] Food and Nutritional Sciences Programme, The Chinese University of Hong Kong.
[§] Food Science Laboratory, The Chinese University of Hong Kong.
[#] Environmental Science Programme, The Chinese University of Hong Kong.
[⊥] Pulmuone Co. Ltd.
[||] Clemson University.

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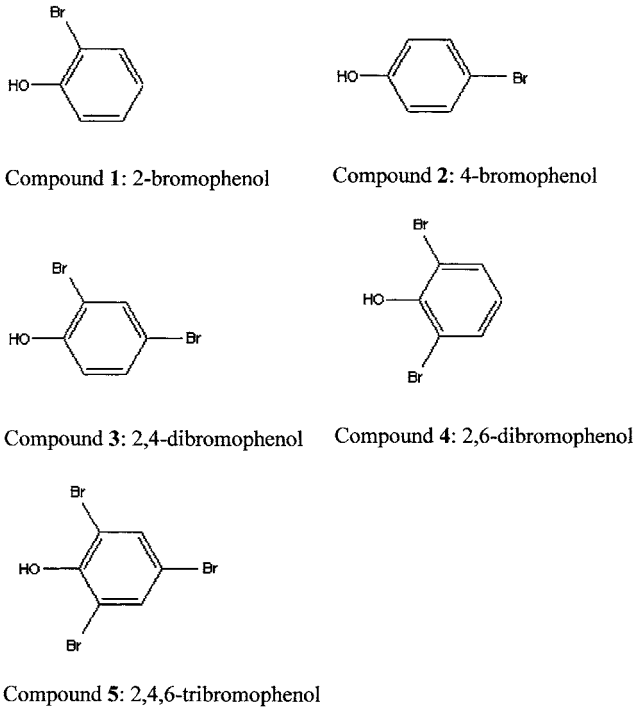

Figure 1. Chemical structures of the five targeted bromophenols.

northeastern part of Hong Kong SAR, China, every 2 months from December 1999 to October 2000. Samples collected were transported to the laboratory immediately. They were gently rinsed with double-distilled water. Excess water was drained out. Samples were packed in plastic bags (23 cm × 30 cm) and stored at −80 °C until extractions of the bromophenols were carried out.

Organic solvents pentane and diethyl ether were purchased from Labscan Ltd. (Ireland) with purities of 99 and 99.5%, respectively. Standard chemical compounds of **1**, **3**, and **4** were bought from Aldrich Chemical Co. (Milwaukee, WI), and **2** and **5** were purchased from Acros Organics (Belgium). The purities of the five bromophenols ranged from 97 to 99%.

Simultaneous Steam Distillation—Solvent Extraction (SDE). The extraction method was adapted from that of Whitfield et al. (*2*, *5*). Each sample, together with 250 mL of double-distilled water, was homogenized with a National blender (MX-T2GM, Matsushita Electric Co. Ltd., Taipei, Taiwan) for 5 min. Treated sample, 1 mL of internal standard (1,3,5-trimethylbenzene, concentration at 84.7 μg/mL), and 250 mL of boiled double-distilled water were transferred to a 5-L round-bottom flask. The sample was then acidified to pH 1 with 96% sulfuric acid. Extraction with 40 mL of pentane/diethyl ether (9:1 v/v) for 2.5 h in a Likens−Nickerson-type SDE apparatus (catalog no. K-523010-0000, Kontes, Vineland, NJ) was carried out. After extraction, the pH value of the residue was measured again to ensure that the acidity was maintained throughout the process. Triplicate extractions of each sample were carried out. Extracts collected were further concentrated to 0.25 mL with a stream of ultrahigh-purity (99.999%) nitrogen gas and were dried by 2.85 g of anhydrous sodium sulfate. Each extract was temporarily stored in a 15-mL conical tube at −80 °C until further analysis.

Gas Chromatography—Mass Spectrometry (GC-MS). A GC-MS system consisting of a Hewlett-Packard 6890 GC coupled with an HP 5973 mass selective detector (MSD) (Hewlett-Packard Co., Palo Alto, CA) was used for qualitative and quantitative analyses. Five microliters of each extract was injected, at splitless mode with the injector temperature at 200 °C, into a fused silica open tubular column (Supelcowax-10, 60 m length × 0.25 mm i.d. × 0.25 μm film thickness, nominal; Supelco, Inc., Bellefonte, PA). Helium gas (ultrahigh-purity grade, 99.999%) was used as the carrier gas with a constant linear velocity of 30 cm/s. Oven temperature was programmed from 100 to 200 °C at a ramp rate of 10 °C/min. The initial and final hold times were 5 and 75 min, respectively. MS interface, ion source, and MS quadrupole temperatures were set at 250, 230, and 106 °C, respectively. Ionization voltage was 70 eV, and electron multiplier voltage was 1200 V. Selected ion monitoring (SIM) mode of the GC-MS was used. Ions monitored for compounds **1** and **2** were at mass/charge (*m*/*z*) 172 and 174; those for compounds **3** and **4** at *m*/*z* 250 and 252; those for compound **5** at *m*/*z* 330 and 332; and those for 1,3,5-trimethylbenzene (internal standard, I.S.) at *m*/*z* 105 and 120 (*2*, *11*, *12*).

Compound Identification and Quantification. The presence of each bromophenol was confirmed by matching a single peak at a corresponding retention time and the presence of their characteristic ions in the selected ion chromatogram with those of the standard compounds under the same chromatographic conditions (*2*).

For quantification, three-point internal standard curves of each bromophenol were established (*2*). Solutions (5 mL) containing 5 mg of each of the five bromophenols were prepared. Serial dilutions at ratios of 1:5 and 1:25 were made. A constant amount of the internal standard, 1,3,5-trimethylbenzene (5 mg), was added to each of the above solutions prepared. Ions chosen for the calculation of response factors among the bromophenols were with *m*/*z* of 172 (monobromophenols), 252 (dibromophenols), 330 (tribromophenol), and 105 (internal standard). The concentration of a bromophenol in a sample was calculated by using the following equation (eq 1):

$$\text{concn of a bromophenol in a sample (ng/g of dry wt)} = \frac{\text{amount ratio of bromophenol/internal standard} \times \text{amount of internal standard (ng)}}{\text{dry wt of sample (g)}} \quad (1)$$

The total bromophenol content (TBC) was calculated by the summation of the concentrations of all five bromophenols.

Extraction Efficiency. The extraction efficiency of the SDE technique on various bromophenols was calculated according to eq 2.

$$\text{extraction efficiency (\%)} = \frac{\text{concn of a bromophenol recovered}}{\text{concn of the bromophenol standard used}} \times 100\% \quad (2)$$

Briefly, it was determined by extracting a known amount of each bromophenol with the SDE method and quantifying the extract and the original standard with the same GC-MS system under the same experimental conditions. The average percentage efficiencies were 99.7 ± 0.4, 38.0 ± 3.2, 99.3 ± 0.9, 93.1 ± 0.3, and 62.5 ± 7.8% for compounds **1**, **2**, **3**, **4**, and **5**, respectively. These values were used to calculate the original amount of bromophenols present in the samples.

Moisture Determination. The percentage moisture of each sample was determined according to the instructions in the operation manual of the Mettler LJ16 moisture analyzer (Mettler-Toledo, Switzerland). The concentration of each bromophenol in marine algae was expressed on a dry weight basis.

RESULTS AND DISCUSSION

Distribution of Bromophenols in Marine Algae. Three species of brown macroalgae, *Padina arborescens*, *Sargassum siliquastrum*, and *Lobophora variegata*, were selected as samples in this study. They were readily available and collected throughout the year of this study except during the dieback period.

Results of the distributions and seasonal variations of the five targeted bromophenols (**1**−**5**) in these three marine algae are shown in **Tables 1**−**3** and **Figures 2**−**4**. Significant amounts of bromophenols were detected in all samples. All five bromophenols were found in both *P. arborescens* and *S. siliquastrum*, but only four bromophenols, **2**−**5**, were found in *L. variegata*. In the majority of the samples investigated, compound **5** was present in the highest amount among other bromophenols. The exception to this was the October 2000 samples of *L. variegata*, in which compound **3** was the highest. In many

Table 1. Seasonal Distribution of Bromophenols in *P. arborescens* Collected from Tung Ping Chau from 1999 to 2000

	bromophenol concn (ng/g of dry wt) in compd				
month	**1**[a]	**2**[a]	**3**[a]	**4**[a]	**5**[a]
December 1999	6.38[b] ± 0.90[c]	25.3 ± 2.6	347 ± 40	10.3 ± 0.68	929 ± 11
February 2000	1.31 ± 0.22	50.5 ± 24.0	116 ± 33	24.3 ± 10.5	547 ± 38
April 2000	59.8 ± 2.9	56.7 ± 3.6	357 ± 20	54.6 ± 3.0	602 ± 54
June 2000	2.33 ± 0.10	9.72 ± 0.86	12.8 ± 3.3	1.54 ± 0.17	14.6 ± 0.9
August 2000	NA[d]	NA	NA	NA	NA
October 2000	44.6 ± 10.0	95.7 ± 14.2	102 ± 7	35.0 ± 7.8	246 ± 37

[a] **1**, 2-bromophenol; **2**, 4-bromophenol; **3**, 2,4-dibromophenol; **4**, 2,6-dibromophenol; **5**, 2,4,6-tribromophenol. [b] Average bromophenol concentration (ng/g of dry weight) from three replicates. [c] Standard deviation of the bromophenol concentration (ng/g of dry weight). [d] Sample not available due to seasonal dieback of algae.

Table 2. Seasonal Distribution of Bromophenols in *S. siliquastrum* Collected from Tung Ping Chau from 1999 to 2000

	bromophenol concn (ng/g dry of wt) in compd				
month	**1**[a]	**2**[a]	**3**[a]	**4**[a]	**5**[a]
December 1999	4.36[b] ± 1.02[c]	1260 ± 30	120 ± 14	15.6 ± 0.8	2430 ± 50
February 2000	0.765 ± 0.051	45.0 ± 4.4	550 ± 32	110 ± 17	1790 ± 210
April 2000	22.9 ± 1.9	109 ± 18	831 ± 134	56.6 ± 8.1	1060 ± 220
June 2000	NA[d]	NA	NA	NA	NA
August 2000	1.18 ± 0.13	45.9 ± 8.5	45.8 ± 5.9	48.0 ± 1.8	345 ± 84
October 2000	13.9 ± 0.5	45.3 ± 3.4	265 ± 15	14.6 ± 2.2	2270 ± 130

[a] **1**, 2-bromophenol; **2**, 4-bromophenol; **3**, 2,4-dibromophenol; **4**, 2,6-dibromophenol; **5**, 2,4,6-tribromophenol. [b] Average bromophenol concentration (ng/g of dry weight) from three replicates. [c] Standard deviation of the bromophenol concentration (ng/g of dry weight). [d] Sample not available due to seasonal dieback of algae.

Table 3. Seasonal Distribution of Bromophenols in *L. variegata* Collected from Tung Ping Chau from 1999 to 2000

	bromophenol concn (ng/g of dry wt) in compd				
month	**1**[a]	**2**[a]	**3**[a]	**4**[a]	**5**[a]
December 1999	ND[b]	73.0[c] ± 19.6[d]	1070 ± 90	23.6 ± 1.3	5870 ± 460
February 2000	ND	244 ± 33	461 ± 141	45.4 ± 4.3	1600 ± 400
April 2000	ND	165 ± 27	340 ± 37	78.8 ± 5.1	975 ± 60
June 2000	NA[e]	NA	NA	NA	NA
August 2000	ND	527 ± 105	736 ± 97	73.5 ± 12.5	1190 ± 150
October 2000	ND	243 ± 14	1280 ± 100	37.4 ± 0.9	1070 ± 130

[a] **1**, 2-bromophenol; **2**, 4-bromophenol; **3**, 2,4-dibromophenol; **4**, 2,6-dibromophenol; **5**, 2,4,6-tribromophenol. [b] Not detected. [c] Average bromophenol concentration (ng/g of dry weight) from three replicates. [d] Standard deviation of the bromophenol concentration (ng/g of dry weight). [e] Sample not available due to seasonal dieback of algae.

samples in which the amount of compound **5** was the most abundant, compound **3** would come next or vice versa. Generally, the concentration of compound **3** among the samples was higher than that of compound **4**. These observations are highly similar to those reported by Whitfield et al. (*6*) in their survey on marine algae from eastern Australia. Brominations at the 2- and 4-positions of the phenolic ring are more favorable than at other positions, and compound **3** is the precursor of compound **5** (*6*).

In *P. arborescens*, all five of the bromophenols were detected in the samples (**Table 1**). The TBCs varied between 40.9 ng/g (June 2000) and 1320 ng/g (December 1999) (**Figure 2**). No sample was available in August 2000 as there was a seasonal dieback of this alga. Among samples of the same species, compound **5** was the most abundant throughout the year. When the TBC concentrations in *P. arborescens* were compared with those of the other two algae, *P. arborescens* always had a significantly lower quantity (ANOVA, $p < 0.05$).

The levels of TBCs in *S. siliquastrum* fluctuated from 486 ng/g (August 2000) to 3830 ng/g (December 1999) (**Table 2**; **Figure 3**). Similarly, sample was unavailable in June 2000 due to seasonal dieback of this algal population. Compound **5** was present in the highest concentration, and compound **3** was the next abundant.

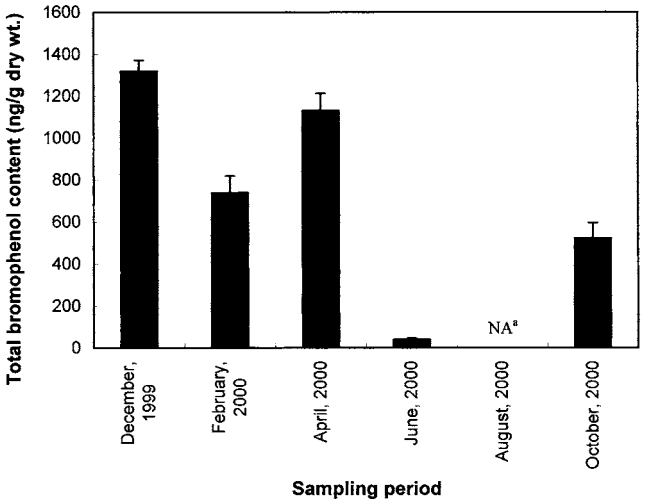

Figure 2. Mean (±SD) total bromophenol contents of *P. arborescens* over time. The mean contents were all significantly different from one another (ANOVA, $p < 0.05$). [a]Sample not available due to seasonal dieback of algae.

The levels of TBCs detected in *L. variegata* collected at different times, except in February and April 2000, were the highest among the three algal samples (**Table 3**; **Figure 4**). The

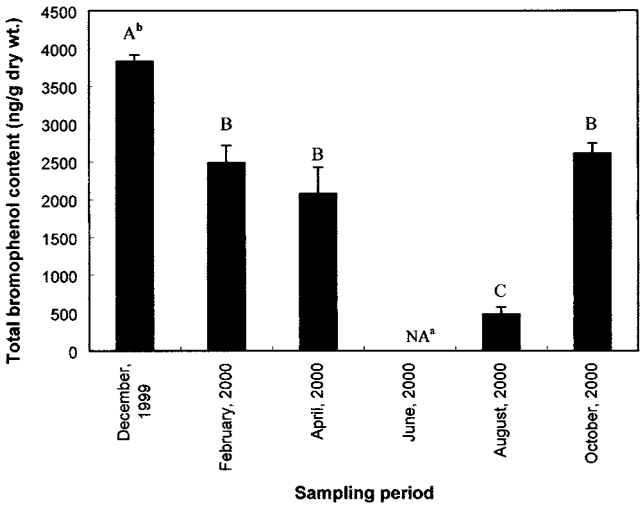

Figure 3. Mean (±SD) total bromophenol contents of *S. siliquastrum* over time. [a]Sample not available due to dieback of plants. [b]Samples marked with different letters were significantly different (ANOVA, $p < 0.05$).

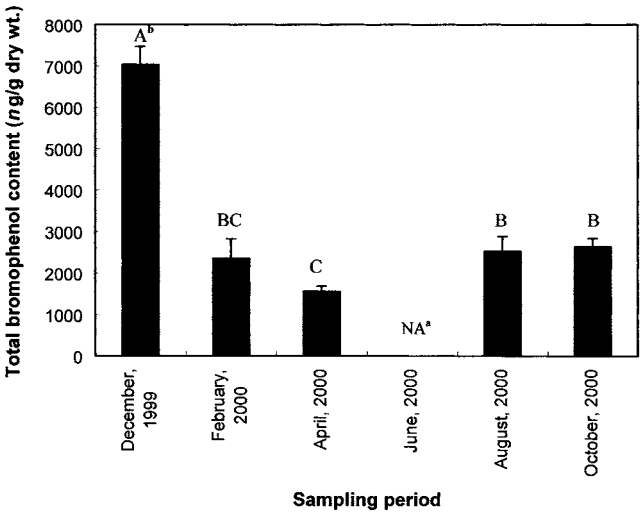

Figure 4. Mean (±SD) total bromophenol contents of *L. variegata* over time. [a]Sample not available due to dieback of plants. [b]Samples marked with different letters were significantly different (ANOVA, $p < 0.05$).

lowest TBC detected in this species was 1560 ng/g in April 2000, and the highest was 7030 ng/g in December 1999. Compound **5** was the most abundant bromophenol for most of the time, and compound **3** was the highest in October 2000.

Seasonal Variations. Concentrations of each bromophenol fluctuated throughout the year of this study, and simple seasonal patterns for most compounds were not observed, except for compound **5** (**Tables 1**−**3**). Compound **5** had the highest level in December 1999 for all three algae and had the lowest in April, June, or August 2000 depending on the species. Among the algae, similar patterns of seasonal variations in the levels of TBC and compound **5** were also observed (**Figures 2**−**4**). The TBC value was used to represent the overall concentration of the five bromophenols. Variation of the TBCs was mainly contributed by compound **5** due to its abundance. All samples had significantly higher TBCs in December 1999 (ANOVA, $p < 0.05$), whereas much lower TBCs were generally detected immediately either before or after the dieback period. This observation might be due to their decreasing rate of bromophenol syntheses coupled with the diffusion of bromophenols from the algae to the surrounding seawater. Dieback of the algae in Hong Kong occurred in summer (June−August) when the temperature was relatively high (28.5 °C). The biosynthesis of bromophenols might be limited during this time. Although some new shoots might also start to emerge during this time from the perennating holdfasts, only a small amount of bromophenols was apparently accumulated in these young algae. Thus, the levels of compound **5** together with TBCs detected were relatively low. When the algae grew again, the levels of compound **5** and TBCs rose simultaneously.

Seasonal variations of bromophenols in the alga *Ulva lactuca* were reported by Flodin et al. (*13*) from Australia. Their bromophenol contents were high in the late Australian summer (February and March) and low during the rest of the year. This is in contrast to the high winter (December) and low summer (June−August) bromophenol concentrations in the three different algal species reported in the present study. Although different species may exhibit different seasonal trends in their bromophenol concentrations, some underlying mechanisms of bromophenol production may be revealed by both of these studies.

Seasonal conditions could likely affect the rate of biosyntheses of various bromophenols. Temperature is an important environmental cue to many phenological developments of marine algae, so it is possible to affect bromophenol synthesis as well (*10*). The average temperature around Sydney, Australia, where the samples of Flodin et al. (*13*) were collected, was relatively lower than that in Hong Kong. For example, the mean temperatures in Sydney in February (summer) and July (winter) were 22.7 and 12.7 °C, respectively (*14*, *15*). With a subtropical climate, the average temperatures in Hong Kong in August 2000 (summer) and December 1999 (winter) were 28.5 and 16.8 °C, respectively (*16*, *17*). High algal bromophenol concentrations were detected in the winter at Hong Kong when the temperature was ∼17 °C, but in the summer at Sydney, Australia, when the temperature was ∼23 °C. A temperature range of ∼17−23 °C may be more suitable for the biosynthesis of the bromophenols in the species of algae studied. Low temperature in the winter (∼13 °C in July) of Australia decreases the production of bromophenols by lowering the activities of bromoperoxidases (*13*). Dieback of the algae occurs every summer in Hong Kong due to overheating and dehydration under strong sunlight and extremely hot weather (∼29 °C in August) (*18*). Thus, the growth of the algae and the biosynthesis of bromophenols are also affected.

Functions of Bromophenols. Marine algae are capable of biosynthesizing bromophenols with the presence of bromoperoxidases (*7*). These enzymes catalyze the bromination of phenol and 2-hydroxybenzyl alcohol to 2,4,6-tribromophenol in red algae (*19*). In green algae, mixtures of the five bromophenols were produced by the bromination of 4-hydroxybenzoic acid and 4-hydroxybenzyl alcohol (*7*, *8*).

Previously, it was believed that these compounds were produced to defend against bacteria, fungi, and grazers (*20*−*23*). However, the results of recent research and the presence of bromophenols in the grazers showed that these compounds were not effective antipredatory or antibacterial agents (*24*, *25*). It is still controversial to make any conclusions on the physiological functions of these compounds in marine algae. When the marine algae are consumed by grazers, these compounds are accumulated. Inadvertently, they become a group of key flavor components perceived by the consumers when the grazers are consumed. Their physiological roles in these grazers are not known.

Bromophenols as Flavor Compounds in Seafood. Bromophenols are found in a variety of marine organisms such as fish (*2*), prawns (*3*), and marine algae (*6*). The organoleptic quality of these compounds in seafood was described as sea-, brine-, or iodine-like (*1*−*4*). It is strongly believed that the detected bromophenols in the seafood were likely derived from the organisms' diet. Marine algae, which can synthesize bromophenols, are a major dietary source of some omnivorous marine animals (*3*, *26*).

In water, the threshold concentrations of compounds **1**, **2**, **3**, **4**, and **5** producing phenolic or iodoform flavor are 3×10^{-2}, 23, 4, 5×10^{-4}, and 6×10^{-1} ng/g, respectively (*3*). At suitable amounts, bromophenols could provide desirable sealike or brinelike flavor and could enhance the intensities of existing seafood flavor (*1*, *2*, *4*). Boyle et al. (*4*) showed that the addition of 10 ng/g of compound **1** in bland marinated whitefish produced a rich, full, and sealike flavor, 0.1 ng/g of compound **4** produced a crab- or shrimplike flavor, and 10 ng/g of compound **5** produced sea saltlike or sea fishlike flavor. However, aquacultured fish and prawns usually contain lower levels of bromophenols than the wild-harvested ones and, thus, they lack such important flavors (*1*, *2*).

As bromophenols are important flavor compounds in seafood (*1*, *4*), their toxicity would be a concern to consumers. These compounds are described as moderately toxic and may cause irritations to skin, eyes, and mucous membranes (*27*−*29*). Lethal doses (LD_{50}) for compounds **1**, **2**, **3**, and **5** administered orally are 6.52×10^5 ng/g (in mouse), 5.23×10^5 ng/g (in mouse), 2.82×10^5 ng/g (in mouse), and 2×10^6 ng/g (in rat), respectively (*30*). However, to produce a lethal effect in humans, it was estimated that 8 million to 600 million prawns should be consumed (*31*). With elevated levels of bromophenols in the seafood, intense iodoform-like off-flavor would be easily recognized by consumers, making them less likely to consume the food (*5*). Therefore, with only a small amount of bromophenols present in seafood (e.g., 9.5−1114 ng/g in Australian prawns) (*2*), the toxic effect may be insignificant.

In this study, we found that bromophenols are present abundantly (TBCs = 40.9−7030 ng/g) among the marine algae investigated in Hong Kong. Besides, seasonal variations in the levels of bromophenols among the algae were observed. The results agreed well with those from previous studies in different areas that marine algae were able to produce bromophenols (*7*, *8*) and were one of the major sources of these compounds in the marine environment (*6*, *25*). As marine alge are food sources for many marine organisms (*9*), the presence of bromophenols in these algae in Hong Kong indicates that these compounds are likely to be accumulated in marine organisms in the local area, providing the sealike or brinelike flavor to these organisms.

Marine algae containing abundant amounts of bromophenols could be utilized to improve the sealike flavor of aquaculture products (*2*, *6*). Aquaculture prawns contained low amounts of bromophenols due to low levels of bromophenols in their diets. This resulted in the absence of sealike flavor in these prawns (*2*). Algae collected in season containing high levels of bromophenols (e.g., December in Hong Kong with TBCs ranging from 1320 to 7030 ng/g of dry weight) can be incorporated into the feeds of aquaculture animals to increase their bromophenol concentrations (*2*, *6*) and, hence, to improve their flavor quality. This approach has not been widely practiced but may soon prove to be a practical way to improve the flavor of aquaculture products.

Overall, to improve and maintain the flavor quality of seafood in the Hong Kong area or nearby locations, protection of the growth of algae for supplying adequate bromophenols to the dwelling organisms and control of the algal growth and harvest for maintaining a suitable accumulation of bromophenols in seafood are essential. By doing so, a balanced supply of the algae and their bromophenols to both the wild marine animals and the farmed seafood can be maintained.

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