JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

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Improved Method for the Extraction and Determination of Bromophenols in Seafoods by High-Performance Liquid Chromatography with Fluorescence Detection

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ABSTRACT: A sensitive precolumn derivatization method using 10-methylacridone-2-sulfonyl chloride (MASC) as derivatizing reagent followed by high-performance liquid chromatography (HPLC) with fluorescence detection has been developed for the determination of naturally occurring bromophenols in seafoods. Instead of using a traditional complex steam distillation solvent extraction method, the extraction method was modified by using sulfuric acid hydrolysis followed by hexane extraction and subsequent alkaline back extraction to obtain good recoveries and fewer interferences. Batch analysis of bromophenols thus became more performable because the sample amount and analysis time were greatly reduced. The recoveries of the five bromophenols were >80% with a noticeable improvement for 4-BP, the recoveries of which were usually <40% when analyzed by traditional methods. The HPLC sensitivity for the determination of bromophenols was greatly enhanced through derivatization. Under optimal conditions, the quantification limits obtained by using 4.0 g of sample were in the range of 0.60−1.0 ng/g. This is the first time that derivatization was applied to enhance the HPLC sensitivity of bromophenols. The proposed method was successfully applied to the determination of the target compounds in seafoods with a much higher sensitivity than traditional HPLC method.

KEYWORDS: *bromophenols, HPLC, fluorescence, seafood*

INTRODUCTION

With the rapid development of modern aquaculture, we have increasing opportunities to consume seafoods. Because the world demand for aquatic products increases every year, many countries utilize aquaculture as an alternative way to increase their productivity and to meet the great demand of consumers. Flavor quality plays an important role in consumer's acceptance of seafood. Seafoods possessing sea-like flavors are often preferred by consumers. Recent studies indicated that simple bromophenols are key flavor compounds occurring in a wide variety of seafood species.[1−4] They are thought to cause the typical sea-like taste and flavor. Although consumers generally appreciate the sea-like taste produced by bromophenols, these compounds have also been associated with off-flavor problems when present at high concentrations.[3,4] Commercial and experimental trials indicate that it is difficult to control the levels of bromophenols in cultivated species such as prawns. A possible solution to overcome the variability of bromophenols in cultivated species was to supplement their diet with a feed high in bromophenols.[5] To obtain a better understanding of the levels of bromophenols in seafoods, and to provide a better guide for aquaculture husbandry, it is desirable to develop a simple and sensitive method for the determination of bromophenols in seafoods.

Determination of low levels of bromophenols has traditionally been a difficult analytical problem.[6] Most of the methods applied gas chromatography−mass spectrometry (GC-MS) in combination with steam distillation solvent extraction (SDE) to analyze trace amounts of bromophenols.[4,6−9] More than 100 g of samples was used in the extraction procedure, but the sensitivities were usually not satisfying. Besides, because bromophenols are semivolatile, SDE may lead to the loss of the target compounds at such a high temperature if the system is not sufficiently gastight. Great loss of the target compounds may also occur during the concentration steps, and the recoveries of 4-BP were usually <40%.[1,3,10] Derivatization is an effective way to enhance the GC or LC sensitivity. For example, the sensitivities of GC-MS methods for bromophenols were greatly enhanced through derivatization with acetic anhydride.[4,9] Containing a benzene ring, bromophenols can also be determined by direct HPLC methods with ultraviolet (UV) or fluorescence detection (FL). However, the sensitivity was relatively low. For example, da Silva et al. determined bromophenols in marine fishes by direct HPLC method with detection limits of 89−232 ng/mL,[1] much lower than the 0.5−50 ng/g of GC-MS methods.[4] Till now, we have not found any trial in derivatization to enhance the HPLC sensitivity of bromophenols.

Received: July 4, 2012
Revised: October 10, 2012
Accepted: October 22, 2012
Published: October 22, 2012

ACS Publications © 2012 American Chemical Society

dx.doi.org/10.1021/jf302882f | *J. Agric. Food Chem.* 2012, 60, 10985−10990

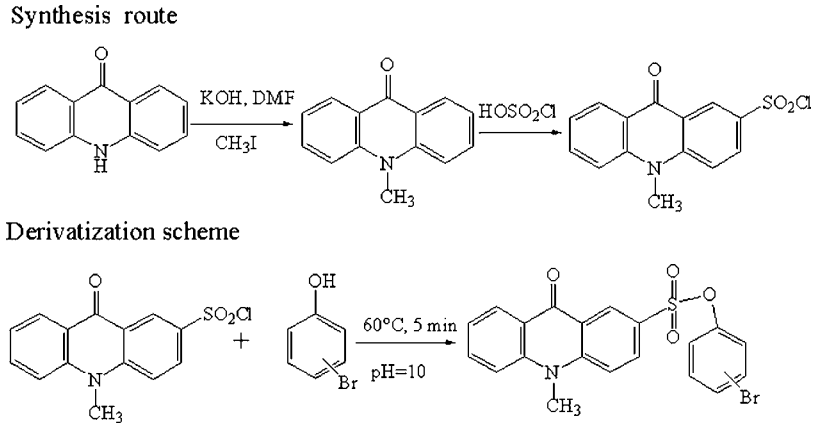

Figure 1. Synthesis route of MASC and derivatization scheme of MASC with bromophenols.

In this paper, bromophenols were derivatized with 10-methylacridone-2-sulfonyl chloride (MASC) and then determined by HPLC with fluorescence detection. The sensitivity of the proposed method was greatly enhanced through the introduction of MASC with excellent fluorescence property into the bromophenol molecules. Besides, the extraction method was also modified and the recoveries were greatly improved. This method shows the merits of simplicity, sensitivity, and lower sample consumption. It can be used as a good alternative to GC-MS for the determination of bromophenols in seafoods.

MATERIALS AND METHODS

Chemicals. Analytical standards of 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) were all obtained from Sigma-Aldrich (USA) with purity >99%. Methanol, dichloromethane, ethyl acetate, *n*-hexane, and acetonitrile were of HPLC grade (Sigma-Aldrich). Water was purified on a Milli-Q system (Millipore, Bedford, MA, USA). All other reagents used were of HPLC grade or at least analytical grade.

Preparation of MASC. MASC was prepared in a way similar to the method previously described by You et al.,[11] and its synthesis route is depicted in Figure 1. In brief, potassium hydroxide (2.6 g) and DMSO (30 mL) were mixed at room temperature for 10 min in a flask (200 mL). Then acridone (5.8 g) in 20 mL of DMSO solution was added and stirred at room temperature for 40 min. A DMSO solution of iodomethane (5.5 mL) was then added dropwise within 10 min. The contents were kept stirring at room temperature for 24 h. The reaction mixture was then poured into water for precipitation. The precipitated solid was recovered by filtration, washed with water, and dried with P_2O_5 under vacuum for 24 h. The crude products were recrystallized three times from acetonitrile to afford a yellow crystal (10-methylacridone). The final product of MASC was prepared by the reaction of 10-methylacridone with chlorosulfonic acid in a way exactly the same as described before.[11] The purity of MASC evaluated by HPLC with FL detection was >99.3%.

Preparation of Standard Solutions and Derivatizing Reagent. Individual stock solutions of 100 mg/L for all bromophenols were prepared in HPLC grade acetonitrile and stored at 4 °C in the dark. Standard solutions containing all compounds were mixed and diluted with acetonitrile. Working solutions of all compounds and calibration concentrations were prepared by appropriate dilution of the stock solutions on the day of analysis.

The derivatizing reagent solution (1.0×10^{-3} mol/L) was prepared by dissolving 3.07 mg of MASC in 10 mL of anhydrous acetonitrile. When not in use, all reagent solutions were stored at 4 °C in a refrigerator.

Extraction Procedure. Fresh Spanish mackerel, prawn, dried sea shrimp, and dried tiny shrimp samples were all purchased from Rizhao fishery market (Rizhao, Shandong province, China). Muscle tissues of aquatic products were homogenized in a blender, and a 4.0 g meat sample (0.50 g for dry sample) was weighed in a 50 mL polypropylene centrifuge tube. Then 8 mL of 50% sulfuric acid solution was added. After vortexing, the samples were placed in a water bath at 60 °C for 30 min. The samples were then cooled to room temperature, and 10 mL of *n*-hexane was added. After vortexing for 2 min, the samples were centrifuged at 4000 rpm for 10 min, then the organic layer was collected, and 4 mL of 0.1 M $NaHCO_3$ buffer (pH 10) was added to back-extract the bromophenols.

Derivatization Procedure. The derivatization of bromophenols with MASC proceeded in basic condition. To a solution containing an appropriate amount of standard or sample solution in a 10 mL tube were added 4 mL of 0.1 M $NaHCO_3$ buffer (pH 10), 900 μL of acetonitrile, and 50 μL of MASC acetonitrile solution. The tube was vortexed for 1 min and then allowed to react at 60 °C for 5 min in a water bath. After the reaction was completed, the mixture was cooled to room temperature; 50 μL of 50% acetic acid solution was then added to adjust the pH to <7.0. The derivatized sample solution was syringe filtered using a 0.22 μm nylon filter and injected directly for HPLC analysis. The derivatization process is shown in Figure 1.

Optimization of Extraction Conditions. The extraction efficiencies of different kinds of extraction solvents were compared by recovery. The influence of temperature on extraction was studied in the temperature range of 40−80 °C with both recoveries and their performances in real sample analysis considered. For the evaluation of the loss caused by the concentration procedure, bromophenol standard solutions were concentrated by nitrogen gas flow or a rotary evaporator to dryness and then redissolved by acetonitrile to their initial volume. The redissolved standard solutions were analyzed simultaneously with standard solutions experiencing no concentration steps. The loss was evaluated by the decrease in peak areas.

Optimization of Derivatization Parameters. Derivatization parameters were optimized by changing only one parameter at a time. The effect of MASC concentration on derivatization was studied from 1- to 10-fold molar reagent excess to total molar bromophenols, whereas the influence of temperature was studied from room temperature to 80 °C. The effect of buffer solution pH on derivatization was studied in the pH range of 7−11.5. Parameters that gave the highest responses were chosen in later experiments.

Instrumental Analysis. The HPLC analysis was performed using an Agilent 1290 series HPLC system, equipped with an online degasser, a quaternary pump, an autosampler, and a thermostated column compartment. A fluorescence detector (model G1321B, Agilent, USA) was adjusted at wavelengths of 262 and 430 nm for excitation and emission. Chromatographic separation was achieved on an SB C18 column (2.1 × 50 mm, 1.8 μm i.d., Agilent) in combination with a gradient elution. Eluent A was 20% methanol solution and eluent B, acetonitrile. The flow rate was constant at 0.25 mL/min, and

the column temperature was kept at 30 °C. The elution conditions were as follows: 10−60% B from 0 to 25 min and 60−90% B from 25 to 28 min. The column was equilibrated with the initial mobile phase for 5 min before the next injection. The injection volume was 3 μL.

Validation of the Proposed Method. The analytical method was validated by linearity, limit of detection (LOD), limit of quantitation (LOQ), accuracy, and precision. A calibration curve was constructed for each compound by plotting peak area versus concentration (0.8−100 ng/mL). All target compounds from extracted samples were injected in this concentration range. Higher concentrations were diluted to meet this standard. LODs and LOQs for all bromophenols were calculated at a signal-to-noise (S/N) ratio of 3 and 10, respectively. Quantitative analysis was carried out by using an external standard method. Recoveries were carried out by spiking blank samples with three different concentrations of standard solutions. Intraday precision was determined by running a sample with spiked standards at three levels with six replicates, and interday precision was determined by running a sample with spiked standards at the same levels with three replicates on three different days over a period of 1 week.

Stability Study. The stability of the MASC solution was studied at room temperature and at 4 °C for 1 week and 1 month, respectively. The stability of the corresponding bromophenol derivatives was studied within 72 h. Peak area deviations were applied to evaluate the stability.

Methods Comparison. The sensitivity (evaluated by LOQ) of the proposed method was compared with those obtained by HPLC without derivatization and GC-MS methods reported before. The signal enhancement effect caused by derivatization with MASC was also depicted by comparing the chromatograms obtained by HPLC with UV detection and this method.

RESULTS AND DISCUSSION

Extraction and Purification. Due to the semivolatile property of bromophenols, SDE became the most often used extraction method. However, the extraction process usually needs a long acidification time because samples should be adjusted to pH <1 and stand at room temperature for several hours to make bromophenols sufficiently convert to their molecular form.[1,5] Besides, the SDE equipment, which is usually composed of a heater, one or two round-bottom flasks, and a condensing tube, is complex and space-occupying. It is difficult to achieve batch analysis because each sample needs an independent SDE equipment. Moreover, a large sample amount is needed and great loss of the target compounds may occur at such a high temperature if the system is not adequately gastight. The low recoveries of bromophenols reported by other authors may be partly attributed to the shortcoming of the extraction method. The extraction of pentachlorophenol could be carried out by hydrolysis with sulfuric acid at 80 °C and then extraction by *n*-hexane.[12,13] Considering the similarity of bromophenols with chlorophenols, the same procedure was applied to the extraction of bromophenols. However, recoveries of 2-BP and 4-BP were <50%. The high vapor pressure of 2-BP and 4-BP at 80 °C might be responsible for this phenomenon because a large quantity of the compounds was converted to the gas state and then evaporated. When the temperature was decreased to 60 °C, the recoveries of the five compounds were >80%. When the temperature was decreased to 40 °C, no decrease in recoveries was observed. However, the determined amounts of bromophenols in real samples were much lower than that obtained at 60 °C. This is probably because bromophenols were not sufficiently converted to their molecular form when hydrolyzed at 40 °C. Therefore, 60 °C was applied for hydrolysis.

Ethyl acetate, *n*-hexane, and diethyl ether were applied to the extraction of bromophenols from the hydrolyzed solution. Almost no recovery was observed when ethyl acetate or diethyl ether was used as extraction solvent. The extraction efficiency of *n*-hexane was satisfying, with recoveries of >80% for all bromophenols. Therefore, *n*-hexane was applied to extract bromophenols from hydrolyzed solution in later experiments.

Loss Caused by Evaporation. Although many methods concentrated their extracts by nitrogen gas flow, we do not think it is feasible. Unlike other phenolic compounds, bromophenols are semivolatile due to the bromine atoms in their molecules. It is well-known that halogenating reaction can greatly enhance the volatility of a compound. For example, heptafluorobutyric anhydride and pentafluorobenzoic chloride are often used as GC-derivatizing reagents due to their high volatile property. The volatility caused by bromine may not be as high as that by fluorine, but nitrogen gas flow, even a gentle gas flow, may lead to great loss of the target compounds. In out study, 50 μL of bromophenol standard solution (1 mg/L) was dried under gentle nitrogen gas flow and then derivatized and analyzed by HPLC. Meanwhile, another 50 μL standard solution of the same concentration was derivatized directly by the same procedure at the same time. The results indicated that >85% of the target compounds was evaporated during the evaporation process, except for 2,4,6-TBP and 4-BP. Slow concentration in a rotary evaporator at a low temperature (<35 °C) seems better than evaporation under a nitrogen gas flow, but great loss of 2-BP was also observed (almost 80%). Therefore, a concentration procedure should be avoided during the whole analysis process to obtain good recovery.

Purification. Most of the methods extracted bromophenols from fishery product by SDE, and then they were analyzed directly without any purification process, but the high level of volatile oils in these extracts may accumulate on the column, causing significant loss of sensitivity.[3,4] In our study, samples were sufficiently purified by the extraction procedure. When *n*-hexane was used to extract the target compounds from sulfuric acid solution, water-soluble impurities were discharged. Organic solvent soluble impurities were also removed when $NaHCO_3$ buffer solution was added to back-extract bromophenols from organic *n*-hexane solution because most of them could not react with the $NaHCO_3$ buffer and remained in the *n*-hexane phase, whereas bromophenols could react with the Na_2CO_3 in $NaHCO_3$ buffer and became soluble in $NaHCO_3$ buffer solution. No expensive solid phase extraction procedure was applied, but the results were satisfying.

Optimization of Derivatization Parameters. *Effects of Buffer Solutions on Derivatization.* Derivatization of bromophenols with MASC should be carried out in basic condition, but strong basic condition is not recommended because MASC may hydrolyze quickly under such conditions. Sodium bicarbonate buffer and borate buffer solutions were tested in this study for their derivatization yields. The results indicated that sodium bicarbonate buffer was superior to borate buffer in derivatization yields. The effect of pH on derivatization reaction was then evaluated with sodium bicarbonate buffer (0.1 M) in the pH range of 7−11.5. Maximum derivatization yields were achieved in the pH range of 9.8−10.5. The detector responses for bromophenol derivatives decreased obviously when the pH value was <8 or >10.5. With pH >10.5, the low responses should be attributed to the hydrolysis reaction of derivatives. As a result, 0.1 M sodium bicarbonate buffer with a pH of 10 was applied for all subsequent derivatization.

Effect of MASC Concentration and Temperature on Derivatization. The concentration of derivatizing reagent plays a vital role in derivatization. Sufficient reaction of the analytes should be guaranteed to fulfill accurate quantitative analysis. In this study, the effects of MASC concentration on derivatization were studied in detail. The results indicated that constant fluorescence intensity was achieved with the addition of a 7-fold molar reagent excess to total molar bromophenols. Further increasing the excess of reagent beyond this level had no significant effect on the yields. The effect of reaction temperature on derivatization was also evaluated. Derivatization of MASC with bromophenols could proceed at room temperature, but the reaction time should be >30 min to obtain steady response. When the temperature was increased to 60 °C, the derivatization could be finished within 5 min. When the temperature was increased to 70 °C, no obvious changes in response were observed. However, when the temperature was increased to 80 °C, the response decreased. This is probably due to the volatilization of bromophenols at high temperature. Hydrolysis of the derivatives at high temperature may also lead to the decrease in response. Maximum and constant peak heights were obtained by the reaction of MASC with bromophenols at 60 °C for 5 min. Thus, a 7-fold molar reagent excess and 60 °C were employed for derivatization.

Stability of Bromophenol Derivatives. An acetonitrile solution of MASC could be stored at room temperature (25 °C) for 1 week without obvious decrease in derivatization yields for bromophenols compared to those newly prepared MASC solution. When placed at 4 °C, it could be stable for 1 month with peak area deviations of <5% for the derivatized bromophenols. The stabilities of the corresponding derivatives were also investigated. A standard solution containing 10 ng/mL bromophenols was derivatized and neutralized according to the procedure described above. This solution was repeatedly analyzed by HPLC after being placed at room temperature for 0, 4, 8, 12, 24, 48, and 72 h, respectively. The corresponding derivatives were stable for normalized peak areas with relative standard deviations (RSDs) of <4.6%. Therefore, it can be concluded that the stability of MASC−bromophenol derivatives is sufficient for chromatographic analysis.

HPLC Separation. It is not difficult to separate the five compounds without derivatization on a reversed phase column. However, after derivatization, the polarities of 4-BP and 2,6-DBP became so similar that coelution appeared. Change in the gradient elution process or the content of organic solvent in mobile phase showed little effect in separation. The fact that no derivatization has ever been applied to the HPLC analysis of bromophenols may be partly attributed to the difficulty in separating them on a normal reversed phase column. Usually, acetonitrile and methanol are used separately due to their different elution performances. In our study, we found a mixture of acetonitrile and methanol showed much better performance in separation than solely using one of them. Complete HPLC separation of the derivatized bromophenols could be achieved by using a C18 column in combination with gradient elution with water−methanol and acetonitrile as mobile phase composition (see Figure 2B).

Method Validation. The linearity, LOD, LOQ, accuracy, and precision of this method were validated by the procedures described above. As can be seen from Table 1, each compound was in good linearity with correlation coefficients of >0.997. Derivatization greatly increased the HPLC sensitivity of bromophenols. As shown in Table 1, the sensitivity of the proposed method was satisfying with LODs ranging from 0.20 to 0.30 ng/g and LOQs ranging from 0.60 to 1.0 ng/g.

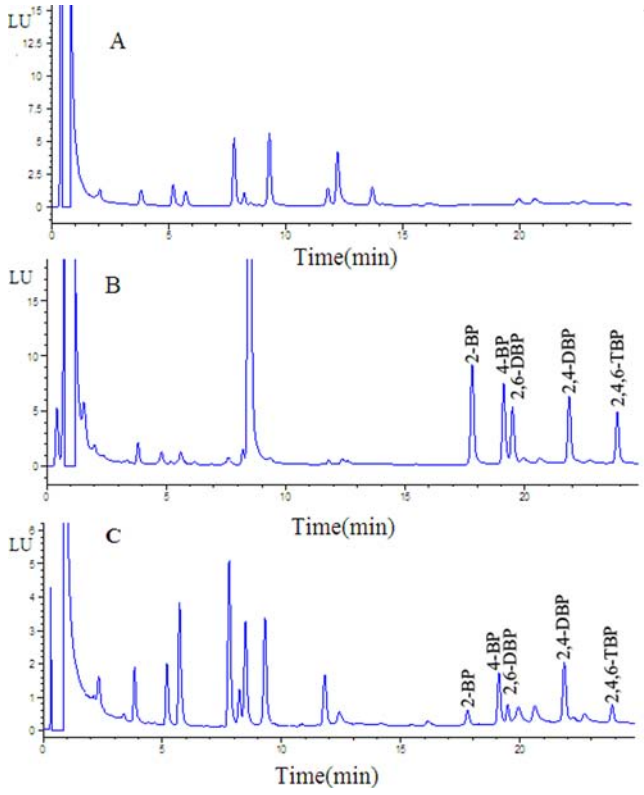

Figure 2. Chromatograms of bromophenol derivatives from (A) a blank sample, (B) 60 ng/mL standard solution, and (C) a prawn sample.

Recoveries were carried out by spiking blank samples with three different concentrations (1.0, 5.0, and 10 ng/g) of standard solutions. Satisfactory recoveries were obtained for all tested compounds (80−95%), and the results are summarized in Table 2. The precision of the proposed method was validated by the RSDs obtained in the intraday and interday analyses of samples spiked at three different concentrations (1.0, 5.0, and 10 ng/g) of bromophenols. As shown in Table 2, the intraday precision for the tested samples was in the range of 4.2−7.5%, whereas the interday precision was between 5.8 and 9.2%.

Comparison of the Proposed Method with Other Methods. The UV or FL properties of bromophenols derived from the benzene ring in their molecules, but their intensities were usually very weak. For example, the LOQs of the five compounds were in the range of 424−898 ng/mL when analyzed by HPLC with UV detection.[1] With the introduction of MASC into the bromophenol molecules, the HPLC sensitivity for bromophenols was greatly enhanced. To clearly demonstrate the signal enhancement effect, chromatographic separation of bromophenols using this method was compared with that obtained by HPLC with UV detection. As can be seen from Figure 3, the sensitivity was enhanced by >2 orders of magnitude through derivatization. HPLC with direct FL detection was also tried, but we could not find obvious peaks at a concentration of 4 mg/L. Although the proposed method is much easier and cheaper, its sensitivity is comparable to that of the sensitive GC-MS method reported by Fuller et al. (0.5−50 ng/g).[4] Another important feature of the proposed method is the small sample amount used. Only 4.0 g of sample was

Table 1. Calibration Curves, LODs, and LOQs for Bromophenol Derivatives

analyte	calibration eq[a]	R	LOD (ng/L)	LOQ (ng/L)
2-BP	$Y = 1.35624783X + 0.0340703$	0.99985	0.20	0.60
4-BP	$Y = 1.03860639X - 0.455267$	0.99968	0.30	0.80
2,6-DBP	$Y = 0.748713375X - 0.8489401$	0.99908	0.40	1.0
2,4-DBP	$Y = 0.920883X - 1.3679625$	0.99715	0.30	0.80
2,4,6-TBP	$Y = 0.7015239X + 0.0135206$	0.99973	0.40	1.0

[a] Y = peak area, X = theoretical concentration of bromophenols.

Table 2. Recoveries and Relative Standard Deviation of Target Compounds in Seafoods

			RSD (%)	
analyte	spike level (ng/g)	recovery (%)	intraday	interday
2-BP	1.0	80	6.6	7.9
	5.0	81	5.8	6.5
	10	84	4.2	5.8
4-BP	1.0	81	7.5	8.5
	5.0	80	6.0	7.0
	10	82	5.8	6.8
2,6-DBP	1.0	84	7.2	8.2
	5.0	86	6.0	7.6
	10	87	6.4	8.4
2,4-DBP	1.0	86	6.2	8.3
	5.0	88	6.0	7.1
	10	88	5.4	6.4
2,4,6-TBP	1.0	90	7.1	9.2
	5.0	93	6.8	6.8
	10	95	4.8	6.1

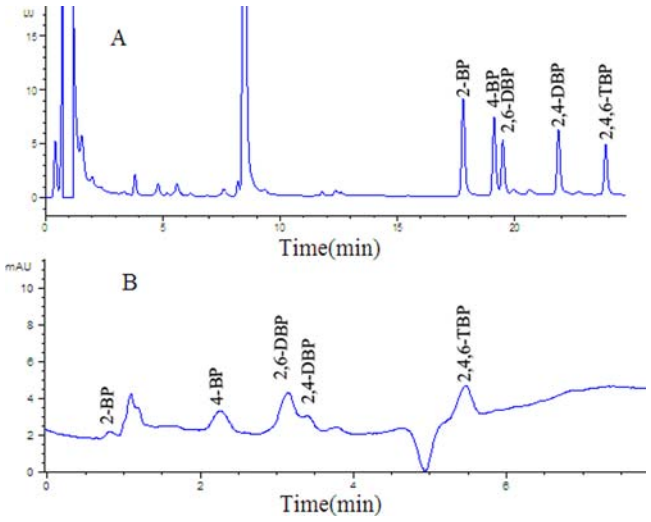

Figure 3. Comparison of bromophenol chromatograms obtained by HPLC with UV detection and HPLC with FL detection: (A) 60 ng/mL standard solution derivatized with MASC and determined by HPLC with FL detection; (B) 4000 ng/mL standard solution without derivatization and determined by HPLC with UV detection.

needed in this method, much less than the often used 100 g in other methods.

Application. The developed method was successfully applied to the determination of bromophenols in aquatic products. The contents of bromophenols from different samples are summarized in Table 3. 2-BP, 4-BP, 2,6-DBP, 2,4-DBP, and 2,4,6-TBP were detected in all of the samples analyzed, ranging from 0.83 to 633 ng/g. The bromophenol concentrations in fresh samples were in the range of 0.83−19.4 ng/g, whereas the highest concentration of bromophenol was found in dried tiny shrimp samples for 4-BP. A typical chromatogram of a prawn sample is shown in Figure 2C.

Table 3. Concentrations of 2-BP, 4-BP, 2,6-DBP, 2,4-DBP, and 2,4,6-TBP in Seafoods

sample		2-BP (ng/g)	4-BP (ng/g)	2,6-DBP (ng/g)	2,4-DBP (ng/g)	2,4,6-TBP (ng/g)
Spanish mackerel	1	2.02	11.3	1.64	19.4	4.23
	2	2.43	7.25	2.32	4.57	1.81
prawn	1	5.71	7.38	1.21	3.46	3.76
	2	0.830	3.26	1.04	1.52	2.36
	3	1.12	2.86	1.54	3.62	3.58
	4	2.44	10.5	5.80	17.1	5.44
dried sea shrimp		42.4	245	129	121	11.8
dried tiny shrimp		94.6	633	106	199	144

In conclusion, a sensitive analytical method was developed for the rapid determination of bromophenols in seafoods. The labeling reaction of bromophenols with MASC is fast and robust and can be carried out under mild conditions. The HPLC sensitivity for the determination of bromophenols was greatly enhanced through derivatization, whereas the sample amount and total analysis time were greatly reduced. The low recoveries that were often encountered in SDE were overcome by the double extraction procedure without any concentration steps. This method also made the batch analysis of bromophenols in seafoods more performable because it was easier to operate. The proposed method could be well applied to the determination of bromophenols in aquatic products with quantification limits between 0.60 and 1.0 ng/g.

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Funding

The work was supported by the 100 Talents Program of the Chinese Academy of Sciences (328) and National Science Foundation of China (No. 21275089).

Notes

The authors declare no competing financial interest.

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NOTE ADDED AFTER ASAP PUBLICATION

This paper was published on the Web on October 30, 2012, with errors to the Results and Discussion Section and the Corresponding Author information. The corrected version was reposted with the issue on November 7, 2012.