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DETERMINATION OF AMYGDALIN IN FIFTEEN DIFFERENT FRUIT KERNELS AND EXTRACTION OPTIMIZATION

Busra Nagihan OZTURK,[a] Serap AYAZ SEYHAN[b,c,*] and Dilek BILGIC ALKAYA[b,c]

[a]Marmara University, Institute of Health Science, 34854, Maltepe, Istanbul, Turkey
[b]Marmara University, Faculty of Pharmacy, Department of Analytical Chemistry 34854, Basıbuyuk, Istanbul, Turkey
[c]Center for Nanotechnology & Biomaterials Application and Research (NBUAM), Marmara University, Istanbul, Turkey

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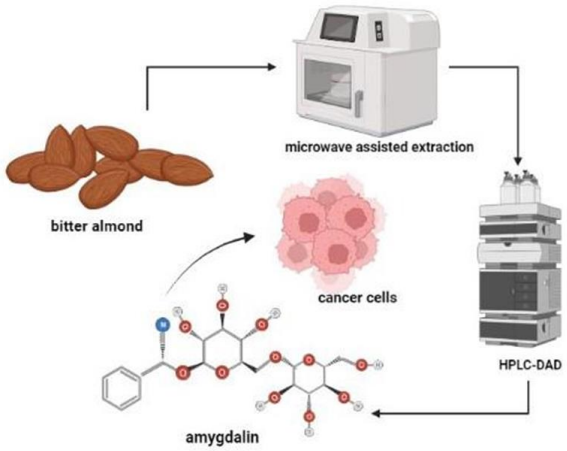
Amygdalin (AMG) is mainly an alternative therapy for traditional cancer treatment. In this study, an effective HPLC-DAD method was developed to investigate the extraction efficiency and determine AMG levels in fifteen fruit kernels belonging to different families. AMG in bitter almond was extracted from using various solvents by different extraction methods (Soxhlet, ultrasonic, orbital shaking and microwave extraction). The most suitable method was determined by the optimized HPLC-DAD method and was applied to fifteen different fruit kernels. The recoveries were in the range 94.68% to 95.26%. The LOD and LOQ values were 0.0097 mg/g and 0.0295 mg/g, respectively. The amygdalin content in kernel fruits was determined ranging from 0.074 to 65.21 mg/g. This study showed that AMG was determined in a short time and with high yield, and the presence of AMG was proven in other fruit kernels belonging to different families that had not been proven before.



INTRODUCTION

Cancer is a major threat to people's health and life worldwide. Despite the advent of various multidisciplinary tumor treatments including surgery, radiotherapy, chemotherapy and immunotherapy, the mortality rate of cancer patients remains high. General anticancer drugs, used in chemotherapy, are associated with severe side effects due to high dosage requirements.[1,2] However, the side effects of conventional drugs and methods on healthy cells have led researchers to seek alternative cancer treatment methods.[3] Cancer treatment using by natural phytochemical compounds is an emerging safe procedure to prevent, and cure cancer. Recent research shows that varied natural products support cancer cell apoptosis, inhibit tumor cell growth and reduce metastasis. As a result, it is revealed that the use of these natural compounds in the medical treatment of cancer will be beneficial. AMG ($C_{20}H_{27}NO_{11}$) is the most commonly encountered cyanogenic di-glycoside found in plants belonging to the especially genus Prunus of the Rosaceae family especially in fruit kernels.[4] Fruit kernels are considered to be unwanted parts of the fruit; but these kernels can represent valuable products with

* Corresponding authors: sayaz@marmara.edu.tr

a large industrial potential.[5] Cyanogenic glycosides are a large group of secondary metabolites including various plants that are commonly consumed by humans. Amygdalin is the most commonly encountered cyanogenic di-glycoside found in plants, for example, in apples, apricots and bitter almonds. An important characteristic of cyanogenic plants is the ability to generate toxic hydrogen cyanide–AMG itself is non-toxic, but its production HCN decomposed by some enzymes is poisonous substance.[6] A number of studies reported that AMG has various activities including anti-tussive, anti-asthmatic, anti-atherogenic, inhibition/prevention of fibrosis, anti-inflammatory, anti-ulcer and anti-cancer activities. Mounting evidence has supported that AMG induces apoptotic cell death of various cancer cells such as promyelocytic leukemia, prostate cancer, cervical and liver cancer cells.[7] Therefore the anti-tumor effect of amygdalin has received more attention in recent years. When the studies of amygdalin are examined in the literature studies, it is seen that AMG is considered to be an alternative antitumor drug which plays a supporting role in the cancer treatment.[8] Considering the aforementioned pharmacological and toxic effects of amygdalin on the one hand, and the consumption of many fruit seeds worldwide (for example, for the production of marzipan and dates) on the other hand, an efficient and simple method for the analysis of amygdalin from natural sources is highly desirable.[9] There are many papers on the determination of amygdalin in different fruit kernels, and most of the publications have used different HPLC methods. [10-13]

Extraction of amygdalin from food plants is a crucial aspect of any analytical procedure due to its potential for rapid degradation. Many of the studies on efficiency of amygdalin extraction were based on the use of water or methanol. Quantification was mostly carried out in almond, apricot and Chinese herbal medicines which contain amygdalin as a major ingredient. Similarly, this research compared the use of ultrasound, Soxhlet extraction with methanol, and reflux extraction with citric acid for extraction of amygdalin from bitter almond kernels. In addition, there are many studies in which AMG was determined by using various extraction methods and solvents in the extraction of AMG from different fruit seeds.[4,6,10,14-29] For example; apricot, cherry, apple,[6] pear, nectarines, plum and bitter almond[4] and quince [14] belong to the *Rosaceae* family; watermelon belongs to the *Cucurbitaceae* family;[15] lemon belongs to the *Rutaceae* family;[16] pomegranate belongs to the *Lythraceae* family;[17] rambutan[18] and longan[19] belong to the *Sapindaceae* family; papaya belongs to the *Cacicaceae* family[20] and guava belongs to the *Myrtaceae* family.[21] Miao *et al.*[22] reported that amount of AMG for apricot kernel as 0.18 mg/mL, Koo *et al*.[23] reported that for apricot as 1.73 mg/g, Lee *et al*.[24] reported that for apricot as 40060.34 mg/kg, Femenia *et al.*[25] reported that for apricot as 5.5 g/100g, Yildirim *et al*.[26] reported that for apricot as 22.53 mg/g, Lv *et al*.[27] reported that for apricot and hairy cherry kernels, respectively as; in ultrasonic extraction, 1.71% and 1.67%; in Soxhlet extraction, 2.03% and 2.11%; in reflux extraction, 4.31% and 4.09%, Bolarinwa *et al*.[10] reported that for apricot as 14.37 mg/g, for cherry as 3.89 mg/g, for nectarine as 0.12 mg/g, for peach as 6.81 mg/g, for plum as 17.49 mg/g, for apple as 2.96 mg/g and for pear as 1.29 mg/g, Zhao[28] reported that for apricot as 0.11%– 3.26%, for peach as 0.13%–3.73%, for plum as 0.28%–4.91%, and for bitter apricot as 0.39%–5.1%, Arrázola *et al*.[29] reported that for bitter almond as 2.44–5.89 mg/100g. The results showed that extraction with water containing citric acid is the best option.[10]

Considering the increase in the effect of amygdalin on cancer treatment in recent years, in this study was conducted to analyze the amounts of amygdalin in fifteen different fruit kernels using various extraction methods (Soxhlet, ultrasonic, orbital shaking and microwave extraction) and different extraction solvents (acetonitrile, hexan, methanol, ethanol, water and citric acid). Bitter almond was chosen in this study to determine the most appropriate extraction method, because of it has high amygdalin content and has been used for medicinal purposes for thousands of years in various countries such as Egypt, China, and India[4]. The most appropriate method determined was applied to fruit kernels.

In the light of this information, this study focused on the determination of the amount of amygdalin in commercial fruit kernels. For this, pear, quince, apricot, apple, cherry, nectarines, plum, watermelon, lemon, pomegranate, bitter almond were bought in common markets in the Turkey; rambutan, papaya, longan and guava were bought from Thailand. Microwave extraction with 2% citric acid determined as the appropriate method was applied to fruit kernels and local differences in content of amygdalin was determined by high performance liquid chromatographic procedure. Finally, the optimized method was

validated to analyze AMG in the other kernels and previously unspecified kernels.

RESULTS AND DISCUSSION

Bitter almond kernels extracts obtained by Soxhlet, orbital shaking, ultrasonic bath and microwave extraction were analyzed by using HPLC-DAD. The highest yielding method was applied to other fruit kernels. There have been previous reports describing the determination of AMG by using different methods in bitter almond kernels.[10] In this study, we use more different methods and solvents and optimize the conditions for quantification of AMG.

Separation of amygdalin by reversed-phase HPLC-DAD

In general, chromatographic separations of AMG in bitter almonds have been carried out using conventional HPLC C18 columns and isocratic elution with methanol:water in the literature. Methanol is a good mobile phase for AMG separation by HPLC.[6] In this study, methanol and water were used at a ratio of 30:70, (v:v). AMG detection was achieved by UV detection in an isocratic elution with an good linearity (correlation, R^2= 0.9994). The LOD and LOQ values were 0.0097 mg/g and 0.0295 mg/g, respectively. 0.2 and 0.5 mg/g were added in watermelon samples and the recoveries of AMG were found respectively as 94.68% and 95.26%. Repeatability RSDs% were found respectively as 0.87% and 0.61%. Reproducibility RSDs% were found respectively as 0.90% and 0.96%

Validation of the HPLC-DAD method

The proposed method was validated with respect to linearity, LOD, LOQ, precision, stability, accuracy, selectivity and robustness according to the the Eurachem Guide: The Fitness for Purpose of Analytical Methods[30]. Validation parameters were given in Table 1.

Linearity

The linearity of the calibration curve was tested for seven concentration of the standart mixtures with three injections per concentration in the range of 1.00–250 mg/L (AMG solution was prepared by dissolving in water). The calibration curve was constructed by plotting peak area against the analyte concentration. Linear regression equations were calculated via the least squares method. This method showed linear regressions at concentrations from 1.00 mg/L to 250 mg/L for AMG. The regression analysis was performed, shows the equation: y = 16831x + 25721 Correlation coefficient was 0.9994.

Limit of detection and limit of quantification

The LOD and LOQ for AMG were obtained by spiked series of low concentrations (15 mg/kg) of standard solution into real samples. LOD is defined as the lowest concentration of analyte that can be detected above baseline noise, S/N=3. LOQ is defined as the lowest concentration of analyte, S/N=10.[8]

Table 1

Validation parameters of proposed HPLC-DAD method

						Intra-day precision (n = 10)		**Inter-day precision (n = 10)**	
Compound	**Regression equation**	**R^2**	**Linear range (mg/L)**	**LOD (mg/g)**	**LOQ (mg/g)**	**Concentration level (mg/kg)**	**RSD (%)**	**Concentration level (mg/kg)**	**RSD (%)**
R-amygdalin	16831x + 25721	0.9994	1 -250	0.0097	0.0295	200	0,87	200	0,90
						500	0,61	500	0,96
						Recovery (%)		**Recovery (%)**	
						94.68		95.26	

Each value is expressed as mean ± standard deviation (n = 3 extractions).

In this study, the LOD and LOQ values were 0.0097 mg/g and 0.0295 mg/g, respectively. LOD and LOQ were determined by making ten readings of the 15 mg/kg amygdalin standard.

Precision

The intra-day precision was determined by ten repetitive injections of samples at the same day. The inter-day precision was determined for ten following days. Ten samples of amygdalin were prepared independently to check the repeatability. Standard solution of amygdalin into a real sample at twice different concentration levels (200 mg/kg and 500 mg/kg). The RSD values of repeatability for all two concentration levels were under 0.88% and for the reproducibility the RSD values were below 0.97%.

Stability

HPLC determination of the AMG standard was performed at 12 h intervals and the peak areas were compared. As a result of the stability study; AMG standard solution remained generally stable for at least 48h.

Accuracy

The accuracy of the method is reported as recovery studies. The recoveries of amygdalin standard were examined at two levels. Recoveries were determined by spiking 200 mg/kg and 500 mg/kg of standard solution of amygdalin into watermelon kernel. Prepared samples were then analyzed by HPLC according to procedure described. Analyses were performed in duplicate at each level. Samples were then analyzed by HPLC according to procedure described above. Analyses were performed in triplicate at each level. The accuracy of the method was evaluated on test solutions prepared as two concentration levels (200 mg/kg and 500 mg/kg of the target concentration) and each solution was injected twice times. Good-to-excellent recoveries (94.68%–95.26%) at each level were achieved within the limit range of 90.0% – 107.0% and repeatability RSDs% were found respectively as 0.87% and 0.61%, reproducibility RSDs% were found respectively as 0.90% and 0.96%. All the values of recoveries and other validation parameters are listed in Table 1.

Selectivity

Two samples were prepared according to the described procedure to identify any compound that might interfere during the quantification process. AMG standards were prepared with water. Water was injected into the system as a blank sample. An aqueous solution of 250 mg/kg of AMG was used as a sample. For all other compounds it was not observed any interfering substances in the chromatogram.

Robustness

The was robustness tested with column temperature and flow rate variations in the HPLC-DAD method parameters. According to the robustness results, the method is not affected by minor changes.

Optimisation of amygdalin extraction from bitter almonds

For optimization, amygdalin was extracted from bitter almond kernel by testing four different methods with variety solvents and the results of analysis were given in Table 2.

Soxhlet extraction

The highest extraction yield was obtained from methanol. The amygdalin content of bitter almond kernel ranged from 0.025 to 44.08 mg/g. In this study, amygdalin was obtained as 0.025±0.001 mg/g from water extract. In another similar study was reported that the amygdalin content as 0.068 mg/g.[10] In our study the obtained amygdalin contents were in 2% citric acid, acetonitrile, 50% ethanol, 50% methanol, methanol, ethanol extracts respectively as; 0.14±0.01 mg/g, 4.29±0.17 mg/g, 9.43±0.38 mg/g, 39.64±1.59 mg/g, 44.38± 1.78 mg/g and 23.41±0.94 mg/g. AMG peak could not be detected from the hexane extract. In a similar study with ethanol, the amount of amygdalin was reported as 0.119 mg/g.[10] In addition, water extracts were cloudy. Protein precipitation may be responsible for the cloudiness of the water extracts.[10] Methanol; it is not preferred because it is toxic, harmful to the environment and human health. Amygdalin contents in bitter almond kernel are given in Fig. 1.a.

Table 2

Results of determination of the amygdalin in bitter almond kernels by HPLC using different extraction methods

	Soxhlet extraction (4h) (mg/g)	Ultrasonic extraction (30m) (mg/g)	Orbital shaking extraction (2h) (mg/g)	Microwave extraction (1h) (mg/g)
Methanol (MeOH)	44.38±1.78	Irregular peak	Irregular peak	-
Ethanol (EtOH)	23.41±0.94	Irregular peak	Irregular peak	-
50% Methanol (MeOH)	39.64±1.59	10.63±0.43	20.04±0.80	25.14±1.01
50% Ethanol (EtOH)	9.43±0.38	Irregular peak	Irregular peak	Irregular peak
2% Citric Acid	0.14±0.01	36.79±1.47	33.60±1.34	65.21±2.61
Acetonitrile	4.29±0.17	Not detected	0.023±0.001	-
Water	0.025±0.001	Not detected	Not detected	24.27±0.97
Hexane	Not detected	-	-	-

Each value is expressed as mean ± standard deviation (n = 3 extractions).

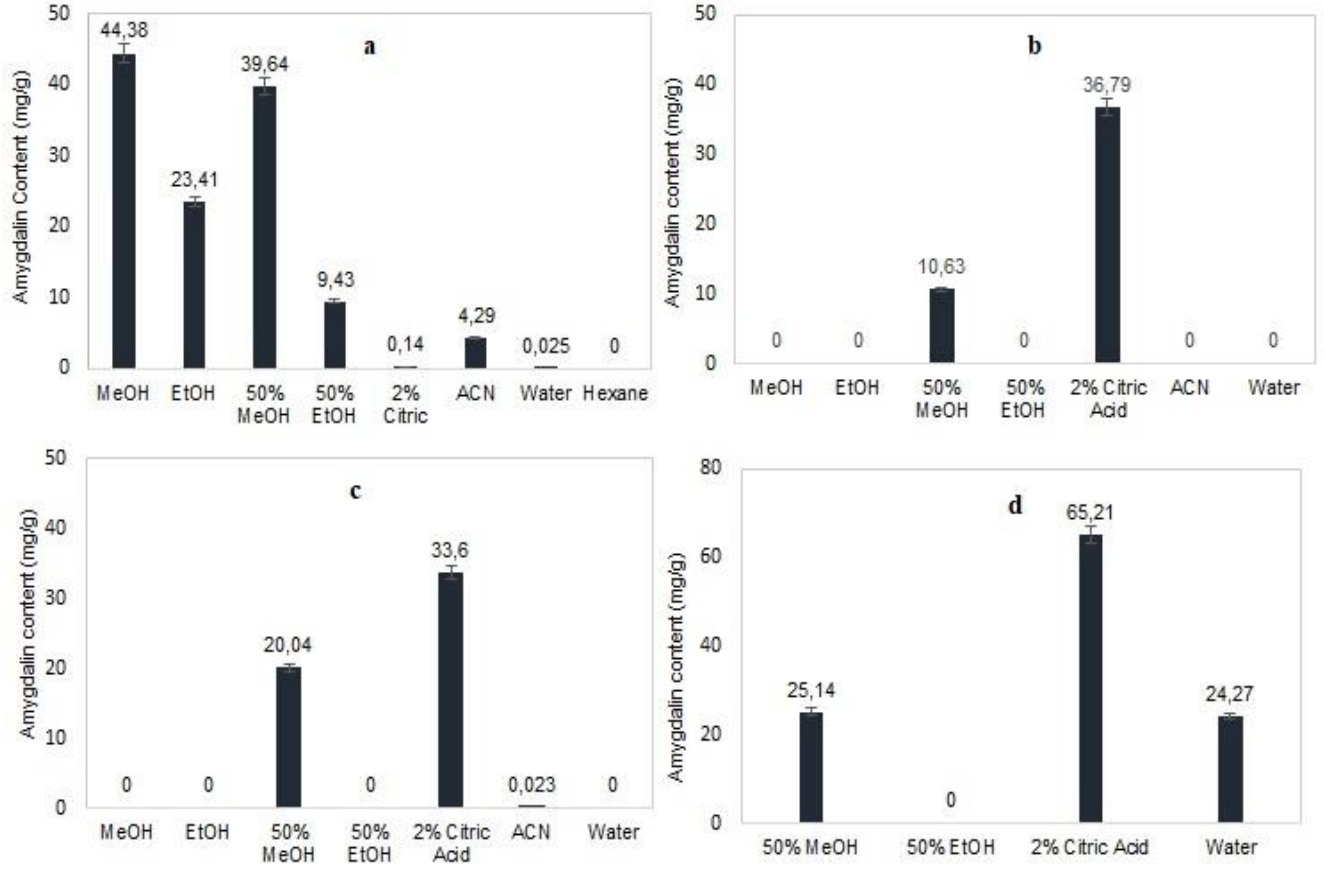


Fig. 1 – Results of determination of the amygdalin in bitter almond kernels by HPLC using different extraction methods
a) Soxhlet extraction, b) Ultrasonic extraction, c) Orbital shaking extraction, d) Microwave extraction.

Ultrasonic extraction

The highest extraction yield was obtained from 2% citric acid. The AMG contents of bitter almond kernel ranged from 10.63 to 34.84 mg/g. The results of the analysis were found respectively as 10.63±0.43 mg/g with 50% methanol; 36.79± 1.47 mg/g with 2% citric acid. Amygdalin peak could not be detected from acetonitrile and water; peak was unregular with methanol, ethanol and 50% ethanol. According to the results, this method is suitable for 2% citric acid solution. Not useful for other solvents. Amygdalin contents in bitter almond kernel are given in Fig. 1.b.

Orbital shaking extraction

The highest extraction yield was obtained from 2% citric acid. The amygdalin content of bitter almond kernel ranged from 0.023 to 33.60 mg/g. The results of the analysis were found respectively as 0.023±0.001 mg/g with acetonitrile; 20.04± 0.80 mg/g with 50% methanol; 33.60±1.34 mg/g with 2% citric acid; amygdalin peak could not be detected from water; peak was not regular with methanol, ethanol and 50% ethanol. This method is similar with ultrasonic extraction method so it is not useful for the other solvents except 2% citric acid. Amygdalin contents in bitter almond kernel are given in Fig. 1.c.

Microwave extraction

The highest extraction yield was obtained from 2% citric acid. The amygdalin content of bitter almond kernel ranged from 24.27 to 65.21 mg/g. The results of the analysis were found respectively as 24.27±0.97 mg/g with water; 25.14±1.01 mg/g with 50% methanol; 65.21±2.61 mg/g with 2% citric acid; peak was not regular with 50% ethanol. It was decided that this method is the best than all methods which were tested. In a shorter time, the highest yield was obtained with 2% citric acid solution. Amygdalin contents in bitter almond kernel are given in Fig. 1.d. This method was also applied to other fruit kernels. The results were given in Table 3 and HPLC chromatograms were given in Figures 2, 3, 4 and 5.

In many previous publications, the amygdalin was extracted from fruit kernels under reflux and Soxhlet which is a time-consuming procedure. These procedures are followed by evaporation and re-dissolving in another solvent.[10,22,23] In our study, the amygdalin was extracted from the samples using 2% citric acid and the solutions were extracted in a microwave for 1 h at 40ºC and under power of 800 W. Advantages of microwave assisted extraction; decrease in solvent and sample amount usage, reduction of extraction time, increase in extraction efficiency and recovery, low cost, ease of use, wide choice of solvent.[31]

In addition, the use of citric acid solution prevents the conversion of amygdalin to its polymer called neo amygdalin.[23] The main problem of the previous studies with the amygdalin analyses in food samples is that often they overlooked the amygdalin epimerisation.[8] In this study, it was observed that microwave extraction prevented the conversion of amygdalin to neoamygdalin.

Sheikh and Saini reported[32] that amount of amygdalin for plum 3.03 ± 0.17 mg/g by using microwave assisted extraction method at power of 450 W for 6 minutes. In another study Vladić *et al*. reported[33] that 0.41 mg/g amygdalin in plum kernels by using cold press and supercritical CO_2 extraction but in our study 40.01±1.0 mg/g amount of amygdalin was obtained from plum kernels. It is showed that analysis time and microwave power was not sufficient in Sheikh and Saini's study.[32] On the other hand, cold press and supercritical CO_2 extraction are not suitable for obtaining amygdalin in high yield in Vladić *et al.* study.[33] The conditions preferred in our study are more suitable for obtaining amygdalin with high efficiency.

Table 3

Results of determination of the amygdalin in the other fruit kernels by HPLC using microwave extraction method

Fruit kernels	**Results of amygdalin (mg/g)**
Pear (*Pyrus communis*)	0.16±0.01
Quince (*Cydonia oblonga*)	7.42±0.30
Apricot (*Prunus armeniaca*)	1.21±0.05
Apple (*Malus domestica*)	13.21±0.53
Cherry (*Prunus avium)*	2.03±0.08
Nectarines *(Prunus persica var. nucipersica)*	0.042±0.002
Plum (*Prunus domestica*)	40.01±1.0
Watermelon (*Citrullus lanatus*)	Not detected
Lemon (*Citrus limon*)	Not detected
Pomegranate (*Punica granatum*)	Not detected
Bitter Almond (*Semen Amygdali amarum*)	65.21±2.61
Rambutan (*Nephelium lappaceum*)	0.15±0.01
Papaya (*Carica papaya*)	Not detected
Longan (*Dimocarpus longan*)	Not detected
Guava (*Psidium guajava*)	0.074±0.003

Each value is expressed as mean ± standard deviation (n = 3 extractions).

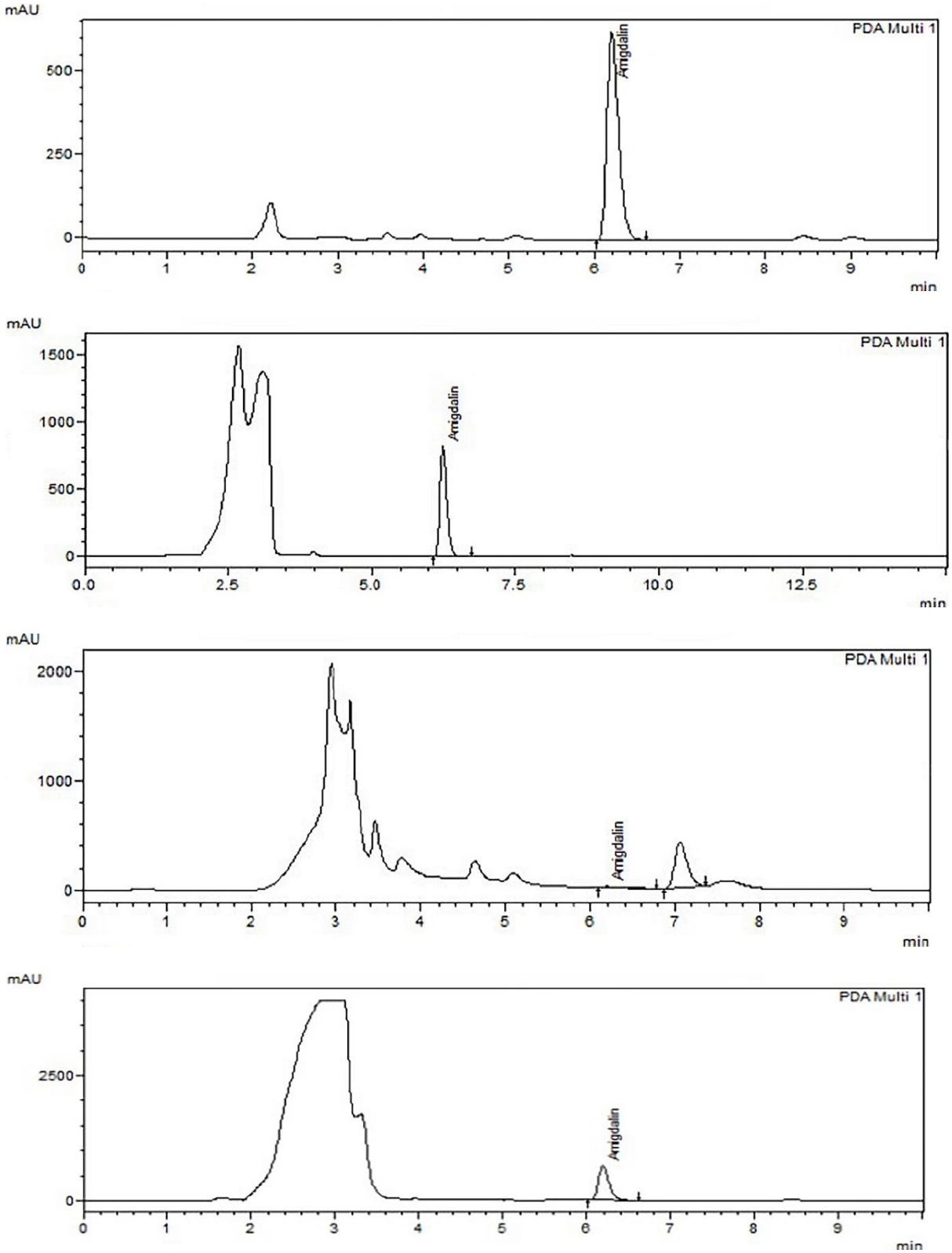


Fig. 2 – Chromatograms of amounts of amygdalin in fruit kernels by microwave extraction
a) Control standard, b) Bitter almond, c) Pear, d) Quince.

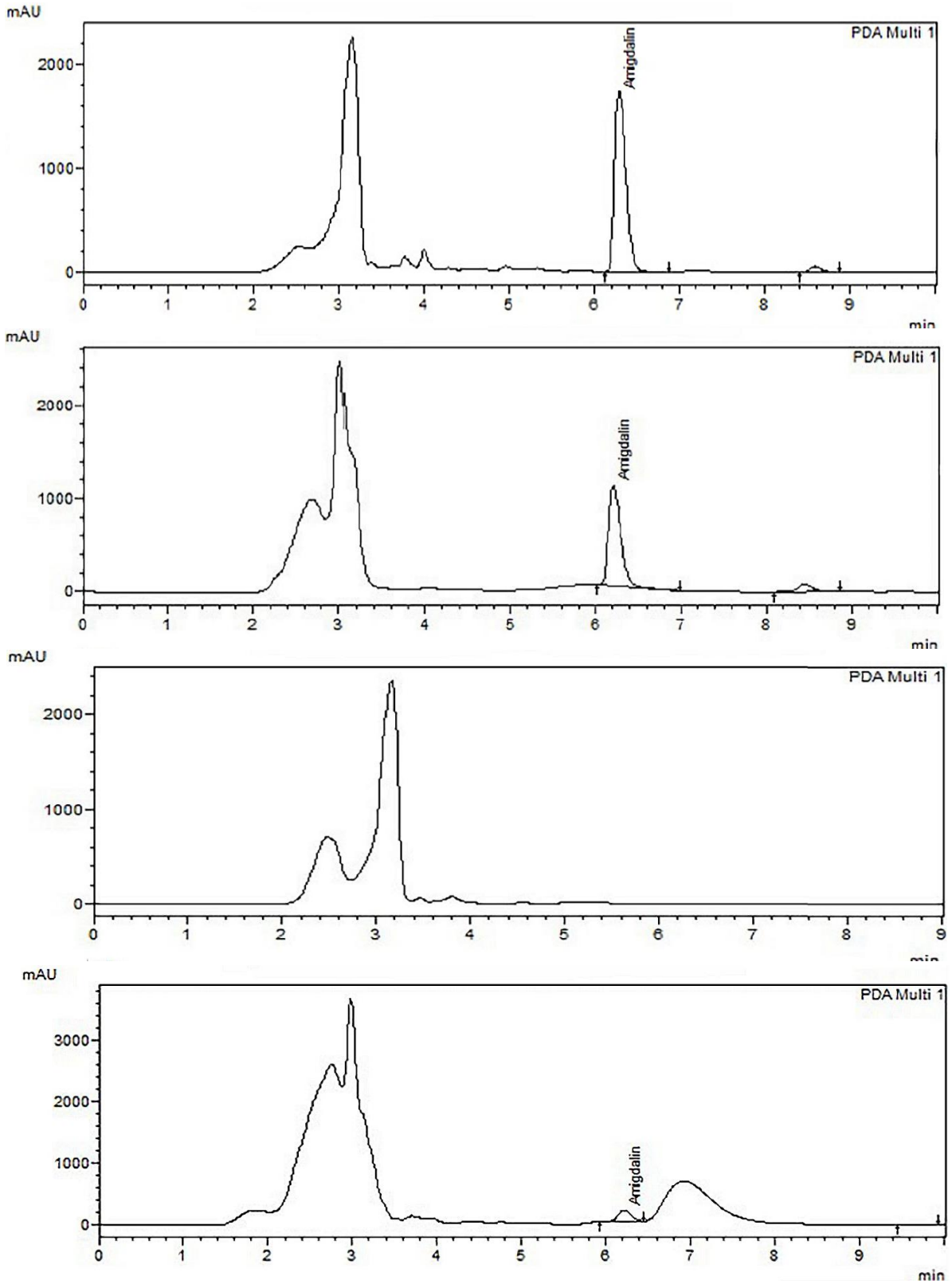


Fig. 3 – Chromatograms of amounts of amygdalin in fruit kernels by microwave extraction
a) Apple b) Plum c) Watermelon d) Apricot.

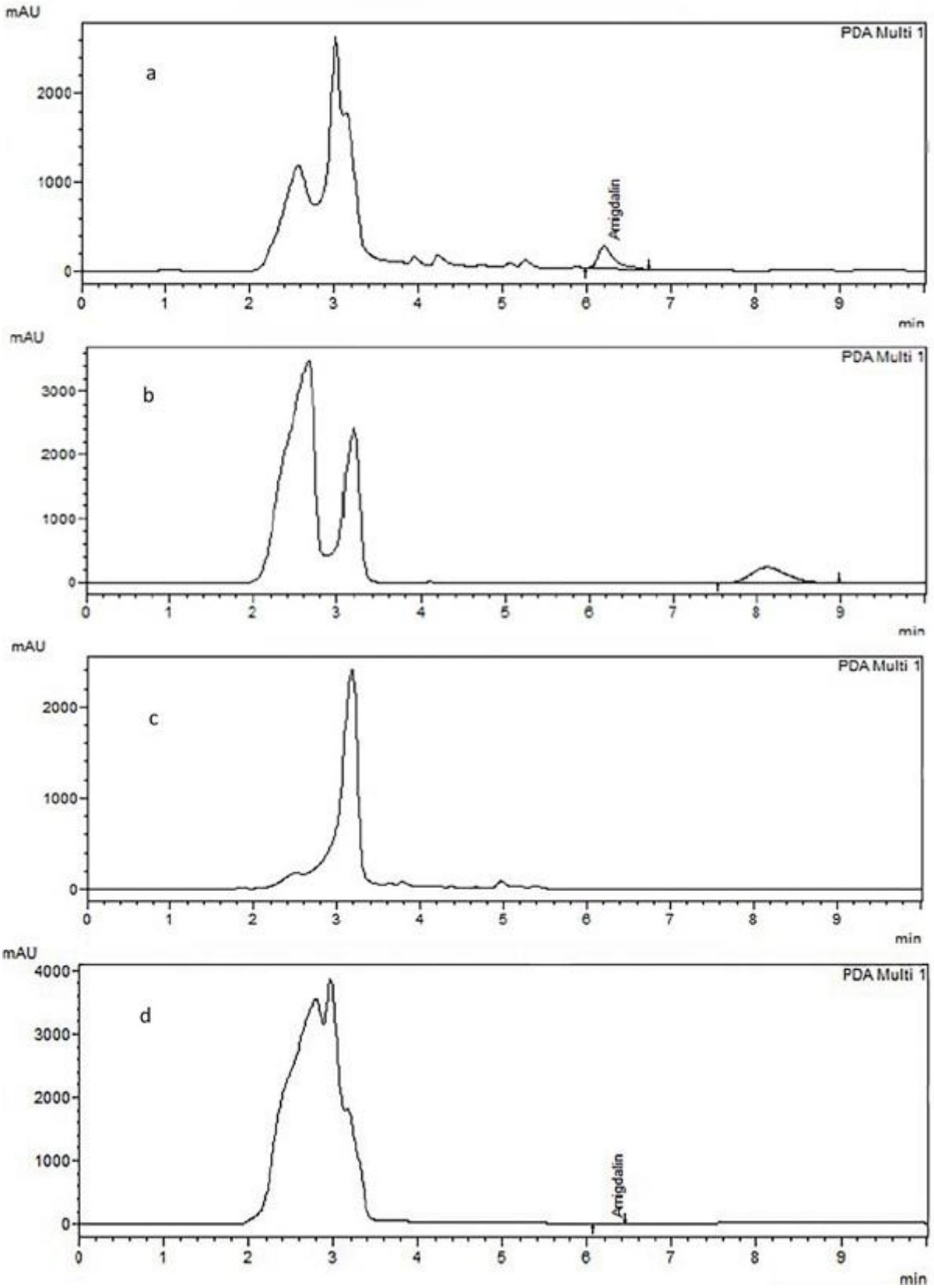


Fig. 4 – Chromatograms of amounts of amygdalin in fruit kernels by microwave extraction
a) Cherry, b) Lemon, c) Pomegranate, d) Nectarine.

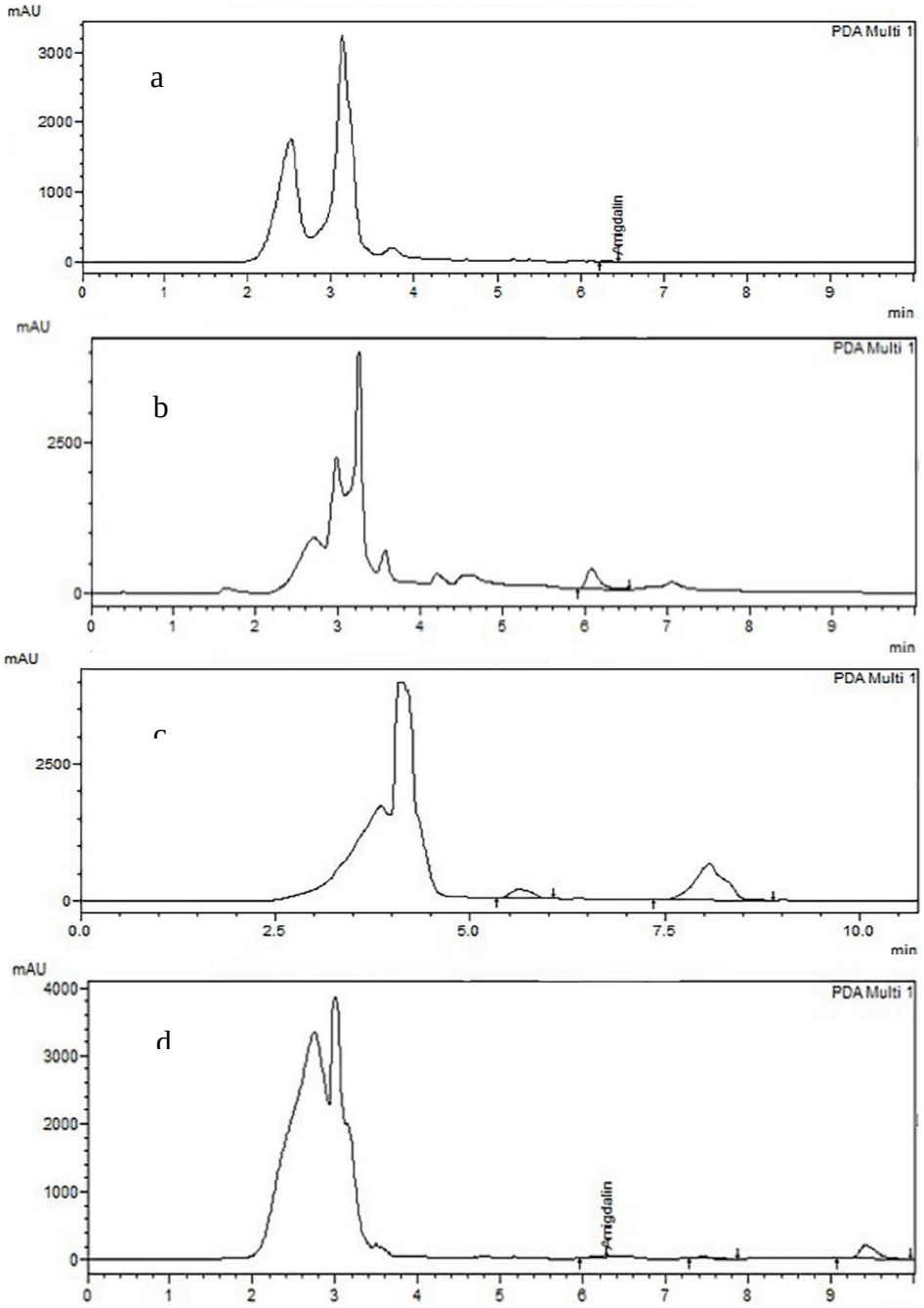


Fig. 5 – Chromatograms of amounts of amygdalin in fruit kernels by microwave extraction
a) Guava, b) Longan, c) Papaya d) Rambutan.

The highest content of amygdalin was detected in kernels of bitter almond. Surprisingly, amygdalin was not detected, although the lemon seed contained bitterness. Maybe it contains another kind of amygdalin derivative. At the same time amygdalin was not detected in watermelon and pomegranate kernels from Turkey and longan, papaya from Thailand. Probably it was a sweet variety with any amygdalin product. Janatová reported[29] that the amygdalin amount increases during the ripening. In this study fresh fruits were used. Probably, amygdalin could be determined if the fruits were analyzed as ripe. The amygdalin was obtained from bitter almond, apricot, sweet cherry, pear, quince, apple, nectarine, plum, rambutan and guava. HPLC analysis of watermelon, lemon, pomegranate, longan and papaya did not show any amount of amygdalin. Amygdalin was detected from kernels of ten species; 65.21 mg/g in bitter almond, 1.21 mg/g in apricot, 2.03 mg/g in sweet cherry, 0.16 mg/g in pear, 7.42 mg/g in quince, 13.21 mg/g apple,

0.042 mg/g in nectarine, 40.01 mg/g in plum, 0.15 mg/g rambutan and 0.074 mg/g guava.

EXPERIMENTAL

Materials

Fruit kernels, which had been analyzed in this study, were obtained from local markets in Turkey, Istanbul and Thailand, Nonthaburi. Pear (*Pyrus communis*), quince (*Cydonia oblonga*), apricot (*Prunus armeniaca*), apple (*Malus domestica*), cherry (*Prunus avium*), nectarines (*Prunus persica* var. *nucipersica*), plum (*Prunus domestica*), watermelon (*Citrullus lanatus*), lemon (*Citrus limon*), pomegranate (*Punica granatum*) and bitter almond (*Semen Amygdali amarum*) were obtained from Turkey, Istanbul local markets; rambutan (*Nephelium lappaceum*), papaya (*Carica papaya*), longan (*Dimocarpus longan*) and guava (*Psidium guajava*) were obtained from Thailand, Nonthaburi local markets. Methanol, ethanol, acetonitrile, hexan, water and citric acid were purchased from Merck (Darmstadt, Germany) and J.T Baker (China). AMG standard was purchased from Sigma-Aldrich Co. (St. Louis, MO, USA).

Equipment

Ultrasonic bath (EFLAB, KUDOS-SK 3310NP), Orbital shaker (EFLAB, EFSH), Soxhlet Extraction (EFLAB, EFYG-4), Microwave Extraction (Terra, Mars 240-50) were used as an extractor of to analyze AMG in the kernels. For assay analysis of AMG, a 20A series Shimadzu high-performance liquid chromatography (HPLC) system with a diode array detector (DAD) was employed. Water Purification Purifier System (NTS, NTS-6, Nisa Teknik) and RE300DB series were used for the evaporation (Stuart Equipment) of solvents after extraction of the AMG.

Sample preparation and extraction

The kernels separated from the fruit parts were dried in an air circulation oven at 40 °C for one night. After drying, it was pounded into small pieces in a mortar. Fruit kernels were not completely pulverized because Koo *et al.* reported that, a larger cutting size showed higher efficiency for amygdalin than cut into powdered form.[23]

Soxhlet extraction

For the preparation of samples, 2.5 grams of dried bitter almond kernels were weighed and placed into an extraction cartridge. 250 mL of solvents were added to extraction flasks. For this, eight different solvents (2% citric acid, methanol, 50% methanol, ethanol, 50% ethanol, hexane, acetonirile, water) were used. The flask contents were boiled during 4 h at 90ºC. After extraction, the solvent was evaporated using a rotary evaporator under the vacuum control and thermostatic bath held at 60ºC. Residue was taken with 5 mL hot water. The extract filtered through a 0.45 µm nylon filter prior to injection into the HPLC system.

Ultrasonic extraction

The extraction of amygdalin was performed in the ultrasonic bath (EFLAB, KUDOS-SK 3310NP) with a nominal power of 100 W and a frequency of 35 kHz. For the preparation of samples, 2.5 grams of dried bitter almond kernels were weighed and placed into volumetric flasks. 25 mL of solvents were added. For this, seven different solvents (2% citric acid, methanol, 50% methanol, ethanol, 50% ethanol, acetonitrile, water) were used. Samples were extracted during 30 m at 40ºC. The flask contents were fullfilled to 50 mL and filtred. The extract filtered through a 0.45 µm nylon filter prior to injection into the HPLC system.

Orbital shaking extraction

For the preparation of samples, 2.5 grams of dried bitter almond kernels were weighed and placed into flasks. 50 mL of solvents were added. For this, seven different solvents (2% citric acid, methanol, 50% methanol, ethanol, 50% ethanol, acetonitrile, water) were used. Samples were extracted during 2 hours. The flask contents were filtred. The extract filtered through a 0.45 µm nylon filter prior to injection into the HPLC system.

Microwave extraction

Microwave oven able to generate 1600 W energy at 2450 MHz was used for heating experiments. The extraction vessels were made from perfluoro alkoxy (PFA) and had a capacity of 55 mL. For the preparation of samples, 2.5 grams of dried bitter almond kernels were weighed and placed into extraction vessels. 10 mL of solvents were added. For this, four different solvents (50% methanol, 50% ethanol, water and 2% citric acid) were used. Samples were extracted during 1 h at 40ºC and under power of 800 W. The vessel contents were filtered. The extract filtered through a 0.45 µm nylon filter prior to injection into the HPLC system.

The reason why pure methanol, ethanol, acetonitrile and hexane are not used in microwave extraction is that pure forms of these solvents can pose a hazard in pressure vessels. Therefore, the preparation of aqueous solutions is safer. In addition, hexane (dipole moment <0.1), which is a non-polar solvent, does not heat up in the microwave. Therefore, the preparation of aqueous solutions is safer. In such cases, it is recommended to mix the non-polar solvent with the polar solvent.[31] However, since hexane has the least dipole moment, it is not preferred much in extractions from especially vegetable sources.

Standard stock solutions

A stock solution of AMG was prepared by dissolving accurately weighed quantity of AMG in water to get the concentration of 50 mg/50 mL. The final working standard solutions were prepared by diluting stock solution with water to get the concentration of 1, 5, 10, 25, 50, 100 and 250 mg/L respectively.

HPLC Analysis

Chromatographic conditions

The analyses were conducted with a Shimadzu series LC-20A HPLC instrument using a diode array detector (DAD). All extracts were analyzed on the HPLC-DAD system. The separation was performed on C18 (250x 4.6 mm i.d, 3µm) column. The column temperature was 35ºC. Isocratic elution using a mobile phase consisted of methanol and water, (30:70) (v/v) achieved the required separation within maximum 6.4 min. For quantitative analysis, various concentrations of AMG was injected in triplicates. Flow rate and injected

volume were 0.9 mL/min and 20µL, respectively. The AMG were detected 207 nm. Calibration curve was constructed using seven different concentrations of standard solution containing 1, 5, 10, 25, 50, 100 and 250 mg/L of amygdalin. Standard solutions were prepared from amygdalin stock solution (50 mg/50 mL) by diluting with water. Quantitative determinations were carried out using calibration curves of the standards. All standard solutions were injected three times and the repeatability of the calibration curve was evaluated by calculating the standard deviations.

Statistical analysis

Values were expressed as mean ± standard deviation and RSD values of, at least, three independent experiments. Linear regression analysis was used to prepare standard lines with amygdalin. All tests were performed in triplicate and repeated 3 times. Results are presented graphically as means with calculated standard deviations (SD) represented by vertical bars. Statistical significance was applied using Microsoft office Excel (2016). Difference in values of recoveries were expressed tested by Student's t-test ($P < 0.05$).

CONCLUSIONS

Amygdalin amounts of bitter almond determined from ultrasonic, orbital shaker, Soxhlet and microwave extractions using 2% citric acid as solvent, amygdalin amounts of bitter almond respectively; 36.79±1.47 mg/g, 33.60 ±1.34 mg/g, 0.14 ±0.01 mg/g and 65.21 ±2.61 mg/g. According to the results, microwave extraction with 2% citric acid gave the best results in this study and this method was also applied to other fruit kernels. Although the results of Soxhlet extraction with methanol were also good, microwave extraction was preferred because duration of Soxhlet extraction is too long and methanol is a toxic solvent. The other advantages of microwave assisted extraction; decrease in solvent and sample amount usage, reduction of extraction time, increase in extraction efficiency and recovery, low cost, ease of use, wide choice of solvent. On the other hand, there are not many studies on the determination of amygdalin by microwave extraction in the literature. In the recent study of Sheikh and Saini, 3.03 ± 0.17 mg/g amygdalin was reported from plum by microwave extraction but in our study, 40.01±1.0 mg/g amygdalin was determined from plum by microwave extraction. This shows that there is a significant difference between the two studies. At the same time, the amounts of amygdalin we determined from other fruit kernels are better than other studies in the literature.[5,10,22,24,28,29] In addition, the proposed HPLC method represents the first report on the amygdalin content of Thai fruits. As a result of the literature review, it was observed that samples of *Rosaceae* species were examined however researches on other families were not focused on. This method was developed using DAD detector for the determination of amygdalin in various fruit kernels grown in Thailand and different families such as *Cucurbitaceae*, *Rutaceae*, *Lythraceae*, *Sapindaceae*, *Caricaceae* and *Myrtaceae*. The obtained results of the levels of amygdalin in the fruit kernels are found comparable to the other fruit kernels in the literature. All samples except watermelon, lemon, pomegranate from Turkey and longan, papaya from Thailand contained measurable value of this compound. The highest median value of amygdalin was found in bitter almond seeds. Method validation results proved the method to be selective, precise, accurate, robust. It is considered that the HPLC method developed in this study will help in determining the amygdalin content in fruit kernels and this study will illuminate cancer researches on the amygdalin.

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