DEVELOPMENT AND VALIDATION OF A UHPLC-DAD METHOD FOR QUANTITATION OF AMYGDALIN IN THE SEEDS OF *PRUNUS ARMENIACA* L.

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Abstract: *In this study, the content of amygdalin in the seeds of Prunus armeniaca L. was quantitatively determined by using a validated UHPLC method. Chromatographic condition was performed on a Halo C_{18} analytical column (100 × 4.6 mm; 5 µm) with a mobile phase consisting of methanol and water. The gradient elution program was set as follows: 0–40 min, 10–100 % methanol; flow rate was maintained at 0.7 mL/min, and detection was carried out using a UV detector at 207 nm. The calibration curve displayed excellent linearity with an R^2 value of 0.9999. The method also showed high sensitivity, with the limit of detection (LOD) and limit of quantification (LOQ) for amygdalin determined to be 0.15 and 0.46 µg/mL, respectively. The chromatographic system exhibited good repeatability with a relative standard deviation (RSD) of 0.07 %. The recovery of method ranged from 100.28 % to 101.41 %, indicating high accuracy and reliability. The results support the potential development of functional foods or nutraceuticals derived from P. armeniaca, where strict control of amygdalin levels is essential.*

Keywords: *Amygdalin; Quantitation; Prunus armeniaca; UHPLC.*

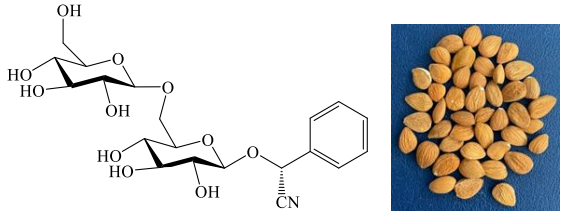
1. Introduction

Prunus armeniaca L., commonly known as the apricot tree, belongs to the family Rosaceae (Bouadid, Akdad, et al., 2023). The seeds of *P. armeniaca* are recognized as herbal medicinal products in the Chinese, Korean, and Vietnamese pharmacopeias (Kitic et al., 2022; *Vietnamese Pharmacopoeia V*, 2017). Many studies have shown that *P. armeniaca* has been widely used in traditional medicine around the world for its anticancer potential, either as primary treatments or as complementary and alternative therapies (Kitic et al., 2022). In addition, *P. armeniaca* has also been investigated for various biological activities, such as antimicrobial, antimutagenic, enzyme inhibitory, cardioprotective, anti-inflammatory, antinociceptive, and antioxidant activities (Bouadid, Moujane, et al., 2023). However, most of the studies conducted to date have focused on the pharmacological investigation of the apricot fruits and seeds (Kitic et al., 2022; Li et al., 2021). Further investigation into other parts of plant, such as leaves and bark, could potentially reveal additional bioactive compounds with anticancer and anti-hypertensive activities (Bouadid, Akdad, et al., 2023; Bouadid, Moujane, et al., 2023; Kitic et al., 2022), thereby broadening the therapeutic applications of *P. armeniaca* (Bouadid, Akdad, et al., 2023; Kitic et al., 2022).

Amygdalin is a naturally occurring compound classified as a *β*-cyanogenic glycoside (Fig.1), abundantly found in members of the Rosaceae family, such as almonds, apricots, apples, and peaches etc. It is proposed that the anti-cancer attributes of amygdalin are mainly due to its active metabolite i.e., hydrocyanic acid (Ioannis et al., 2015; Saleem et al., 2019). Many studies have demonstrated that amygdalin inhibits tumor cell growth by stimulating the apoptotic process, primarily through the upregulation of pro-apoptotic proteins such as Bax and caspase-3, and the downregulation of the anti-apoptotic protein Bcl-2. These findings suggest that amygdalin suppresses cell proliferation and tumor progression by effectively slowing down

the cell cycle. In addition, amygdalin has traditionally been used for its anti-inflammatory, antipyretic, and antitussive properties (Chang et al., 2005). Other studies have demonstrated that amygdalin treatment is effective in alleviating alcohol-induced gastric ulceration. The protective effects on the gastric mucosa are believed to be mediated through the suppression of tumor necrosis factor-alpha (TNF-α) and the enhancement of nitric oxide production in the gastric tissue (Fatemeh et al., 2011). Based on the reported biological effects and potential therapeutic benefits of amygdalin, this study aimed to quantify the amygdalin content in the seeds of *P. armeniaca* using a validated UHPLC method.

Fig.1. The structure of amygdalin and seeds of *P. armeniaca*



2. Research overview

P. armeniaca L. has been used in folk medicine as a remedy for various diseases. *P. armeniaca* L. has been extensively studied for its diverse biological activities, including antimicrobial, antioxidant, hepatoprotective, anti-inflammatory, antimutagenic, antinociceptive, and enzyme inhibitory effects. Among these, its antimicrobial and antioxidant properties have been the focus of significant research and have demonstrated high efficacy under *in vitro and in vivo* conditions (Rai et al., 2016). The seeds of *P. armeniaca* L. have been identified as a medicinal plant material of considerable pharmacological interest. They have traditionally been utilized in the management of gynecological disorders, rheumatic pain, headaches, and cutaneous hyperpigmentation. Furthermore, the oil extracted from the seeds has been applied in the treatment of dermatological conditions, otitis, and tinnitus (Wang et al., 2010). In Korea, the seeds are used as a therapeutic agent for cough, phlegm, and the common cold, while in Vietnam, they are used to treat respiratory and digestive disorders (Kshirsagar & Magno, 2011; Lim, 2012). The phytochemical composition of the seeds has been the subject of extensive investigation by researchers worldwide. They comprise potential bioactive components and nutrients, including cyanogenic glycosides, carotenoids, carbohydrates, vitamins, phenols, terpenoids, esters, and volatile compounds. Mohamed Amine El-Hajjaji et al assess the properties of aqueous and methanol extracts of *P. armeniaca* seeds using high-performance liquid chromatography with a diode array detector (HPLC-DAD) (El-Hajjaji et al., 2024). These results indicate that the seeds contain a high concentration of bioactive compounds, especially tocopherol and resveratrol, and may be useful in treating various diseases due to the promising antioxidant and antimicrobial activities of its extracts. In 2005, amygdalin in apricot seeds was isolated and quantified using HPLC combined with solid-phase extraction (Lv et al., 2005). Xingjun et al. compared second-derivative spectrophotometry and HPLC for the determination of amygdalin in apricot seeds (Miao et al., 2013). Therefore, future studies on the isolation and quantification of amygdalin in the seeds of *Prunus armeniaca* L. can incorporate the principles of green chemistry and employ high-precision analytical techniques such as UHPLC-DAD or HPLC-DAD.

3. Material and methods

3.1. Material

- *General Experimental Procedure*

Agilent 1290 Infinity UHPLC system (Santa Clara, CA 95051, US); Halo C18 column 4.6 × 100 mm, part No. 92814-602.

- *Reagents and Chemicals*

Methanol were purchased from Honeywell, Korea. Deionized water was obtained from a Milli-Q water purification system (Millipore, Billerica, MA, USA). Amygdalin was purchased from Sigma-Aldrich (CAS-No.: 29883-15-6).

- *Plant Materials*

The seeds of *P. armeniaca* were purchased in 2025 from Nongboo Mind Co. Ltd. Korea and were identified by Prof. Jung Huyn-Ju (College of Pharmacy, Wonkwang University). A voucher specimen (WKU-25-1) has been deposited at the Pharmacognosy Laboratory, College of Pharmacy, Wonkwang University, Chon-bukllo,

Korea.

3.2. Methods

Preparation of sample solutions: The sample was accurately weighed 1.0 g and then transferred to a 100 mL Erlenmeyer flask. 50 mL of MeOH 100% was added and sonicated for 60 minutes in an ultrasonic bath. If necessary, supplying MeOH for enough volume. This solution was also filtered through a 0.22 µm membrane filter before analysis.

Preparation of standard solution: A stock solution of amygdalin was prepared at a concentration of 1000 µg/mL in methanol. For calibration purposes, a series of working solutions were prepared by serial dilution of the stock solution with methanol to obtain various concentrations.

Method validation: Linearity was assessed by preparing a series of at least five appropriate concentrations of the stock solution, each analyzed in duplicate. Method validation for the quantification of amygdalin was performed in accordance with the International Conference on Harmonisation (ICH) guidelines, evaluating key parameters including linearity, precision (intra- and inter-day), accuracy, limit of detection (LOD), and limit of quantification (LOQ).

Specificity and selectivity: Specificity and selectivity of the analytical method were evaluated by assessing potential interference from the blank sample at the analyte's retention time. This was done by preparing and analyzing both blank samples and standard solutions. The absence of detectable peaks at the analyte's retention time in the blank sample confirmed the lack of interference and demonstrated the method's specificity and selectivity.

Selection of UV wavelength: Amygdalin has a λ_{max} at 207 nm.

Linearity and range: The stock solutions of amygdalin was diluted in the concentration range of (50, 100, 200, 400, 800, and 1000 µg/mL) in triplicate (n=3). The linearity curve was prepared by plotting the area of peak obtained versus concentration of amygdalin. Least-squares linear regression analysis using Microsoft Excel 365 MSO was used to determine the slope, intercept, and correlation coefficient values.

LOD and LOQ: LOD and LOQ for amygdalin was calculated from the linear regression equation based on the standard deviation of the intercept and the slope using the formula:

$$LOD = 3.3\times \frac{\sigma}{S'}$$

$$LOQ = 10\times \frac{\sigma}{S'}$$

where σ: the standard deviation of the intercept, S': slope of the calibration curve.

Precision, accuracy, and recovery: Precision (intra and inter-day) of the proposed method was performed on three replicate sets of three concentration samples of amygdalin (50, 100, and 200 µg/mL each). Intraday precision was assessed by injecting 3 independent combined samples on the same day, and inter-day precision was injected using same concentration solutions by comparing the results on 3 different days under the same operating conditions for reproducibility. The precision of the method was expressed as the % relative standard deviation (RSD), and values of %RSD within 3% were acceptable. The accuracy of the proposed method was performed through a recovery experiment performed by adding the spiked standard solutions of three different concentrations (25, 50, and 100 µg/mL) into the MeOH extract from the seeds of *P. armeniaca* by analyzing three injections of each concentration. Recovery (%) was evaluated according to the following equation:

$$\text{Recovery (\%)} = \frac{found\ amount - original\ amount}{spiked\ amount}\times 100$$

4. Results

4.1. Chromatographic condition

The mobile phase condition in UHPLC was also studied, indicating that the gradient eluting system of methanol-water mixture (Table 1) provided excellent separation in a reasonable time. The chromatographic peaks in the sample solutions were identified by comparing their retention times with amygdalin standards as well as UV shapes (Figure 2).

4.2. Specificity and Selectivity

The optimized analytical method was able to detect and quantify amygdalin. Chromatographic specificity of the method was demonstrated by the absence of significant interfering peaks at the retention time of amygdalin (t_R 7.10 min) as shown in Figure 2. The obtained result indicate that the developed method was specific for

amygdalin.

4.3. Repeatability

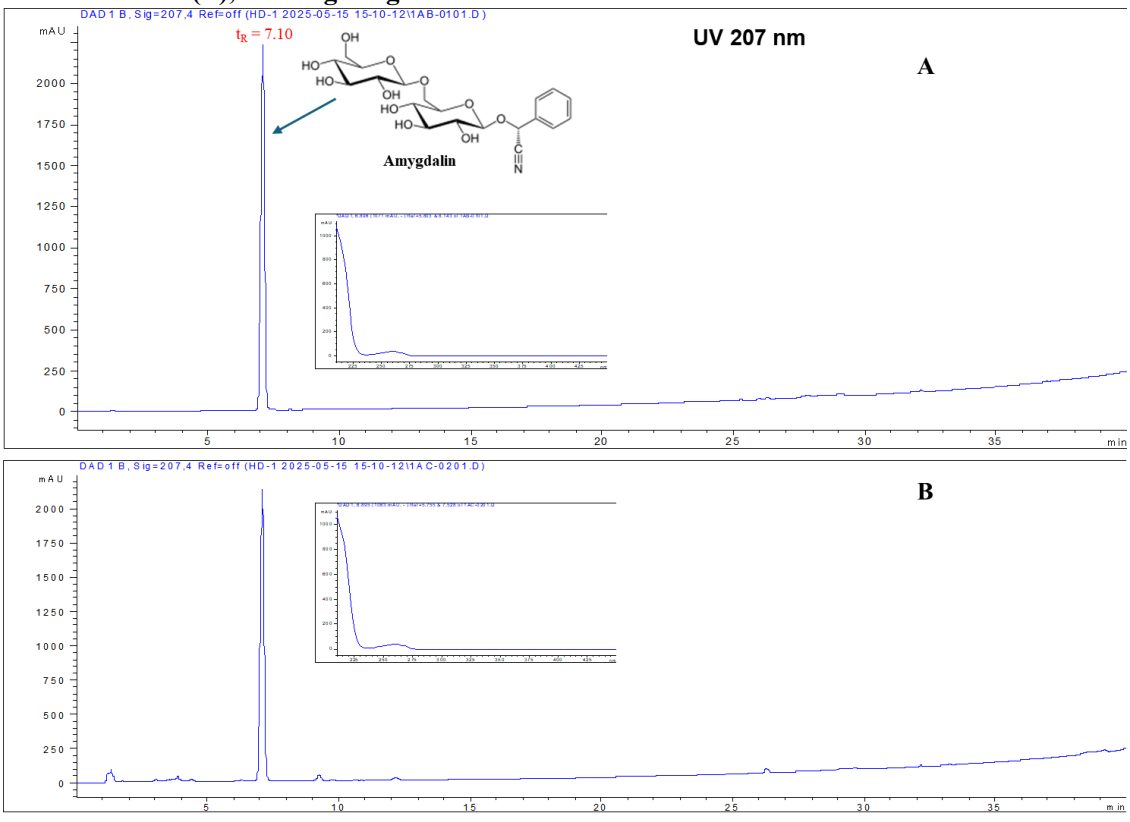
Tab.1. Repeatability data

Injection	t_R (min)
1	7.101
2	7.121
3	7.109
4	7.100
5	7.109
6	7.108
RSD (%)	0.07

Tab.2. UHPLC conditions for analysis

Parameters	Conditions		
Analytic column	Halo C18 column 4.6 x 100 mm		
UHPLC system	Agilent 1290 Infinity UHPLC system		
Detector	G4212A 1290 DAD (Diode Array Detector)		
Mobile phase	**Time (min)**	**% MeOH**	**% DW**
	Initial	10	90
	40	100	0
Flow rate	0.7 mL/min		
Column temperature	25°C		
Injection volumn	10 µL		

Fig.2. Representative chromatographic profiles of amygdalin (A), MeOH extract from the seeds of *P. armeniaca* (B), UV wagelength at 207 nm



The repeatability of the method was evaluated by implementing six individual injections of the amygdalin standard solution at a concentration 100 µL. The results are shown in Table 2,

demonstrate the consistency and precision of analytical procedure. These results allow applying the program to quantify amygdalin in the seeds of *P. armeniaca*.

4.4. Linearity

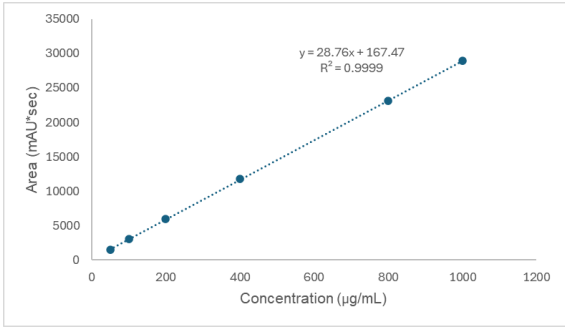
The calibration curves were achieved from five different concentrations of at ranging from 50 to 1000 µg/mL as shown in Figure 3. A high correlation coefficient ($R^2 > 0.999$) value for each calibration curve indicated excellent linearity in this study (Table 3).

Tab.3. Regression Equations, Linearity, LOD and LOQ of amygdalin

Sample	Amigdalin
Linear range (µg/mL)	50.00-1000.00
Regression equation	y = 28.76x + 167.47
Coefficient of determination	0.9999
LOD (µg/mL)	0.15
LOQ (µg/mL)	0.46

y: peak area of compound; x concentration ((µg/mL) of compound.

Fig.3. Calibration curves of amygdalin



4.5. Precision

The intra-day precision was assessed by analyzing thrice a day and % RSD values ranging from 0.09 % to 0.31 %. The inter-day precision was evaluated over three consecutive days, with % RSD values ranging from 0.13 % to 0.47 %. These results indicated that the developed analytical method is precise, as confirmed by the repeatability tests. The detailed precision data are presented in Table 4.

Tab.4. Analytical results of intra- and inter-day of amygdalin

Sample	Conc. (µg/mL)	Intra-day*		Inter-day*	
		Observed conc. (µg/mL)	RSD (%)	Observed conc. (µg/mL)	RSD (%)
Amygdalin	50.00	50.29 ± 0.16	0.31	50.41 ± 0.24	0.47
	100.00	100.13 ± 0.15	0.15	100.22 ± 0.13	0.13
	200.00	200.15 ± 0.17	0.09	200.27 ± 0.26	0.13

*Intra- and inter- day: three times per day and two times analysis of amygdalin for three days, respectively

Tab.5. Recovery results for amygdalin (n = 3)

Analyte	µg/mL in sample	Spiked amount (µg/mL)	Measured amount (µg/mL)	Recovery (%)[a]	RSD (%)
Amygdalin	647.60	25.00	25.35 ± 0.39	101.41 ± 1.56	1.54
		50.00	50.14 ± 0.28	100.28 ± 0.56	0.56
		100.00	100.35 ± 0.41	100.35 ± 0.41	0.41

[a]Recovery (%) = (found amount – original amount)/spiked amount × 100

4.6. Recovery

The accuracy of the method is determined by spiking an exact concentration of amygdalin (25, 50, and 100 μg/mL) into the test sample, which is exactly known content of amygdalin. The results were analyzed with the proposed method (Table 5). The results accepted that the % recovery of amygdalin at three levels ranged from 100.28 % to 101.41 %. Thus, this method has a high degree of accuracy.

4.7. Quantitative analysis of amygdalin in P. armeniaca

Tab.6. Contents of amygdalin in *P. armeniaca*

Weight of sample (g)	Analyte	Contents*	
		mg/g	% (w/w)
1.0	Amygdalin	32.38 ± 0.04	3.238 ± 0.04 %

*Values are mean ± SD in triplicate (n = 3)

The validated UHPLC method was successfully applied to quantify amygdalin in the MeOH extract from the seeds of *P. armeniaca*. The peak areas of triplicate samples were analyzed by the regression equation obtained from calibration curves, yielding an amygdalin content of 3.238 % (Table 6).

5. Discussions

According to both Korean Pharmacopoeia XII and the Vietnamese Pharmacopoeia V, the amygdalin content in the seeds of *P. armeniaca* must exceed 3.0 % (The Korean Pharmacopoeia, 2024; Vietnamese Pharmacopoeia V, 2017). The quantitative analysis conducted in this study confirmed that the seed samples meet the required standard, thereby indicating their compliance with official quality specifications. Amygdalin is not a toxic compound. However, it can be hydrolyzed by the *β*-glucosidase enzyme in the human small intestine to produce hydrocyanic acid (HCN), a highly toxic compound that may cause poisoning (Wang et al., 2010). Therefore, the quantification of amygdalin content is essential for safety assessment. This also contributes to ensuring the safety and efficacy of herbal formulations containing amygdalin. In addition, the results support the potential development of functional foods or nutraceuticals derived from *P. armeniaca*, where strict control of amygdalin levels is essential. However, UHPLC-DAD, while effective for quantifying amygdalin, may lack the sensitivity and specificity needed to detect structurally similar compounds or potential degradation products. Therefore, further investigation using advanced techniques such as LC-MS or LC-MS/MS is recommended to provide more detailed qualitative and structural information, especially in complex herbal matrices.

6. Conclusions

In this study, we have completely developed a validated UHPLC method for the quantitation of amygdalin in the seeds of *P. armeniaca*. The results obtained contribute to the standardization and quality control of amygdalin in medical plants. Furthermore, the established method was also applied to detemine the amygdalin content amygdalin in the samples analyzed.

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PHÁT TRIỂN VÀ THẨM ĐỊNH PHƯƠNG PHÁP UHPLC-DAD ĐỂ ĐỊNH LƯỢNG AMYGDALIN TRONG HẠT CỦA LOÀI *PRUNUS ARMENIACA* L.

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Tóm tắt: *Trong nghiên cứu này, hàm lượng amygdalin trong hạt Prunus armeniaca L. được xác định bằng phương pháp định lượng UHPLC. Điều kiện sắc ký được thực hiện trên cột phân tích Halo C18 (100 × 4,6 mm; 5 µm) với pha động gồm methanol và nước. Quá trình rửa giải gradient được thiết lập như sau: 0–40 phút, 10–100 % methanol; tốc độ dòng chảy được duy trì ở mức 0,7 mL/phút và phát hiện được thực hiện bằng đầu dò UV ở bước sóng 207 nm. Đường cong hiệu chuẩn cho thấy độ tuyến tính tuyệt vời với giá trị R^2 là 0,9999. Phương pháp này cũng cho thấy độ nhạy cao, với giới hạn phát hiện (LOD) và giới hạn định lượng (LOQ) đối với amygdalin được xác định lần lượt là 0,15 và 0,46 µg/mL. Hệ thống sắc ký có độ lặp lại tốt với độ lệch chuẩn tương đối (RSD) là 0,07 %. Tỉ lệ thu hồi của phương pháp dao động từ 100,28 % đến 101,41 %, cho thấy độ chính xác và độ tin cậy cao. Kết quả nghiên cứu củng cố tiềm năng phát triển các sản phẩm thực phẩm chức năng hoặc dược liệu có nguồn gốc từ Prunus armeniaca, trong đó việc kiểm soát nghiêm ngặt hàm lượng amygdalin là yếu tố then chốt.*

Từ khóa: *Amygdalin; Định lượng; Prunus armeniaca; UHPLC.*