Qualitative and Quantitative Analysis of Amygdalin in Sweet and Bitter Almond Kernel in Erbil City Using GC-FID Technique

Kamal M. Mahmoud[1] & Roza T. Yasin[2]

[1&2]Department of Chemistry, College of Science, Salahaddin University, Erbil, Iraq
Correspondence: Kamal M. Mahmoud, Salahaddin University, Erbil, Iraq.
Email: kamal.mahmoud@su.edu.krd

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Abstract: This paper described the determination of amygdalin in sweet and bitter almond kernel in Erbil City using GC-FID technique. The effect of wavelength, selecting the best solvent as a mobile phase, flow rate, injection volume, pH, and temperature had been studied, in which the flow rate 10.0 ml min^{-1} at the concentration range (20-600) µg ml^{-1} with correlation coefficient and relative error (0.9990, 1.501%) respectively.

Keywords: Determination, Almond, Amygdalin, GC-FID

1. Introduction

Amygdalin is a cyanogenic diglucoside (D-mandelonitrile-β-D-gentiobioside) which belongs to Rosaceae family (Moertel, 1982). It has the following empirical formula ($C_{20}H_{27}NO_{11}$) with the chemical structure shown in Figure 1 (Yan, Tong & Li 2006), which has diglucoside (A and B) combine with the cyanogenic group, while monoglucoside (prunasin) present in vegetative organs (Miao et al. 2013).

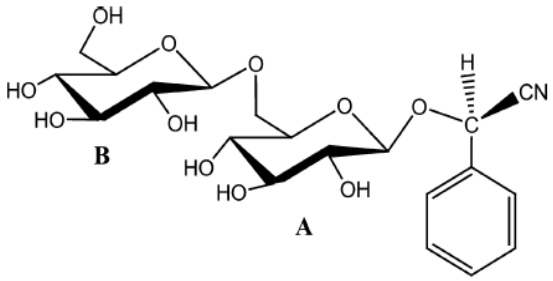

Figure 1: Chemical structure of amygdalin

Amygdalin is found, for example, in apples, apricot, and almonds. Therefore, it is necessary to find sensible, economical, accurate, rapid and precise analytical methods for amygdalin quantitatively to investigate determination of the extracted amygdalin from sweet and bitter almond kernel using high performance liquid chromatography (HPLC) and gas chromatography (GC) techniques (Agunbiade & Olanlokun, 2006; Hajslova & Cajka, 2007; Hale, 2012).

Chromatographic techniques are important for quantitative analysis, especially gas and high performance liquid chromatography (Agilent 2002). Few methods were found in the literature for the determination of amygdalin using GC technique. Well, it was used for analysis of amygdalin from apricot kernel that combined GC with electron impact and chemical-ionizations mass spectrometry.

Carrier gas was He, with a flow rate of 40 ml min^{-1}, and ionization energy was 70 eV (Godteredsen et al., 1978; Rood, 2007). Funazo et al. (1981) and Moertel et al. (1982) were determined plasma and urine concentration of amygdalin, cyanide concentrations increased to values as high as 2.1 µg ml^{-1} serum.

2. Experimental

2.1 Chemicals and Reagents

The chemicals that used for the detections were of analytical grade. Deionized water was used throughout. The standard amygdalin was purchased from Scharlau Company- Germany. Different solvents were used with very high purity.

2.2 Instruments and Apparatuses

GC-FID: A (Agilent 7890A, USA) gas chromatography was performed on (DB-17 30 m, 0.25 mm, film 0.25 µm) capillary column, flame ionization detector (FID) 300 ^{o}C attached to a gas chromatograph. Soxhelt apparatus with heating Electro thermal MO 500/30 K1, (ENGLAND).

2.3 Sampling

Almond (*Prunus dulcis*) is a member of the Rosaceae family (Nurhan et al. 2010). It measures (3.5-6.0) cm (1.0-2.0) in long. It's called drupe in botanical terms. The almond kernel food (with both types (sweet and bitter)) was taken from Kurdistan Region in Hawler- market, valley of Balesan, between November and December 2013. The samples were stored in airtight containers protected from light at room temperature. Dried plant material (almond kernel) was grounded in an electrical granular to obtain a fine powder.

2.4 Extraction and Fractionation of the Almond

2.4.1 Ultrasonic Bath System

From (unpilled sweet and bitter) Plant material (15.0 g) of each sample were immersed in 150 ml of solvents (using different types of solvent such as diethylether for de-fatting, ethanol and methanol respectively according to their different polarity), 0.1 g of active carbon (Norit CNR 115) was added to the solute, and put it in an ultrasonic bath for 30 min at room temperature, then soaking for 30 min to reach the equilibrium between solvent and extraction. Extraction repeated three times. The extract was obtained after filtration using a Buchner funnel (Lv et al., 2005). The solvent was evaporated in a vaccumm rotary evaporator (Hussain, 2008).

2.4.2 Soxhlet Extraction

A powder of (18.0 g) of each unshells sweet and bitter almond kernels was extracted three times with 250 ml methanol for 5.0 h with 0.1 g of active carbon (Norit CNR 115) using soxhlet apparatus. The mixture was heated by electromantle heating. The extracted solvent was obtained after filtration (Lv et al. 2005) through a filter paper (Whatman 42). The filtrate was concentrated using a rotary evaporator.

2.4.3 Binary Solvent Extraction

Binary solvents were used for extraction of unpilled sweet and the bitter almond kernel. In this study 1.0 g of the samples was put in a small beaker (250 ml) then added 10 ml of methanol-water mixture in a different ratio [(10:0), (8:2), (6:4), (5:5), (4:6), (2:8), (0:10)] respectively, the contents of the beaker were shacked continuously using a magnetic stirrer for increasing the extraction process and efficiency (Liang, and Fang 2006) for (30 min) at room temperature. Finally, add 0.1 g of active carbon (Norit CNR 115) to the extracted samples, to ensure the freedom from pigments, which may be interfaced with the chromatography (Arrazola et al., 2012).

2.5 Qualitative Analysis of Extracted Amygdalin

2.5.1 Thin Layer Chromatography (TLC)

TLC was used for the identification of amygdalin in the almond. The stationary phase, which was an adsorbent made of silica gel (SiO_2). Reference solution was prepared according to European pharmacopoeia (Zhao 2012), 10 mg from amygdalin was dissolved in 2-3 ml water and diluted to 10 ml with methanol. Sample solution was prepared also as the reference solution, then they spotted onto the TLC plate and placed in a chamber with eluting fluid (methanol: water (50:50)) as the best mobile phase (after pretested for different ratios). The eluting fluid, or (mobile phase) rise up the plate via capillary action, carrying the analytes with it. After ascending development, staining and drying, silica gel plates were scanned by UV detector. Table 1 shows the R_f values of standard and sample, which indicate the same R_f values.

Table 1: R_f values of amygdalin

Compound	R_f	Color with UV-lamp at 254 (nm)
Reference: Amygdalin	0.53	Green
(Sweet and Bitter) Sample	0.53	Green

2.6 Quantitative Analysis of Extracted Amygdalin

2.6.1 Effect of Temperature on the Quantity of Extracted Amygdalin

Extraction of amygdalin was performed by mixing the sample (unpilled) sweet and bitter almond kernel with the quantity of water (1:10) for a fixed period of time. A heated magnetic stirring plate was used to preheat the water to 25, 50, 90^{o}C, for stirring the suspension and maintaining the temperature throughout the extraction. However, the common techniques for extracting amygdalin from foods for the analysis of amygdalin in the literature are ultrasonic or Soxhlet extraction with methanol, this is because some amygdalin was decomposed into benzaldehyde, HCN and glucose by emulsion a hydrolysis enzyme, and some are converted into its epimers, neoamygdalin (L-mandelonitrile-β-D-gentiobioside), during the process of decoction in water (Lv et al., 2005). Therefore, in this study the extraction of amygdalin was performed by three procedures: ultrasonic, soxhlet and extraction of solvent at room temperature.

2.6.2 Effect of Time on the Quantity of Extracted Amygdalin

The pH and temperature were stabilized for studying the effect of duration of extraction (ultrasonic 30 min, soxhelt 3.0 hours and binary solvent extraction 15 min). The main factor that influenced the amygdalin extraction yield is extraction time; a short extraction time would decrease the extraction yield of amygdalin. A long extraction time would cause some amygdalin to convert into its epimers or decompose in the aqueous solution (Lv et al., 2005) and also consuming much more solvent. In this study the extraction has been repeated three times to make sure that the analyte is achieved and it is much better than extracting once but for a long time (e.g. more than 3.0 hours).

2.6.3 Effect of Solvents on the Quantity of Extracted Amygdalin

A number of different solvents were tested, by changing the volume ratio of the solvent, to obtain the optimum result. Here, for three of extracting processes a different solvent in a different ratio for both ultrasound and soxhlet extraction (diethyl ether, ethanol and methanol) were used, respectively, but for binary solvent extraction water and methanol were used in a different ratio, according to their polarity extraction performed. In this study the analyte is polar, so by increasing the polarity of solvent the extraction yield increased and gave the best result.

2.7 Preparation of Standard Amygdalin

A stock solution of standard amygdalin was prepared by dissolving (1.0 g) of amygdalin in methanol and then completed to 1.0 L with methanol in a volumetric flask and stored in refrigerators before use. A serial standard solution was prepared by a suitable dilution of the stock solution with methanol (Arrazola et al., 2012) ranged from (10-600 μg ml^{-1}).

2.8 Determination of Amygdalin by GC-FID

2.8.1 Carrier Gas

The sample was transported in a mobile phase, which was a helium gas, purity 99.9999%, delivery pressure (0-6.9 bar) and forced through a stationary phase held in a column that does not react with the mobile phase. The carrier gas system also contains a molecular sieve to remove water and other impurities.

2.8.2 Injection Port

The sample was injected manually with a micro syringe (Agilent PN 5190-1490, 10 μl, Australia) to inject sample through a rubber septum into a flash vaporizer port at the head of the column syringe into the analyzer.

2.8.3 Chromatograph Column

This part is the heart of the GC system, a capillary fused silica open tubes column (FSOT) (DB-17, 30 m, 0.25 mm, and 0.25 μm) was used which was stainless steel columns, and the sample gas was carried through the column by the carrier gas. These columns have much thinner wall than the glass capillary column, and are given strength by the Polyimide coating. It is flexible and wound into coils. It has the advantages of physical strength, flexibility and low reactivity.

2.8.4 Detector

The FID detector was used in this study in which indicates a substance by the generation of an electrical signal. It is very sensitive down to 10^{-12} g. It has a higher linear dynamic range 10^{7} and is a very robust and reliable.

3. Results and Discussion

3.1 Identification of Amygdalin

3.1.1 Extraction Methods for Amygdalin

Three extraction methods, including ultrasonic extraction of different solvents (methanol and ethanol). Soxhlet extraction using the same solvents, and binary solvent extraction using (water and methanol mixture), were compared for amygdalin both sweet and bitter almond kernels. The extraction yield of amygdalin with ultrasonic extraction using methanol was the maximum, and soxhlet extraction using diethyl ether was the minimum. Table (2) shows this result. Methanol is a good solvent for extracting ingredients of crude herb medicine, because it is known that it can extract almost all of the ingredients, and amygdalin can be easily dissolved in it too. Ultrasonic extraction by methanol is the best choice for the almond kernels, because it gave the highest amount of both sweet and bitter almond kernels which are (24.45 and 563.66 µg ml^{-1}) respectively.

Table 2: Amygdalin yields using different extraction methods

Extraction Methods	Yield of sweet (µg ml^{-1})		Yield of bitter (µg ml^{-1})	
	Ethanol	Methanol	Ethanol	Methanol
Ultrasonic	22.40	24.45	525.50	563.66
Binary solvent	17.40	15.45	432.20	443.75
Soxhelt	9.98	10.44	269.83	273.21

3.1.2 Identification of Amygdalin with TLC

TLC test was used for identification of amygdalin from sweet and bitter almond kernels. In Figure 2 fraction (S) is the standard for amygdalin and gave a dark-brown spot, fraction (A) gave smooth line with no spot for amygdain extract from unpilled bitter almond by soxhlet extraction which indicated having many compounds in this fraction that could not be separated well, fraction (B) gave broad dark-brown spot which indicated that the extract containing different compounds which may be amygdalin one of these extract from unpilled bitter almond by ultrasonic, fraction (C) gave one dark-brown spot which indicated that the extract containing only one compound which was amygdalin from unpilled bitter almond by binary solvent extraction, and fraction (D) gave no spot for sweet unpilled almond which indicated that the extract containing no and/or less amount of amygdalin. Figure 2 shows the TLC at wavelengths 254 nm. The spots were detected at wavelength 254 nm but were absence at 366 nm. The R_f value for the visualization method was (0.53) for three spots of fraction S, B, and C.

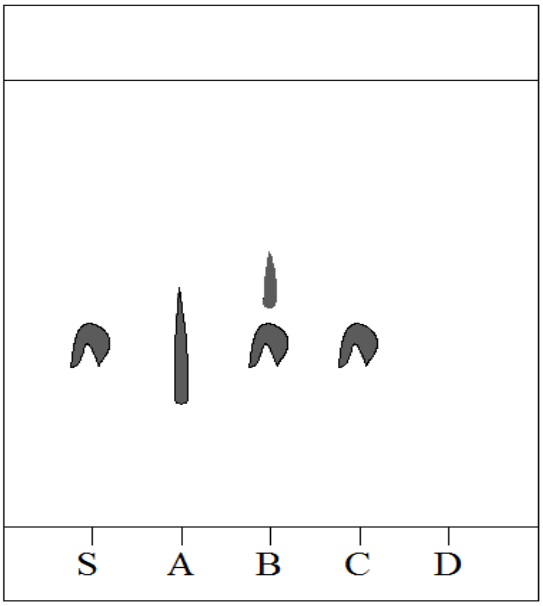

Figure 2: TLC of extract amygdalin (A-D) and standard amygdalin (S) using a UV detector at 254 nm.

3.2 Determination of Amygdalin by GC-FID

3.2.1 Effect of Temperature Column

Generally, increasing the temperature of the column in the GC system could be causing to decrease the retention time (t_R) for the analytes (Urias 2002). However, in this study the temperature ranged between (150-300^{o}C) doesn't affect on the t_R, because amygdalin is a non-volatile material so it requires high temperature for vaporization.

When it compares the (t_R) value of the GC- system with that of (t_R) value of the HPLC - system (Roza, 2015), it's seen that the (t_R) for the former system is greater than the (t_R) for the latter, this is due to the length of the column in the GC - system is much longer than the column in HPLC, in addition the separation for HPLC done under high pressure. Therefore, the optimum temperature for separation of amygdalin in the GC - system was found to be 180^{o}C, while, by increasing the temperature, the amount of amygdalin led to decrease high temperature may be causing the decomposition of amygdalin.

3.2.2 Effect of Flow Rate

The effect of flow rate between (10-50 ml min^{-1}) of mobile phase on the (t_R) was studied when 5.0 µl of (1000 µg ml^{-1}) of amygdalin injected into the GC column. Responses (t_R) and flow rates are recorded. The obtained results show that the (t_R) remain constant by increasing the flow rate, while, in general increasing the flow rate may cause decreases in (t_R). Results found in Figure 3, and for the subsequent work 10 ml min^{-1} was selected as an optimum flow rate.

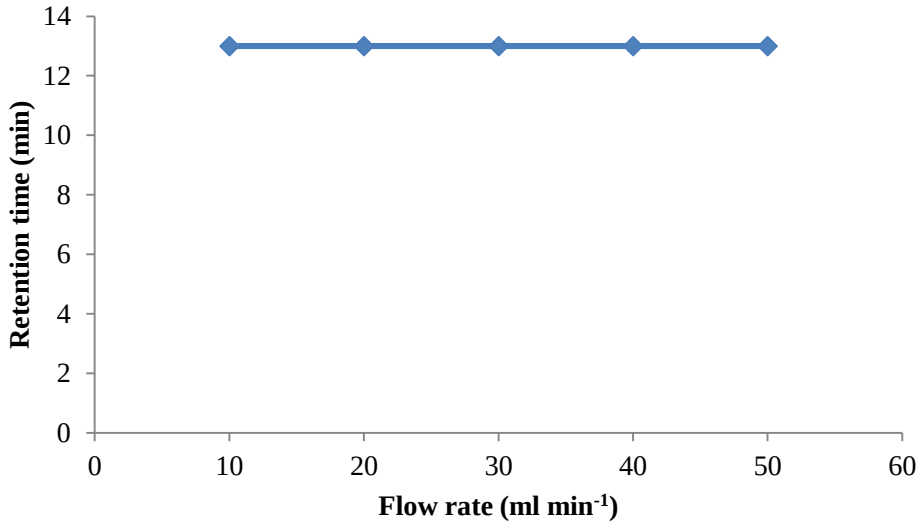

Figure 3: Effect of flow rate on the t_R in GC system

3.2.3 Effect of Column Length

5.0 µl aliquots of a solution of (1000 µg ml^{-1}) amygdalin injected, with a flow rate of (10 ml min^{-1}), and varying the column length between (30-90 m). It was found that by increasing the length of the column the (t_R) value will be increased also, in addition, the theoretical plates will be increased. Figure 4 shows the relation between (t_R) and column length, the column length of 30 m was selected in order to get shorter (t_R) value.

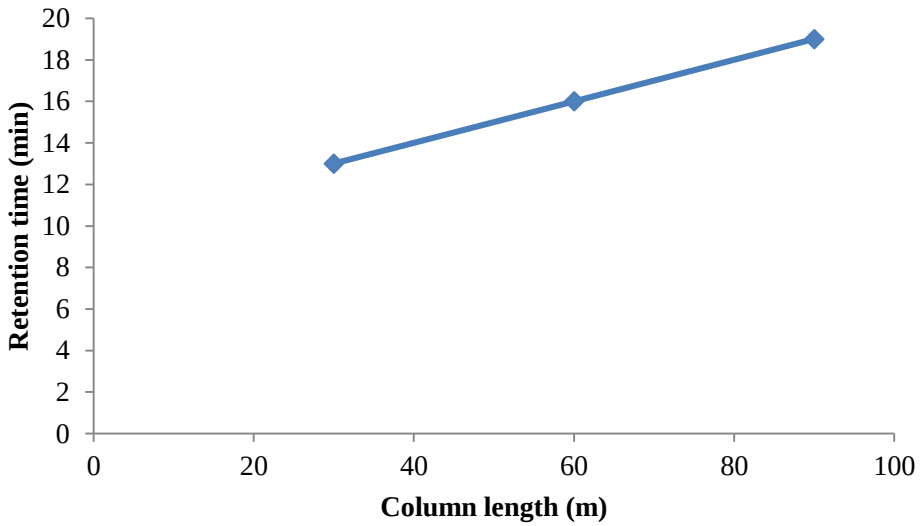

Figure 4: Effect of column length on the t_R in GC system

3.2.4 GC Condition for Analysis of Amygdalin

The extracted amygdalin from almond kernel and pure standard amygdalin were analyzed using GC-FID technique for both qualitative and quantitative analyses. Separation was carried out for 5.0 µl

samples, using DB-17 column (30 m, 0.25 mm, film 0.25 µm) at 180°C, eluted the sample with the carrier gas (helium) at (flow rate =10 ml min^{-1}). Separation was detected by FID at 200°C (detector temperature). The extracted solutions (mentioned before) were run by GC-FID, the retention time was more than those which done by HPLC because amygdalin is a non-volatile material and it required time to vaporize, the retention time for pure amygdalin which was done by HPLC was 2.404 min but for GC it was 13.356 min. Figures 5-7 show the chromatogram for pure amygdalin and extracts.

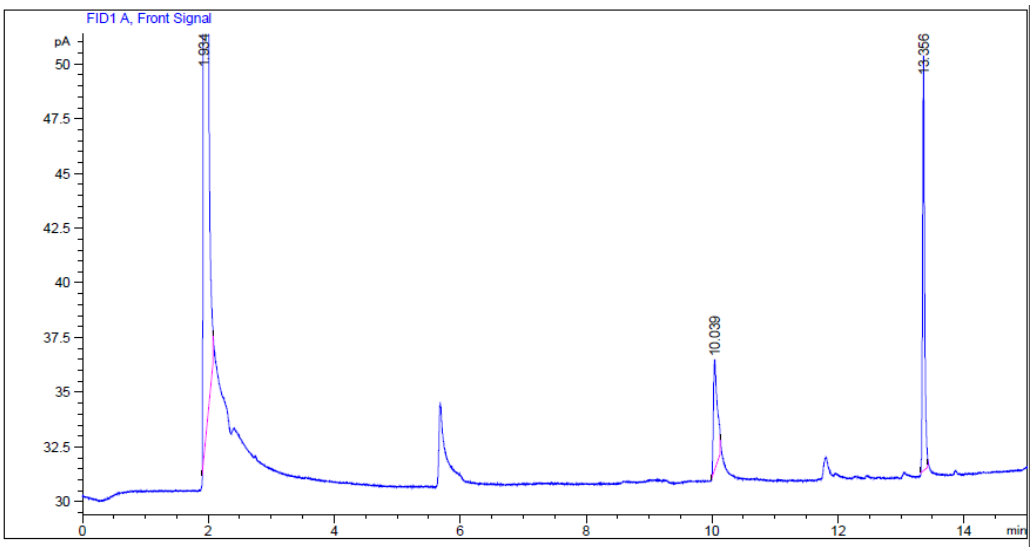

Figure 5: GC Chromatogram for standard amygdalin using GC system

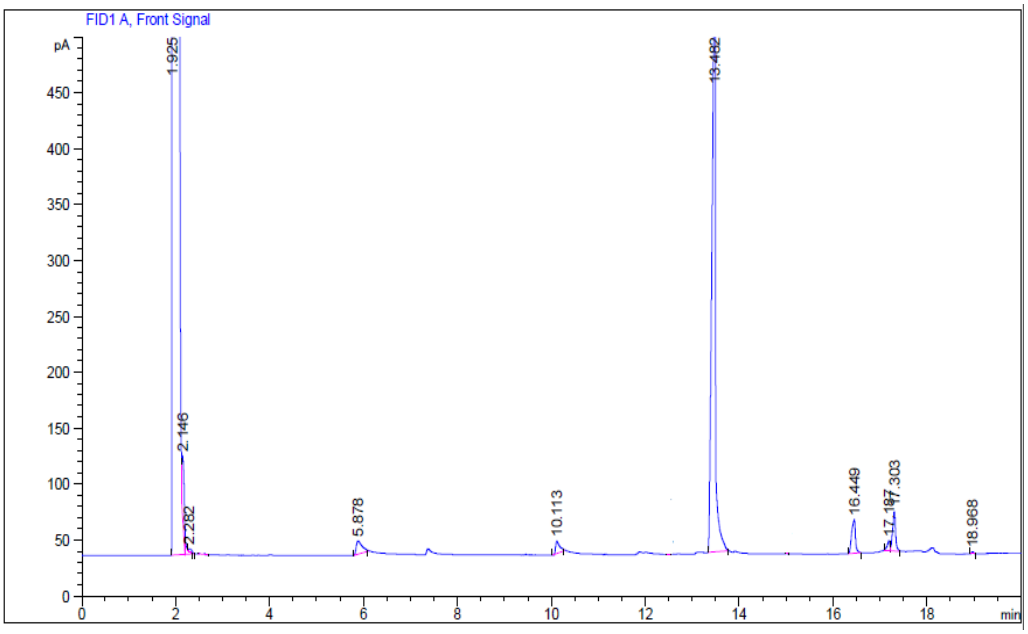

Figure 6: GC Chromatogram for bitter almond kernel extract by ultrasonic extraction using GC system

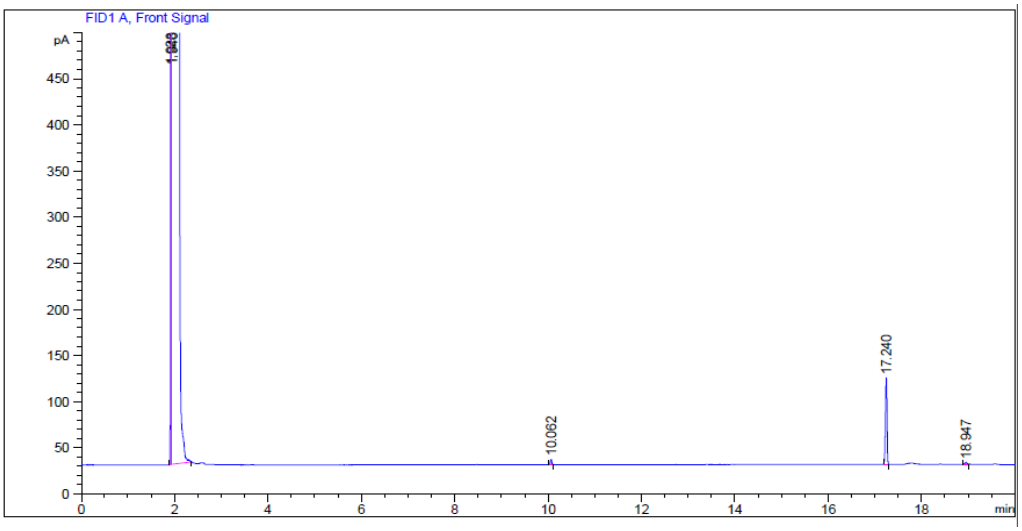

Figure 7: GC Chromatogram for a blank solution using GC system

3.2.5 Calibration Graph

The GC-FID system was used for the determination of amygdalin from both sweet and bitter almond kernels using the optimum experimental conditions (mentioned before) and the chromatographic conditions for the determination of amygdalin, the linear calibration graph was established by plotting the peak area versus the concentration of amygdalin ranged from (20-600 µg ml^{-1}) as shown in Figure 8, the statistical results for calibration curve were shown in Table (3). The precision and accuracy are shown in Table (4). The result showed that the maximum concentration of amygdalin in bitter almond was (507.61 µg ml^{-1}), but in sweet almond was very low (42.69 µg ml^{-1}), in which extracted by ultrasonic and determined by GC-FID. The same result obtained for RP-HPLC and GC-FID, but for HPLC/MS the result is much better because it almost gained the high amount of amygdalin with a less time (retention time).

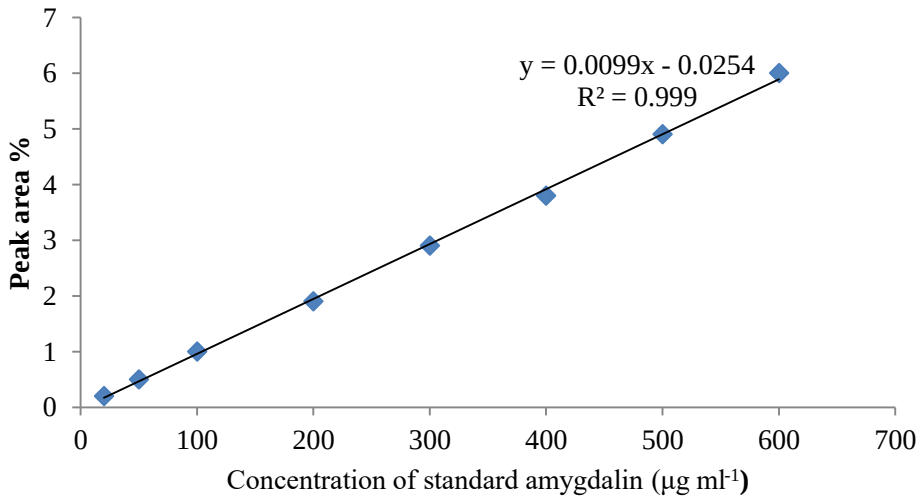

Figure 8: Calibration graph for amygdalin using GC system

Table 3: Statistical data for the calibration graph of the determination of amygdalin by GC-FID

Analyte	Linear range (μg ml^{-1})	Detection limit (μg ml^{-1})	Correlation Coefficient (R^2)
Amygdalin	20-600	0.520	0.9990

Table 4: Precision and accuracy by GC-FID

Analyte	Analyte Conc. (μg ml^{-1}) (standard solution)	Practical conc. for standard sol. (μg ml^{-1})	S.D	RSD%	E%
Amygdalin	300	295	0.033	1.16	1.66

3.3 Discussion

Both species of the almond kernel (sweet and bitter) in the present study have shown high concentrations of amygdalin especially bitter almond kernel. GC- derived amount of amygdalin content in the sweet almond kernel was lower compared to the amount of amygdalin in the same species with the bitter almond kernel, and the amount of amygdalin of the GC-FID was less than both HPLC and HPLC-MS (Roza, 2015), due to the non-volatility of amygdalin and time requirement.

3.4 Conclusion

The use of advanced analytical instruments such as HPLC, HPLC/MS (Roza, 2015) and GC-FID had led to the identification of different phytochemical plant compounds. From this study, it is suggested that consumption of the sweet almond kernel can be as good as the bitter almond kernel.

The extraction by ultrasonic system gave the best result (high amount) with methanol solvent for HPLC (Roza, 2015) and GC-FID, but for the HPLC-MS (Roza, 2015) the extraction with binary solvent (8 ml methanol + 2 ml H_2O) were chosen for detection of amygdalin. The HPLC method (Roza, 2015) is found to be highly sensitive, reproducible, specific accurate precise and rapid. This method can be used for the routine analysis of other extracted compounds from plants. The result showed that the HPLC presented better result than GC-FID because the amygdalin was non-volatile material.

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