



Purification of Two Flavones from *Chamaecyparis obtusa* by Solid-Phase Extraction

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Received: 21 May 2013;

Accepted: 17 September 2013;

Published online: 10 May 2014;

AJC-15127

Solid phase extraction was used to purify two compounds obtained by *Chamaecyparis obtusa*. Commercial silica and amino silica, MCM-41 and amino MCM-41, respectively, which have a regular structure, were applied as sorbents to increase the purification efficiency. The adsorption isotherm confirmed amino MCM-41 to have higher absorption ability. Moreover, the two target compounds could be separated and purified by solid phase extraction using amino MCM-41 with different washing and elution solvents. Under optimal conditions, 4.93 $\mu\text{g/g}$ of myricetin and 5.91 $\mu\text{g/g}$ of amentoflavone were extracted from *Chamaecyparis obtusa*.

Keywords: Purification, Flavones, MCM-41, Solid phase extraction.

INTRODUCTION

Chamaecyparis obtusa (*C. obtusa*) is a species of *cupressaceae* native to central Japan that is distributed mainly in Korea, Japan, North America, Taiwan and north eastern China^{1,2}. *C. obtusa* wood is generally used as a structural material, such as floor coverings³. The essential oils extracted from the leaves and twigs of *C. obtusa* are generally used as functional additives with a good fragrance in soaps, toothpaste and cosmetics⁴. *C. obtusa* essential oil has antibacterial, antifungal, antimite, antitermite and acaricidal activities^{5,6}. Flavones are the other major active compounds in *C. obtusa*, including myricetin and amentoflavone (Fig. 1) and have antioxidant, anticancer and antiinflammatory activities⁷. Myricetin has a range of biological effects, such as spasmolytic, antiphlogistic, antiallergic, antiatherosclerotic and antithrombotic agents⁸. Amentoflavone has been evaluated for its analgesic and antiacetylcholinesterase activities^{9,10}.

Adsorption isotherms are normally used to determine the separation efficiency¹¹. The complexity of adsorption and the errors in the experimental adsorption data have a significant influence on the applications and development of adsorption technology¹². The Langmuir-Freundlich model is the most popular equilibrium isotherm among the many non-linear isotherm models available¹³. The Langmuir-Freundlich isotherm equation has an additional three parameters compared to the other equations and a higher correlation accuracy has been reported¹⁴.

Solid-phase extraction (SPE) and is used widely for the preconcentration and clean-up of analytical samples, as well as

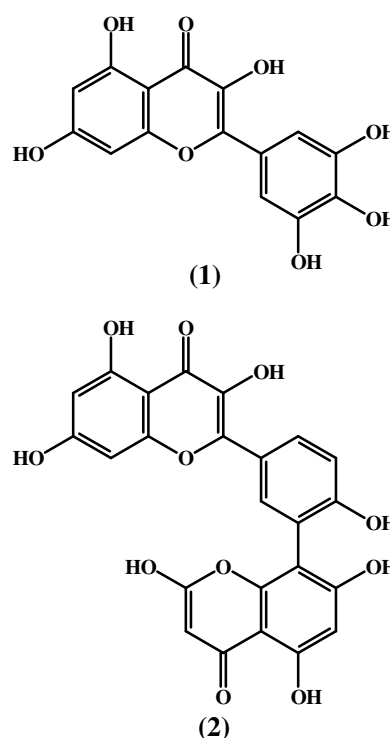


Fig. 1. Structures of (1) myricetin and (2) amentoflavone

for the purification of range of chemicals and the removal of toxic or valuable substances. Solid-phase extraction avoids or minimizes the disadvantages of liquid-liquid extraction (LLE). Compared to liquid-liquid extraction, solid-phase extraction

reduces the time required, particularly if automated methods are used. Solid-phase extraction can handle small samples (50–100 μL) and requires small solvent volumes. For analytical purposes, solid-phase extraction is generally performed using a small column or cartridge containing an appropriate packing¹⁵. The powdered plant is packed above the sorbent in a solid-phase extraction cartridge. A fixed volume of extraction solvent is then percolated through the cartridge, which transfers the target compound to the sorbent. The solvent is then collected and used to repeat the process (extraction stage). After full extraction, the unnecessary compounds are removed from the sorbent and the purified target compound remaining are eluted (washing and elution stage)¹⁶.

This paper reports the use of different materials as solid-phase extraction sorbents to extract and purify myricetin and amentoflavone from *C. obtusa*. The interactions between the target compounds and sorbent were compared using the adsorption isotherms.

EXPERIMENTAL

Myricetin and amentoflavone were obtained from Sigma-Aldrich (Milwaukee, WI, USA). Acetonitrile, methanol and acetic acid were purchased from Duksan Pure Chemical Co., Ltd. (Ansan, Korea). All other reagents used in the experiment were of the highest grade. Double distilled water was filtered using a vacuum pump (Division of Millipore, Waters, USA) and filter (HA-0.45, Division of Millipore, Waters, USA) prior to use. All samples were filtered through a filter (MFS-25, 0.2 mm TF, Whatman, USA) before being injected into the high performance liquid chromatography system.

Chromatographic condition: HPLC was performed with a Waters 600s multisolvent delivery system, Waters 616 liquid chromatography and waters 2487 variable wavelength, dual-channel UV detector (Waters Associates, Milford, MA, USA). A commercial C18 column (4.6 \times 250 mm, 5 μm) purchased from RStech Co. (Daejeon, Korea) was used.

The flavones were separated by HPLC using acetonitrile/water/acetic acid (40:60:0.5, v/v/v) as the mobile phase. The flow rate, UV wavelength and injection volume were set to 0.5 mL/min, 372 nm and 10 μL , respectively.

Preparation of standard solutions and sample solution: Stock solutions of the two target compounds (100 $\mu\text{g}/\text{mL}$) was prepared in 1 mL methanol. *C. obtusa* leaves were powdered and 10 g of the resulting powder was weighed and extracted with 100 mL of methanol by heating under reflux at 60 $^{\circ}\text{C}$ for 2 h. After centrifugation and filtration, the extract was collected and stored for later use.

Solid-phase extraction procedure: Commercial solid-phase extraction cartridges (diameter 0.9 cm, 3.0 mL) with 0.2 g C18, silica and NH_2 -silica sorbent were purchased from Alltech (Deerfield, IL, USA) and MCM-41 (3.0 nm pore diameter, surface area = 800 m^2/g) was supplied by Hailong Tech Development Co. Ltd (Binzhou, China). 2.0 g of MCM-41 and NH_2 -MCM-41 were packed separately at the bottom of the empty polypropylene cartridge and preconditioned with methanol. The sample solution (0.2 mL) was loaded into the solid-phase extraction cartridge, washed sequentially with methanol, water and *n*-hexane and eluted with methanol, acetonitrile and acetonitrile-acetic acid (95:5, v/v).

Preparation of absorption isotherm: The static method was performed on the NH_2 -MCM-41 particles. 2.0 mg NH_2 -MCM-41 was placed into microtubes and mixed with 1.0 mL of the standard flavone solutions (0.5, 1.0, 2.0, 4.0, 5.0 $\mu\text{g}/\text{mL}$) at room temperature for 72 h until it reached equilibrium. The adsorption solution was centrifuged and the supernatant solvent was collected and filtered (0.2 μm). The experimental adsorption isotherms were fitted to three models: linear, Freundlich and Langmuir-Freundlich models. This process was accomplished using the solver function in OriginPro 7.5 software (Origin Lab Corporation, MA, USA) by varying the fitting parameters until the squared correlation coefficient (r^2) reached 1 unity.

RESULTS AND DISCUSSION

A series of mixtures of standard solutions containing myricetin and amentoflavone were diluted (0.5, 1, 2, 4 and 5 $\mu\text{g}/\text{mL}$) with methanol. The resulting linear regression equations of the two compounds were $Y = 596371 \times -172977$ ($r^2 = 0.9994$) for myricetin and $Y = 233484 \times -47316$ ($r^2 = 0.9999$) for amentoflavone. Where, Y and X represent the peak area of the analytes and concentration of the analytes in the *C. obtusa* leaves, respectively. Assays of the repeatability calculated as the relative standard deviations (RSDs) were performed by injecting standard solutions 5 times in a 5-day period. RSDs lower than 8.55 % showed acceptable precision and accuracy.

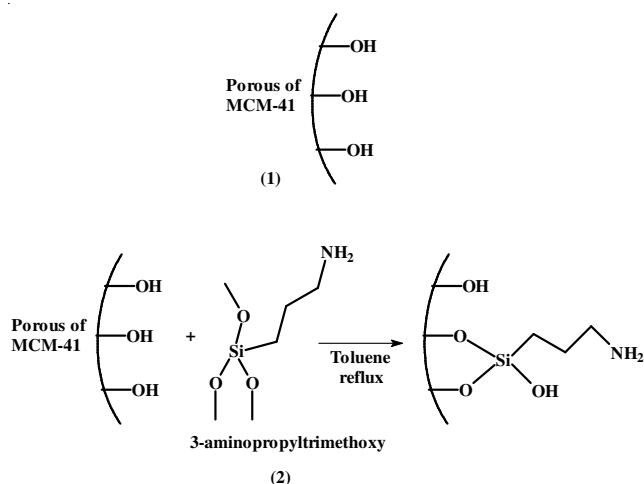
The amount (Q) of myricetin and amentoflavone adsorbed on NH_2 -MCM-41 was determined using the following equation:

$$Q = \frac{(C_0 - C)V}{m} \quad (1)$$

where Q ($\mu\text{g}/\text{g}$) is the amount absorbed, C_0 ($\mu\text{g}/\text{mL}$) is the initiator concentration, C ($\mu\text{g}/\text{mL}$) is the free concentration, V (mL) is the volume of the sample solvent and m (g) is the dry mass of the sorbent. Q was calculated using equation (1), The experimental parameters were compared by non-linear regression analysis using the equilibrium isotherms.

Purification of myricetin and amentoflavone by solid-phase extraction: Commercial C18, silica, NH_2 -silica, MCM-41 sorbent prepared. The NH_2 -MCM-41 sorbent is synthesis for compared of amount of adsorption with commercial sorbent. MCM-41 is a popular material known as a mesoporous material. On the other hand, the surface of unmodified MCM-41 contains only hydroxyl groups (-OH). Therefore the adsorption and selectivity of the target compounds on MCM-41 cannot be controlled. The separation efficiency can be increased by surface modification of the sorbent^{17,18}. Fig. 2 shows structure of MCM-41 and NH_2 -MCM-41.

Selecting the washing solvent and elution solvent is an important process in solid-phase extraction. The removal of interfering compounds in the washing stage is important for obtaining the target compounds in the elution stage. Table-1 lists the amounts of target compounds in the different washing solvents. Large or weakly interacting molecules can be removed due to the sorbent structure and pore size of the sorbents. Hydrophobic and hydrophilic interference could be washed out without the target compound using *n*-hexane and water, respectively. Only water could remove the interference compounds

Fig. 2. Structure of (1) MCM-41 and (2) NH₂-MCM-41

Washing solvent	Sorbent	Amount (µg/g) Myricetin Amentoflavone	
Methanol	C ₁₈	2.900	10.475
	Silica	2.927	13.302
	NH ₂ -silica	2.946	2.199
	MCM-41	2.972	6.427
	HN ₂ -MCM-41	5.067	6.782
Water	C ₁₈	ND	ND
	Silica	ND	ND
	NH ₂ -silica	ND	ND
	MCM-41	ND	ND
	HN ₂ -MCM-41	ND	ND
<i>n</i> -hexane	C ₁₈	3.006	2.572
	Silica	3.063	3.904
	NH ₂ -silica	2.989	11.290
	MCM-41	3.030	6.648
	HN ₂ -MCM-41	ND	ND

ND: not detected

with the lowest concentration of target compounds. The target compounds were soluble in organic solvents, such as ethanol and ether, but insoluble in water. Therefore, water was selected as the washing solvent and fixed at 2 mL. Table-2 lists the amounts of target compounds remaining in the different elution solvents. After the washing stage, the highest amounts of target compounds were extracted in the elution stage using 2 mL methanol as the elution solvent. The optimal solid-phase extraction conditions were as follows: water as the washing solvent and methanol as the elution solvent. Under this condition, the quantities of myricetin and amentoflavone obtained were 4.93 µg/g and 5.91 µg/g, respectively. Fig. 3 shows the solid-phase extraction process for purifying the target compounds.

Analysis of the adsorption isotherms: Fig. 4 shows the amount of myricetin and amentoflavone adsorbed on NH₂-MCM-41. More myricetin than amentoflavone was adsorbed on the same surface area of the solid phase. This was attributed to the smaller molecular size of myricetin than amentoflavone¹⁹.

Elution solvent	Sorbent	Amount (µg/g) Myricetin amentoflavone	
Methanol	C ₁₈	2.913	3.347
	Silica	2.909	3.279
	NH ₂ -silica	3.060	4.644
	MCM-41	2.910	3.160
	HN ₂ -MCM-41	4.933	5.911
Acetonitrile	C ₁₈	2.925	2.947
	Silica	2.917	2.237
	NH ₂ -silica	2.953	4.344
	MCM-41	2.922	2.506
	HN ₂ -MCM-41	2.960	3.469
Acetonitrile: acetic acid (98:2 v/v)	C ₁₈	ND	0.495
	Silica	ND	ND
	NH ₂ -silica	ND	1.838
	MCM-41	ND	0.742
	HN ₂ -MCM-41	ND	1.945

ND: not detected

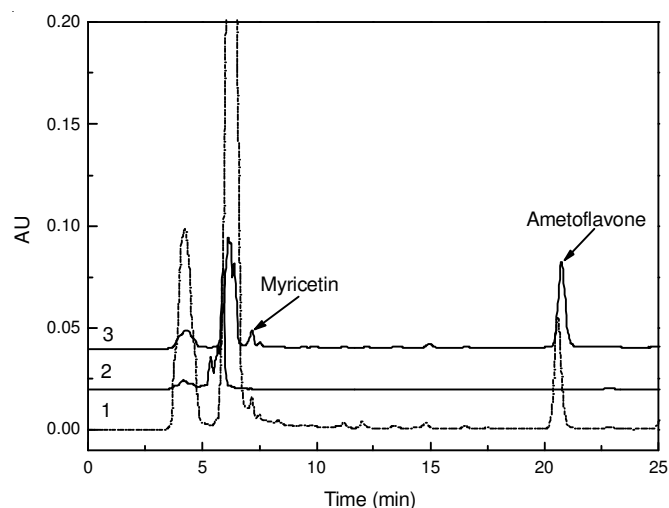


Fig. 3. Chromatograms of the water washing solvent and methanol elution solvent in NH₂-MCM-41 (Mobile phase: acetonitrile-water-acetic acid = 40:60:0.5 (v/v/v), flow rate: 0.5 mL/min, column: C18 (4.6 × 250 mm, 5 µm), UV: 372 nm, injection volume: 10 µL, 1: *C. obtusa* extraction sample, 2: water washing stage, 3: methanol elution stage)

The experiment data of myricetin and amentoflavone was fitted to the following three adsorption isotherm models:

$$Q = \frac{aC_E}{1 + bC_E} \quad (2)$$

$$Q = aC_E^b \quad (3)$$

$$Q = \frac{abC_E^c}{1 + bC_E^c} \quad (4)$$

where *a*, *b* and *c* are parameters and *C_E* (µg/mL) is the concentration of myricetin and amentoflavone in the standard solution samples. The adsorption isotherm models used were the Langmuir (eqn. 2), Freundlich (eqn. 3) and Langmuir-Freundlich (eqn. 4). Table-3 lists the adsorption parameters using the three adsorption isotherm models. The Langmuir-Freundlich isotherm

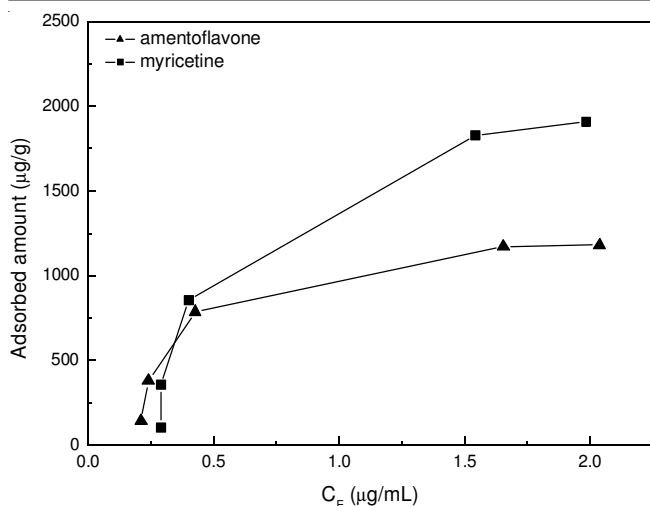


Fig. 4. Quantities of the target compounds adsorbed on $\text{NH}_2\text{-MCM-41}$

was used to better compare the competitive adsorption. The regression coefficient (r^2) of the competitive Langmuir-Freundlich of myricetin and amentoflavone was 0.9888 and 0.9858, respectively.

Target compounds	Parameters	Parameters			Regression coefficient
		a	b	c	
Myricetin	Langmuir	2157.44	1.27	-	0.9018
	Freundlich	1239.61	-	0.91	0.9472
	Langmuir-Freundlich	1137.67	0.13	2.39	0.9888
Amentoflavone	Langmuir	2157.55	1.27	-	0.9018
	Freundlich	928.03	0.64	-	0.9074
	Langmuir-Freundlich	1181.44	30.07	3.133	0.9858

Conclusion

This study compared the ability of C18, silica, amino-silica, MCM-41 and amino-MCM-41 to separate the two

flavones from *C. obtusa*. Amino MCM-41 showed better separation and purification of the target compounds in the solid-phase extraction process. Under the optimized conditions of 2 mL of water as the washing solvent and 2 mL of methanol as the elution solvent, 4.93 µg/g of myricetin and 5.911 µg/g of amentoflavone were obtained.

ACKNOWLEDGEMENTS

This study was supported by the Basic Science Research Program through the National Research Foundation (NRF) of Korea funded by the Ministry of Education, Science and Technology (2012-0005250).

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