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Separation of Camelliaside C from Tea Seed by RP-HPLC

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The plant *Camellia sinensis* has been cultivated widely in oriental countries as a source of various types of tea. The main flavonoids in these teas are glycosides of kaempferol including camelliasides. Using the pure water, the Camelliaside C contained in tea seed was extracted. The pretreatment steps were composed of solvent extraction, filtration, concentration and membrane filtration. The procedures of extraction separation were proposed and the extraction effects of various extractive solvents were compared. As a potential skin diseases agent, the Camelliaside C contained in tea seed (Korea) was considered for isolation by reversed-phase high-performance liquid chromatography. The mobile phase was linearly varied from an A/B ratio of 85/15 to 65/35 vol. % over a period of 50 min (A: water/acetic acid, 99.9/0.1 vol. % and B: acetonitrile/acetic acid, 99.9/0.1 vol. %). It was shown that Camelliaside C is contained in water and *n*-butanol extracts, but ethyl acetate layer did not contain the target material.

Key Words: Camelliaside C, Tea seed, Extraction, Separation, RP-HPLC.

INTRODUCTION

Medicinal herbages are an important source of natural products for agricultural and food science, human nutrition and medicine. They include various chemical components ranging from liposoluble to water-soluble compounds. Traditional oriental medicine is an extremely rich source of the experience acquired over a long period of time. In order to make greater use of traditional Chinese and Korean nutrition and medicine, modern scientific methods are used to find the bioactive compounds in the conventional drugs and to use them as leading compounds for new drug design. In recent years, cosmetic, pharmaceutical and chemical industries have become increasingly interested in antioxidants. Current research efforts on antioxidants have focused on flavonoids that show strong free radical scavenging effects and metal ion chelating properties. In addition to their antioxidant activity, flavonoids have been reported to inhibit various enzymes. As a result, nutritional flavonoids have attracted attention for potential beneficial effects on humans[1-4] and new medical products developed in this way include the flavonoids agents.

Various kind of tea has been cultivated widely in Asia for centuries. Medical studies suggest that the consumption of tea provides protection against cancers in humans[5-8]. Tea is generally consumed in one of three forms: green, oolong or black. To produce green tea, after the leaves are picked, the young leaves are rolled and steamed to minimize oxidation[9]. Recently, as the popularity of green tea has increased, the production of green tea seed has also improved. As tea contains a number of chemical constituents possessing medicinal and pharmacological properties, green tea seed contains many biologically active compounds such as flavonoids, vitamins and oils[8]. *Camellia sinensis* has been cultivated widely in oriental countries as a source of various types tea. The main flavonoids in these teas are glycosides of kaempferol including camelliasides[10]. The isolation of the target bioactive components is the starting point of further research in chemistry, pharmacology and food science as well as in the utilization of these compounds. Concerning the commercial use of flavonoids for other industrial purposes, the production of pure separated flavonoids from plant sources such as tea is not easy.

For separating and purifying bioactive compounds from medicinal herbs including green teas, modern chromatographic techniques, such as gas chromatography, high performance liquid chromatography, thin-layer chromatography and electrophoresis have significantly raised the technical level and have shortened the time required for research projects. Reversed-phase high performance liquid chromatography (PR-HPLC) has been recognized as an effective means for separation and purification of a wide variety of bioactive components[11,12].

In this study, we report the separation of Camelliaside C from tea seed extracts by RP-HPLC. For the optimum analytical separation condition, several experiments were performed. Also, the extraction effects of various extractive solvents were compared.

EXPERIMENTAL

The standard of Camelliaside C and tea seed were obtained from Amore Pacific Cosmetic Co. (Korea). HPLC-grade solvents such as acetonitrile, ethyl acetate (EtOAc), *n*-butyl alcohol (*n*-BuOH) were purchased from Duksan Co. (Seoul, Korea). Water was filtered by a millipore ultra pure water system (Millipore, Bedford, MA, USA).

An extracted sample was concentrated by using a rotary-evaporator (LABO-THERM SW 200, Resona Technics Co.). The HPLC system was equipped with Waters 600S solvent delivery system including 616 solvent delivery pump and 600S controller and the 486 UV dual channel detector,

an injector (0.02 mL sample loop) of Rheodyne injector (Waters, Milford, MA, USA). The data acquisition system was Millenium32 (Waters) installed in a HP Vectra 500 PC. The finally extract of tea seed was filtered with HA-0.2 μm membrane.

Extraction method: The procedures to purify Camelliaside C from tea seeds comprise the following stages (Fig. 2) extraction by water solvents, partition step and development of the analytical conditions. The powder (2 g) of tea seed was added to water (100 mL) and macerated for 0.5 h at 80 °C. It was cooled after the aqueous extract was concentrated in vacuum to about 10 mL and partitioned with EtOAc (10 mL × 3 times) and *n*-BuOH (10 mL × 3 times), respectively. After evaporation of the solvent, the EtOAc extract (10 mL) and *n*-BuOH (10 mL) were obtained.

Analysis: The standard stock solution was prepared as 2 mg of Camelliaside C in 4 mL of water. The concentration was adjusted to 500 ppm by water. The solutions were stored at 4 ºC and the working standards were re-prepared every 2 d to avoid the potential errors from decomposition of the targets. Tea seeds (2 g) were macerated with 100 mL of boiling water[11,12] for 0.5 h. Fig. 1 showed the chemical structure of Camelliaside C. Sufficient times were allowed for the stabilization of the column and detector signal after each injection and the solvents in reservoirs were continuously stripped with helium to degas the mobile phase. The wavelength was fixed at 254 nm. The chromatographic columns used in analytical

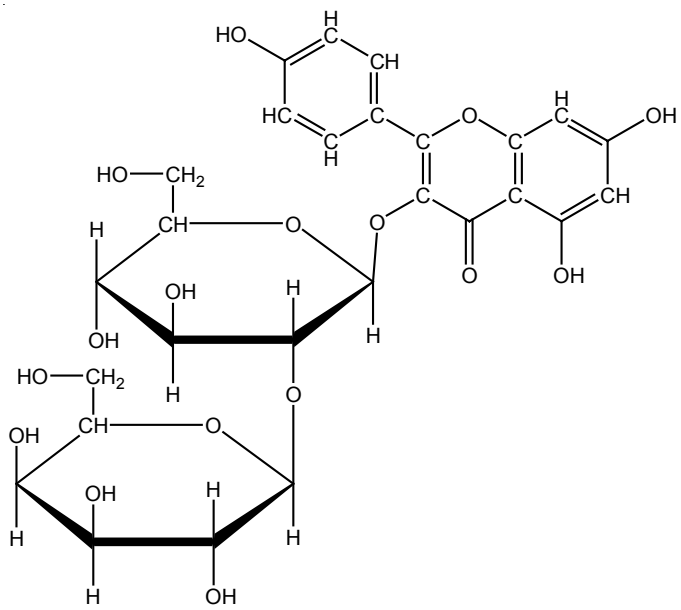

Fig. 1. Structure of Camelliaside C (m.f.: $C_{27}H_{30}O_{16}$, m.w. 610)

experiments was a commercially available from RS-tech (0.46 × 25 cm, 5 μm, C_{18}, Daejeon, Korea) and the column (0.39 × 30 cm, 12 μm, C_{18}, Merck, Germany) was used for semi-preparative isolations. The flow rate was 1 mL/min and the injection volume was fixed at 20 μL. The binary mobile phases were composed of water and acetonitrile with acetic acid (0.1 % vol). The adjustable experimental variables were the conditions of gradient modes and mobile phase compositions. It was linearly varied from an A:B ratio of 85:15 to 65:35 (vol. %) over a period of 50 min [A: water:acetic acid, 99.9:0.1 (vol. %) and B: acetonitrile:acetic acid, 99.9:0.1 (vol. %)]. All chromatographic procedures were performed at ambient temperature. The qualitatively identification of Camelliaside C was carried out using commercial standard.

RESULTS AND DISCUSSION

No single liquid chromatographic separation method is able to solve all separation problems and moreover it is very common to find multi-step operations for the isolation of pure natural products. Although it is possible to obtain a target compound by a one- or two-step procedure, combination of separation techniques is normally required. Of course there are many different ways of putting together all the possible separation techniques, but in reality the choice of strategy is limited by a number of constraints: the sample preparation, the extraction methods and the separation techniques.

Generally, solvents of increasing polarity extract the plant materials. This extraction step is very important because it allows a first rough fractionation of the plant constituents. Initial extraction with low polarity solvents yields the more lipophilic components, while alcoholic solvents give a larger spectrum of apolar and polar material. The plant extracts are usually very complex mixtures, which contain hundreds or thousands of different constituents.

The concentrations of flavonoids in the plants are quite low, typically. Furthermore, the sample matrices in which flavonoids are usually determined are very complex in most cases. Consequently, extensive sample extraction, clean-up and preconcentration are often required prior to the analysis. The most important and time-consuming step in the determination of flavonoids in samples is the extraction of the analytes from the matrix. Extraction using water or an organic solvent, such as alcohols, has traditionally extracted hydrophilic compounds, such as Camelliaside C. One of the most common solvents, removing the majority of hydrophobic analytes, is ethyl acetate. Samples with high water content are best extracted with polar solvents such as methanol, acetone or acetonitrile. Usually, a typical extraction sequence consists of routine steps. First, the tea seed is prepared

to receive a soluble sample, by wetting with an organic solvent or by conditioning with water. Then, the tea seed extract is applied and often the analytes of interest are retained together with interferences from the sample matrix. Some of these interferences can then be removed by application of a washing solution. In the last step, the concentrated analytes are desorbed with a small volume of organic solvent, which can then be partially evaporated to increase the enrichment factor. This approach has been used to isolate and determine Camelliaside C from natural tea seed extracts and the detection with an ultraviolet detector. The combination of different separation techniques for the isolation of Camelliaside C from tea seed is presented in Fig. 2. At first, the tea seed were extracted with water at 80 ºC for 0.5 h. The obtained water extract was concentrated by evaporation. Then, the part of water concentrate was partitioned in parallel with EtOAc and *n*-BuOH. Finally, the water extract and acquired organic layers were chromatographed. RP-HPLC of complex mixtures such as plant extracts is usually carried out on reversed-phase columns. For the separation of the analytes, acetonitrile-water gradients elution is applied. Most often, for the suppression of tailing due to flavonoids constituents, modifier such as acetic acid was added. If the isolation of a given constituent is required, a scale-up of the analytical separation conditions to preparative or semi-preparative chromatography techniques is needed. Preparative-scale separation is one of the most important operations carried out in a natural products laboratory. It is often tedious and time-consuming, especially when the mixture to be separated is complex. Fig. 3 shows the separation of

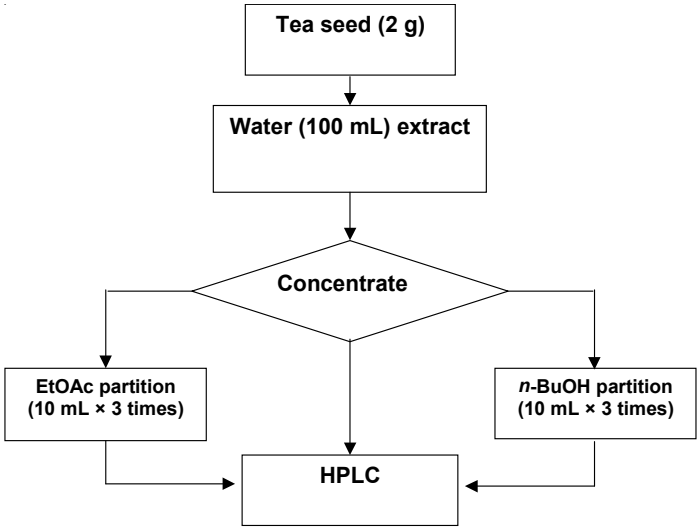

Fig. 2. Strategy for the isolation of Camelliaside C from tea seed

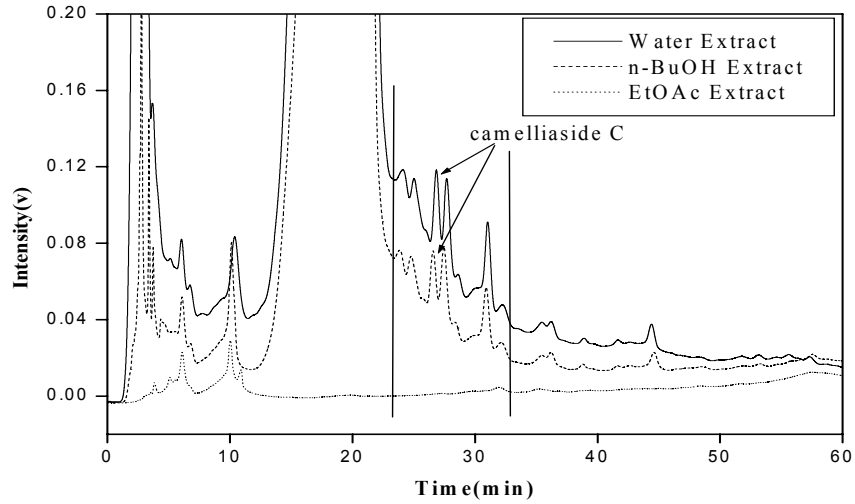

Fig. 3. Chromatograms of extracts from tea seed with semi-preparative column (C_{18}, 12 μm column, water:acetonitrile 85:15-65:35 vol. % for 50 min, inj. vol. 20 μL)

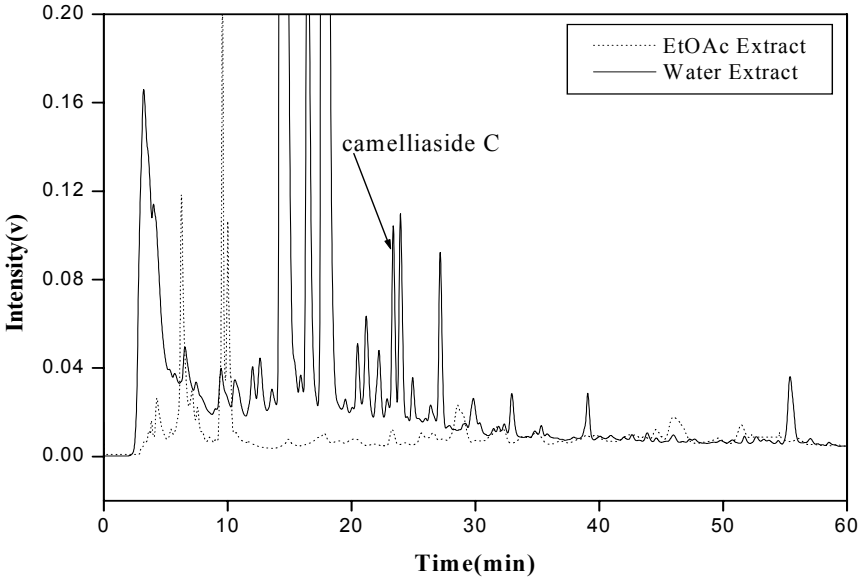

Fig. 4. Chromatograms of extracts from tea seed with analytical column (C_{18}, 5 μm column, water:acetonitrile 85:15-65:35 vol. % for 50 min, inj. vol. 20 μL)

analytes from teas seeds extracts by semi-preparative RP-HPLC. As is obvious, the peak of Camelliaside C is identified in water and *n*-BuOH fractions. The EtOAc layer showed an absence of Camelliaside C. Analogous results with analytical column demonstrated Fig. 4. These chromatograms verified availability of Camelliaside C in the water extract and missing of it in the

EtOAc fraction. The semi-preparative fractions eluted from 22-32 min were collected, concentrated and analyzed with analytical column. A typical chromatogram of the water fraction after semi-preparative separation is shown in Fig. 5. The separation was achieved by applying a linear acetonitrile-water gradient on a C_{18} column. A satisfactory isolation of the Camelliaside C was achieved.

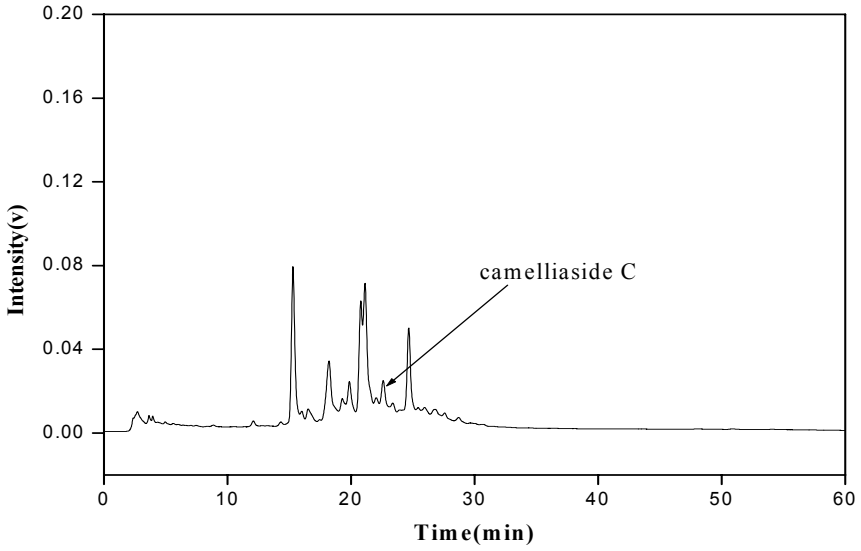

Fig. 5. Chromatograms of water fraction from tea seed as Fig. 3 (22-32 min) (C_{18}, 5 mm column, water:acetonitrile 85:15-65:35 vol. % for 50 min, inj. vol. 20 µL)

Conclusion

In this work, we report the qualitative determination of Camelliaside C in tea seed. The procedures of extraction separation were proposed and the extraction effects of various extractive solvents were compared. The semi-preparative and analytical isolations of Camelliaside C were performed by RP-HPLC. It was shown that Camelliaside C is contained in water and *n*-butanol extracts, but ethyl acetate layer did not contain the target material.

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