Reversed-phase vortex-assisted liquid–liquid microextraction: A new sample preparation method for the determination of amygdalin in oil and kernel samples

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Abstract

A novel, simple and rapid reversed-phase vortex-assisted liquid–liquid microextraction coupled with high-performance liquid chromatography has been introduced for the extraction, clean-up and pre-concentration of amygdalin in oil and kernel samples. In this technique, deionized water was used as the extracting solvent. Unlike the reversed-phase dispersive liquid–liquid microextraction, dispersive solvent was eliminated in the proposed method. Various parameters that affected the extraction efficiency, such as extracting solvent volume and its pH, vortex and centrifuging times were evaluated and optimized. The calibration curve shows good linearity (r^2=0.9955) and precision (RSD<5.2 %) in the range of 0.07–20 μg mL^{-1}. The limit of detection and limit of quantitation were 0.02 and 0.07 μg mL^{-1}, respectively. The recoveries were in the range of 96.0–102.0 % with relative standard deviation values ranging from 4.0–5.1%. Unlike the conventional extraction methods for plant extract no evaporative and re-solubilizing operations were needed in the proposed technique.

Keywords: Amygdalin; High-performance liquid chromatography; Liquid-liquid microextraction; Reversed phase ;Sample preparation;

1 Introduction

Amygdalin is a cyanogenic glycoside widespread in vegetables that exhibits antitussive and antinociceptive activities. Also, amygdalin interferes with tumor growth by both anti-angiogenesis and induction of apoptosis [1, 2]. The kernels of *Prunus persica*, *Prunus armeniaca* and almond seeds that possess amygdalin are used in many pharmaceutical formulations in traditional medicines [1].

Conventional extraction of natural compounds by using maceration, Soxhlet extraction (SE) and distillation techniques need large volumes of organic solvents (usually toxic), long extraction times and high temperatures, which destroy the natural compounds [3–7]. In conventional solvent extraction (CSE) methods, due to the large volume and incompatibility of extracting solvent with analytical instruments, evaporation to dryness and reconstitution of the extract in a very small volume of appropriate solvent is essential [8–10]. As a result, an increasing demand for the extraction of natural molecules by using a clean and green extraction method with safe solvents at low temperatures is observed.

LLE and related microextraction techniques are the most common techniques for the extraction of compounds from liquid samples [11–15]. Normally, the sample is an aqueous phase and the extracting phase is an organic solvent. The fundamental of the extraction process is that the more polar hydrophilic compounds prefer the aqueous phase and the more non-polar hydrophobic compounds prefer the organic solvent [16]. Usually, in LLE analytes were transferred from the aqueous phase to the organic phase. When the target analyte is hydrophilic, the extraction process can be done in reverse mode, which means the extracting solvent is aqueous phase. Recently, a new design of dispersive liquid–liquid microextraction (DLLME) termed as reversed-phase DLLME has been developed for the pre-concentration and determination of phenolic compounds from olive processing wastewater and virgin olive oil [17–19]. In other reports, several methodologies for the determination of pyrethroid pesticides, cadmium, lead and different selenium species present in edible oils are introduced by using DLLME [20–22]. In all studies, a low volume of an aqueous solution in the presence of water-miscible organic solvent as disperser solvent was used as the extracting solvent. In the present study, extraction was performed by using water without any disperser solvent.

In most cases, the determination of compounds from complicated matrices was achieved in two steps. Initially, analytes were extracted by using solid sorbents, surfactants or organic phases and then back extracted into a small volume of appropriate solvent compatible with the analytical instrument [23–27]. In this work, extraction, clean-up and pre-concentration were performed in one step.

In previous reports, amygdalin was extracted from solid samples by using traditional extraction methods such as reflux and maceration [28–31]. The disadvantages of these methods are large solvent volume and long extraction time. On the other hand, due to high volume of extraction solvent and nonselective extraction, pre-concentration and clean-up are essential. Recently, the amygdalin content of several seeds, kernels and food products available commercially was determined by HPLC [32, 33]. In these studies, four extraction procedures including water extraction at 37°C, water extraction at 100°C, ethanol extraction at 37°C and ethanol extraction at 78.5°C were applied for amygdalin extraction from almond kernels. The results were shown that the optimum extraction time with water and ethanol at 37°C, and for reflux extraction with water (100°C) and ethanol (78.5°C) is 100 min. In the other hand, several steps such as removing the fat, evaporative of extracting solvent and reconstitute the extract in water for injection to HPLC system are needed.

The aim of our work was to develop a novel, simple and rapid reversed-phase vortex-assisted liquid–liquid microextraction (RP-VALLME) technique for the determination of amygdalin in almond oils and several fruits kernel. Unlike the RP-DLLME method, extraction was performed without using disperser solvent. After mixing the sample solution and extraction solvent by using vortex, the cloudy mixture was subjected to centrifugation. Finally, the lower aqueous phase was removed and injected into HPLC system. The influences of the various experimental parameters on the extraction efficiency of amygdalin are studied and optimized.

2 Materials and methods

2.1 Chemicals and samples

Methanol (HPLC grade), cyclohexane, ethanol, tetrahydrofuran (THF), acetonitrile (ACN), sodium hydroxide and orthophosphoric acid were purchased from Merck (Darmstadt, Germany). Amygdalin (purity≥99%) was obtained from Sigma–Aldrich (USA). All solutions were prepared with deionized water from a Milli-Q system (Millipore, USA).

Fruits and oil samples were purchased from local supermarkets in Khorramabad (Lorestan, Iran). Fruits of *Amygdalus Scoparia* were collected from the mountainous regions of Lorestan and Yasouj provinces in Iran.

The stones from these fruits were removed and dried in an oven (37°C) for 4 h. Then stones were broken to obtain the seeds. The seeds were kept dry overnight in an airtight container and stored at room temperature until extraction process.

2.2 Chromatographic conditions

The HPLC system (Shimadzu Corporation, Kyoto, Japan) which consisted of a quaternary pump (LC-10ATvp), UV-Vis detector (SPD-M10Avp), vacuum degasser and system controller (SCL-10Avp) was used. A manual injector with a 10 μL sample loop was applied for loading the sample. A class VP-LC workstation was employed to acquire and process chromatographic data. A reversed-phase C_{18} analytical column (Shim-Pack VP-ODS, 250 mm × 4.6 mm i.d., Shimadzu, Japan) was used.

The mobile phase consisted of water and methanol (80:20, v/v). Before preparation of the mobile phase, water and methanol were degassed separately using a Millipore vacuum pump. The UV detector was set at 218 nm. The flow rate was adjusted at 1.0 mL min^{-1}.

2.3 Standard solution preparation

A stock standard solution (100 μg mL^{-1}) was prepared by dissolving amygdalin in methanol. Working standard solutions at a concentration range of 0.07–20 μg mL^{-1} were prepared by diluting the suitable volume of the stock standard with cyclohexane. Standard solutions were subjected to the optimized proposed method for construction of calibration curve.

2.4 Sample preparation for solid and oil samples

Powdered samples (50 mg) were sonicated in 5.0 mL of cyclohexane at 40°C for 30 min and centrifuged for 5 min. Then 1.0 mL of the extract was transferred to a microtube and subjected to RP-VALLME.

0.5 mL of oil sample was added to a microtube containing 0.5 mL of cyclohexane and subjected to RP-VALLME.

2.5 Reversed-phase vortex-assisted liquid-liquid microextraction procedure

1 mL of standard or sample solution was transferred into a 1.5 mL conical polypropylene microtube. 75 µL of deionized water as extracting solvent was added to the microtube and the mixture was subjected to vortex for 2 min. Phase separation was completed by centrifuging the mixture at 12000 rpm for 2 min. Finally, 10 µL of water phase

was withdrawn and injected into the HPLC system for analysis. The schematic diagram of sample preparation using RP-VALLME is illustrated in Fig. 1.

3 Results and discussion

To find the optimum conditions for solid–liquid extraction by using UAE, several preliminary experiments were performed. In this step, the liquid phase and ultrasonic time were investigated. Three organic solvents including cyclohexane, octanol and cyclohexane/octanol mixture (50:50 v/v) were used for the extraction of amygdalin from solid samples by using UAE. As observed from the results in Fig. 2, cyclohexane exhibits the highest extraction efficiency for amygdalin. Therefore, cyclohexane was used as the extraction solvent in UAE process. Also, optimum extraction time was 30 min.

3.1 Optimization of reversed-phase vortex-assisted liquid–liquid microextraction

3.1.1 Selection of extracting and disperser solvents

To clean-up and pre-concentration of extracted amygdalin by UAE, cyclohexane extract was subjected to reversed-phase vortex-assisted liquid–liquid microextraction. Similar to previously reversed-phase extraction methods [17–19], an aqueous phase was used as extracting solvent. In the other hand, 100 µL of several organic solvents such as ethanol, methanol, acetonitrile, tetrahydrofuran and mixture of acetonitrile/tetrahydrofuran (50:50 v/v) were used as disperser solvent. The addition of a disperser solvent to water, reduces the sedimented water volume and extraction efficiency (Fig. 3). Therefore, in this study disperser solvent was not used. Removing the disperser solvent from extraction process is advantage of the proposed method.

3.1.2 Volume of extracting solvent

The volume of extracting solvent can be affected on the extraction efficiency and enrichment factor of analyte. To find the optimum volume of extracting solvent various volumes of deionized water were tested. The results from Fig. 4 illustrate the analyte peak area decreases with increasing water volume. This phenomenon can be attributed to dilute the amygdalin concentration in extracting phase.

3.1.3 The effect of water pH

The pH of aqueous phase influences the distribution coefficient of the ionizable analytes between aqueous and organic phases. The effect of water pH was examined in the range of 2–10. As can be seen from Fig. 5, the extraction efficiency increases with increasing water pH up to 4 and then remains constant. The reason of this behavior may be related to the charge distribution of amygdalin as a function of pH. However, pH 4 was chosen as the optimum pH.

3.1.4 Vortex time

To increase the contact area between aqueous and organic phases, solution was vortexed. Vortex enhances the contact between extraction solvent and analyte which can be affected on the analyte extraction. Therefore, various experiments were performed by using different vortex times in the range of 30–180 s. Fig. 6 illustrates the effect of vortex time on the extraction efficiency. The maximum peak area was obtained at vortex time of 120 s. Hence, 120 s was chosen as the optimum vortex time in subsequent experiments. Also, miniaturization via reducing the size of microtube and volume of cyclohexane leads to the fast mass transport of analyte from the organic phase to the aqueous phase.

3.1.5 Centrifuging time

The effect of centrifuging time on the extraction efficiency and phase separation was studied in the range of 1–5 min. After centrifuging of sample solution for 2 min at 12000 rpm aqueous phase was settled at the bottom of the tube and its volume reached a constant value. In the other hand, the peak area of amygdalin reaches its maximum at 2 min and then levels off. Therefore, 2 min was selected as the optimum centrifuging time for subsequent experiments.

3.2 Method evaluation

Chromatograms of blank extract, standard solution and extracted amygdalin under the optimized conditions are shown in Fig. 7. It is clear, the RP-VALLME technique is an effective method for extraction and pre-concentration of amygdalin. Under the optimized conditions, validation parameters of the proposed method such as linearity, LOD, LOQ, precision (repeatability and reproducibility) and accuracy were determined. The linearity of the RP-VALLME–HPLC–UV method was evaluated by using extracting and injecting standard solutions of amygdalin at different concentrations under the optimized conditions. R-square value of calibration curve was 0.9955 which approved the linearity of the proposed method. The LOD and LOQ were defined as concentrations with S/N=3 and 10, respectively. The LOD and LOQ values were 0.02 and 0.07 µg mL^{-1}, respectively.

Results of repeatability and reproducibility of the proposed method at three concentration levels are detailed in Table 1. Intraday and interday RSD values for amygdalin were less than 5.1 and 7.2 %, respectively.

The accuracy of the proposed method was investigated by determining the relative recovery of spiked amygdalin in oil and solid samples at three concentration levels. Table 1 lists the obtained relative recoveries from the analysis of spiked samples. As can be seen, relative recoveries were in the range of 96.0–102.0 %. The results show that the oil and solid matrixes does not influence on the extraction process and appropriate recoveries are obtained at the working range.

To investigate of method performance amygdalin content of several oil and kernel samples was determined by using the proposed method under the optimized conditions. The results were listed in Table 2.

The extraction parameters of the proposed method such as extraction time, volume of extraction solvent, sample amount and LOQ were compared with several reported methods in the literature (Table 3). The results show that the sample amount, extraction solvent volume and LOQ were decreased by the proposed method. In addition, the extraction time by this method was shorter than that for other methods. The proposed method can be certainly used to extract, clean-up and pre-concentrate of amygdalin in solid and oil samples.

4 Conclusion

The present study describes the development of a miniaturized, simple and rapid reversed-phase vortex-assisted liquid–liquid microextraction (RP-VALLME) technique for determination of amygdalin in almond oils and several fruit kernels. Miniaturization relates to the downscaling of physical dimension of sample preparation devices and instrumentation. An important general aspect of miniaturization is the drastic reduction of sample and reagent consumption during sample preparation. Although in the miniaturized methods the amount of analyte is reduced, analyte detection can be done without interferences due to the high pre-concentration. Compared to other reported methods, the main advantages of the proposed method are the use of small volume of organic solvent, simplicity, speed and lower cost. In the proposed method several steps in natural product extraction methods such as removing the fat, evaporation of extracting solvent and reconstitute the extract in a suitable solvent for injection to HPLC system were removed.

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Figure 1. Schematic diagram of the proposed RP-VALLME procedure.

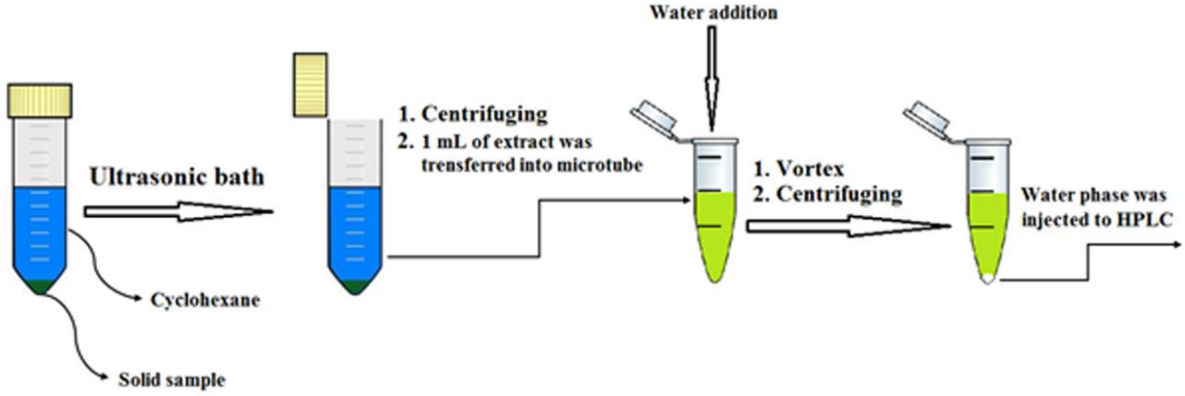


Figure 2. Effect of extraction solvent on the extraction efficiency of amygdalin by using UAE. UAE conditions: sample, 50 mg *amygdalus Scoparia* (Yasouj); ultrasonic time, 30 min; temperature, 40°C, solvent volume, 5 mL. RP-VALLME conditions: water volume, 75 μL; water pH, 4; vortex time, 120 s; centrifuging time, 2.0 min.

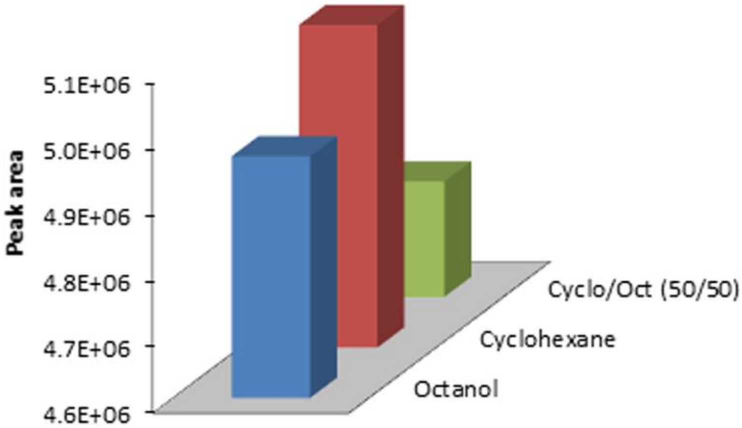


Figure 3. Effect of extracting and disperser solvents on the amygdalin extraction by RP-VALLME. Extraction conditions: water volume, 75 µL; disperser volume, 100 µL; water pH, 4; vortex time, 120 s; centrifuging time, 2.0 min.

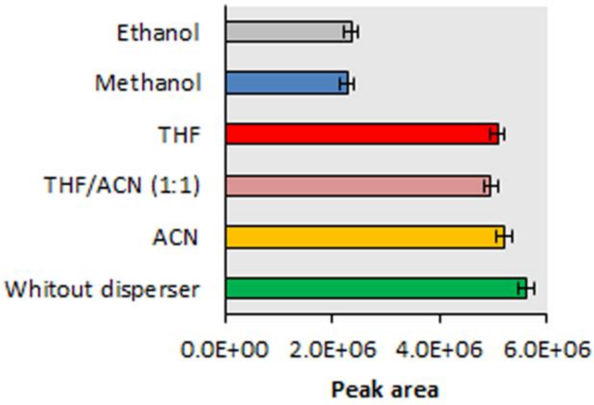
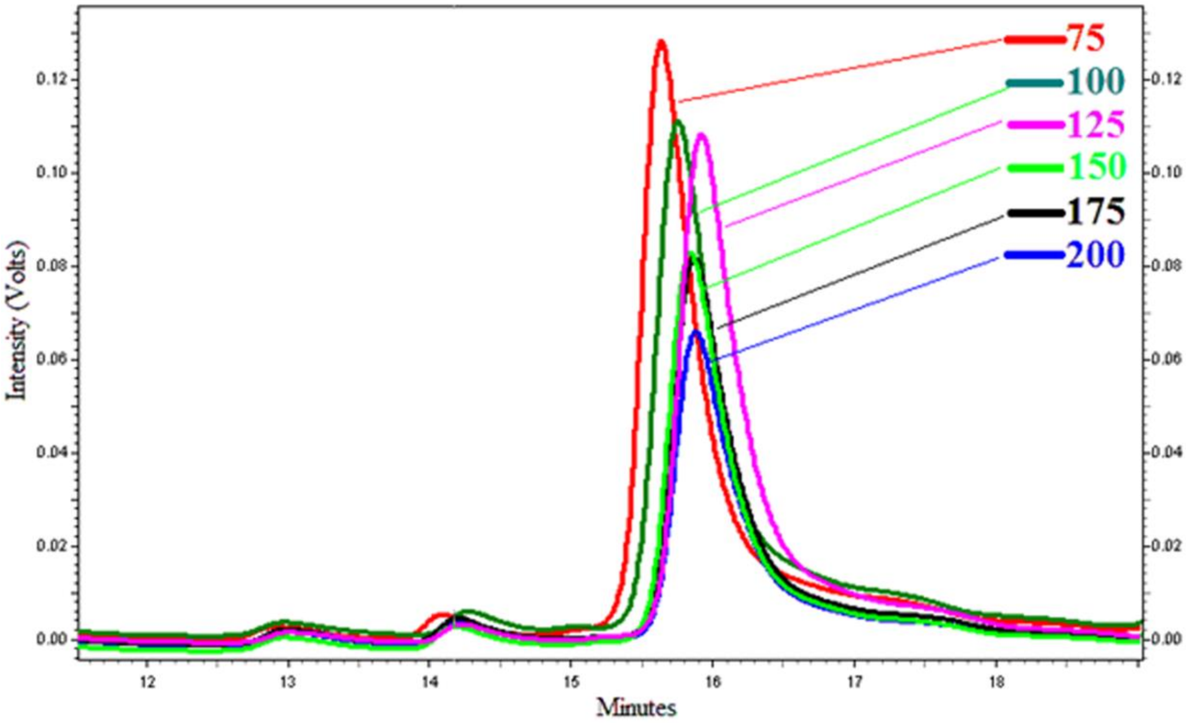


Figure 4. Effect of extracting solvent volume on the amygdalin extraction by RP-VALLME. Results are expressed as amygdalin chromatograms (a) and column chart (b). Extraction conditions: water pH, 4; vortex time, 120 s; centrifuging time, 2.0 min.



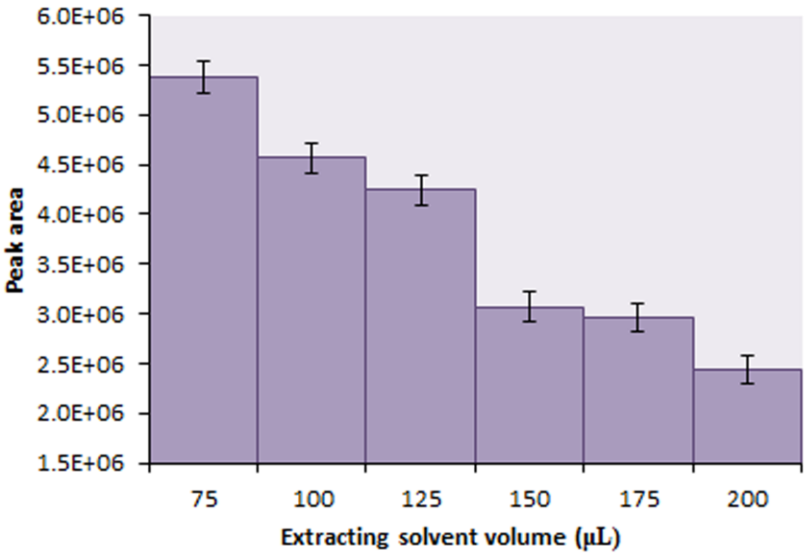


Figure 5. Effect of water pH on the amygdalin extraction by RP-VALLME. Extraction conditions: water volume, 100 µL; vortex time, 120 s; centrifuging time, 2.0 min.

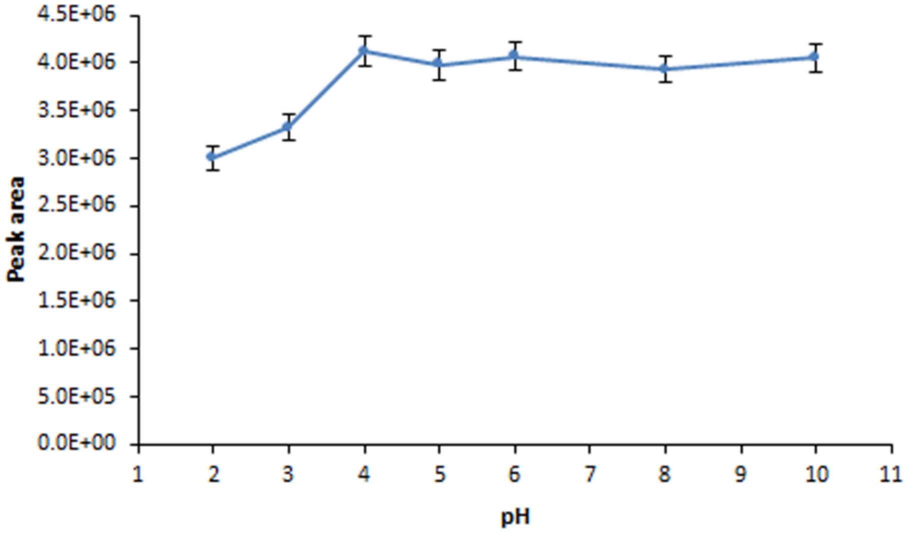


Figure 6. Effect of vortex time on the amygdalin extraction by RP-VALLME. Extraction conditions: water volume, 100 µL; water pH, 4; centrifuging time, 2.0 min.

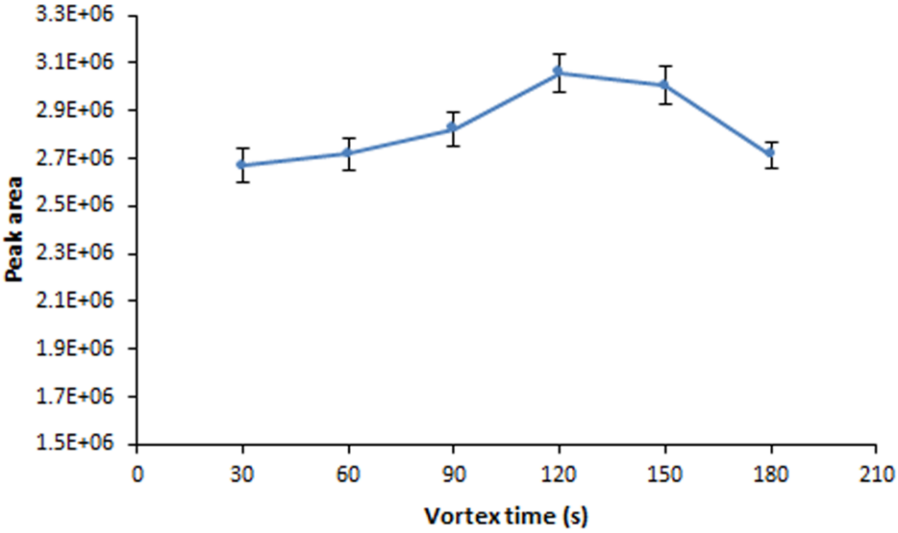


Figure 7. HPLC chromatograms of blank extract, direct injection of standard solution and RP-VALLME. Concentration of amygdalin in standard and RP-VALLME were 20 and 5 µg mL^{-1}, respectively. Extraction conditions: water volume, 75 µL; water pH, 4; vortex time, 120 s; centrifuging time, 2.0 min.

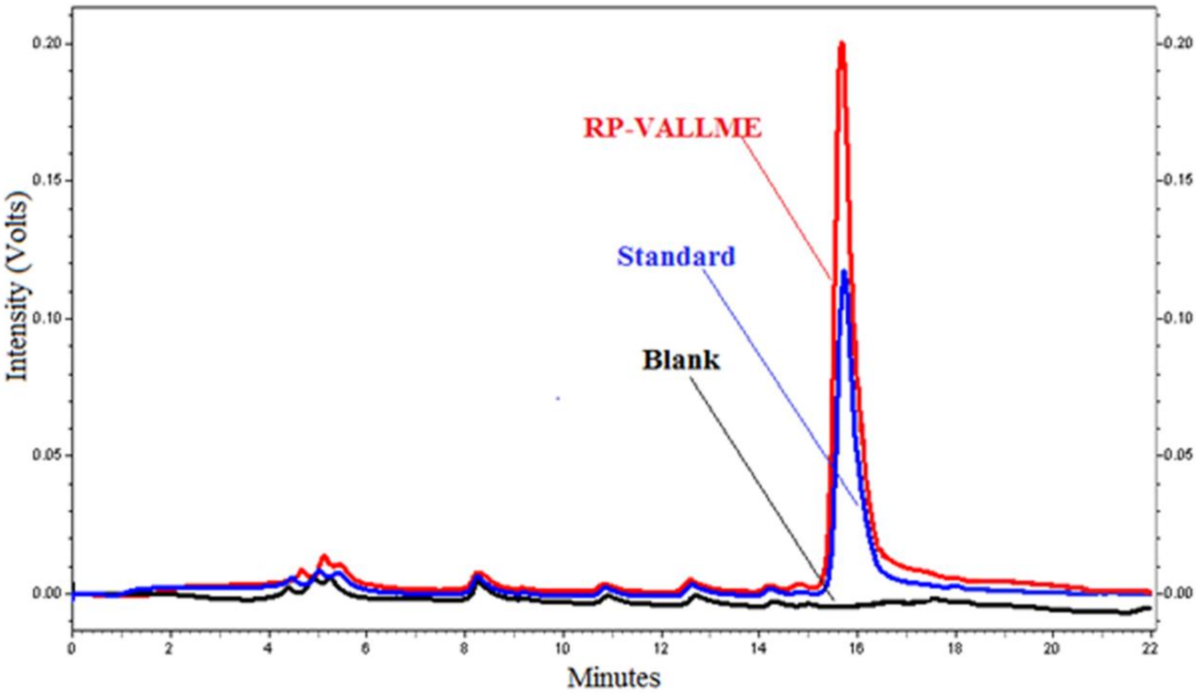


Table 1. Precision and accuracy data for amygdalin spiked in oil and solid samples by using RP-VALLME method.

Matrix	Accuracy				Precision	
	Concentration added (μg mL^{-1})	Concentration found (μg mL^{-1})	Recovery (%)	RSD (%), (*n*=3)	Intraday (RSD (%), *n*=3)	Interday (RSD (%), *n*=9)
Bitter almond oil	0.5	0.48	96.0	4.8	5.1	7.2
	5.0	5.1	102.0	5.1	4.8	6.6
	10.0	9.9	99.0	4.5	4.9	5.6
Prunus Persica	0.5	0.49	98.0	4.8	4.9	6.5
	5.0	4.8	96.0	5.1	5.0	7.0
	10.0	10.1	101.0	5.0	4.8	6.4

Table 2. Amygdalin content of real samples.

Sample	Amygdalin content (mg g^{-1})
Prunus Persica	0.020±0.001
Prunus Subg. Padus	0.063±0.002
Amygdalus Scoparia (Khorramabad)	0.176±0.004
Amygdalus Scoparia (Yasouj)	0.370±0.003
Prunus Armeniaca	0.022±0.001
Prunus Avium	0.051±0.002
Sweet oil almond	0.047±0.002
Bitter almond oil	0.092±0.003

Results are expressed as mean ± SD (*n* = 3).

Table 3. Comparison between extraction parameters of the proposed method and other methods in the literature

Extraction method	Pretreatment techniques	Extraction time (min)	Extraction solvent volume (mL)	Sample amount (g)	LOQ	Detection system	Ref.
Reflux and UAE	Solvent evaporation and reconstitute of extract	30	70	0.5	102 µg mL^{-1}	HPLC	[28]
Reflux	Solvent evaporation and reconstitute of extract	3×60	250	5.0	0.05 mM	HPLC	[30]
Maceration	SPE & centrifuge	12×60	10	0.4	NR [a]	HPLC	[31]
Reflux	Solvent evaporation and reconstitute of extract	100	50	1.0	0.3 µg mL^{-1}	HPLC	[32]
Reflux	Solvent evaporation and reconstitute of extract	100	50	2.0	1.0 µg mL^{-1}	HPLC	[33]
Maceration and UAE	Solvent evaporation and reconstitute of extract	150	40	0.5	50 µg mL^{-1}	HPLC	[34]
UAE	RP-VALLME	30	5	0.05	0.07 µg mL^{-1}	HPLC	This work

[a] NR; not reported.