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Notes

Mass Spectrometric Quantification of Amphipathic, Polyphenolic Antioxidant of the Pacific Oyster (*Crassostrea Gigas*)

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A novel amphipathic phenolic compound, 3,5-dihydroxy-4-methoxybenzyl alcohol (DHMBA), that can be isolated from the Pacific oyster (*Crassostrea gigas*) has been found to protect human hepatocytes against oxidative stress. This study aims to establish a method for the measurement of DHMBA for industrial application. Liquid chromatography-tandem mass spectrometry using deuterated DHMBA as an internal standard and a polar end-capped ODS (Hypersil GOLD aQ) as the solid phase was validated. The limit of detection was 0.04 pmol (S/N = 5), and the limit of quantitation was 0.1 pmol (S/N = 10). The calibration curve was linear throughout the range of 0.1 – 16 pmol (r^2 = 0.9995). This method successfully quantified DHMBA in oysters from 11 sea areas in Japan. The results showed that the yield of DHMBA was variable from 9.8 to 58.8 μg g^{-1} whole oyster meat wet weight but not affected by the seawater temperature. The proposed LC-MS/MS method is useful in quantitative studies for DHMBA and potentially for other amphipathic substances.

Keywords Amphipathicity, polyphenol, LC-MS/MS, antioxidant, Pacific oyster, polar end-capped ODS

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Introduction

Recent studies have focused on marine organisms as a source of polyphenols.[1] As for oysters, small molecule antioxidants such as ascorbic acid,[2] α-tocopherol, glutathione[3] and carotenoids[4] have been reported. We have reported 3,5-dihydroxy-4-methoxybenzyl alcohol (DHMBA) as a new phenolic antioxidant derived from the Pacific oyster (*Crassostrea gigas*) extract.[5] DHMBA can strongly inhibit copper-mediated oxidation of human low-density lipoproteins,[5] and effectively prevent hepatocyte from lipid peroxidation by 2,2′-azobis (2-amidinopropane) dihydrochloride.[6] Moreover, DHMBA has anti-apoptotic effects on cultured human hepatocytes under oxidative stress, without showing significant cytotoxicity up to high concentrations.[7] Hence, industrial utilization of DHMBA as a source of functional foods can be expected.

The amphipathic property of DHMBA may partly explain its strong antioxidant property in the cell-based experiments. In a recent report, two water soluble antioxidants, chlorogenic acid and rosmarinic acid, were converted to amphipathic alkyl esters and found to acquire increased antioxidative potential.[8,9] Although amphipathic antioxidants are attracting more attention in food science and industry, it is difficult to measure amphipathic compounds including DHMBA by means of chromatographic separation. Amphipathic compounds are hardly retained on solid phases for conventional reversed-phase chromatography and hydrophilic interaction chromatography (HILIC).

In this study, several kinds of solid phases were examined with the aim of constructing a liquid chromatography-tandem mass spectrometry (LC-MS/MS) for DHMBA. Here, we report that a polar end-capped octadecylsilyl (ODS) solid phase and a graphite carbon solid phase are useful for this purpose. Analytical validation of the proposed method and its practical application to a study concerning the yield of DHMBA of the oysters from various sea areas in Japan are described in this report. This report might include useful information for convenient quantification of DHMBA and also other amphipathic antioxidant substances.

Experimental

Chemicals

Methyl gallate, ammonium acetate, ultrapure water, tetrahydrofuran, potassium carbonate, *N*,*N*′-dimethylformamide, lithium aluminum hydride, HPLC grade methanol and acetonitrile were purchased from Wako Pure Chemical Industries, Ltd., Osaka, Japan. Methyl-d_3 iodide (99.5 atom% D) was obtained from Cambridge Isotope Laboratories Inc. (Tewksbury, MA).

Apparatus

Hypersil GOLD (5 μm, 100 × 2.1 mm), Hypersil GOLD C8 (5 μm, 100 × 2.1 mm), Hypersil GOLD aQ (5 μm, 100 × 2.1 mm), Hypercarb (5 μm, 100 × 2.1 mm) and Accucore HILIC (2.6 μm, 50 × 2.1 mm) columns were purchased from Thermo Fisher Scientific, Inc. (Waltham, MA). Inertsil CN-3

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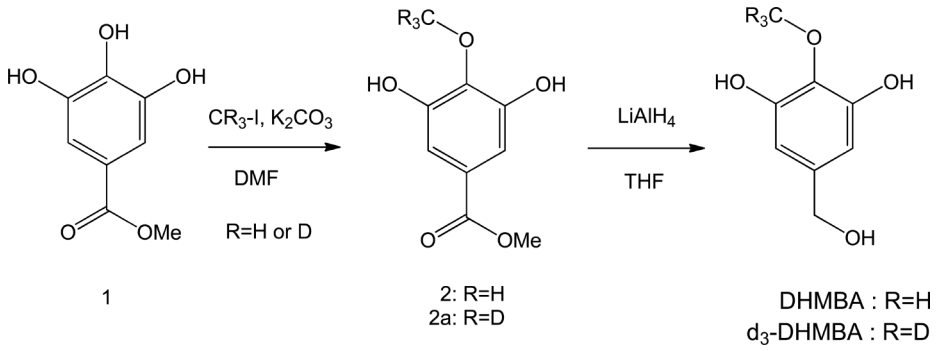

Fig. 1 Synthetic pathway of 3,5-dihydroxy-4-methoxybenzyl alcohol and [d_3]-methyl 3,5-dihydroxy-4-methoxybenzyl alcohol.

(5 μm, 100 × 2.1 mm), Inertsil Diol (5 μm, 100 × 2.1 mm) and Inertsil NH2 (5 μm, 100 × 2.1 mm) columns were purchased from GL Sciences Inc., Tokyo, Japan. The LC-MS/MS system consisted of an LC-2010HT (Shimadzu Corp., Kyoto, Japan) coupled with TSQ Quantum Access (Thermo Fisher Scientific, Inc.) for quantitative analysis of DHMBA.

Synthesis of DHMBA and d_3-DHMBA

DHMBA and d_3-DHMBA as internal standard (IS) compounds were chemically synthesized from methyl gallate by a two-step reaction, as described previously.[5] The synthetic scheme is shown in Fig. 1. Briefly, potassium carbonate was added to a solution of methyl gallate **1** in *N*,*N*′-dimethylformamide and the mixture was stirred at 85°C for 1 h. After cooling the reaction mixture, methyl(-[d_3]) iodide was added and the reaction mixture was stirred at 0°C for 30 min and stirring was continued at room temperature for another 24 h. To the solution of 4-methoxy compound **2/2a** in tetrahydrofuran was added lithium aluminum hydride and the mixture was refluxed for 4 h. Standards and internal standards were stored in plastic tubes (methanol) at –20°C until use. Both compounds were stable for at least 12 months.

LC-MS/MS system

The electrospray voltage was set at –4.0 kV in negative ion mode. Nitrogen was used as the sheath gas and the auxiliary gas (set at 30 psi and 5 psi). The capillary temperature was set at 330°C. DHMBA was monitored by setting *m*/*z* 169 as the precursor ion, setting *m*/*z* 154 as the product ion and by setting 13 V as collision energy. The internal standard (d_3-DHMBA) was monitored by setting *m*/*z* 172 as the precursor ion, setting *m*/*z* 154 as the product ion and by setting 15 V as collision energy. Gradient elution was performed using a mobile phase composed of 5 mM aqueous ammonium acetate (solvent A) and acetonitrile (solvent B). The gradient was programmed as follows: 0.0 – 0.1 min 100% solvent A and 0% solvent B; 0.1 – 1.0 min 100% solvent B; 1.0 – 1.1 min 100% solvent A. The flow rate was 0.2 mL min^{-1}.

Samples and extraction of DHMBA

Fresh Pacific oysters (*Crassostrea gigas*) cultured in 11 sea areas of Japan, from the northernmost (the Hokkaido area) to the southernmost (the Kyushu area), were collected commercially during a winter season (November 2014 to March 2015). Data of sea temperature for each area were obtained from the Japan Oceanographic Data Center. The seawater temperatures at the time and location of sampling ranged from 8.4 to 21.4°C (16.7 ± 3.8°C, mean ± SD).

About 150 g (wet weight) of oyster meat and 2 L of water were boiled for 36 h. After cooling, the boiled liquid (100 μL) was mixed with 100 μL of acetonitrile using a vortex mixer. The mixture solution was then filtered by a PVDF membrane (0.45 μm). The filtrate was diluted 10-fold with water and then mixed with 20 μL of aqueous solution of IS (4 nmol mL^{-1}). Two microliters of the resulted mixture solution was injected to LC-MS/MS.

Statistics

Quantification was performed using Xcalibur 2.0.7. The correlative coefficient between the yields of DHMBA and the seawater temperatures was calculated using Microsoft Excel. The *p*-value of the correlative coefficient was based on a two-sided test and, if less than 0.05, considered statistically significant.

Results and Discussion

The ODS silica gel solid phase (Hypersil GOLD) and the octylsilyl silica gel solid phase (Hypersil GOLD C8) were tested for retaining DHMBA in 5 mM ammonium acetate/acetonitrile, 9/1 (v/v). Further, the cyanopropyl silica gel solid phase (Inertsil CN-3), the aminopropyl silica gel solid phase (Inertsil NH2), and the diol silica gel solid phase (Inertsil Diol) phase were also tested under the same conditions. Additionally, the HILIC (Accucore HILIC) in 5 mM ammonium acetate/acetonitrile, 1/9 (v/v), was also tested. DHMBA was not retained in any of the tested solid phases in these conditions.

On the other hand, the phase of the ODS silica gel, which has polar groups at residual silanols (Hypersil GOLD aQ), well retained DHMBA. This solid phase possesses both non polar ODS and polar groups, which might explain its better performance for amphipathic compounds. According to a previous study, the retention behavior of this phase will be partly explained by hydrophobic interactions that occur between the ODS phase and the phenyl group of DHMBA.[10] Further, the polar and ionic interactions that may result between the polar end-capped groups and the hydroxyl groups of DHMBA will give another explanation.

Not only the solid phase but also the mobile phase appears to play a part in determining the retention behavior. In this study, acetonitrile was used as a mobile phase (Solvent B). We tested methanol as well as acetonitrile, and found that the peak shape of DHMBA in acetonitrile was better than that in methanol.

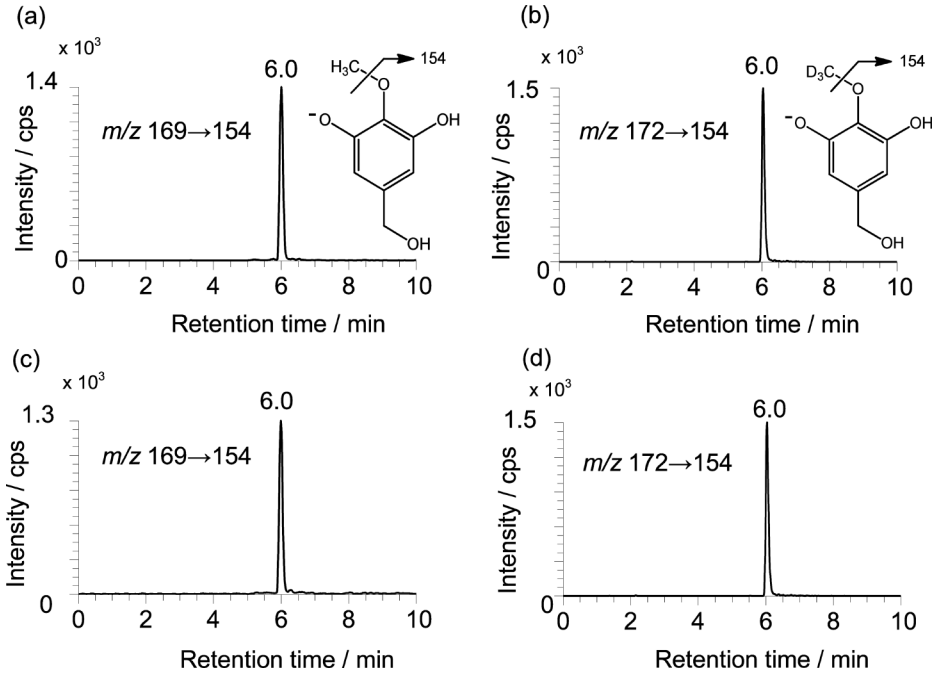

Fig. 2 Representative chromatograms of DHMBA (a), d_3-DHMBA (b), DHMBA from the oyster extract (c) and d_3-DHMBA added in the oyster extract (d). For abbreviation see text.

After optimizing the gradient elution condition for Hypersil GOLD aQ, the retention of DHMBA was attained successfully giving the retention time at 6.0 min (Fig. 2a). Particle size, pore size and carbon load of the packing material in the Hypersil GOLD aQ column used in this study were 5 μm, 17.5 nm and 12%, respectively. The comparatively high theoretical plate of the peak as high as 15000 was obtained.

In addition to Hypersil GOLD aQ, a Hypercarb column filled up with graphite carbon also showed good retention. In this case, a charge-induced interaction between graphite carbon and DHMBA might explain the retention.[11-13] The theoretical plate of the peak for the Hypercarb column was 13000. We selected the Hypersil GOLD aQ column for quantitative analysis because of its better theoretical plate than that for the Hypercarb column. The chromatograms of DHMBA standard and IS were comparable with those for DHMBA and IS from the oyster extracts (Fig. 2).

The constructed quantitative method was validated according to the Food and Drug Administration (FDA) guidelines. The recoveries for six samples were 84.0 – 101.2% (0.1 pmol) and 100.5 – 117.0% (16 pmol). The intra-assay coefficient of variation (CV) for the six samples was 7.6% (0.1 pmol) and 5.2% (16 pmol). The inter-assay CV for the six samples during five days was 13.0% (0.1 pmol) and 8.0% (16 pmol). The limit of detection was 0.04 pmol (S/N = 5). The limit of quantitation was 0.1 pmol (S/N = 10). The calibration curve showed good linearity throughout the range from 0.1 to 16 pmol ($y = 2.3739x - 0.1949$, $r^2 = 0.9995$).

To show the utility of the established method, quantification of DHMBA in Pacific oysters (*Crassostrea gigas*) sourced from major aquafarming sea areas in Japan was performed. The yields of DHMBA were variable among locations, ranging between 9.8 and 58.8 (mean ± SD, 32.2 ± 13.5) μg g^{-1} of whole oyster meat wet weight (Fig. 3). This result is similar with our previous estimate, 67 μg g^{-1} of whole oyster meat wet weight.[5] The observed variation will be explained by environmental factors, such as nutrient salts, organic substances, and planktons, and seawater temperatures. In this study, we only examined the contribution of seawater temperatures. The plot of the yields of DHMBA from the oysters and the average temperature of seawater for each location, provided from the Japan Oceanographic Data Center, is shown in Fig. 3. No significant contribution of the seawater temperatures to the yield of DHMBA was observed (r^2 = 0.0589, p = 0.788).

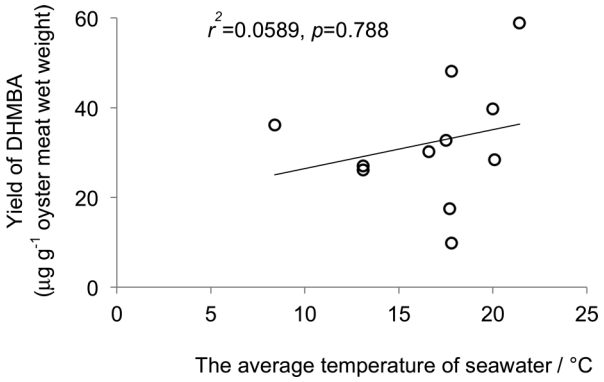

Fig. 3 Correlation between the average temperatures of seawater and the yields of DHMBA from oyster meat.

Conclusions

A quantitative method for DHMBA by LC-MS/MS using a Hypersil GOLD aQ column (a polar end-capped ODS) was established. The proposed method was successfully applied to quantification of DHMBA in Pacific oyster extract. The Hypersil GOLD aQ column, and secondarily the Hypercarb

column, might serve as an excellent solid phase for measurement of amphipathic substances including DHMBA. The yield of DHMBA from the Pacific oyster was found to be variable among sea areas, without significant correlation with the sea water temperatures.

Acknowledgements

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