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Separation of Three Flavones in *Chamaecyparis obtusa* by Using Solid-Phase Extraction

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Three primary flavones components, amentoflavone, quercitrin and myricetin were extracted from *Chamaecyparis obtusa* leaves by refluxing in methanol and separated by gradient elution by RP-HPLC. Solid-phase extraction with different polarity solvents was applied to separate the components from the extract of methanol. 3.040 mg/g for myricetin, 0.0908 mg/g for quercitrin and 0.514 mg/g for amentoflavone can be obtained.

Key Words: Amentoflavone, Quercitrin, Myricetin, *Chamaecyparis obtusa*, Solid-phase extraction.

INTRODUCTION

Natural plants have been used in many domains including medicine, nutrition, flavourings, beverages, dyeing, repellents, fragrances, cosmetics and other industrial purposes. Since the prehistoric era, plants have been the basis for nearly all medicinal therapy until synthetic drugs were developed in the 19th century[1,2]. *Chamaecyparis obtusa*, a kind of common tree to beautiful the environment, distributes in Japan and Taiwan primary. It has been preserved as a valuable wood for the building materials of such nationally important building as the empire place, famous shrines and temples and for general use in hygienic woodenware[3]. The primary composition of *Chamaecyparis obtusa* is essential oil, which are antioxidative and antimicrobial and can be used for beauty. Flavonoid is also an important component in *Chamaecyparis obtusa*. There are several papers are available in literature about the separation of flavonoid[4].

Liquid-liquid extraction is used in multiresidue methods based on hydrophilic solvent extraction to remove water and water soluble co-extractives and to bring the residues into a low-boiling medium polarity solvent that is amenable to subsequent clean-up steps. However, this technique is a time consuming operation[5] and the disadvantages are different extraction efficiencies for various compounds with various extracting agents and low sensitivity[6].

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Nowadays, because of its advantages of high recovery, short extraction time, high enrichment factor, low cost and low consumption of organic solvents[7-10], solid-phase extraction (SPE) has developed as an alternative to liquid-liquid extraction for the separation, purification, concentration and/or solvent exchange of solutes from solution[11] because of the different interactions between target compounds and the sorbents.

In this study, three flavone components amentoflavone, quercitrin and myricetin will be extracted from *Chamaecyparis obtusa* by solid-phase extraction and appropriate separation condition will be determined.

EXPERIMENTAL

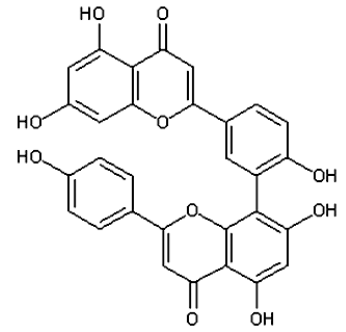
HPLC analysis was performed using a liquid chromatography system containing a Waters 600s Multisolvent Delivery System and a Waters 616 pump (Waters, Milford, MA, USA), a Waters 486 Tunable Absorbance UV detector (Waters, Milford, MA, USA) and a Rheodyne injection valve (20 µL sample loop). Autochro-2000 software (Younglin Co. Ltd., Korea) was used as data acquisition system. Separation was accomplished on C_{18} column (4.6 mm × 250 mm, 5 µm). The flow rate was set at 0.5 mL/min, and the column temperature was maintained at room temperature.

Amentoflavone, quercitrin and myricetin were bought 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 of the highest grade commercially available. 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. All the samples were filtered by using a filter (MFS-25, 0.2 µm TF, Whatman, USA) before injection into the HPLC system.

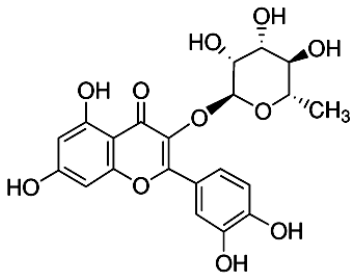
Preparation of standard solutions: Stock standard solutions of amentoflavone, quercitrin and myricetin were prepared in methanol (Fig. 1). Working standard solutions containing each of the three compounds were prepared by diluting the stock solutions with methanol to proper volumes. The standard stock solutions and working solutions were all prepared in dark brown calibrated flasks and stored at 4 °C until analysis.

Preparation of extract sample: In order to get the most amounts of target components from *Chamaecyparis obtusa*, two extract methods (stirring and reflux distillation) were used and compared.

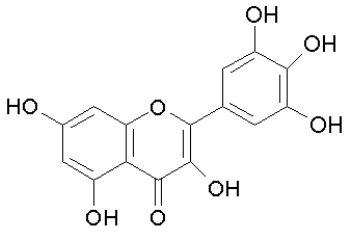
5.0 g powder of *Chamaecyparis obtusa* leaf was added into a glass bottle (100 mL capacity) with 50 mL of methanol. Then, the materials were stirred for 24 h at room temperature or reflux distilled in a reflux unit for 5 h. After that, aliquot of the solution was filtered through a 0.20 µm disposable syringe filter. Finally, the samples were filled in dark brown calibrated flasks and stored at 4 °C until analysis.



Amentoflavone



Quercitrin



Myricetin

Fig. 1. Structures of the three flavones in *Chamaecyparis obtusa*

Solid-phase extraction: Two kinds of cartridges (C_{18} and silica) were used and 4 mL of different polarity solvents including water, methanol, ethanol, acetonitrile, *n*-hexane, *n*-octanol and chloroform were used to select the elution solvent.

RESULTS AND DISCUSSION

Effect of different analytical conditions: In order to optimize the composition of the mobile phase, different concentrations of acetonitrile from 20-80 % in water were compared. The results showed that the standard samples can be separated completely in 20-40 % aqueous acetonitrile and based on the retention time and resolution, but 27 % aqueous acetonitrile found to be the optimal composition of the mobile phase for standard samples. But when the extract was analyzed by 27 % aqueous acetonitrile, myricetin can not be separated with peripheral impurities. So 22 % aqueous acetonitrile were used for the optimum concentration.

Effect of mobile phase additive: In order to obtain the optimum separation condition, different concentrations of acetic acid were added to the mobile phase to increase the resolution of the three compounds. The result showed that until the concentration of acetic acid was 0.2 % (v/v), the resolution was not improved obviously. When the concentration increased to 0.3 % (v/v), all the three components can be separated completely. Hence, 0.3 % (v/v) acetic acid was selected for the experiment as additive.

Gradient eluted scheme: When acetonitrile/water/acetic acid (22:78:0.3, v/v/v) was used as the mobile phase, three components in sample solution can be separated, but the retention time of amentoflavone was 70 min longer than quercitrin. Therefore, a gradient eluted program was applied to shorten the time. The best resolution and shortest analysis time was obtained when the mobile phase was changed linearly from acetonitrile/water/acetic acid (22:78:0.3, v/v/v) to acetonitrile/water (45:55, v/v) within 10 min. Chromatograms of the extraction of *Chamaecyparis obtusa* leaves and the mixture of standard myricetin, quercitrin and amentoflavone is shown in Fig. 2.

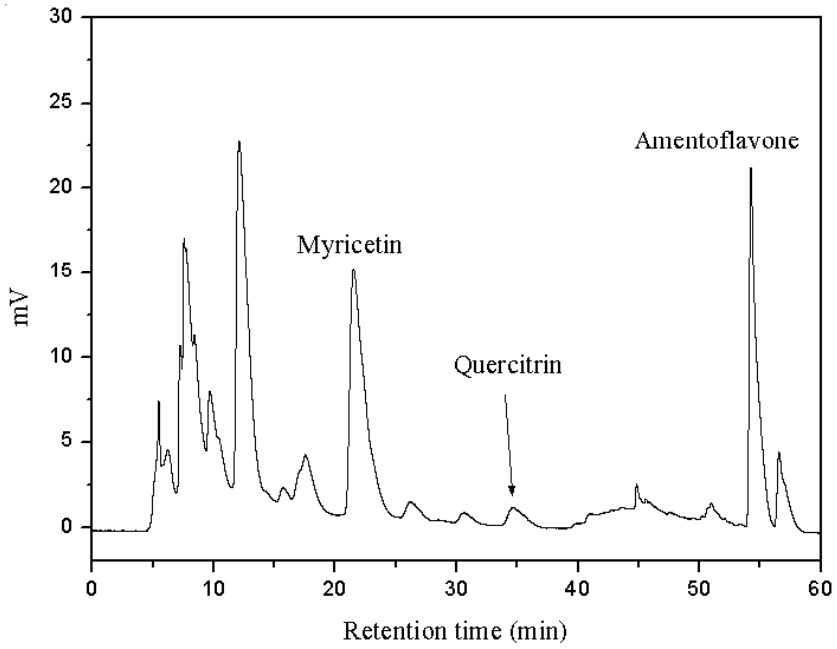


Fig. 2. Chromatograms of the extract of *Chamaecyparis obtusa* leaves

Method validation: Linear regression equation is the foundation of quantitative experiments, which shows the relationship between the peak area and the concentration of target component. All the calibration graphs were plotted based on linear regression analysis of the integrated peak areas (Y) *versus* concentrations (x, from 0.2-200.0 μg/mL) of the three components in the standard solution at five different concentrations. Y = 886.01x + 77.20 for amentoflavone, Y = 166.62x - 162.25 for quercitrin and Y = 203.90x - 57.17 for myricetin with $r^2 > 0.99$, which revealed good linear relationship between the peak areas of the three target components and their concentrations.

According to the linear regression equations and the chromatogram peak areas, the concentrations of the three components were 3.040 mg/g for myricetin, 0.0908 mg/g for quercitrin and 0.514 mg/g for amentoflavone.

Solid-phase extraction: Elution abilities of different solvents were shown in Table-1. It is clear that the methanol is the most and least effective elution solvent, so methanol/water (v/v) would be used for elution, and C_{18} cartridge is better than silica cartridge.

TABLE-1
ELUTION ABILITIES OF SEVERAL SOLVENTS

Component	C_{18} cartridge (%)				Silica cartridge (%)		
	Methanol	Ethanol	Acetonitrile	*n*-Octanol	Methanol	Chloroform	*n*-Hexane
Amentoflavone	100	35.0	28.3	12.6	18.6	1.5	–
Quercitrin	100	94.9	81.4	37.4	66.7	23.8	–
Myricetin	100	42.3	27.3	23.4	25.7	28.5	–

15 % and 50 % aqueous methanol solutions were used as elution solvents. Figs. 3 and 4 show the chromatograms of solid-phase extract samples washed by 15 % and 50 % aqueous methanol solutions, respectively. In Fig. 3, myricetin and quercitrin can be washed completely and several impurities can also be washed together. While in Fig. 4, just amentoflavone and several impurities after 1 h could be washed out.

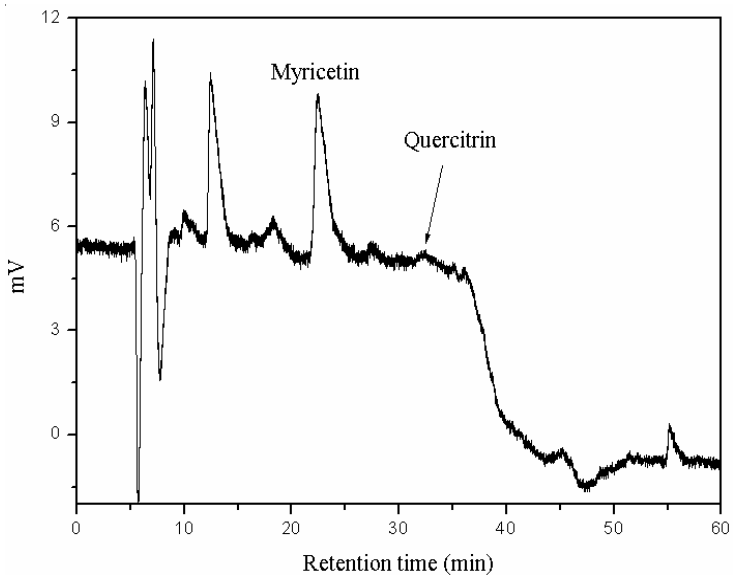


Fig. 3. Solid-phase extraction of extract by 15 % methanol aqueous solution at first step

Conclusion

Three primary flavones components, amentoflavone, quercitrin and myricetin were extracted from *Chamaecyparis obtusa* leaves by refluxing in methanol and separated by gradient elution by RP-HPLC. The elution mobile phase was changed

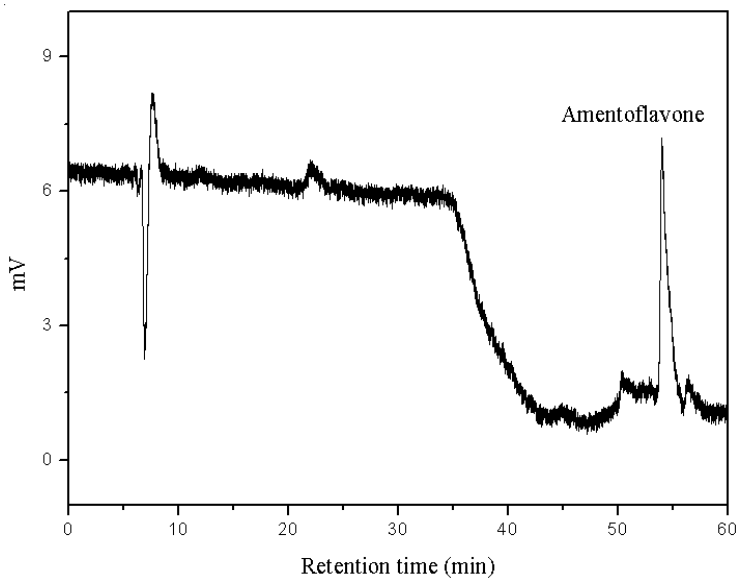


Fig. 4. Solid-phase extraction of extract by 50 % methanol aqueous solution at second step

linearly from acetonitrile/water/acetic acid (22:78:0.3, v/v/v) to acetonitrile/water (45:55, v/v) within 10 min. The contents of the components in *Chamaecyparis obtusa* leaf powder were calculated: 3.040 mg/g for myricetin, 0.0908 mg/g for quercitrin and 0.514 mg/g for amentoflavone.

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