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ARTICLES

Improved Approach for Analyzing Bromophenols in Seafood Using Stable Isotope Dilution Analysis in Combination with SPME

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An analytical method for the measurement of five naturally occurring bromophenols of sensory relevance in seafood (barramundi and prawns) is presented. The method combines simultaneous distillation−extraction followed by alkaline back extraction of a hexane extract and subsequent acetylation of the bromophenols. Analysis of the bromophenol acetates was accomplished by headspace solid phase microextraction and gas chromatography−mass spectrometry using selected ion monitoring. The addition of $^{13}C_6$ bromophenol stable isotope internal standards for each of the five congeners studied permitted the accurate quantitation of 2-bromophenol, 4-bromophenol, 2,6-dibromophenol, 2,4-dibromophenol, and 2,4,6-tribromophenol down to a limit of quantification of 0.05 ng/g of fish flesh. The method indicated acceptable precision and repeatability and excellent linearity over the typical concentration range of these compounds in seafood (0.5−50 ng/g). The analytical method was applied to determine the concentration of bromophenols in a range of farmed and wild barramundi and prawns and was also used to monitor bromophenol uptake in a pilot feeding trial.

KEYWORDS: Bromophenols; halophenols; seafood flavor; barramundi; prawns

INTRODUCTION

The natural occurrence of the simple bromophenols 2-bromophenol (2-BP), 4-bromophenol (4-BP), 2,6-dibromophenol (2,6-DBP), 2,4-dibromophenol (2,4-DBP), and 2,4,6-tribromophenol (2,4,6-TBP) in some species of wild-caught ocean crustacea and finfish has been found to contribute to the desirable marine flavors that are absent in the same species produced by aquaculture (*1, 2*). Of the five commonly found congeners, 2-BP, 2,6-DBP, and 2,4,6-TBP are the most sensorily potent, with flavor taste thresholds in water reported at 5×10^{-4}, 3×10^{-2}, and 0.6 ng/g, respectively (*3*).

In fish, bromophenols vary in flavor potency and perception. Studies using bland whitefish as a matrix found the flavor of 2,6-DBP at 0.1 ng/g as crab- or shrimp-like, 2,4,6-TBP at 10 ng/g as sea salt- or sea fish-like, and 2-BP at 10 ng/g as rich, full-flavored and sea-like (*4*). The same study found that when these compounds were present below their individual threshold levels, but in combination, they produced similar flavor nuances in whitefish, suggesting an additive sensory effect.

Although consumers generally appreciate the flavor-enhancing qualities of this group of halophenols, the compounds have also been associated with off-flavor problems when present at elevated levels. Consumer rejection of Endeavor prawn, *Metapenaeus endeavori* (Schmitt), caught commercially in the Exmouth Gulf, Western Australia, is one example of excess dietary uptake of bromophenols by a commercial species having an adverse economic impact on the fishing industry (*3*).

With the exception of two species of marine polychaetes, commonly used as bait by amateur fishers (*5*), bromophenols are not synthesized by marine animals of commercial significance. However, bromophenols are considered to be ubiquitous in the lower levels of ocean food chains, and analytical investigations on a range of marine fish species from Australian and northern Atlantic waters indicate that the presence of these compounds in fish muscle is related to dietary intake (*1, 6*).

Bromophenols can potentially enhance the flavor quality of some marine and freshwater species currently produced by aquaculture. The inclusion of bromophenols in commercial feeds is regarded as essential for their uptake and assimilation in edible

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Table 1. Summary of $^{13}C_6$ Bromophenol Stable Isotope Internal Standard Concentrations ($n = 2$)

$^{13}C_6$ congener	mean concn (μg/mL)	SD
$^{13}C_6$ 2-bromophenol	5.8	0.14
$^{13}C_6$ 4-bromophenol	5.3	0.07
$^{13}C_6$ 2,6-dibromophenol	6.3	0.07
$^{13}C_6$ 2,4-dibromophenol	4.3	0.07
$^{13}C_6$ 2,4,6-tribromophenol	76.1	2.61

tissue. Recent research on cultured prawns and silver seabream (*7, 8*) has shown mixed results, highlighting the need for further work in feed development and aquaculture husbandry. Underpinning this work is the requirement to accurately and reliably measure bromophenol levels in a range of commercial seafood species.

In previous studies, bromophenols have been quantified by gas chromatography−mass spectrometry (GC-MS) analysis of the crude extract obtained by combined steam distillation−solvent extraction (SDE) (*3*). Although this method has proved to be sensitive, it requires the injection of an aliquot of concentrated organic solvent extract directly onto the GC column or precolumn. The high level of volatile oils in these extracts rapidly accumulate on the front of the GC column, causing significant loss of sensitivity (*8*). As a consequence, frequent column trimming is required to maintain analytical performance, thereby reducing the applicability of such methods to routine analysis.

In the current study, we investigate a simple extraction and cleanup method to overcome the analytical inefficiencies associated with the analysis of fatty samples, to allow for routine analysis of bromophenols in seafood samples. The method is based on an alkaline back-extraction of the bromophenols from an organic extract and their subsequent derivatization to their corresponding acetates with quantitative analysis by headspace solid phase microextraction (SPME) and GC-MS using stable isotope internal standards. Data on bromophenol levels in a range of farmed and wild caught barramundi (*Lates calcarifer*) and prawns are presented, and some factors affecting the uptake of bromophenols by barramundi are discussed.

MATERIALS AND METHODS

Chemicals. All reagents were of analytical grade unless otherwise stated. Bromophenol reference standards, antifoam emulsion, and acetic anhydride were supplied from Sigma Aldrich Chemicals Co. Inc. (Australia). Chromatography grade hexane (*Proanalsi*), sodium chloride, and potassium carbonate were obtained from Merck (Australia). Distilled and deionized water was used throughout. Stable isotope $^{13}C_6$-phenol was purchased from Cambridge Isotope Laboratory (Andover, MA).

Preparation of Stable Isotope Bromophenol Standards. The $^{13}C_6$-labeled internal standards were prepared according to the following general procedure [adapted from Blythe et al. 2006 (*9*)]. A solution of $^{13}C_6$-phenol (0.55 mg/mL) was prepared in sodium tetraborate (9.52 g/L in 25 mM HCl) buffer. The $^{13}C_6$-phenol solution (100 mL) was cooled to −2 °C prior to the addition of bromine water (325 μL, 0.29% v/v). After ∼15 s, the solution was quenched with sodium thiosulfate solution (5 mL, 4 g/L) and then acidified with hydrochloric acid (32% w/v) to pH 5. The bromophenols were extracted using dichloromethane (2 × 5 mL), which was subsequently combined, dried (sodium sulfate), and then stored at −18 °C. Quantification of the $^{13}C_6$-bromophenol mixture was achieved in water-spiking experiments with high-purity $^{12}C_6$-analogue reference solutions (**Table 1**).

Barramundi Samples from Commercial Survey. All sample preparation was carried out in a food grade kitchen. Barramundi, whole chilled and frozen fillets or portions packed in 5−10 kg lots, were obtained from a number of commercial sources representing a range of production environments including wild ocean-caught and pond-reared aquacultured product. Upon receipt, the whole chilled fish samples were skinned, deboned, filleted, and then blast frozen (−40 °C) and stored at (−28 °C) until mincing. Fillets to be minced were removed from the freezer 1−3 h (depending on size) prior to mincing and placed on large trays to facilitate thawing. Semifrozen fillets were cut into 2 cm strips and combined thoroughly in a chilled container to obtain a homogeneous bulk sample. Portions of the semifrozen composite sample were passed through the mince attachment (mesh size approximately 4 mm) on a Kenwood KM201 mixer operated at medium speed. The minced fish muscle was subsequently collected in a large prechilled metal bowl, thoroughly mixed, and then dispensed (125 g) into whirl-top bags. The homogeneous minced fish muscle was stored at −18 °C until the time of chemical analysis.

Prawn Samples from Commercial Survey. Fresh samples of farmed and wild-caught prawns were obtained from the Sydney Fish Market (Pyrmont, Sydney, Australia) and transported on ice to the laboratory. Fresh uncooked prawns were used except for the 'Royal Reds', which were obtained in peeled and frozen form. For the fresh prawns, the heads and the muscle tissue from the tails were separated by hand and treated as two separate samples. After dilution (2 parts water to 1 part prawn, w/w), the prawn samples were homogenized in a Waring blender until a fine slurry was obtained. Portions of the prawn slurries were then transferred into cryo-vac plastic bags, heat-sealed, and immediately stored at −18 °C until the time of analysis.

Extraction of Bromophenols. On the day of extraction, frozen minced barramundi or frozen homogenized prawns were thawed in a water bath at 40 °C in sealed plastic bags. For the extraction of barramundi, 100 g of sample was weighed accurately into a 2 L round-bottom flask, and 1000 mL of high-purity deionized water was added. Prawn homogenate (300 g) was accurately weighed into the extraction flask followed by the addition of 800 mL of water.

For both prawn and barramundi samples, the contents of the flask were blended for 40 s using an Art-Miccra D8 blender set at 33000 rpm. After initial blending, 50 μL of a stable isotope internal standard solution (**Table 1**) was added and the mix blended for an additional 20 s. The homogenate was acidified to pH <2 with sulfuric acid and 10 mL of saturated sodium chloride added to the acidified homogenate together with antifoaming agent (2 drops) and a few fused alumina antibumping granules. The sample flask was attached to the distillation head, and the volatile compounds were extracted by combined steam distillation−solvent extraction with 50 mL of hexane for 2 h. Following extraction, the solvent extract was removed from the distillation head and dried over 6 g of anhydrous sodium sulfate for 1 h. The dried extract was then concentrated to a final volume of 5 mL using a Kuderna-Danish apparatus and stored at −18 °C in brown glass screw-cap vials until required.

Back Extraction and Derivatization. Prior to back extraction and derivatization, the sample extracts were removed from −18 °C storage and allowed to come to room temperature before transfer to a tapered glass reaction tube. A 5 mL volume of a 5% (w/v) potassium carbonate solution was added to the concentrated hexane extract in the tapered glass tube. The tube was capped, and the contents were shaken vigorously by hand for 2 min; the phases were allowed to partition overnight. The lower alkaline layer containing the bromophenols was transferred to a clean 20 mL glass headspace vial.

Derivatization of extracted bromophenols to their corresponding acetates was performed using an adaptation of the procedure described by Insa et al. (*10*). Briefly, 200 μL of acetic anhydride was added to the 20 mL headspace vial containing the alkaline extract, and the vial was capped using a crimp seal. A 23 gauge needle was inserted through the septum to allow venting of carbon dioxide produced during the acetylation reaction, and the tube contents were mixed vigorously for 2 min using a vortex mixer. Venting of gas buildup was essential to prevent possible explosion of sample vials. To minimize loss of the volatile bromophenol acetates, the needle vent was removed from the sample vial septum immediately after mixing. The resulting acetylated extracts were subsequently analyzed by headspace SPME-GC-MS.

Instrumental Analysis. Analysis of bromophenols was undertaken using a Shimadzu GC-17A gas chromatograph (GC) coupled to a Shimadzu QP5050A mass selective detector (MSD) operated in selected ion monitoring (SIM) mode. The GC and MSD were controlled by Shimadzu GC-MS Solutions software (version 1.10). Headspace

Table 2. Analysis Parameters for the Determination of Bromophenol Compounds in Fish Using Selected Ion Monitoring

internal standard	RT (min)	target ion (*m*/*z*)	qualifier ion[a] (*m*/*z*) (%)	analyte	RT (min)	target ion (*m*/*z*)	qualifier ion (*m*/*z*) (%)[a]
$^{13}C_6$ 2-bromophenol	25.03	178	180 (99)	2-bromophenol	25.03	172	174 (98)
$^{13}C_6$ 4-bromophenol	25.77	178	180 (99)	4-bromophenol	25.77	172	174 (98)
$^{13}C_6$ 2,6-dibromophenol	28.87	258	260 (47)	2,6-dibromophenol	28.87	252	254 (48)
$^{13}C_6$ 2,4-dibromophenol	29.33	258	260 (50)	2,4-dibromophenol	29.33	252	254 (47)
$^{13}C_6$ 2,4,6-tribromophenol	32.82	336	338 (85)	2,4,6-tribromophenol	32.82	330	332 (98)

[a] Abundance of qualifier ion relative to the target ion.

equilibration and sampling was performed using a Combi-PAL autosampler (CTC Analytics, Zwingen, Switzerland) controlled by Cycle Composer software (CTC Analytics, version 1.5.2).

A 50/30 μm Carboxen/divinylbenzene/polydimethylsiloxane (Car-DVB-PDMS StableFlex, Supelco, Bellefonte, PA) SPME fiber was used for all analyses. In preliminary investigations, PDMS fibers were also evaluated. These fibers were considerably less sensitive than the Car-DVB-PDMS (data not shown), in agreement with other reports, and hence not further investigated.

Prior to sampling, the vials were equilibrated at 60 °C for 40 min. During extraction, the SPME fiber was exposed to the sample headspace for 30 min at 60 °C, then inserted into the heated GC inlet, and desorbed at 260 °C in splitless mode. After 4 min, a 1:50 split ratio was programmed and maintained for the duration of the analysis. The SPME fiber was then moved to the fiber cleaning port for 30 min at 270 °C. The GC column oven was fitted with a DB-1 capillary column (50 m $\times$ 0.22 mm i.d., 1 μm phase) SGE, Australia. The carrier gas was ultrahigh-purity helium (BOC, Australia) set to a flow rate of 0.7 mL/min, linear velocity = 23.7 cm/s. The initial oven temperature was 40 °C for 4 min, then ramped at 10 °C/min to 280 °C, and held for 28 min. The interface temperature was set to 300 °C. Detection of 2-BP, 4-BP, 2,6-DBP, 2,4-DBP, and 2,4,6-TBP as their corresponding acetate derivatives was achieved with the MSD in positive electron ionization mode (70 eV and electron multiplier at 1350 V). A SIM program was developed to monitor the most intense target and qualifier ions for both the ^{12}C and ^{13}C bromophenol congeners (**Table 2**). Scan width and scan interval were set to 0.3 μ and 0.1 s, respectively, and voltage relative to tune was 0.3 kV. The ratio of integrated area counts for the target ions of both ^{12}C and^{13}C bromophenol congeners was used for quantitation. Positive identification was confirmed by the presence of both target and qualifier ions at the correct retention time with the correct ratio as shown in **Table 2**.

Method Validation. A five-point calibration was performed in duplicate by spiking aquacultured barramundi mince that was found to contain very low detectable background levels of bromophenols. Defined quantities of $^{13}C_6$ internal standards and the target $^{12}C_6$ bromophenols were added to fish homogenates prior to final blending. The concentrations of labeled internal standard were constant for each calibration level and were 2.9, 2.7, 3.2, 2.2, and 38.1 ng/g for 2-BP, 4-BP, 2,6-DBP, 2,4-DBP, and 2,4,6-TBP, respectively. The amounts of $^{12}C_6$ bromophenols added at each calibration level were 0.0, 0.05, 0.5, 5.0, and 50 ng/g for all five congeners. To ascertain the repeatability and precision of the method, six extractions and analyses were performed on the 5.0 ng/g spiking level of $^{12}C_6$ bromophenols.

Method Sensitivity. The limits of detection (LOD) based on a factor of 5 times background noise, in prawn, barramundi, and mantis shrimp were 0.008, 0.039, 0.006, 0.003, and 0.023 ng/g for 2-BP, 4-BP, 2,6-DBP, 2,4-DBP, and 2,4,6-TBP, respectively. The limit of quantification (LOQ) was set at 0.05 ng/g on the basis of the lowest addition of the $^{12}C_6$ bromophenol congeners in the calibration range.

Barramundi Feeding Trials. Feeding trials were performed in compliance with the appropriate guidelines for aquaculture trials at Queensland Department of Primary Industries and Fisheries. A batch of 50 live barramundi fingerlings (average size = 100 g) were sourced from a commercial hatchery in July 2006. The fish were housed in two 700 L recirculating seawater tanks with pH, salinity, and temperature maintained at 8 ($\pm$0.2), NaCl 36 ppm ($\pm$0.5 ppm), and 27 °C, respectively.

One tank of 25 fish (treatment 1) was assigned to a high-bromophenol feeding regimen using a batch of frozen Royal Red (*Haliporoides sibogae*) prawn tails previously found to be high in bromophenols (see **Table 8**). The fish in the second tank (treatment 2, 25 fish) were maintained on a diet of frozen Banana prawns (*Fenneropenaeus merguiensis*), found to contain low levels of bromophenols (see **Table 8**). All fish from both treatments were fed until they were satiated. Both high and low bromophenol feeding treatments were maintained for a 5 week period, after which a random sample of three fish from both treatments (average fish weight for treatment 1 was 200 g and that for treatment 2, 166 g) were sacrificed, and the fillets were minced and stored at −18 °C until the time of bromophenol analysis. The remaining fish from both treatments were retained and fed a commercial pellet diet. After a period of 7 days, an additional random sample of three fish from treatment 1 only was sacrificed, and the fillets were treated as described above.

Mantis shrimps (*Squila mantis*) were shown to have a naturally high concentration of bromophenols (**Table 6**) and therefore considered to be a good candidate as a natural source of bromophenols for introduction into barramundi diets. Approximately 100 days after the conclusion of the prawn feeding trials, the remaining live barramundi fingerlings (average weight = 416 g) were divided into two feeding treatments and fed Mantis shrimp for a period of 2 weeks. All Mantis shrimp used as part of this trial were obtained frozen from a local commercial fisher and stored at −18 °C until used for feeding. For treatment 1, six fish were fed Mantis shrimp pieces injected with 0.1 mL of a 1 mg/mL ethanol solution of bromphenols. Treatment 2 consisted of 29 barramundi fed Mantis shrimp only. Both treatments were maintained for a 2 week period with subsequent sampling, fillet mincing, and storage as described above.

RESULTS AND DISCUSSION

Bromophenol Analysis Method. Although the method of extraction of bromophenols is similar to the method described by Whitfield et al. (*3*), this method introduces a novel sample cleanup step as well as the use of the full range of bromophenol stable isotope standards for quantitation of target bromophenols. The addition of the stable isotope standards at the initial stage in the sample preparation process ensures that any subsequent loss of target compounds is accurately accounted for by the corresponding labeled analogue. This effectively negates the need to correct results for recovery levels, which are likely to be variable, particularly during the SPME headspace sampling of the bromophenol acetates.

A linear response was observed for all five congeners across the typical range of concentrations and encompasses previously published flavor thresholds (*3, 4, 11*). **Table 3** demonstrates very good linearity of response for all five congeners over 4 orders of magnitude. These data demonstrate the general applicability of the method to complex matrices such as fish.

Data that support the precision of the method are shown in **Table 4**. Relative standard deviations (RSD) for the recovery of added BPs from fish muscle ranged from 1.18% for 2-BP to 8.12% for 4-BP, indicating that repeatability was acceptable. Whereas a relatively low signal strength for both internal standard and target ions probably accounted for the higher RSD value for 4-BP, method precision for the most flavor active bromophenols (2-BP, 2,6-DBP, and 2,4,6-TBP) were all below

Table 3. Calibration Range Equations and R^2 Values for Bromophenol Compounds in a Fish Matrix [$y = m/z$, x = Concentration of $^{12}C_6$ Congener (ng/g)]

analyte	flavor threshold[a] (ng/g)	calibration range[b] (ng/g)	m/z ratio (peak area)	calibration eq	R^2
2-bromophenol	2 (*3*)	0.05−50	172/178	$y = 0.3138x + 0.0655$	0.9997
4-bromophenol	no information	0.05−50	172/178	$y = 0.2858x + 0.2119$	0.9983
2,6-dibromophenol	0.06 (*3*)	0.05−50	252/258	$y = 0.3421x + 0.0063$	0.9996
2,4-dibromophenol	no information	0.05−50	252/258	$y = 0.5207x - 0.0146$	0.9998
2,4,6-tribromophenol	50 (*11*)	0.05−50	330/336	$y = 0.0256x + 0.0055$	1.0000

[a] Flavor threshold determined in prawn meat. [b] Calibration performed using barramundi muscle.

Table 4. Repeatability Data ($n = 6$) for Quantitation of Bromophenols in a Fish Muscle Matrix

analyte	spike level (ng/g)	mean concn measured (ng/g)	SD	RSD (%)
2-bromophenol	5.2	5.3	0.019	1.18
4-bromophenol	5.5	5.4	0.436	8.12
2,6-dibromophenol	5.0	5.8	0.227	3.88
2,4-dibromophenol	5.0	5.9	0.244	4.14
2,4,6-tribromophenol	5.1	5.0	0.238	4.73

5%. The mean measured concentrations observed were found to be slightly higher than the known spiked level for 2-BP, 2,6-BP, and 2,4-BP. This is likely to be due to existing trace levels of these congeners in the fish matrix.

^{13}C Bromophenol Stable Isotopes. Positive ion mass spectra for both $^{12}C_6$ 2,6-dibromophenol and $^{13}C_6$ 2,6-dibromophenol acetates are shown in **Figure 1**. During electron impact (EI) fragmentation, the acetate group is removed and the characteristic bromophenol parent ion clusters are produced. Natural bromine isotopes ^{79}Br and ^{81}Br are present in almost equal abundance. It can be clearly seen that the two clusters at mass/charge ratios (m/z) 250, 251, 252, 253, and 254 for the ^{12}C compound and m/z 256, 257, 258, 259, and 260 for the ^{13}C analogue share no common ion fragments. This was also the case with the other ^{12}C and ^{13}C bromophenol congeners. The lack of overlap of ion fragments between the ^{12}C and ^{13}C bromophenol congeners represents a considerable analytical advance compared with previously reported methods employing the use of deuterated standards. Deuterated bromophenol standards share common ions with their nondeuterated congeners, making reliable quantification more complicated (*11*). Furthermore, in the same study, chromatographic conditions needed to be optimized to maximize the separation of the mass spectral clusters. The use of ^{13}C bromophenols obviates the need for any special chromatographic separation.

The present work demonstrates the improvement of bromophenol analysis using $^{13}C_6$-labeled standards. An additional advantage of using $^{13}C_6$-labeled standards as opposed to partially deuterated standards is that fragmentation patterns are, other than for a shift in mass, identical. Consequently, equivalent fragmentation ions can be considered to have identical responses for the same concentrations in labeled and unlabeled bromophenols.

The inclusion of a $^{13}C_6$ stable isotope analogue for each of the five bromophenols simplifies the calculation of bromophenol levels in fish by eliminating the requirement to develop extensive standard addition calibration curves. The value of bromophenols in fish muscle (ng/g) was easily determined by the ratio of integrated areas of the target analyte quantitative ion and the corresponding stable isotope quantitative ion.

The problems associated with the direct injection of an organic extract of fish muscle (*8*) have been avoided. This has been achieved by the partitioning of the bromophenols, from the fatty solvent extracts, into an alkali solution before acetylation. The inclusion of this cleanup step in the sample workup procedure greatly minimized inlet and column contamination and loss of sensitivity. Moreover, during the course of this study, the GC inlet was regularly inspected, and no evidence of contamination was observed. The derivatization of bromophenols to their corresponding less polar acetates was a necessary step to avoid the poor chromatographic performance encountered when underivatized halophenols are assayed by GC (*12*).

Analysis of Bromophenols in Prawns and Barramundi. Bromophenol concentrations for a range of commercially produced wild and aquacultured barramundi are given in **Table 5**. The concentrations of bromophenols found in these barramundi varied minimally, with the exception of 2,6-DBP, which was present at a significantly higher concentration in the "wild 4" sample (0.1 ng/g) compared to the other samples. All of the measured bromophenol concentrations were below detectable flavor or sensory thresholds (*11*). It was expected that bromophenol concentrations would be significantly higher in the wild-caught samples, compared to aquacultured, in agreement with similar investigations in the literature (*2*). In the 2006 season, there had been irregular cyclone activity and heavy flooding around the time of harvest. Under such conditions, large inflows of fresh water into the normally saltwater-dominated estuarine habitats can occur, and barramundi are often caught in these estuarine or semiestuarine environments. Because bromophenols in barramundi are derived exclusively from marine diets, the shift to a freshwater diet would likely be accompanied by a diet with minimal concentrations of bromophenols. Importantly, the sensorially significant bromophenols have low octanol−water partition coefficients (*1*)and do not bioaccumulate in fish lipids. As suggested by previous research (*11, 13*), the equilibrium concentration of bromophenols in seafood flesh at the time of capture reflects the balance between dietary intake and depuration processes.

Levels of bromophenols in feed used in the preliminary feeding trial are given in **Table 6**. The influence of dietary levels of bromphenols and uptake in barramundi is demonstrated by the data in **Table 7**. Initial feeding trials compared the uptake of bromophenols in fish fed Royal Red prawns containing relatively high levels of endogenous bromophenols with those fed a low bromophenol diet based on Banana prawns. Despite the differences in dietary bromophenol content, almost no differences in bromophenol concentrations were measured immediately postfeeding. Both feeding regimens resulted in low but measurable concentrations of bromophenols. These measured concentrations were generally much lower than typical data published on ocean fish (*1, 4, 14*). After 7 days on the normal commercial pellet feed, no detectable bromophenols were measured, indicative perhaps of rapid depuration.

Despite the relatively high concentration of bromophenols measured in Mantis shrimp, very similar bromophenol uptake to the Royal Red and Banana prawn diets was measured. Similarly, upon cessation of the Mantis shrimp feed, the concentration of bromophenols rapidly decreased. In contrast, higher concentrations of bromophenols were found in fish that had been fed Mantis shrimp spiked with a 1.0 mg/mL solution

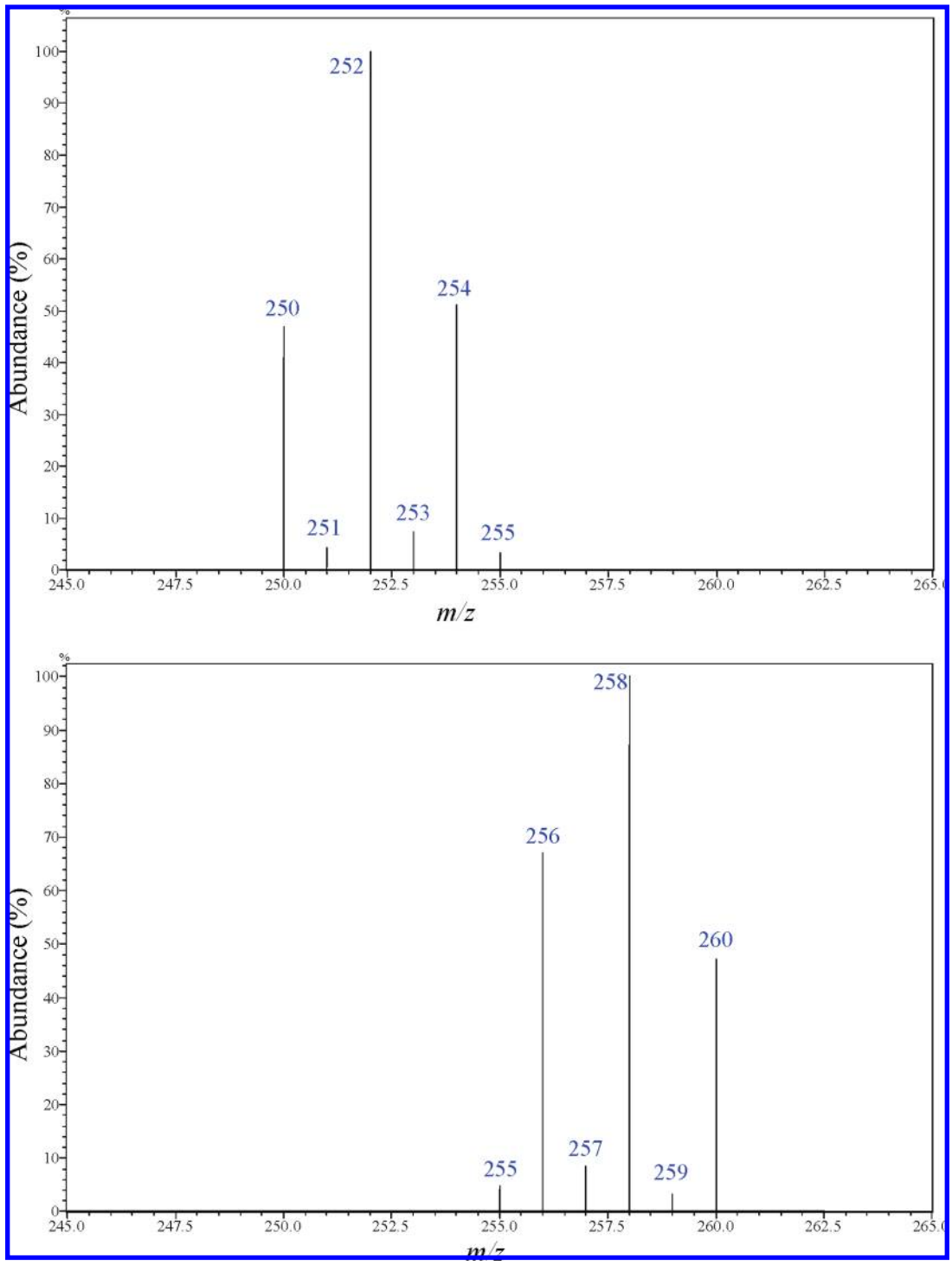

Figure 1. Positive ion electron impact mass spectra (*m*/*z* 240−275) of $^{12}C_6$ 2,6-dibromophenol acetate (top) and $^{13}C_6$ 2,6-dibromophenol acetate (bottom).

of bromophenols (**Table 7**). It was noteworthy that the apparent rate at which the accumulated bromophenols were depurated following their exclusion from the fish's diet was similar to the rate of depletion from fish on a natural diet. From these data, the 2-BP, 4-BP, and 2,4-DBP accumulated in the muscle tissue by dietary uptake were completely depurated after 7 days. Depuration of 2,6-DBP and 2,4,6-TBP was also substantial with only 2.3 and 2.6%, respectively, remaining in the muscle tissue after this time. Similar results have been reported for the loss of bromophenols from Pacific salmon as they cease feeding during their transition from a marine to a freshwater river environment (*1*). We attribute the depuration of bromophenols to their octanol−water partition coefficients (log *P*). Although the log *P* values for the bromophenols are sufficiently low (<6) to promote rapid uptake, none of the congeners would be expected to strongly bioaccumulate as log *P* values of >5 are required. It can be speculated that a mechanism similar to that reported for salmon may be driving the uptake and depuration rates of bromophenols in barramundi and may explain the low levels found in the wild-caught fish evaluated in this work.

Bromophenol concentrations measured in the prawns purchased from Sydney Fish Market are listed in **Table 8** None of the farmed prawns exhibited levels of bromophenols in the edible flesh sufficient to influence flavor. In contrast, the

Table 5. Bromophenol Levels in Commercially Sourced Barramundi (*Lates calcarifer*) Fillets

	bromophenols (ng/g)				
sample	2-BP	4-BP	2,6-DBP	2,4-DBP	2,4,6-TBP
farmed 1	0.05	<0.05	<0.05	<0.05	0.16
farmed 2	<0.05	<0.05	<0.05	<0.05	0.09
farmed 3	<0.05	<0.05	<0.05	<0.05	0.11
wild 1	<0.05	<0.05	<0.05	<0.05	0.04
wild 2	<0.05	<0.05	<0.05	0.14	<0.05
wild 3	<0.05	<0.05	<0.05	<0.05	0.09
wild 4	<0.05	<0.05	0.10	<0.05	0.11

Table 6. Bromophenol Levels in Farmed Barramundi Feed

	bromophenols (ng/g)				
feed type	2-BP	4-BP	2,6-DBP	2,4-DBP	2,4,6-TBP
prawn, Royal Red	<0.05	<0.05	13.42	5.54	6.34
prawn, Banana	<0.05	<0.05	<0.05	<0.05	0.18
Mantis shrimp	1.46	10.35	3.41	143.99	113.59

Table 7. Bromophenol Levels in Farmed Barramundi Fed a Range of Diets

	sampling time post-treatment	bromophenols (ng/g)				
barramundi feed	(days)	2-BP	4-BP	2,6-DBP	2,4-DBP	2,4,6-TBP
prawn, Royal Red	0	<0.05	<0.05	<0.05	<0.05	0.15
prawn, Royal Red	7[a]					
prawn, Banana	0	<0.05	<0.05	<0.05	<0.05	0.13
prawn, Banana	7[a]					
Mantis shrimp	0	<0.05	<0.05	<0.05	0.05	0.17
Mantis shrimp	7[a]					
Mantis shrimp + BPs	0	3.07	0.80	3.47	2.16	19.52
Mantis shrimp + BPs	7	<0.05	<0.05	0.08	<0.05	0.51

[a] Bromophenol analysis not performed.

Table 8. Bromophenol Distribution in Prawn Samples Collected from the Sydney Fish Market

	bromophenols (ng/g)									
	2-BP		4-BP		2,6-DBP		2,4-DBP		2,4,6-TBP	
sample[a]	heads	flesh	heads	flesh	heads	flesh	heads	flesh	heads	flesh
a	<0.05	<0.05	0.06	<0.05	0.22	<0.05	0.12	<0.05	0.49	0.14
b	<0.05	<0.05	0.12	0.16	0.25	<0.05	0.40	0.31	0.48	0.17
c	<0.05	<0.05	0.36	0.10	0.26	<0.05	0.30	0.09	0.49	0.14
d	<0.05	<0.05	0.19	<0.05	0.22	0.09	0.44	0.08	1.16	0.16
e	0.08	<0.05	5.33	1.83	3.31	0.50	16.06	3.86	10.83	2.79
f	0.13	0.07	18.03	22.07	0.58	0.07	20.73	6.08	40.08	3.40
g	0.33	<0.05	153.18	7.57	1.12	0.08	215.22	14.41	13.77	1.25
h	na[b]	0.58	na	1.40	na	35.69	na	8.45	na	5.68

[a] a, Black Tiger (*Penaeus monodon*), farmed, uncooked; b, Banana (*Fenneropenaeus merguiensis*), farmed; c, Vannamei *(Litopeneaus vannamei*), farmed, cooked; d, School (*Metapenaeus macleayi),* wild, uncooked; e, King (Coffs Harbour) (*Melicertus latisulcatus*), wild, cooked; f, King, Clarence River (*M. latisulcatus*), wild, uncooked; g, King (Spencer Gulf) (*M. latisulcatus*), wild, uncooked; h, Royal Red (*Haliporoides sibogae*), wild, cooked. [b] na, not assayed.

concentration of the potent 2,6-DBP congener was found to be close to the reported sensory threshold of 0.06 ng/g in the flesh of wild samples d, f, and g and significantly higher than the flavor threshold in samples e and h. For the majority of the evaluated samples, the levels of bromophenols found in the head of the animal were significantly higher than those found in the flesh. This is in agreement with a previous study that reported bromophenol levels for a range of wild and farmed prawns (*2*).

The method described here provides a significant improvement over previous analytical approaches to the quantitative measurement of bromophenols in seafood at concentrations that are of sensory significance. Without compromising the extraction efficiency of SDE, the use of alkaline back-extraction and subsequent headspace analysis of bromophenol acetates ensures clean samples free of matrix contaminants and eliminates the problems associated with previously described solvent extraction methods. Additionally, the novel application of $^{13}C_6$ bromophenol internal standards increases the overall accuracy and ease of quantification of bromophenols in fish and potentially other complex matrices.

Results from the preliminary feeding experiment suggested that prawns and Mantis shrimp alone appear not to be an adequate vehicle to introduce bromophenols into barramundi flesh above accepted sensory threshold concentrations. Although the findings of this investigation were disappointing, there may be physiological reasons for the low uptake of bromophenols by barramundi. The high-fat prawn matrix may have hindered the passage of bromophenols (at natural concentrations) through the gut lining into the flesh. Although prawns are a natural constituent of wild barramundi diet, there are likely to be other sources of dietary bromophenols in the wild, such as algae (*15*), polychaetes, and other marine organisms (*5, 7*). The experimental addition of synthetic bromophenols at a relatively high concentration in the feeding trial did, however, illustrate that it is feasible to introduce bromophenols into barramundi at a sensorially relevant concentration. The preliminary feeding experiments were devised to ascertain in a simple experiment whether dietary manipulation could be used to increase bromophenol concentrations. Clearly, different strategies need to be evaluated to determine the appropriate natural feed components for the enhancement of bromophenol levels in barramundi.

Within a defined range of concentrations, bromophenols contribute in a positive way to the flavor of many commercial seafood species. In general, aquacultured species are devoid of these compounds, and further research on feed development and husbandry practices will be required to bridge the quality gap between wild and farmed products. Accurate analytical methods to measure bromophenols will be an important component underpinning future work in this area.

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LITERATURE CITED

(1) Boyle, J. L.; Lindsay, R. C.; Stuiber, D. A. Bromophenol distribution in salmon and selected seafoods of fresh- and saltwater origin. *J. Food Sci.* **1992**, *57* (4), 918–922.

(2) Whitfield, F. B.; Helidoniotis, F.; Shaw, K. J.; Svoronos, D. Distribution of bromophenols in Australian wild-harvested and cultivated prawns (shrimp). *J. Agric. Food Chem.* **1997**, *45*, 4398–4405.

(3) Whitfield, F. B.; Last, J. H.; Shaw, K. J.; Tindale, C. R. 2,6-Dibromophenol: the cause of an iodoform-like off-flavour in some Australian crustacea. *J. Sci. Food Agric.* **1988**, *46*, 29–42.

(4) Boyle, J. L.; Lindsay, R. C.; Stuiber, D. A. Contribution of bromophenols to marine-associated flavours of fish and seafood. *J. Aquat. Food Prod. Technol.* **1992**, *1* (3/4), 43–63.

(5) Whitfield, F. B.; Drew, M.; Helidoniotis, F.; Svoronos, D. Distribution of bromophenols in species of marine polychaetes

and bryozoans from eastern Australia and the role of such. J. Agric. Food Chem. **1999**, *47*, 4756–4762.
(6) Whitfield, F. B.; Helidoniotis, F.; Shaw, K. J.; Svoronos, D. Distribution of bromophenols in species of ocean fish from eastern Australia. J. Agric. Food Chem. **1998**, *46*, 3750–3757.
(7) Ma, W. C. J.; Hau Yin, C.; Put, O. A.; Joo-Shin, K. Enhancement of bromophenol levels in aquacultured silver seabream (*Sparus sarba*). J. Agric. Food Chem. **2005**, *53*, 2133–2139.
(8) Whitfield, F. B.; Helidoniotis, F.; Smith, D. Role of feed ingredients in the bromophenol content of cultured prawns. Food Chem. **2002**, *79* (3), 355–365.
(9) Blythe, J. W.; Heitz, A.; Joll, C. A.; Kagi, R. I. Determination of trace concentrations of bromophenols in water using purge-and-trap after in situ acetylation. J. Chromatogr., A **2006**, *1102* (1−2), 73–83.
(10) Insa, S.; Salvado, V.; Antico, E. Development of solid-phase extraction and solid-phase microextraction methods for the determination of chlorophenols in cork macerate and wine samples. J. Chromatogr., A **2004**, *1047* (1), 15–20.
(11) Whitfield, F. B.; Helidoniotis, F.; Drew, M., The role of diet and the environment in the natural flavours of seafoods. *Flavour Science: Recent Developments*; Taylor, A. J., Mottram, D. S., Eds.; The Royal Society of Chemistry: Cambridge, U.K. 1996; pp 3−12.
(12) Martinez-Urunuela, A.; Gonzalez-Saiz, J. M.; Pizarro, C. Optimisation of the derivatisation reaction and subsequent headspace solid-phase microextraction method for the direct determination of chlorophenols in red wine. J. Chromatogr., A **2004**, *1048* (2), 141–151.
(13) Boyle, J. L.; Lindsay, R. C.; Stuiber, D. A. Occurrence and properties of flavour-related bromophenols found in the marine environment. J. Aquat. Food Prod. Technol. **1993**, *2* (2), 75–112.
(14) Hau Yin, C.; Joyce Ma, W. C.; Joo-Shin, K. Seasonal distribution of bromophenols in selected Hong Kong seafood. J. Agric. Food Chem. **2003**, *51* (23), 6752–6760.
(15) Whitfield, F. B.; Helidoniotis, F.; Shaw, K. J.; Svoronos, D. Distribution of bromophenols in species of marine algae from eastern Australia. J. Agric. Food Chem. **1999**, *47*, 2367–2373.

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