

Isolation and Quantitation of Amygdalin in *Apricot-kernel* and *Prunus Tomentosa Thunb.* by HPLC with Solid-Phase Extraction

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Abstract

Apricot-kernel and *Prunus Tomentosa Thunb.* are traditional Chinese herb medicines that contain amygdalin as their major effective ingredient. In this report, three methods for the extraction of amygdalin from the medicinal materials are compared: ultrasonic extraction by methanol, Soxhlet extraction by methanol, and reflux extraction by water. The results show that reflux extraction by water containing 0.1% citric acid is the best option. The optimal reflux time is 2.5 h and water bath temperature is 60°C. The solid-phase extraction method using C₁₈ and multiwalled carbon nanotube as adsorbents is established for the pretreatment of reflux extract, and the result shows that the two adsorbents have greater adsorptive capacity for amygdalin and good separation effect. In order to quantitate amygdalin in *Apricot-kernel* and *Prunus Tomentosa Thunb.*, a reversed-phase high-performance liquid chromatography method using methanol–water (15:85, for 30 min and pure methanol after 30 min) as mobile phase is developed and a good result is obtained.

Introduction

Apricot-kernel is the dry and mature seed of *Prunus armeniaca L.* and *Prunus armeniaca L. var. Ansu Maxim.* It has been prescribed in many traditional Chinese medicines for its antitussive, expectorant, and laxative functions (1). *Prunus Tomentosa Thunb.* is the dry and mature seed of *Prunus humilis Bge.*, *Prunus japonica Thunb.*, and *Prunus pedunculata Maxim.* It has diuresis and moistening dryness activities and is used in treating oedema and dermatophytosis (2). The major effective ingredient of these two herb medicines is amygdalin (D-mandelonitrile-β-D-gentiobioside) (as shown in Figure 1), which has antitussive and lubricant activities. It is decomposed by the action of β-D-glucosidase to yield hydrocyanic acid, which reflexively stimulates the respiratory center and produces antitussive and antiasthmatic

effects (3). Recently, it has been reported that amygdalin can kill cancer cells selectively at the tumor site without systemic toxicity (4).

Amygdalin can be dissolved in water and methanol easily. Traditionally, the extraction method of the chemical ingredients in the medicinal materials is decoction in boiling water. But the common techniques for extracting amygdalin from *Apricot-kernel* and *Prunus Tomentosa Thunb.* for the analysis of amygdalin are ultrasonic extraction or Soxhlet extraction by methanol (5,6). This is because some amygdalin is decomposed into benzaldehyde, HCN, and glucose by emulsion (a hydrolysis enzyme both in *Apricot-kernel* and *Prunus Tomentosa Thunb.*), and some are converted into its epimers, neoamygdalin (L-mandelonitrile-β-D-gentiobioside) (Figure 1) during the process of decoction in water. Recently, Hwang et al. established an optimum condition for inhibiting the conversion of amygdalin to neoamygdalin in Tonin (*Persicae Semen*) by changing the pH. They added 0.1% citric acid in the boiling water and prevented the loss of content of amygdalin (7). They also studied the optimum extraction condition of amygdalin without enzymatic hydrolysis from Tonin. They concluded that the extraction yield of amygdalin was highest when using the size larger than half (8).

The determination of amygdalin was performed mainly by

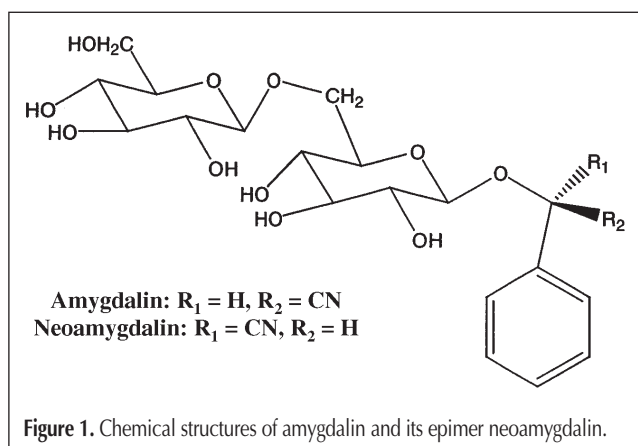


Figure 1. Chemical structures of amygdalin and its epimer neoamygdalin.

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high-performance liquid chromatography (HPLC) (9). Although other analytical methods are reported, such as gas chromatography (10) and capillary electrophoresis (3). HPLC is the most convenient method, usually with a C₁₈ column as the stationary phase and a mixture of methanol (acetonitrile) and water as the mobile phase.

Solid-phase extraction (SPE) is a universal sample pretreatment method. Its advantages include solvent retention, high selectivity, and easy operation. It was widely applied in the separation and concentration of target compounds in complex samples, such as environmental water and herb medicine (11,12).

The aim of this study was to establish an optimal method for the extraction of amygdalin from *Apricot-kernel* and *Prunus Tomentosa Thunb.*; a sample pretreatment method using SPE, C₁₈, and multiwalled carbon nanotube (MWNT) as adsorbents; and a separation and analysis method using HPLC. This is the first report to separate amygdalin from herb medicines using SPE with MWNT as the adsorbent.

Experimental

Chemicals and reagents

Apricot-kernel and *Prunus Tomentosa Thunb.* were purchased from Tong Ren Pharmacy (Beijing, China). Amygdalin was purchased from Beijing Chemical Reagent Company (Beijing, China). MWNT was obtained from Reaction Engineering Lab of Chemical Engineering Department, Tsinghua University (Tsinghua, China). HPLC-grade methanol was used, and the other reagents and solvents were of guaranteed or analytical grade.

Apparatus and conditions

HPLC

An HP 1100 chromatographic system consisting of a quaternary pump, degasser, diode array detector, and HP ChemStation Data system (Hewlett-Packard, Palo Alto, CA) was used. Separation was achieved on a Agilent C₁₈ column (250 × 4.6-mm i.d., 0.45 μm). The mobile phase consisted of methanol–water (15:85 for 30 min and pure methanol after 30 min) and the flow rate was 0.8 mL/min. The mobile phase was filtered before use by a Millipore vacuum filter system equipped with a 0.45-μm filter (Billerica, MA). Because the maximal absorption of amygdalin was at 215 nm, the detection wavelength was set at 215 nm. The column temperature was 30°C. The injected volume of samples was 20 μL, by loop.

SPE

The SPE instrument was a RapidTrace SPE workstation (Zymark Company, Hopkinton, MA).

Calibration curve and detection limit

Amygdalin was dissolved in methanol and six different standard solutions containing 50, 100, 150, 200, 250, and 300 μg/mL of it were obtained. The calibration curve was constructed according to the peak area and the concentration of amygdalin. Then the standard solution of the lowest concentration was diluted gradu-

ally and injected into the instrument to determine the detection limit when the signal-to-noise ratio is 3.

Extraction method

Ultrasonic extraction

Apricot-kernel and *Prunus Tomentosa Thunb.* were prepared in the form of small pieces. Each sample (1 g) was immersed in 10 ml methanol and ultrasonicated for 30 min. Extraction was repeated two times. The combined methanol extract was obtained after filtration and made volume up to 25 mL.

Soxhlet extraction

Small pieces of *Apricot-kernel* and *Prunus Tomentosa Thunb.* (3 g of each) were Soxhlet extracted by 100 mL methanol for 5 h, in a water bath at 70°C. The methanol extract was obtained after filtration and diluted to 100 mL.

Reflux extraction

Small pieces of *Apricot-kernel* and *Prunus Tomentosa Thunb.* (3 g of each) were reflux extracted by 100 mL water with 0.1% citric acid for 2.5 h in the condition of a 60°C water bath. The water extract was collected after filtration. The residue was dissolved in 100 mL water with 0.1% citric acid and the previously mentioned process was repeated. The two times water extract was then combined and diluted to 200 mL.

SPE method

Prior to a preconcentration step, the C₁₈ and MWNT packed cartridge was washed with 5 mL of methanol at a flow rate of 4 mL/min and activated with 5 mL of water at a flow rate of 4 mL/min. Then, 5 mL sample solution was passed through the preconditioned cartridge at a flow rate of 4 mL/min. After the sample solution had passed through, the cartridge was washed with 5 mL of 10% methanol aqueous solution at a flow rate of 4 mL/min to remove coadsorbed matrix materials from the cartridge. The analytes retained on the SPE packing materials were then eluted with 5 mL of methanol at a flow rate of 4 mL/min, and the eluate was diluted to 5 mL. Finally, 20 μL of methanol eluate was injected into the HPLC system for determination.

Results and Discussion

Separation of amygdalin by reversed-phase HPLC

It was necessary to establish a suitable HPLC method to determine amygdalin in the herb medicines. An ODS column (C₁₈, Agilent) and methanol–water as mobile phase were used. It was found that when the ratio of methanol and water was 15:85, amygdalin could be completely separated with the other ingredients. But if the ratio of methanol was a little large, amygdalin would not be separated well. If the ratio of methanol was small, it would lead to long analytical time. Because amygdalin was the only analyte, other ingredients had to be eluted as quickly as possible to cut down on analytical time when the peak of amygdalin was finished. For this reason, a gradient elution method (methanol–water is 15:85 for 30 min and pure methanol after 30 min) was also attempted, and a good chromatographic separation was obtained within 50 min (Figure 2).

The calibration curve between peak area (A) and concentration (C) of amygdalin showed excellent linearity ($r^2 = 0.9986$), and the detection limit was $0.2 \mu\text{g/mL}$ (Figure 3).

Comparing three solvent extraction methods for amygdalin

Three extraction methods—including ultrasonic extraction by methanol, Soxhlet extraction by methanol, and reflux extraction by water—were compared for amygdalin. Figure 4 shows the extraction yield, presented as the mass ratio of amygdalin to medicinal materials. Among the extraction yield of amygdalin, reflux extraction by water was the maximum, and ultrasonic extraction by methanol was the minimum. Water is a good solution for extracting ingredients of crude herb medicine, because it is known that it can extract almost all of the ingredients. Though methanol is also a common solution for extraction, and amygdalin can be easily dissolved in it too. However, if all the factors are contemplated (such as economy, accurate quantitation, and traditional method of decoction in boiling water), reflux extraction by water is the best choice for the *Apricot-kernel* and *Prunus Tomentosa Thunb.*

Optimum conditions on the reflux extraction of amygdalin by water

The main factors that influenced the amygdalin extraction yield by refluxing on a water bath are reflux time, water bath temperature, and extraction times. Citric acid (0.1%) was added in the aqueous solution to prevent amygdalin from decomposition and inhibit the conversion of amygdalin to neoamygdalin (7).

Several different reflux times and water bath temperatures were selected. The extraction yield of amygdalin, according to reflux

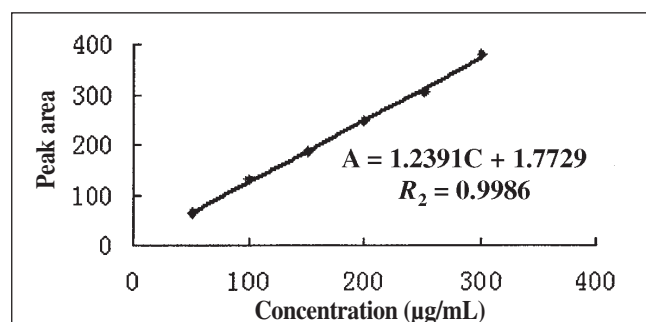


Figure 2. The calibration curve between peak area (A) and concentration (C) of amygdalin.

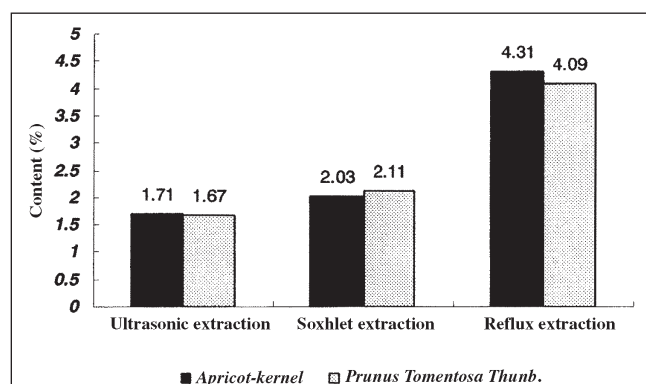


Figure 3. Extraction yield of amygdalin by triple extraction method.

time and water bath temperature, are shown in Figures 5 and 6. It could be observed that a 2.5-h reflux time and 60°C water bath temperature are the most suitable reflux extraction conditions. A short reflux time and low water bath temperature would decrease the extraction yield of amygdalin. A long reflux time and high water bath temperature would cause some amygdalin to convert into its epimers or decompose in the aqueous solution.

Extraction times were also investigated. Table I lists the extraction yield of amygdalin according to extracting times. It can be concluded that amygdalin was almost completely extracted after two extraction processes.

SPE

The solution extracted by water pollutes the separation column so as to reduce the column effect and also make it more difficult to separate the ingredients of the herbal medicine, because the components in it are too complex. Therefore, an SPE was used to attempt to solve this problem.

C_{18} is one of the most common adsorbents used as the solid phase of SPE. It is applied to many compounds from non-to medium polarity. The principle of extraction is similar to that of LC. Figure 2A is the chromatogram of an aqueous solution after extraction using a C_{18} SPE cartridge. It could be found that the chromatographic peaks of other ingredients, especially those that elute prior to amygdalin, are weakened greatly, compared with those of the original (Figure 2C), and the content of amygdalin is almost the same. The result indicated that the C_{18}

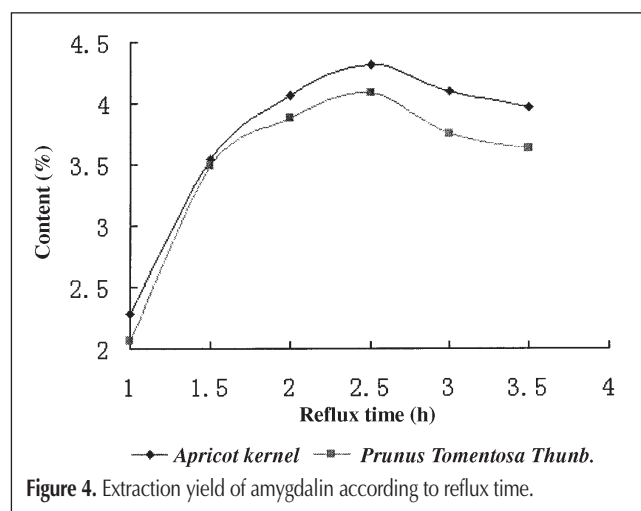


Figure 4. Extraction yield of amygdalin according to reflux time.

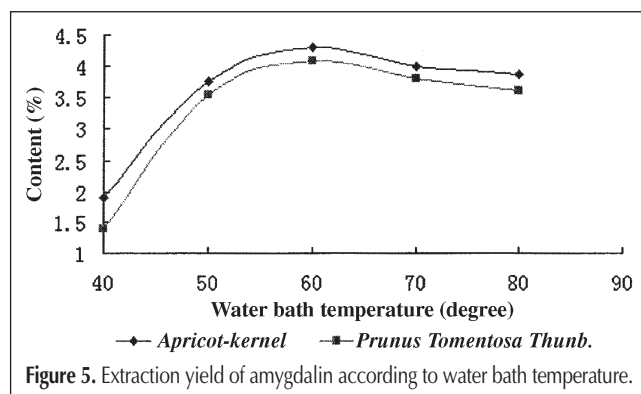


Figure 5. Extraction yield of amygdalin according to water bath temperature.

SPE cartridge is a good adsorbent for amygdalin and it could be applied in the pretreatment method for the separation of amygdalin from herb medicine.

MWNT is a new material. Recently, Cai et al. used MWNT as SPE adsorbent firstly for determination of bisphenol-a, 4-*n*-nonylphenol, and 4-*tert*-octylphenol (13). The compound

Table I. Extraction Yield of Amygdalin According to Extraction Times

Extraction times	Content (<i>Apricot-kernel</i>) (%)	Content (<i>Prunus Tomentosa Thunb.</i>) (%)
1	3.07	2.87
2	1.37	1.30
3	0.017	0.011

Table II. Extraction Yield of Amygdalin

SPE cartridge	Content (<i>Apricot-kernel</i>) (%)	Content (<i>Prunus Tomentosa Thunb.</i>) (%)
None	4.44	4.18
C ₁₈	4.13	4.00
MWNT	4.32	4.09

that has a delocalized π -bond structure, such as benzene ring, could easily combine with MWNT and achieve good adsorptive effect, for the π bond at the surface of the benzene ring has an interaction with the π bond at the surface of the MWNT. MWNT was used as the adsorbent in the pretreatment method for the separation of amygdalin from other ingredients in the *Apricot-kernel* and *Prunus Tomentosa Thunb.*, because amygdalin has a benzene ring structure (Figure 1), according to the absorptive capability of MWNT (that it is strong to the compounds that have a benzene ring while weak to others). Figure 2B is the chromatogram of aqueous solution after extracting using MWNT packed cartridge. The result indicated that MWNT is a better adsorbent because amygdalin can be adsorbed in it more strongly and than other ingredients, compared with C₁₈, and MWNT could be used as the solid phase for SPE to obtain better HPLC analysis result for isolation and quantitation of amygdalin in *Apricot-kernel* and *Prunus Tomentosa Thunb.* Table II lists the extraction yield of amygdalin in *Apricot-kernel* and *Prunus Tomentosa Thunb.*

Conclusion

An HPLC method for quantitative analysis of amygdalin in *Apricot-kernel* and *Prunus Tomentosa Thunb.* is developed. An SPE method that uses C₁₈ and MWNT as adsorbents after refluxing by water was established in order to obtain a better separation effect and to protect the chromatographic column. The results showed that these two adsorbents were effective, especially MWNT. This method could be used to determine amygdalin in *Apricot-kernel* and *Prunus Tomentosa Thunb.* because it is convenient and rapid.

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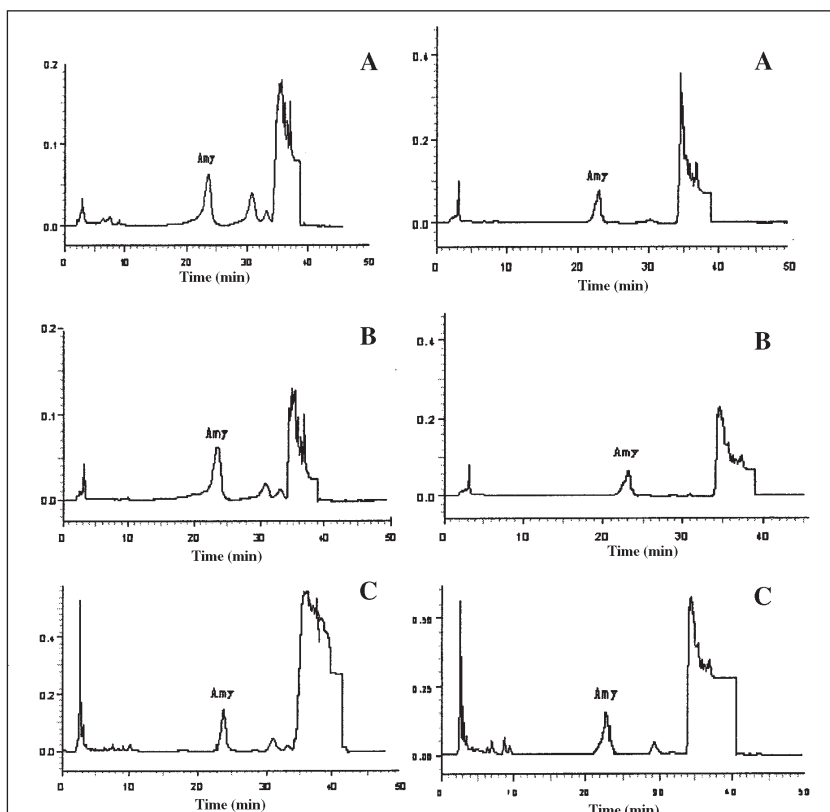


Figure 6. Chromatograms of aqueous solution after extracting using a C₁₈ SPE cartridge (A), MWNT packed cartridge (B), and original (C). The left column is chromatograms of *Apricot-kernel*, and the right column is that of *Prunus Tomentosa Thunb.* Chromatographic conditions were: column, Agilent C₁₈ (250 × 4.6-mm i.d., 5 μ m); mobile phase, methanol–water (15:85 for 30 min and pure methanol after 30 min); flow rate, 0.8 mL/min; UV detection, 215 nm; and peak, amygdalin (Amy).

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