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Determination of simple bromophenols in marine fishes by reverse-phase high performance liquid chromatography (RP-HPLC)

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Abstract

Brominated phenols 2- and 4-bromophenol (2-BP and 4-BP); 2,4- and 2,6-dibromophenol (2,4-DBP and 2,6-DBP) and 2,4,6-tribromophenol (2,4,6-TBP) have been identified as key flavor compounds found in seafoods. Depending on their concentrations, they were responsible for marine or ocean flavor (shrimp/crab/fish/sea salt-like) or for phenolic/iodine/iodoform-like off-flavor. In this work a new analytical methodology was developed to determine, simultaneously, such bromophenols in fish meats, based on reversed-phased high-performance liquid chromatographic separation (RP-HPLC). The separation of bromophenols was made onto a Lichrospher 100 RP-18 column using water:acetonitrile gradient at a flow rate of 1.0 mL min^{-1}, using absorbance detection at 286 nm, were the 2-BP, 4-BP, 2,4- and 2,6-DBP show significant absorbtivity values and at 297 nm for 2,4,6-TBP. They were separated in 20 min with a good chromatographic resolution (Rs) for the isomeric compounds: 2- and 4-BP, Rs = 1.23; 2,4- and 2,6-DBP, Rs = 1.63. The calibration curves were linear in the bromophenols concentration range of 200.0–1000 ng mL^{-1}. Under optimized conditions, the detection limit of the HPLC method was 127 ng mL^{-1} for 2-BP; 179 ng mL^{-1} for 4-BP; 89.0 ng mL^{-1} for 2,4-DBP; 269 ng mL^{-1} for 2,6-DBP and 232 ng mL^{-1} for 2,4,6-TBP. This method has been applied in determination of bromophenols, isolated by combined steam distillation-solvent extraction with 2 mL of pentane/diethyl ether (6:4), from Brazilian fishes samples, collected on the Atlantic coast of Bahia (13°01′S and 38°31′W), Brazil. The concentration range determined were 0.20 ng g^{-1} (2-BP) to 299 ng g^{-1} (2,4,6-TBP). The method proposed here is rapid and suitable for simultaneous quantification of simple bromophenols in fish meat. As long as we know, it is the first analytical methodology, using RP-HPLC/UV, which was developed to determine simple bromophenols in fish meat.

Keywords: Bromophenols; RP-HPLC/UV; Fish; Seafood

1. Introduction

The halogenated marine natural products represent a wide variety of chemical compounds; since simple volatile organic halides to sophisticated terpenoids. The biologic role of these compounds is not well understood, probably is related to defense. Among the marine halo compounds special attention has been done to the bromophenols due to their iodoform-like odor. At first they were considered anthropic pollutants, but it is well known that they can be produced naturally by a large variety of marine organisms [1–4].

In the last decade especial attention has been done to the marine bromated compounds, above all to the simple bromophenols, like 2-bromophenol (2-BP), 4-bromophenol (4-BP); 2,4-dibromophenol (2,4-DBP) 2,6-dibromophenol (2,6-DBP) and 2,4,6-tribromophenol (2,4,6-TBP) (Fig. 1). These compounds are semi-volatiles, have a strong odor and have flavoring properties. The intensity and characteristic of the odor and flavor, varies significantly depending upon the bromophenol isomer present in the marine specie, their concentration and the local characteristics of the marine environment [3,5–7].

Recently, the simple bromophenols have been considered an important group of key flavor compounds occurring in a wide variety of seafood species like, fishes, mollusks, crustaceans and algae [2,4,7–9]. When present in high concentration, in seafood, the bromophenols produces an undesirable flavor and

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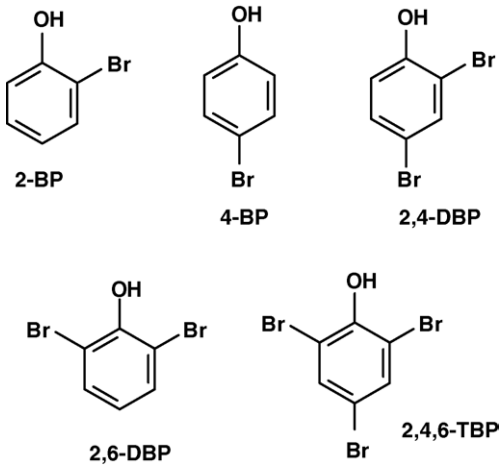

Fig. 1. Simple bromophenols: 2-bromophenol (2-BP); 4-bromophenol (4-BP); 2,4-dibromophenol (2,4-DP); 2,6-dibromophenol (2,6-DP) and 2,4,6-tribromophenol (2,4,6-TBP).

are associated of inferior quality. Meanwhile, when present in low concentration levels (e.g. ng g^{-1}) these compounds produce a desirable marine- or ocean-like flavor and enhance the existing flavor in seafood [2,3]. Indeed, simple bromophenols are wide spread in seafood but were virtually absent in freshwater fish [3,4,7,9–12].

Among the bromophenols, the 2-BP and 2,6-DBP generally has the lower threshold values and as such have the more potent flavors [3,7,13]. For example, in aqueous solution, sensory assessment showed that 2,6-DBP, had an iodoform-like flavor at an extremely low threshold of 5.0×10^{-4} μg L^{-1} and in prawn meat it was found to have a flavor threshold concentration of 6.0×10^{-2} μg kg^{-1} [3,10]. This compound was found in concentrations as higher as 96 μg kg^{-1} in feather bryozoans; 250 μg kg^{-1} in endeavor prawns; 8.99 μg kg^{-1} in mollusks, and 15.3 μg kg^{-1} in fish [3,4,10].

These simple bromophenols have high molar masses and boiling points, which make difficult their isolation from the marine species by the usual techniques employed in the characterization of flavor compounds, such as headspace. The analyses of these compounds in seafood has been carried out using simultaneous steam distillation-solvent extraction (SDE), based on Likens-Nickerson apparatus [14], followed by high resolution gas chromatography with mass spectrometry detection (GC-MS) and multiple or selected ion monitoring mode, by comparison with internal and external standards [3,4,9–12,15,16]. The bromophenols separation usually requires temperature as high as 280 °C and chromatography run for more than 60 min [3,4,9–12,15,17]. High performance liquid chromatography was used in the determination of red algal bromophenols [18]. Meanwhile, we could not find simple bromophenols in the list of analyzed bromophenols [18].

In this work a new analytical methodology was developed to determine, simultaneously, five simple bromophenols: 2-BP, 4-BP, 2,4-DBP, 2,6-DBP and 2,4,6-TBP, based on reversed-phased high-performance liquid chromatographic separation (RP-HPLC) with UV detection. This method has been applied in determination of bromophenols, in Brazilian fishes samples, collected on the Atlantic coast of Bahia (13°01′S and 38°31′W), Brazil.

2. Experimental

2.1. Reagents and standards

The bromophenols standards were obtained from Aldrich (Milwaukee, WI), their purities ranged from 97 to 99%. Purified water was obtained by distillation and filtration through an E-pure Alltech (Deerfield, IL). Acetonitrile (HPLC grade) were obtained from Aldrich and was filtered in 0.45 μm membrane. The other reagents (sulfuric acid, pentane and ethyl ether) were analytical grade and obtained from Merck (Darmstadt, Germany).

2.2. Sample collection and preparation

Two fish species of the family Lutjanidae were studies: *Lutjanus synagris* and *Ocyurus chrysurus*. Fresh fish, collected on the Atlantic coast of Bahia, Brazil (13°01′S and 38°31′W), were brought from the local market and were obtained from the same shop throughout the study. For each species, three fishes were purchased each time, with an average weight of 1.0 kg and 30 cm length (total samples = 30 fishes). After their arrival at the laboratory, each species was washed with distilled water. The flesh was removed, washed with a saturated NaCl solution, and thus transferred to a food processor (Triton-Arno), until blended to a fine purée. Samples of flesh (in portions of 100 g–250 g) were stored in a sealed polyethylene bag at −15 °C until required for analysis.

2.3. Bromophenols extraction

Representative samples of flesh (100–250 g) were separately homogenized in purified water (1000 mL) and the homogenates were acidified to pH 1 with 10 M sulfuric acid (the pH control is necessary to keep the phenolic compounds protonated, turning difficult the ionization of the hydroxyl group.) and were left to stand at ambient temperature (26 ± 3 °C) to circa 12 h. The volatile components were isolated by combined continuous hydrodistillation-solvent extraction with 2 mL of pentane/diethyl ether (6:4) using a modified Clevenger apparatus (Vidrosel Inc. Vidros, Brazil) adapted for this study (Fig. 2). Those modifications include the use of a distilling trap, to avoid mechanical carrier of the sample due to heated boiling and foam formation and cooling the arm which conducts the solvent vapor. After 4 h the hydrodistillation process was finished and the pH of the residues was measured. The collected extract was concentrated at 20 °C, with a gentle stream of ultrahigh purity (99.999%) nitrogen. Due to the high boiling points of bromophenols (194–286 °C), losses by vaporization, in this step, were minimized. The concentrated extract was them dissolved in acetonitrile (500 μL) and stored in 2 mL dark glass vials at −15 °C until required for analysis.

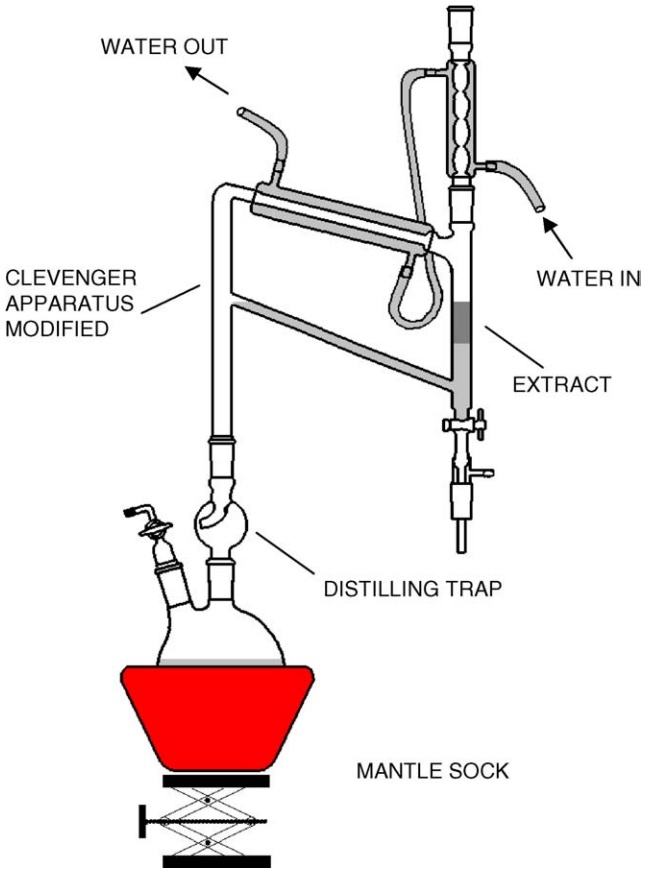

Fig. 2. The modified Clevenger apparatus adapted for this study, combines hydrodistillation-solvent extraction.

2.4. Preparation of bromophenols standards and calibration solutions

The stock solutions (100 mg mL^{-1}) were prepared by first weighting each bromophenol and them dissolving it in acetonitrile. The calibrations standards solutions were prepared by dilution in acetonitrile of the bromophenol stock solutions, in the concentration range from 200 to 1000 ng mL^{-1}. They were stored at 4 °C in the dark-flasks glass. The standard solutions, even when stored under refrigeration, had their chromatographic signal depleted by circa 20% after ten days, for all the bromophenols. Hence, standards solutions should be prepared, at least, weekly.

2.5. Compounds separation

A Perkin-Elmer liquid chromatograph series 200 equipped with a Rheodyne (Cotati, California, USA) injector valve with a 20 μL sample loop and a Perkin-Elmer series 200 model UV–visible detector were used. Chromatographic separation of bromophenols was performed on a LiChrospher 100 RP-18 (244 mm × 4.4 mm i.d., 5 μm; Merck) column linked to a LiChrospher guard column of similar characteristic (4 × 4 mm i.d.; Merck). The mobile phase degassed in sonicator under vacuum, was a mixture of water:acetonitrile pumped in gradient mode (Table 1) at a flow rate of 1.0 mL min^{-1} at ambient temperature. The detection was performed at 286 nm, were the 2-BP, 4-BP, 2,4- and 2,6-DBP show significant absorbtivity values and at 297 nm for 2,4,6-TBP.

Table 1
RP-HPLC solvent gradient varying the mobile phase water–acetonitrile composition used in the bromophenols separation

Time (min)	Solution with 45% CH_3CN:55% H_2O	CH_3CN (%)
0	100	0
10	100	0
20	45	55
22	45	55
27	100	0

2.6. Calibration curve and quantification

Calibrations curves were constructed by plotting the observed peak height against the bromophenol amount injected (200–1000 ng mL^{-1}). Quantification of bromophenols was performed by external standard by measuring peak height at each retention time and calculated with the calibration curve. Spikes of each bromophenol were done in the samples to ascertain exactly the retention times.

2.7. Extraction efficiency

The accuracy of the method was examined by adding an aliquot (1 mL) of a solution containing each of the five bromophenols (1 μg mL^{-1} in acetonitrile) in 100 g of fish meat. The extractions were performed in triplicate, under the same experimental conditions of combined continuous hydrodistillation-solvent extraction (session 2.3).

3. Results and discussion

3.1. Optimization of the chromatographic separation

The chromatographic separation was optimized by development of univariate methodology (one variable at each time), using reversed-phase (C_{18})-HPLC, with isocratic or gradient elution and binary eluent system (organic modifier/water). Two experimental variables were considered: organic modifier and the composition of mobile phase. The optimization was established considering a complete separation (baseline resolution) of the chromatographic peaks of the isomeric bromophenols (2, and 4-BP; 2,4- and 2,6-DBP) and also total analysis time.

Firstly, an isocratic-mode method with UV detection was run to optimize the experimental conditions. For this, methanol, ethanol and acetonitrile were used as organic modifiers in the mobile phase with the following composition: organic modifier/water (50/50%, v/v). The better responses for resolution (Rs) – a measure of the degree of separation between adjacent peaks – and total analysis time were achieved with the acetonitrile as modifier. For two compounds A and B in a chromatographic run is expressed as $Rs = 2(t_A - t_B)/(w_A + w_B)$ in which t_A and

t_B are the retention times and w_A and w_B refers to the width at the base of the component peaks [19]. The minimum value of chromatographic resolution (Rs), between two peaks, which leads to a complete baseline separation is 1.0, and the maximum value, without impairment to the analysis time, is 1.5. Ethanol led to problems of high pressure in the chromatographic system, and use of the methanol implied an analysis time of 29 min, with can constitute disadvantage for the method.

The next step was determining the optimal composition of the mobile phase. In this way, the percent of the acetonitrile were varied (45–55% of the organic modifier). The flow rate was changed from 0.8 to 1.0 mL min^{-1}.

The best separation was obtained with the following conditions: acetonitrile/water (55/45%, v/v), as mobile phase at a flow rate of 1.0 mL min^{-1} and UV detection at 286 nm (AUFS = 0.03). Under these conditions the retention times were: 2-BP (rt = 6.93 min); 4-BP (rt = 7.72 min); 2,6-DBP (rt = 12.93 min); 2,4-DBP (rt = 14.56 min) and 2,4,6-TBP (rt = 28.0 min). Hence, the total separation has occurred in 30 min.

In order of reduce the total analysis time the gradient elution was studied. When the gradient elution was used, the separation of bromophenols was made onto a Lichrospher 100 RP-18 column using water:acetonitrile gradient (Table 1), at a flow rate of 1.0 mL min^{-1}, using absorbance detection at 286 nm, where the 2-BP, 4-BP, 2,4- and 2,6-DBP show significant absorbtivity values and at 297 nm for 2,4,6-TBP. The experiment performed under the optimised conditions of flow rate and mobile phase gives the chromatogram of Fig. 3, in which, within 20 min, all five bromophenols are separated in both a standard solution and a sample of fish, with a good chromatographic resolution (Rs) for the isomeric compounds: 2- and 4-BP, Rs = 1.23; 2,4- and 2,6-DBP, Rs = 1.63. The use of gradient elution and different wavelengths allowed for shortening the analysis time in circa of ten minutes, while improved the peak symmetry and the detection conditions for 2,4,6-TBP, without use of acidified water, normally employed in the separation of phenolic compounds using reversed phase HPLC [20].

3.2. Validation parameters

The RP-HPLC/UV method was validated in terms of limit of detection (LOD), limit of quantitation (LOQ), linearity and precision calculated as relative standard deviation (RSD) [21,22].

3.2.1. Linearity

To check the linearity of the response of detector, a linear regression analysis of height peak versus concentration of the bromophenols was used. The linearity was determined by the square correlation coefficients of the calibration curves generated by three repeated injections of standard solutions at six concentrations levels. The analytical curves were linear for the five bromophenols in the concentration range of 200–1000 ng mL^{-1}, as is shown in the Table 2. The correlation values of all the calibration curves were higher than 0.99, thus confirm the linearity of the method developed (Table 2).

3.2.2. Detection and quantitation limits

Limits of detection and quantitation decide about the sensitivity of the method. LOD is the lowest concentration of the analyte detected by the method; LOQ is the minimum quantifiable concentration. The limits were estimated from the residuals of calibration curve as recommended by IUPAC [21,22]. The LOD was taken as the ratio between three times three the standard deviation of the signal assigned to the "zero concentration" and the slope of the analytical curve. In these conditions, the LOD was 127 ng mL^{-1} for 2-BP, 179 ng mL^{-1} for 4-BP, 89 ng mL^{-1} for 2,4-DBP, 269 ng mL^{-1} for 2,6-DBP and 232 ng mL^{-1} for 2,4,6-TBP. The LOQ, defined as ten times that ratio was 424 ng mL^{-1} for 2-BP, 596 ng mL^{-1} for 4-BP, 297 ng mL^{-1} for 2,4-DBP, 878 ng mL^{-1} for 2,6-DBP and 774 ng mL^{-1} for 2,4,6-TBP.These results are shown in the Table 2. These results suggest

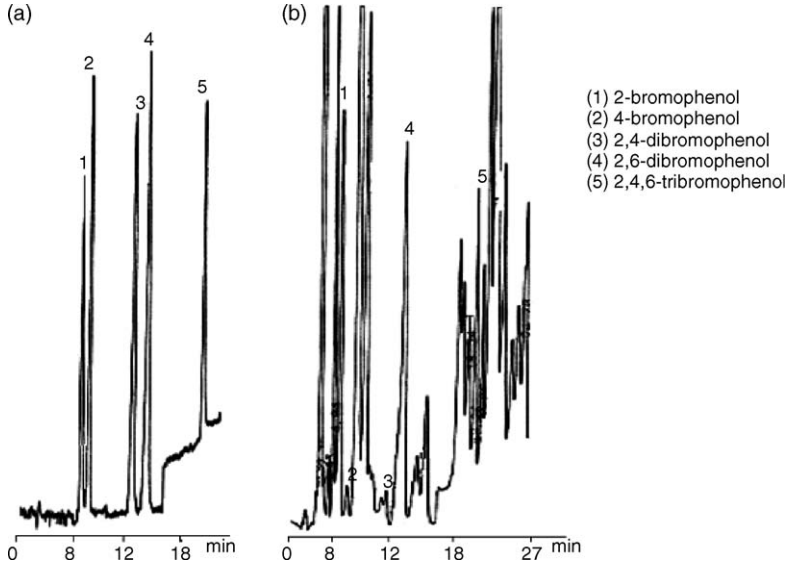

Fig. 3. Chromatograms obtained in the separations of the simple bromophenols under the optimized conditions for (a) standards and (b) a fish sample.

Table 2
Performance characteristics

Bromophenol	Calibration curves (X, concentration ($ng\,mL^{-1}$); y, peak height)	r^2 (determination coefficient)	LOD ($ng\,mL^{-1}$)	LOQ ($ng\,mL^{-1}$)
2-BP	$y = 0.030X + 0.073$	0.994	127	424
4-BP	$y = 0.023X + 3.265$	0.993	179	596
2,4-DBP	$y = 0.017X + 4.359$	0.999	89	297
2,6-DBP	$y = 0.014X + 1.281$	0.999	269	898
2,4,6-TBP	$y = 0.015X + 1.653$	0.999	232	774

that proposed method is sensitive enough for the determination of bromophenols in seafood, in accordance with previous amounts reported [4,7,11].

3.2.3. Precision

Precision was studied in a real sample and standard solution for the five bromophenols in two ways: retention times and peak heights. The repeatability of retention times and peak heights were calculated by the RSD of six injections carried out on the same day. The RSD for the peak heights of all peaks, in a real sample and standard solution was <0.32%. A high repeatability in the retention time was obtained with RSD values lower than 0.012%.

3.2.4. Accuracy

The accuracy of the method was determined by analyzing the percentage of recovery of the bromophenols. The average recoveries under combined continuous hydrodistillation-solvent extraction were 2-BP – 89.0 ± 7.5%; 4-BP – 39.3 ± 4.0%; 2,4-DBP – 58.4 ± 3.3%; 2,6-DBP – 69.4 ± 5.3%; 2,4,6-TBP – 55.3 ± 3.6%. These results are in agreement with those obtained elsewhere using SDE extraction [4,11,23].

3.3. Selectivity of the method

To improve the selectivity, different wavelengths were used for quantify the five bromophenols: 286 nm for 2-BP; 4-BP; 2,4-DBP; 2,6-DBP and 297 nm for 2,4,6-TBP. The identified peaks were then confirmed by spiking samples with standard mixtures.

3.4. Bromophenols content in fish samples

The method has been applied in two fish species of the family Lutjanidae: *Lutjanus synagris* and *Ocyurus chrysurus,* collected on the Atlantic coast of Bahia, Brazil (13°01′S and 38°31′W). In the Table 3 are shown the results obtained in the brominated phenols 2-BP, 4-BP, 2,4-DBP, 2,6-DBP and 2,4,6-TBP analysis in fish samples. The highest concentration $229\,ng\,g^{-1}$ was determined for the 2,4,6-TBP.

The results presented in the Table 3 are in agreement with those obtained by Whitfield et al. [11,23] and Chung et al. [4] in marine fish when were found high total bromophenol concentrations (2.72–462 $ng\,g^{-1}$), especially for 2,4,6-TBP, which was the more frequent and abundant bromophenol [4]. The concentrations of 2,6-DBP, 2,4-DBP and 2,4,6-TBP determined in seven fish species (*Branchiostegus wardi*, *Girella tricuspidata*, *Nemadactylus douglassi*, *Rhabdosargus sarba*, *Acanthopagrus australis*, *Meuschenia trachylepis*, *Pseudorhombus jenynsii*) [20] were, respectively, in the range of 0.4–18 $ng\,g^{-1}$, 112–150 $ng\,g^{-1}$ and 5.7–170 $ng\,g^{-1}$. The concentrations reported here (Table 3), shows similar predominance of the three bromophenols in the two fish species studied.

Table 3
Bromophenols concentrations[a] ($ng\,g^{-1}$) determined in 15 different samples of marine fish species (*Lutjanus synagris* and *Ocyurus chrysurus)* collected from January to September

Bromophenols[b]	*Lutjanus synagris* (15)	*Ocyurus chrysurus* (15)
2-BP	1.08–34.8	0.20–18.9
4-BP	Nd – 19.8	Nd – 13.9
2,4-DBP	11.5–110	6.62–158
2,6-DBP	3.22–28.9	Nd – 28.4
2,4,6-TBP	15.2–171	35.5–299

Nd = not detected; () numbers in parenthesis represent the number of samples.
[a] Concentrations calculated based on fresh weight of fish.
[b] Highest concentrations registered in the literature ($ng\,g^{-1}$): 2-BP (38), 4-BP (46), 2,4-DBP (23), 2,6-DBP (0.6) and 2,4,6-TBP (12) [4,7,11].

4. Conclusions

The RP-HPLC/UV method proposed shown to be appropriate for the separation and simultaneous quantification of 2-bromophenol, 4-bromophenol, 2,4-dibromophenol, 2,6-dibromophenol and 2,4,6-tribromophenol in fish samples, presenting high sensibility. The use of gradient elution and different wavelengths allowed for shortening the analysis time and also enhance the detection conditions, permitting to reach very low limits of detection. The present work, as long as we know, it is the first analytical methodology, using RP-HPLC/UV, that was developed to determine simple bromophenols in fish meat.

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