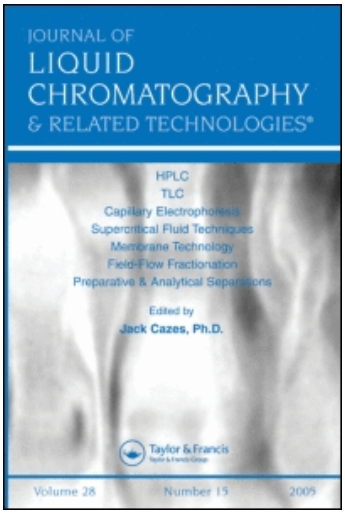

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Hee Ju Lee[a]; Min Cheol Kim[a]; Byung Hun Um[a]; Chul Young Kim[a]
[a] Natural Products Research Center, KIST Gangneung Institute, Gangneung, Gangwon-do, Korea

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Determination of Phyllodulcin from Sweet Hydrangea Leaves by Subcritical Water Extraction and High Performance Liquid Chromatographic Analysis

Hee Ju Lee, Min Cheol Kim, Byung Hun Um, and Chul Young Kim

Natural Products Research Center, KIST Gangneung Institute, Gangneung, Gangwon-do, Korea

Abstract: The subcritical water extraction was developed for extraction of phyllodulcin, well known as a sweetener in *Hydrangea macrophylla* var. *thunbergii* Makino (Saxifragaceae). Several temperatures (50, 100, 125, and 150°C) and static extraction times (5, 10, and 20 min) were studied for optimization of the extraction protocol. The optimized conditions are as follows: temperature, 150°C; static extraction time, 20 min. In addition, extraction efficiency of the subcritical water extraction for phyllodulcin was compared with ultrasonic extraction with methanol. The extraction yields of phyllodulcin were 10.41 ± 2.02 mg/g by subcritical water extraction (temperature, 150°C; static extraction time, 20 min) and 17.40 ± 2.02 mg/g by ultrasonic extraction with methanol. As results in this experiment show, subcritical water extraction was an alternative environmentally friendly extraction method for phyllodulcin in *H. macrophylla* var. *thunbergii*.

Keywords: Sweet hydrangea leaves, Subcritical water extraction, Phyllodulcin, Ultrasonic extraction

INTRODUCTION

Sweet hydrangea leaves (*Hydrangea macrophylla* var. *thunbergii* Makino, Saxifragaceae) contained phyllodulcin, which is a dihydroisocoumarin

Correspondence: Chul Young Kim, Natural Products Research Center, KIST Gangneung Institute, Gangneung Techno Valley, Gangneung, Gangwon-do 210-340, Korea. E-mail: kcy98@kist.re.kr

derivative. Phyllodulcin is well known as a sweetener, since it is 600–800 times sweeter than sucrose.[1,2] It is also used to make tea served during the Buddhás Birthday celebration. Several biological activities have been observed for phyllodulcin, including antiallergic, effects,[3] inhibition of microsomal lipid peroxidation induced by NADPH,[4] and inhibition phosphodiesterase in bovine adernocortical cells.[5]

Conventionally, the isolation of plant derived compounds involves an extraction process with an organic solvent, either with magnetic stirring or by using a Soxhlet apparatus. Water is non-flammable, harmless, readily available, and an eco friendly solvent. However, it has not yet received much attention as an extraction solvent for plant materials because it is too polar to efficiently dissolve most organic compounds that are associated with botanicals. Subcritical water refers to water at the temperatures between its boiling temperature (373.15 K) and its critical temperature (547.3 K), and at the pressure high enough to maintain it in the liquid state. At such conditions, the water dielectric constant decreases, and thereby decreasing its polarity. As a result, the solubility of organic compounds in subcritical water increases.[6–8] Subcritical water extraction has recently been investigated for extraction of various plant secondary metabolites.[6,7,9] These include essential oils from various plants such as coriander seeds, oregano, and *Thymbra spicata*.[10–12] Extraction of other compounds including hypericin and pseudohypericin from St. John's wort,[13] iridoid glycosides from *Veronica lonifolia*,[14] and kava lactones from kava roots[15] were also investigated.

In this study, subcritical water extraction for phyllodulcin was developed and its extraction yield was evaluated using high performance liquid chromatography.

EXPERIMENTAL

Material and Reagents

The fermented and dried leaves of *Hydrangea macrophylla* var. *thunbergii* Makino, (Saxifragaceae) were obtained from a herb farm (Sugukdawon) near Gangneung city, Korea. The voucher specimen (HM-GN-011) was stored at the Natural Products Research Center, KIST Gangneung Institute, Gangneung, Korea. Standard phyllodulcin was purchased from Wako Chemicals (Osaka, Japan). HPLC grade solvents were purchased from Fisher Scientific (Pittsburgh, PA, USA). Sea sand (15–20 mesh) for pressurized liquid extraction was purchased from Junsei (Tokyo, Japan). Trifluoroacetic acid of analytical reagent grade was purchased from the Sigma-Aldrich Company (St. Louis, MO, USA). All other chemicals were analytical grade.

Pressurized Liquid Extraction

Pressurized liquid extractions were carried out using a Dionex ASE 300 Accelerated solvent extractor (Sunnyvale, CA, USA). The dried powder of *H. macrophylla* (1 g) was placed in a 34 mL stainless steel extraction cell. A filter paper was placed at the bottom of the extraction cell. Sea sand (35 g) has been used as the supporting material in the extraction cell. Extractions were performed at four extraction temperatures (50, 100, 125, and 150°C) and three extraction times (5, 10, and 20 min).

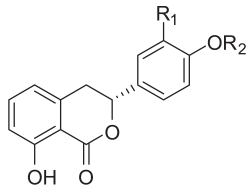
The extraction procedure was as follows: (i) the extraction cell was loaded into the oven; (ii) the cell was filled with solvent; (iii) initial heat up time was applied; (iv) a static extraction with all system valves closed was undertaken; (v) the cell was rinsed with 60% of the cell volume with extraction solvent; (vi) solvent was purged from the cell with N_2 gas for 120 s; and (vii) depressurization took place. The extractions were collected into glass collection vials. The extract was transferred to a 100 mL volumetric flask, which was brought up to its volume with methanol and filtered prior to injection into the HPLC system.

Ultrasonic Extraction

The ultrasonic extractions were carried out in an ultrasonic cleaning bath (model RK 158s, Bandelin, Germany). A 1.0 g of ground sample were extracted with 30 mL of methanol at room temperature for 1 h and filtered through Whatman No. 1 filter paper into a 100 mL volumetric flask. The extract was brought up to its volume with methanol and filtered prior to injection into the HPLC system. The procedures were repeated three times.

HPLC Analysis

All of the extracts were analyzed in an Agilent Series 1200 liquid chromatograph, equipped with a G1379B vacuum degasser, G1312A binary pump, G1329A autosampler, G1316A column oven, and G1315B DAD detector, connected to Agilent ChemStation software. A Zorbax Eclipse XDB C-18 column (4.6 × 150 mm i.d., 5 μm, Agilent) was used for the analysis of phyllodulcin. The mobile phase was acetonitrile 0.1% TFA in gradient mode as follows: acetonitrile 0.1% TFA (30:70 v/v at 0 min to 50:50 v/v at 20 min). The flow rate was 1.0 mL/min and the oven temperature was set at 40°C. Detection was at 254 nm and the injection volume of standards and extracts was 20 μL, respectively.



phyllodulcin (**1**), R_1=OH, R_2=Me
hydrangenol (**2**), R_1=H, R_2=H

Figure 1. Structures of phyllodulcin and hydrangenol.

HPLC-DAD-ESI/MS Analysis of Phyllodulcin and Hydrangenol from *H. macrophylla* Extract

HPLC-DAD-ESI/MS was obtained by the Agilent 1100 Series HPLC-MSD System equipped with an autosampler, a column oven, a quaternary pump, a DAD detector, and a degasser (Agilent, Waldbronn, Germany) was used. A 5 μL volume of sample solution was directly injected on a Zorbax Eclipse XDB-$C_{18,}$ (150 mm × 4.6 mm, 5 μm). The mobile phase was acetonitrile 0.1% TFA in gradient mode as follows: acetonitrile 0.1% TFA (30:70 v/v at 0 min to 50:50 v/v at 20 min). The flow rate was 0.3 mL/min and the oven temperature was set at 40°C. The ChemStation software was used to operate this HPLC system. Mass spectrometer conditions are as follows; positive ion mode; mass range, m/z 100–1000; the capillary voltage, 3000V; nebulizing gas pressure (N_2), 35 psi; drying gas (N_2) flow rate, 12.0 L min^{-1}; drying temperature, 350°C (Figure 1).

Statistics

The data presented are means ± SD of three determinations.

RESULTS AND DISCUSSION

HPLC-DAD-ESI/MS Analysis of *H. macrophylla* Extract

H. macrophylla extract was investigated by HPLC-DAD-ESI/MS. The UV and ESI-MS spectra of HPLC peaks of phyllodulcin and hydrangenol are shown in Figure 2. The UV spectra of compounds **1** and **2** were similar in the ranges 240–260 nm (shoulders), and 290–340 nm (maxima). Phyllodulcin showed base ion peak at m/z 287.1 $[M + H]^+$ and other ion peaks were detected at m/z 595.2 $[2M + Na]^+$, 309.1 $[M + Na]^+$, and 269.1 $[M\text{-}OH]^+$. Mass spectrum of hydrangenol exhibited base ion peak at m/z 257.0

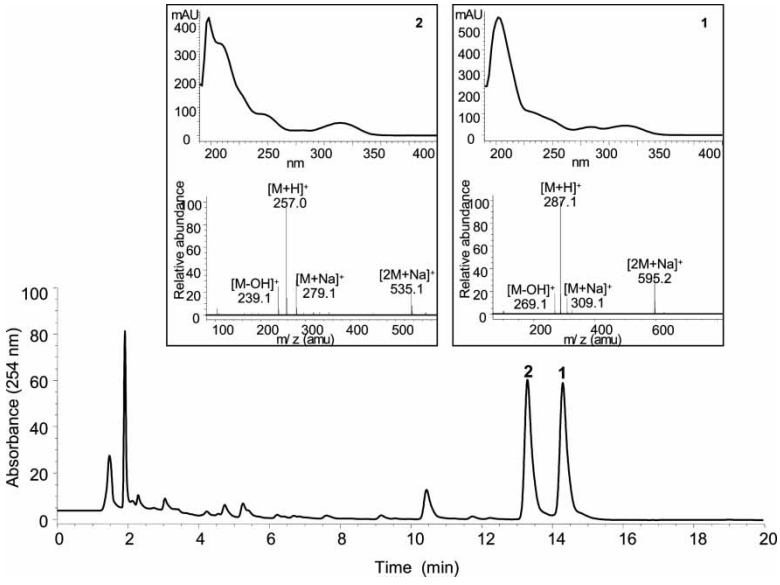


Figure 2. HPLC chromatogram, UV and MS spectra of phyllodulcin (**1**) and hydrangenol (**2**). Chromatographic conditions for HPLC and HPLC-DAD-ESI/MS are described in Experimental section.

$[M + H]^+$ and other ion peaks were identified at m/z 535.1 $[2M + Na]^+$, 279.1 $[M + Na]^+$, and 239.1 $[M\text{-}OH]^+$. Phyllodulcin was identified by retention time, UV and MS spectrum compared with those of authentic sample using HPLC-DAD-ESI/MS. Hydrangenol was determined by UV spectrum and ESI-MS data.[2]

Quantitative Analysis of Phyllodulcin in *H. macrophylla*

The standard curve of phyllodulcin was calibrated by using the linear least squares regression equation derived from the peak area. The concentration of phyllodulcin in the samples was calculated according to the regression parameters derived from the standard curve. Three replicate measurements were carried out. The calibration curve for phyllodulcin was obtained and expressed as $Y = 16.184\ X - 28.421$. A good linearity was achieved with a correlation coefficient of 0.9999 over the concentration ranges 4–500 μg/mL. The limits of detection (LOD) and quantification (LOQ) for phyllodulcin were determined at the standard deviation of the response and the slope (σ/S) of about 3.3 and 10. The LOD and LOQ values for phyllodulcin were 1.45 and 4.38 μg/mL. The overall precision of the analysis was satisfactory. For the intra-day variability test, the samples were analyzed in triplicate for three

times within 1 day; while for the inter-day variability test, the solution was examined in triplicate for a consecutive 3 days. Variations were expressed by the relative standard deviations (R.S.D.). The intra–day and inter-day precision of phyllodulcin were 1.5% and 2.5%

Optimization of Subcritical Water Extraction Conditions for Phyllodulcin Extraction

Subcritical water extraction conditions of temperature and time can affect the extraction amount of phyllodulcin from *H. macrophylla.* In subcritical water extraction, the temperature is a key factor affecting the dielectric constant of extraction solvent. The experiments used 1.0 g of powdered samples extracted by water at various temperatures such as 50, 100, 125, and 150°C at 1500 psi (pressure), static extraction time of 5 min, 60% of flush volume, and one extraction cycle. The extraction yield of phyllodulcin is shown in Figure 3. As shown in Figure 3, the extraction efficiency of phyllodulcin (0.64–4.98 mg/g) increased when increasing the temperature. Higher temperature resulted in higher extraction contents.

Figure 4 shows the function for static extraction time between 5, 10, and 20 min at temperature of 150°C. The highest extraction yield of phyllodulcin was 10.41 mg/g, which was achieved at 150°C (extraction temperature) and 20 min (static time) by subcritical water extraction; however the ultrasonic extraction with methanol yielded 17.41 mg/g.

Resulting from this study, the optimum subcritical water extraction conditions to obtain the highest extraction efficiency of phyllodulcin were selected as: temperature, 150°C; static extraction time, 20 min.

Although subcritical water extraction efficacy was approximately 40% lower than that of conventional organic solvent extraction with methanol,

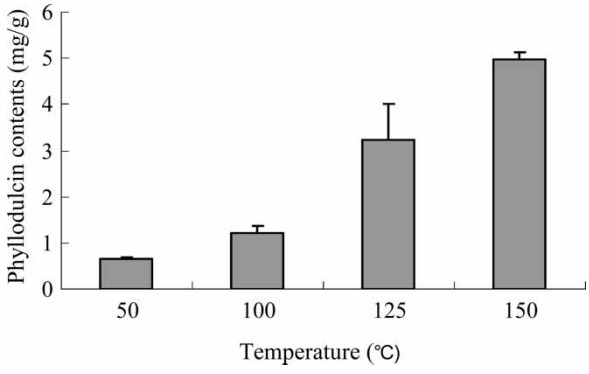


Figure 3. Phyllodulcin contents of crude water extracts from *H. macrophylla* by subcritical water extraction with variation of temperature at 1500 psi.

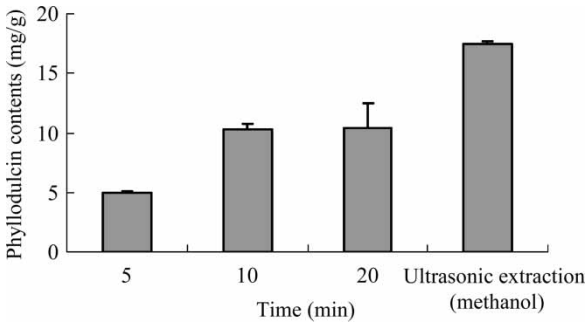


Figure 4. Phyllodulcin contents of crude water extracts from *H. macrophylla* by subcritical water extraction with variation of static extraction time at 1500 psi and 150°C.

the subcritical water extraction was safe, non-toxic, readily available, and environmentally friendly.

CONCLUSION

HPLC-DAD-ESI/MS analysis of phyllodulcin and hydrangenol in *H. macrophylla* extract was accomplished and, subsequently, the extraction conditions of phyllodulcin by subcritical water extraction were optimized. The optimized extraction conditions are as follows: temperature, 150°C; static extraction time, 20 min. The results showed that subcritical water extraction could be an alternative to soxhlet and ultrasonic extraction for phyllodulcin.

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