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Optimized Extraction and Separation Conditions of Four Bioactive Compounds from *Gelidium amansii*

M. TIAN, S. LI and K.H. ROW*

Department of Chemical Engineering, Inha University, Incheon 402-751, South Korea

*Corresponding author: Fax: +82 32 8720959; Tel: +82 32 8607470; E-mail: rowkho@inha.ac.kr

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The simultaneous extraction and separation of four bioactive compounds from *Gelidium amansii* were carried out through liquid-liquid extraction with a liquid chromatography separation. The optimum extraction conditions were established using different extraction solvents, procedures and times. The extracts were separated and determined using an analytical C_{18} column with a mobile phase consisting of acetonitrile-water (containing 1.0 % acetic acid) for a gradient elution of 0-30 min from 15:85-50:50 (v/v). Finally, total amount of 0.239 mg of four bioactive compounds was obtained from 1.0 g of *Gelidium amansii*.

Key Words: *Gelidium amansii*, Bioactive compounds, Extraction, High performance liquid chromatography.

INTRODUCTION

Algae are important sources of various bioactive compounds, including phenols, with different physiological effects (toxic or curative) on human health. Many of these compounds possess antioxidant, antimicrobial and antiviral activities that are important for the protection of the algal cells against stress conditions[1-4]. *Gelidium amansii* possesses a comparable bioenergy production potential to land plants and contains a large number of other organic compounds including various important phenolic compounds, namely *p*-hydroxybenzaldehydes, *p*-hydroxybenzoic acids, 3,4-dihydroxybenzaldehyde, 2,3-dihydroxybenzoic acid, salicylic acid and so on[5].

Phenols are an important group of natural products with antioxidant and other biological activities. These compounds play an important role in the algal cell defense against abiotic and biotic stress[6,7]. All of these phenolic compounds are used as flavoring in the food, confectionary, beverage, perfumery, cosmetics and pharmaceutical industries. Although synthetic phenolic compounds are cheap and widely produced, the consumers have shown an increased demand for high quality natural products and related phenolic compounds because of food safety concerns[8].

Phenolic compounds are indicators of a good quality *Gelidium amansii* extract for commercial purposes[9] and so far, more than 25 phenolic compounds are known to be present in different species of algae. The halogen derivatives of *p*-hydroxybenzoic acid were also identified in algae[10]. Cinnamic acid esters (*n*-butyl 3, 5-dimethoxy-4-hydroxycinnamate and isopropyl 3,5-dimethoxy-4-hydroxycinnamate) and methyl 3,4,5-trihydroxybenzoate were studied using ^{1}H and ^{13}C NMR in brown algae *Spatoglossum variabile*[11]. Some of the first polyphenols that were found in algae (*Fucus* and *Ascophyllum* spp.) were phlorotannins which form from the oligomeric structures of phloroglucinol (1,3,5-trihydroxybenzene)[12]. Flavonoids or similar polyphenols have not yet been found in algae. However, the compounds with a flavonoid skeleton were analyzed using atmospheric pressure chemical ionization, ESI-mass spectrometry and other methods in the supercritical-fluid extracts of *Spirulina platensis*[13]. Hence, the purpose of this study is to develop a simple method to simultaneously extract the four bioactive compounds and isolate using analytical chromatography.

EXPERIMENTAL

Authentic standards of 3,4-dihydroxybenzaldehyde (DB), *p*-hydroxybenzoic acids (HA), salicylic acid (SA) and 2,3-dihydroxybenzoic acid (DA) (Fig. 1) were purchased from Sigma-Aldrich (USA). Acetonitrile and acetic acid were obtained from Duksan Pure Chemical Co. Ltd., (Ansan, Korea). All the other reagents used in the experiment were HPLC or analytical grade. Double distilled water was filtered with a vacuum pump (Division of Millipore, Waters, USA) and filter (HA-0.45, Division of Millipore, Waters, USA) before use.

Chromatography was performed with a Waters 600s multisolvent delivery system, a Waters 616 liquid chromatography and a Waters 2487 variable wavelength, dual-channel

UV detector (Waters Associates, Milford, MA, USA). A six-port Rheodyne injector (with 20 μL and 25 μL sample loops) was also used. Data processing was performed with Millennium 3.2 software resident in an HP Vectra 500PC. Compounds were separated on a 150 mm × 4.6 mm, 5 μm particle, OptimaPak C_{18} column (RStech, Daejeon, Korea). The flow rate was set at 0.5 mL/min. The wavelength was fixed at 245 nm. An injection volume of 10 mL was applied throughout the experiments. All procedures were carried out at ambient temperature.

Samples preparation: *Gelidium amansii* was oven-dried, sliced and crushed into powder for use in the extraction experiments. Four standards compounds were dissolved in methanol to yield a final concentration of 0.025 mg/mL.

Different extraction solvents used in the experiment were water, methanol, ethanol, acetone, *n*-hexane and chloroform. 50.0 mLs of each solvent was used to extract 0.5 g of *Gelidium amansii* using the same dipping time (5 h) under room temperature, respectively. Then, different assisted methods, dipping time (20-300 min) and temperature (30-80 ºC) were investigated.

RESULTS AND DISCUSSION

Effect of different extraction solvents: Table-1 shows the extracted amounts of four compounds by the different solvents, respectively. As the Fig. 1 shows, the four compounds all have several hydroxyls, which make them easily dissolved and extracted by polar solvents. All of them have the highest polarity and have the largest solubility in water, so using water as extractant can get more amount than using organic solvent.

TABLE-1
EXTRACTION AMOUNT OF FOUR COMPOUNDS BY USING LIQUID-SOLID EXTRACTION

Extraction solvent	Extraction amounts (mg/g)			
	DA	HA	DB	SA
Water	0.0094	0.0063	0.079	0.012
Methanol	0.0029	0.0047	0.0027	0.00
Ethanol	0.00	0.083	0.00	0.00
Acetone	0.00	0.00	0.00	0.00
n-Hexane	0.0060	0.0016	0.020	0.0
Chloroform	0.00	0.012	0.00	0.046
Water (containing HCl), pH < 3.0	0.028	0.00	0.00	0.00
Water (containing NaOH), pH > 10.0	0.040	0.018	0.082	0.018

Furthermore, three of them are organic acid, so the pH of water was modified with hydrochloric acid and sodium hydroxide. According to the results in Table-1, water with NaOH additive can extract the largest amount of the four compounds simultaneously (Fig. 2). So it was used in the subsequent experiments.

Effect of different extraction methods: In order to obtain the optimum extraction conditions, several methods were established. The dipping method was used first. The amounts of four compounds increased as the dipping time was increasing from 20-180 min and there was no obvious increase after 180 min, as shown in Fig. 3. Then 1 h dipping extraction with different temperature were investigated. Fig. 4 shows that the amounts of four compounds increased with the temperature increasing until 60 ºC, but three of them were decomposed

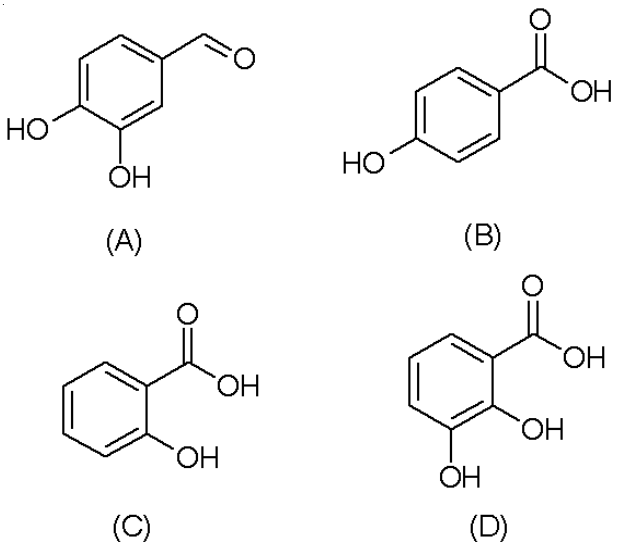

Fig. 1. Chemical structures of four bioactive compounds. (A) 3,4-Dihydroxybenzaldehyde (DB), (B) *p*-hydroxybenzoic acid (HA), (C) salicylic acid (SA), (D) 2,3-dihydroxybenzoic acid (DA)

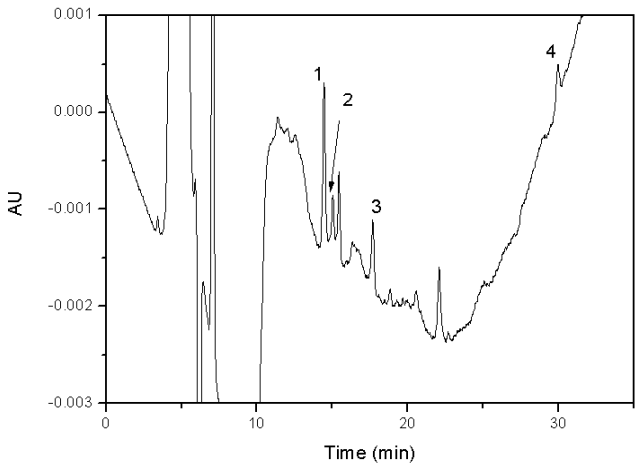

Fig. 2. Chromatogram of extract by water (containing 0.1 mol/L NaOH). (Mobile phase: acetonitrile/water (v/v, containing 1 % acetic acid) from 15/85-50/50 with 0-30 min gradient elution, flow rate: 0.5 mL/min, column: C_{18} (5 μm, 250 mm × 4.6 mm, RStech), UV: 245 nm, concentration: 0.01 mg/mL, injection volume: 10 mL). (1) 2,3-Dihydroxybenzoic acid, (2) *p*-hydroxybenzoic acid, (3) 3,4-dihydroxybenzaldehyde, (4) salicylic acid

when the temperature was higher than 60 ºC. So 1 h dipping under 60 ºC was selected as the optimum extraction condition.

Equivalent samples also were prepared by using ultrasonic-assistant and microwave-assistant method. In Table-2, the extracted amounts of the four compounds by using 0.5 h ultrasonic were less than dipping method but cost much more energy. With the microwave method, the extraction solvent was heated rapidly and the high temperature decomposed the target compounds. So in further experiment, assistant methods were unnecessary.

Effect of concentration of NaOH extraction solvent: As Fig. 5 shows, the extracted amounts of all target compounds increased as the concentration of NaOH increasing. However, no obvious increase was observed when the concentration was higher than 1.0 mol/L. Therefore, the use of 1.0 mol/L of NaOH in water was determined.

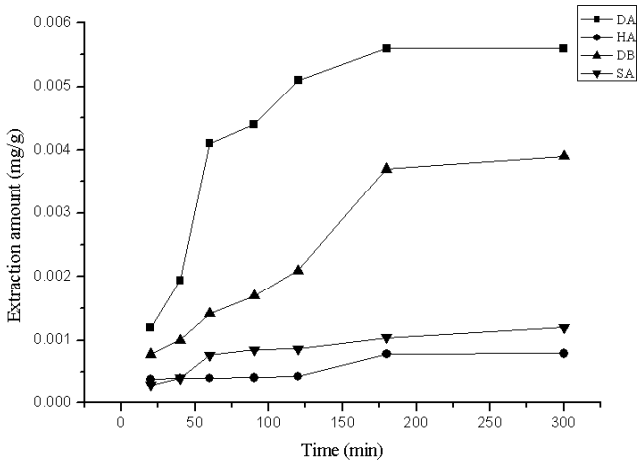

Fig. 3. Effect of different dipping time on the extraction amount of four compounds

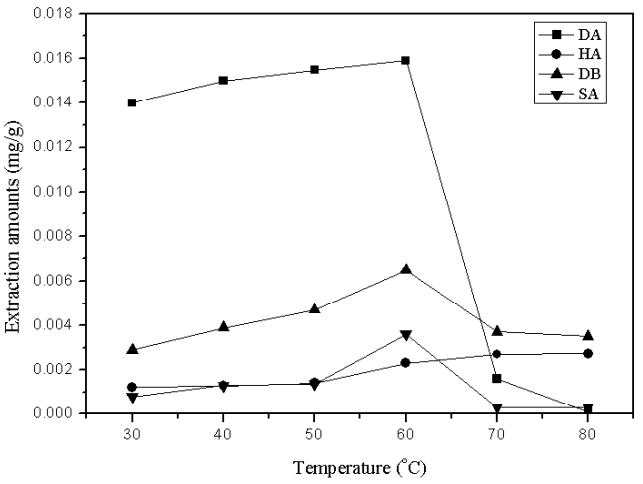

Fig. 4. Effect of different dipping temperature on the extraction amount of four compounds

TABLE-2
EXTRACTION AMOUNT OF FOUR COMPOUNDS BY USING ULTRASONIC-ASSISTANT AND MICROWAVE-ASSISTANT EXTRACTION

Method	Extraction amounts (mg/g)			
	DA	HA	DB	SA
Ultrasonic (0.5 h)	0.013	0.0030	0.046	0.00
Microwave (1 min)	0.0081	0.0014	0.0086	0.00
Microwave (2 min)	0.0046	0.00	0.0044	0.00

Method validation: To ensure the specificity and selectivity of the method, concentrations from 0.01-0.2 mg m/L were applied to each standard solution of the four compounds. The analyte peak area values were plotted against the corresponding concentrations of the analytes and the calibration curves were constructed by means of the least-square method. Calibration curves of the four compounds showed good linearity (r^2 > 0.999); the regression equations were $Y = 1.33 \times 10^7x - 607.97$ for DA, $Y = 3.85 \times 10^7x - 5252.22$ for HA, $Y = 1.70 \times 10^7x + 11480.91$ for DB and $Y = 1.49 \times 10^7x + 1181.72$ for SA.

Assays of repeatability calculated as relative standard deviations (RSDs) were performed by injecting standard solutions of target compounds 5 times in a 5-day period. Also, the standard solutions were diluted and injected until the limit of detection (LOD) was obtained at a signal/noise ratio of 3. The RSD was less than 0.9 % and the lowest LOD was 460 ng/mL. Comparison with the real sample analysis verified that the values noted above were of acceptable precision and accuracy.

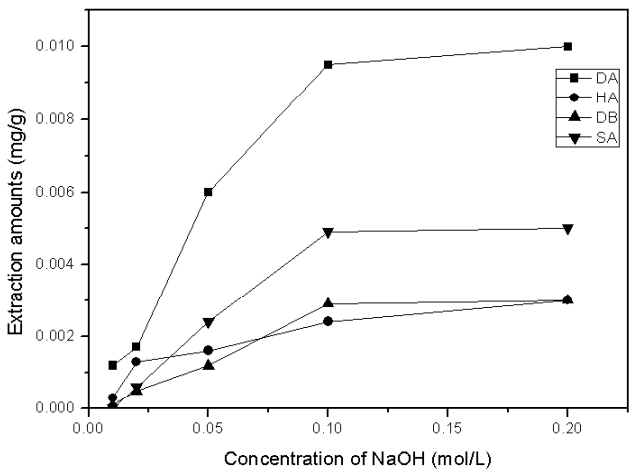

Fig. 5. Effect of different concentration of NaOH on the extraction amount of four compounds

Conclusion

In this study, a simple and convenient method for the simultaneous extraction and separation of four bioactive compounds from *Gelidium amansii* is described. Under the optimum extraction conditions (0.1 mol/L NaOH solvent dipping under 60 ºC for 1 h), the extracted amounts of DA, HA, DB and SA were 0.056, 0.083, 0.082 and 0.018 mg/g, respectively.

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