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IDENTIFICATION OF FLAVONOIDS IN IRAQI HONEY AND COMPARISON WITH OTHERS KIND BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY WITH UV DETECTION

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Abstract

Honey is rich in phenolic acids and flavonoids, which exhibit a wide range of biological effects and act as natural antioxidants. The analysis of polyphenols has been regarded as a very promising way of studying floral and geographical origins of honeys. The aim of this study was to determine, flavonoid in honey samples collected from different region in Iraq and Comparison with others kind. The flavonoids which measured in honey samples are (Myricetin, Quercetin, Hesperdin, Naringenin, Apigenin, Kaempferol, Chrisin). Chromatographic Separation was performed using a reversed phase column C18 and acetonitrile 60% (v/v) flow rate 1.0 ml/min, pH 5.5 at 280 nm. All calibration curves of the flavonoid compounds showed good linearity (r - 0.9999) within the test range. Concentration of flavonoids ranged from Myricetin (1.903- 18.5) mg/kg, Quercetin (3.31-22.01) mg/kg, Hesperdin (4.55-16.2) mg/kg, Naringenin (3.2-24.68) mg/kg, Apigenin (6.76-28.81) mg/kg, Kaempferol (3.31-38.13) mg/kg, Chrisin (2.0-28.0) mg/kg respectively.

Keywords: Determination; HPLC; Flavonoids; Identification.

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1. Introduction

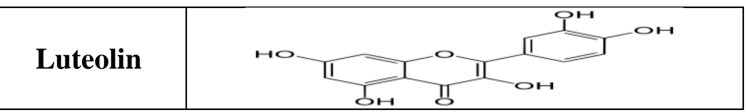
Honey is a natural substance produced by bees and is a nutritious food of economic Importance worldwide [(1-3)]. Honey is a sweet and viscous fluid created by honeybees from the nectar of flowers. It is important to note that honey created by insects other than honeybees has very different properties .Interestingly, honey has been cited in the Quran, a Holy book for Muslims (Section 16 surat 68-69), in reference to its medicinal properties [(4,5)]

The use of honey in folk medicine is thought to be as old as civilization, but in recent times there has been a renaissance in interest in its use as a medicinal product. Honey is one of the oldest medicines known, its recorded use going back more than 4 millennia. It was used to treat wounds and ulcers, sunburn, and infections of the eyes, throat and gut. These uses have continued into present-day folk medicine and are increasingly becoming part of modern professional medicine [6-8]. Honey has been reported to be active in gastrointestinal disorders in restoration of wounds and burns as an anti-microbial agent and to provide gastric protection versus acute and chronic gastric lesions.[9]. One of the essential features for honey is its antibiotic properties, where it can be kept for long periods of time without becoming mushy. Polyphenol represent group of components that possess more than one phenolic hydroxyl group linked to one or more benzene ring. They come in free and restrictive form as esters or glycosides in plants [10].

Phenolic compounds as antioxidants are also recommended as dietary complement to improve human health; Phenolic compounds are one of the important groups of compounds that occur in plants [11]. Flavonoids have been reported to exert broad range of biological activities. These contain: anti-inflammatory, antibacterial, antiviral, anti-allergic, cytotoxic antitumor, curing of neurodegenerative diseases, vasodilatory action. Resistance to antimicrobial agents has become a more important and pressing universal problem. Of the 2 million people who gained bacterial contagion in US hospitals each year, 70% of cases do not take part strains that are resistant to at least one drug [12]. Several studies have focused on the analysis of honey flavonoids by using the high performance liquid chromatography (HPLC) mode. HPLC was first used for the determination of flavonoids in 1976 by fisher and Wheaton. UV with photodiode array (PDA) detection is the standard method used for the detection of flavonoids. Two UV absorption bands are characteristic of this kind of compounds. Band with a maximum in the 240-285 nm range [13, 14]. Table (1) shows Structure of Some Flavonoids found in Honeys bee.

Table 1: Structure of Some Flavonoids found in Honeys bee

Flavonoids	Structure
Kaempferol	
Myricetin	
Quercetin	
Apigenin	
Chyrisn	
Pinoncebrine	

Luteolin	

2. Materials and Methods

Honey samples: honey samples were collected from different regions and market in Iraq As shown in Table 1. Samples were collected in glass bottles and stored in dark prior 25 C° to analysis.

Table 2: The sours of honey bees

Honey name	Source
Flower	Arable
Trefoil	Babble
Seder(1)	College of Science of Women
Seder(2)	Alnajef
Eucalyptuses(1)	Alnajef
Nigella sativa	Baghdad
Mountain	Sulaymaniyah
Eucalyptuses(2)	College of Science of Women
Citrus	Baghdad
Eucalyptuses(3)	ALaniber
Olive honey	Southern of Baghdad
Sunflower	West of Baghdad
Germany	Germany
American	American
India	India

3. Reagents

All reagents and standard solutions were of the highest purity available and at least of analytical-reagent grade. Deionizer water is used for all purposes. Glass ware, tubes, volumetric flasks, pipettes, tips and other glass were immersed in HNO_3 (5% V/V) for 24 hr. the visuals were rinsed with deionizer water about three times. Stock standard solution amble with a concentration of 1000 mg/L for element measurement was manufactured BDH, Supelco company (USA) and sigma Company.

4. Preparation Standard Solutions of Flavonoids

StandardmixtureFlavonoids (Quercetin, Myricetin, Hesperdin, Naringenin, Apigenin, Kaempferol, Chrisin) was 2ml ampoules contained the above mixtures with concentration of 100µg/ml each and diluted to 25 µg/ml as working standard solution.
Standard solution of Flavonoids (25 µl) Separated in to column ODS-C_{18} (50 mm x 4.6 mm i.d.)

5. Extraction Samples

The fresh honey samples 0.5 gm each was extracted for determination Flavonids. The aqueous extracted were obtaining by using boiling deionize water (200ml), the homogenized mixture with an ultrasonic bath for 20 min and passed through a filter (Whitman No.1) .after filtration 100ml of aqueous extract were acidified with 0.5 ml of 98% acetic acid to pH 3.0 and applied to the octadecyl column (2×5 cm, shimadzu, Japan to) which was then washed with 100 ml of methanol . the elute was evaporated using (Buchi system ,Germany)evaporator to dryness then the extract re-dissolve in 1ml of the mobile phase to obtained (1ml) of the final extracted ,then 20 µl were subjected to HPLC analysis (15-16)

6. Result and Dissection

Food was the main source of phenols, including honey, fruits and vegetables many research studies suggest the estimate of phenolic compound of interest in human body which estimated about 1 gm /day. Flavonoids multiple polyphenolic compound human diet, which were naturally found in plant source, the compound received wide attention in medical fields as it work protection against heart ,blood vessels and cancer disease (17-18).

Optimization of the Separation of Flavonoids

1) Effect of pH on the retention time

The effect of mobile phase pH on in reversed phase HPLC for separation a mixture Flavonoids was intensively studied. To observe the effect of various pH on the retention time of standers. The pH of the system was then varied between ranges of (2.5-7.5), it was adjusted by a few drops of either (0.1 M)HCl or (0.1 M) NaOH solution for flavonoids The effect of pH changes on the retention time of flavonoids , Table (3).A plot of adjusted retention time (tR) for flavonoids , versus pH were present in figure (1). The optimum pH obtained for best baseline separation of Flavonoids, pH 5.5.

Table 3: Variation retention time (tR) for Flavonoids at flow rate1.0 ml /min, at different pH values.

No	Flavones	Retention time (tR min)					
		pH					
		2.5	3.5	4.5	5.5	6.5	7.5
1.	Myricetin	4.2	3.3	2.51	1.6	1.72	2.22
2.	Quercetin	5.83	4.72	3.61	2.5	2.1	3.5
3.	Hesperdin	6.74	5.65	4.85	3.95	4.82	5.16
4.	Naringenin	7.92	6.81	5.71	4.6	5.62	6.36
5.	Apigenin	9.12	7.82	6.64	5.5	6.12	7.06
6.	Kaempferol	10. 35	8.65	7.51	6.85	7.02	8.12
7.	Chrisin	11.24	9.61	8.52	7.82	8.62	9.42

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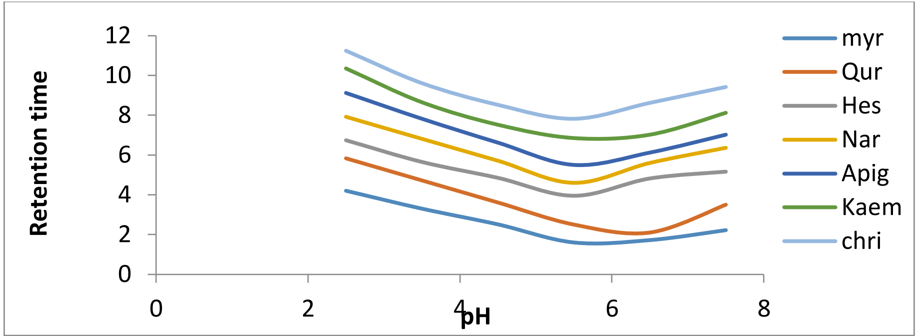

Figure 1: Plot of adjusted retention time of Flaovonids versus pH at Flow rate 1ml/min

2) Optimization of concentration of Mobile Phase in on elution of Flavonid

The results show in table (4) generally that the retention time was decreased with increasing the % of acetonitrile, this effect has been attributed to decrease of the surface concentration of the counter-molecule because of the competition by solvent. In Figure (2), collated Naringenin with Hesperdin at 60% acetonitrile that means it is not possible to get complete baseline separation of Flavonide mixture.

Table 4: Variation of retention time (tR) of Flaovonids on reversed phase at different % acetonitrile flow rate 1 ml/min.

No	Flaovonid	Retention time (min.)					
		Concentration of acetonitrile (%)					
		10%	20%	30%	40%	50%	60%
1.	Myricetin	4.62	3.51	2.41	1.58	1.23	1.12
2.	Quercetin	5.61	4.71	3.61	2.51	2.0	1.85
3.	Hesperdin	6.31	5.21	4.12	3.96	3.12	2.5
4.	Naringenin	7.12	6.31	5.21	4.61	3.55	2.7
5.	Apigenin	8.41	7.21	6.38	5.51	4.5	3.41
6.	Kaempferol	10.12	8.91	7.81	6.84	5.23	4.21
7.	Chrisin	11.21	9.95	8.91	7.71	6.51	5.32

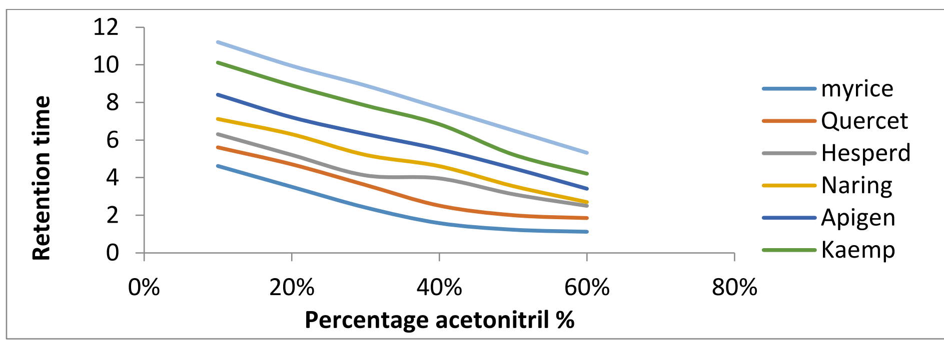

Figure 2: Effect of percentage of acetonitrile of separation Flaovonids

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7. Effect of Flow Rate

Flow rate may adversely affect the quality of the chromatography not giving the analyte sufficient time to interact with the stationary phase. Faster is not always better. A lower than usual flow rate may leave the analyte waiting for the peak to appear at the detector Table (5) and figure (3) shows retention time as a function of flow rate for Flavonoids .

Table 5: Effect of flow rate on retention time of Flavonoids

Flow rate ml/min		Retention time (min.)				
No	**Flavonid**	**0.6**	**0.8**	**1.0**	**1.2**	**1.4**
1.	Myricetin	2.16	1.85	1.6	1.41	1.2
2.	Quercetin	3.85	2.98	2.5	1.92	1.42
3.	Hesperdin	5.52	4.5	3.94	3.12	2.16
4.	Naringenin	7.62	5.52	4.6	3.65	2.59
5.	Apigenin	9.16	7.3	5.5	4.25	3.25
6.	Kaempferol	12.7	9.81	6.8	5.69	4.32
7.	Chrisin	15.2	14.6	7.8	6.61	5.16

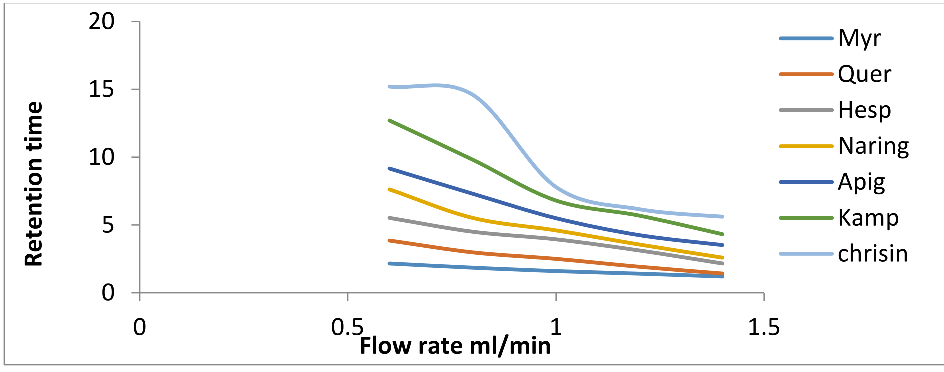


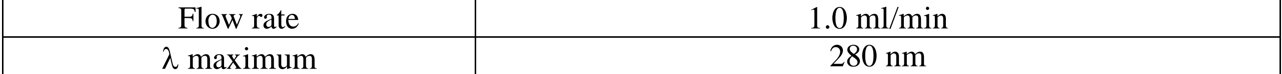
Figure 3: Retention time as a function of flow rate for Flavonoids

8. Calibration grave

The optimum conditions for separation of Flavonoids. On reversed phase column were listed in table (6) and figure (4) shows Calibration curve of Flavonoids.

Table 6: The optimum working conditions for the determination of flavonoids and phenolic acid

Parameters	Value of Flavonoids
Sample volume	20 µL
Column	ODS (50x 4.6 mm i.d.)
Organic modifier	60% acetonitrile
pH	(5.5)



Flow rate	1.0 ml/min
λ maximum	280 nm

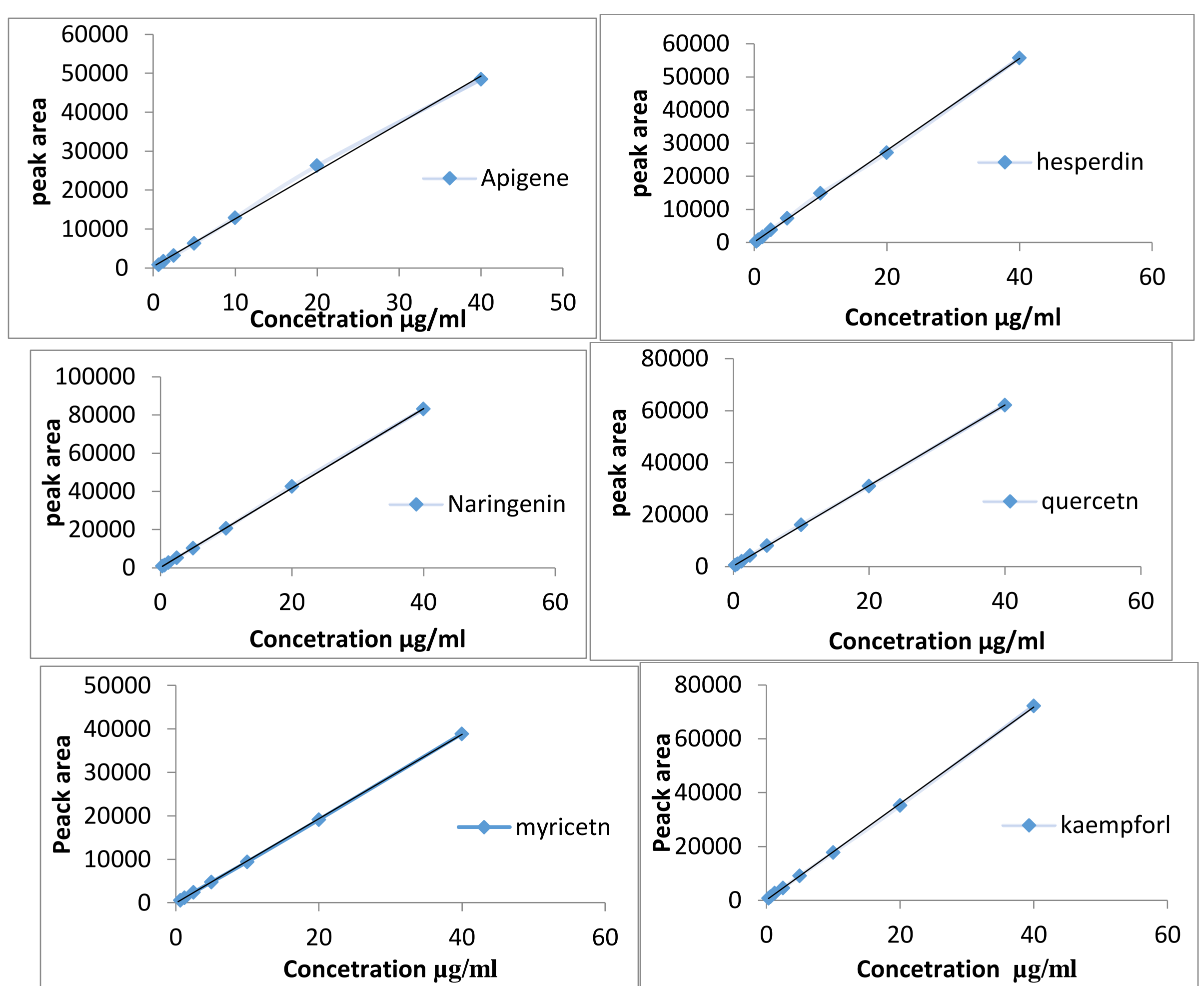


Figure 4: Calibration curve of Flavonoids

Table 7: Linear equation, correlation coefficients R 2, and detection limits for flavonoids

No.	Compound	Linear Equation	R^2	Detection limit µg/ml
1.	Myricetin	971.67×+100.21	0.9999	0.083
2	Quercetin	1548×+204.66	0.9998	0.013
3	Hesperdin	1383.9×+178.42	0.9995	0.025
4	Naringenin	2055.3×+79.701	0.9995	0.046
5	Apigenin	1221.2×+412.94	0.9983	0.031
6	Kaempferol	1792.3×+67.287	0.9998	0.013
7	Chrisin	1154.9×-55.19	0.9999	0.017

9. Accuracy and Precision

The accuracy and precision for determination of flavonoids, was depending upon the percentage value of the relative error (E %), recovery (Rec %), and relative standard deviation respectively. The mean value for each concentration of flavonoids was obtained by three replicates analysis, through all analysis at different concentration. The results tabulated in Table (8).

Table 8: Error % and Rec % results for analysis of flavonoids in standard solutions

Flavonodis	Conc. of favonoids µg/ml					
	Present	Add	Found	Error %	Rec %	RSD%
1. Myricetin	3	1	3.1	3.3	103	3.2
	10	2	9.88	1.2	99	1.39
	18	3	16.99	6.7	95	0.78
2. Quercetin	5	1	4.85	4	97	0.32
	9	2	8.9	1.12	99	0.17
	22	3	21.3	3.3	97	0.47
3. Hesperdin	4.5	1	4.3	4.7	97	0.48
	8.6	2	8.1	6.2	96	1.23
	16	3	15.7	1.9	98	0.62
4. Naringenin	3	1	2.88	4.2	96	0.88
	6.5	2	6.6	1.6	102	3.1
	20	3	19.63	1.9	98	0.18
5. Apigenin	10	1	9.8	2.0	98	1.0
	20	2	20.3	1.5	101.5	0.76
	30	3	29.1	3	103	0.68
6. Kaempferol	4	1	3.72	7.5	93	0.83
	7	2	6.83	2.5	97	0.36
	11	3	11.3	2.6	103	1.37
7. Chrisin	2	1	1.93	3.6	97	0.74
	6	2	6.3	4.8	105	0.30
	16	3	15.6	2.5	98	0.1

Application of the Optimum Conditions for the Separation and Determination of Flavonoids

The optimum conditions which found in experimentally from the above mentioned investigations have been applied for the separation of Flavonoids. After optimization of the HPLC conditions for separation of the standard mixture. This was applied to analysis flavonoids. By variation the organic modifier. pH and flow rate the experimental results for these studies observe the optimum conditions. which gave base line separation for the whole mixture were 60 % acetonitrile, pH5.5and flow rate 1ml /min for Flavonoids shown in typical chromatography(5)and (6), for some sample honey and standard solution

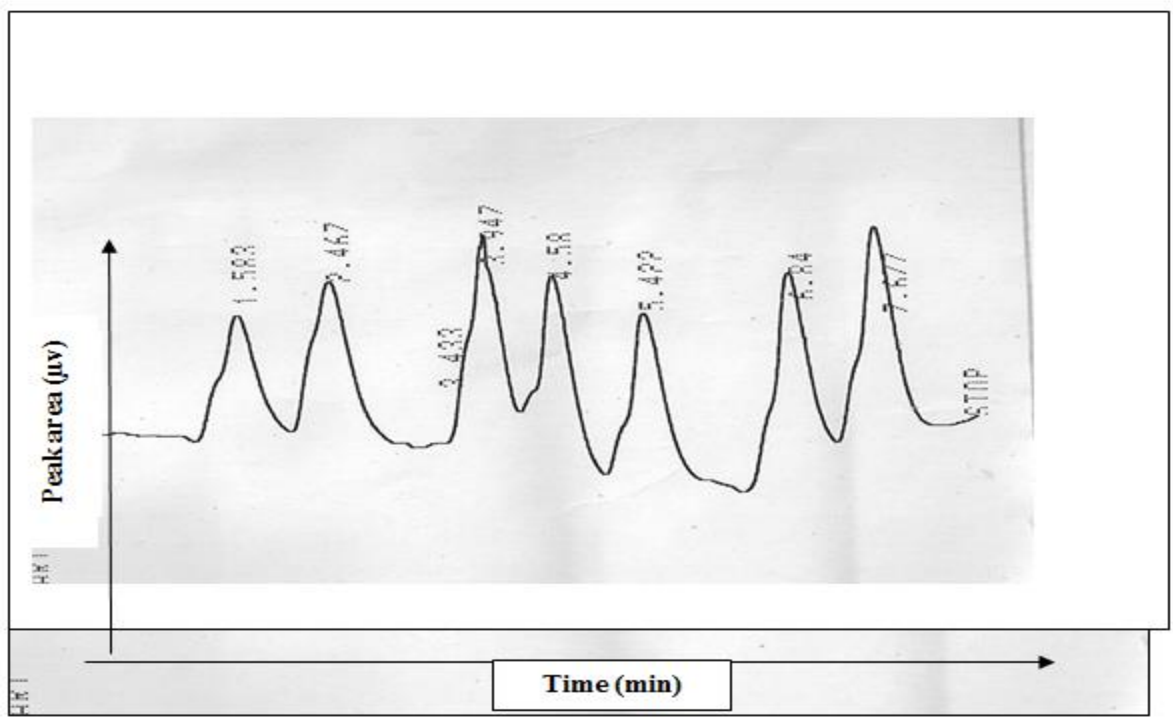


Figure 5: Standard mixture of Flavonoid reveres phase separation on ODS (50×4.6 I'd) column,3µm Particle size pH 5. 5, flow rate 1 ml /min, UV detector 280 nm

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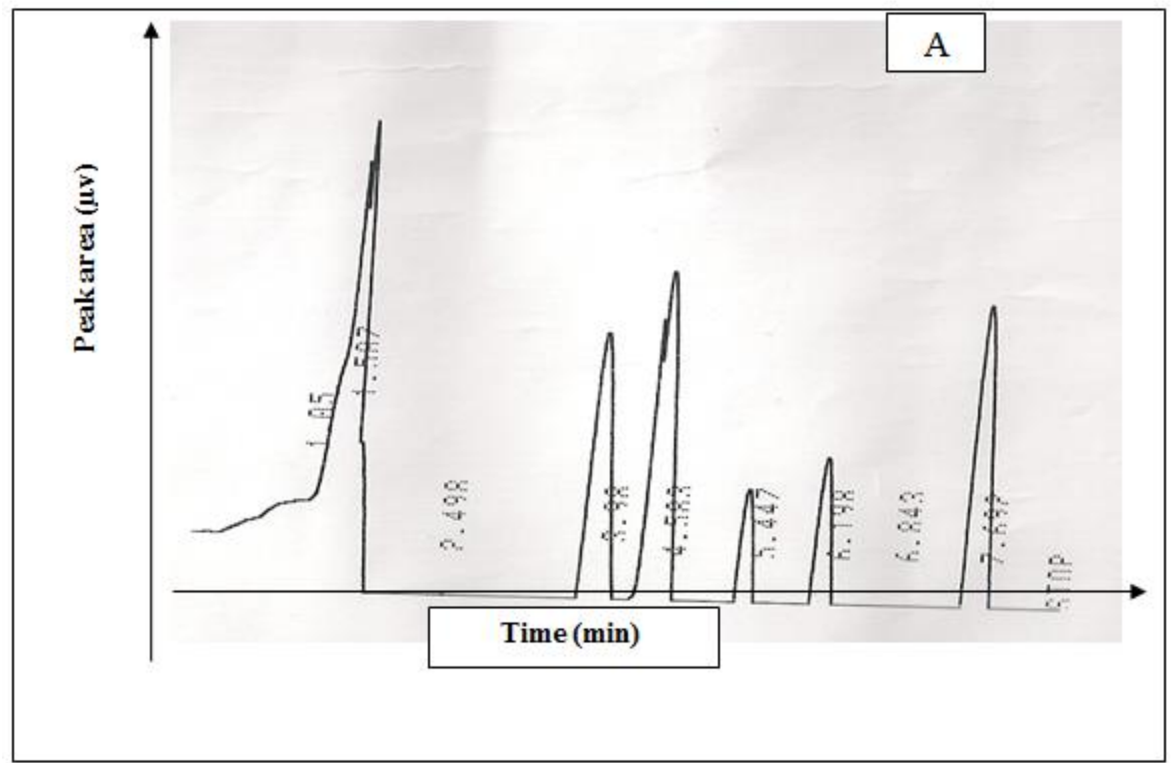

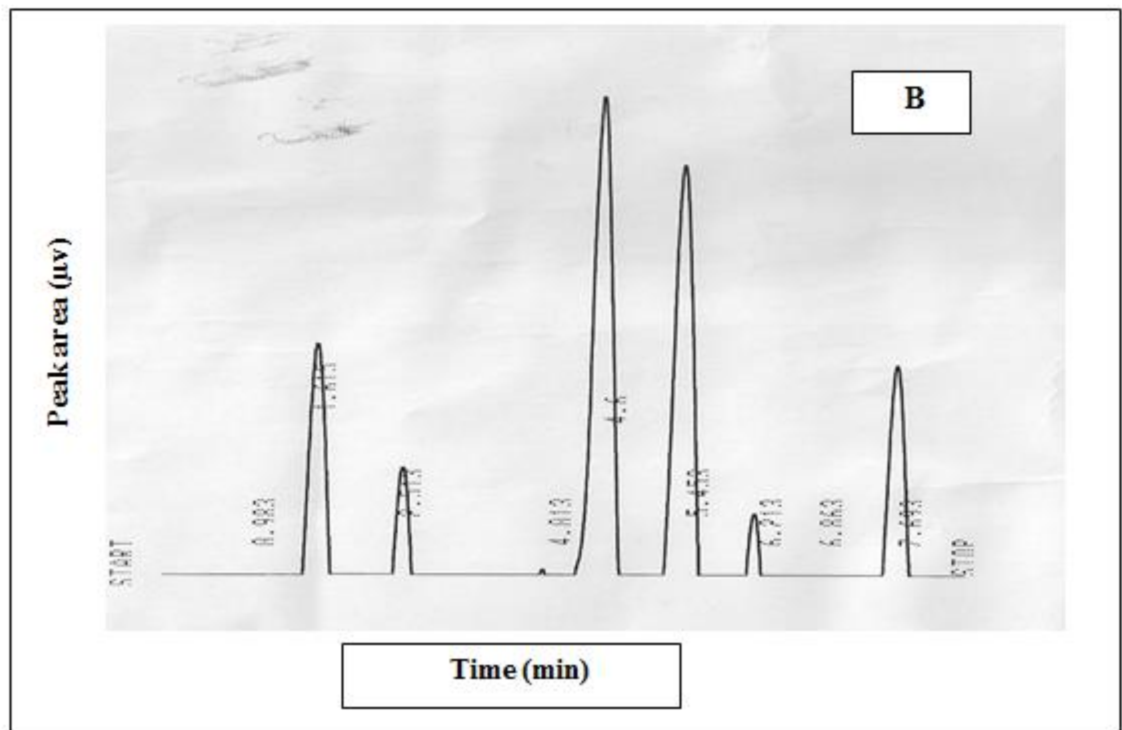

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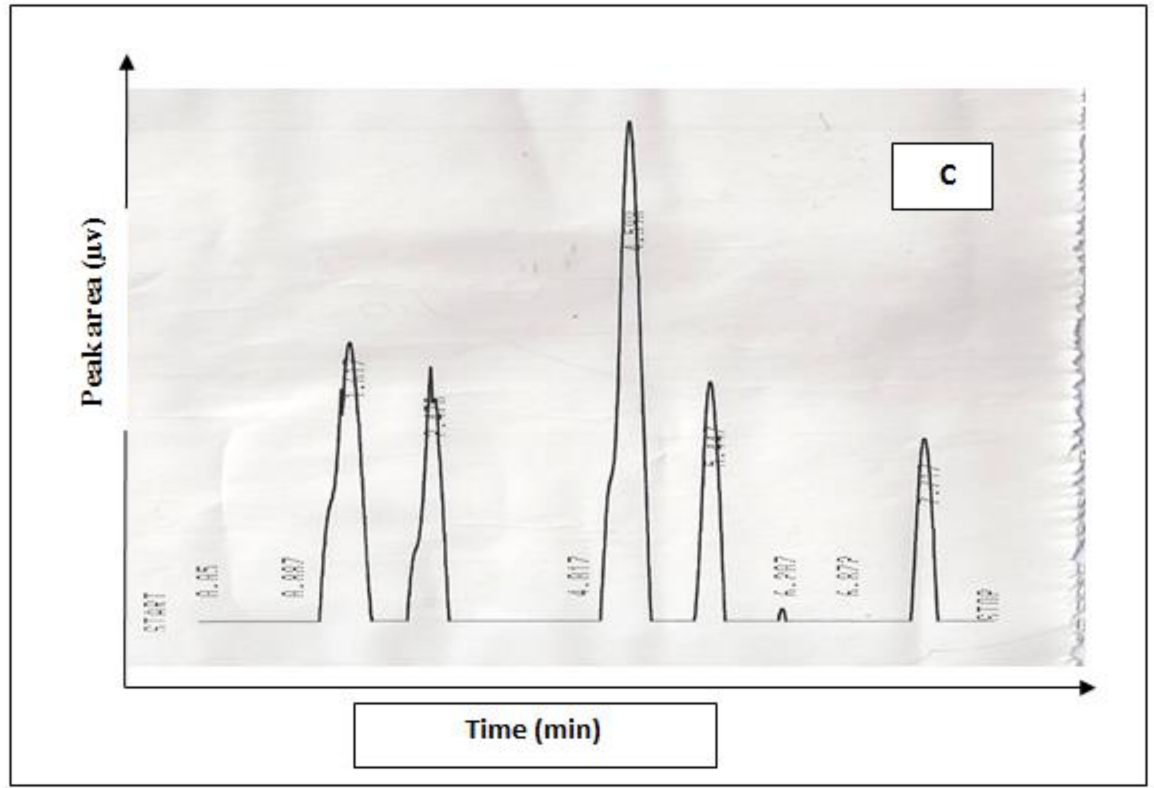

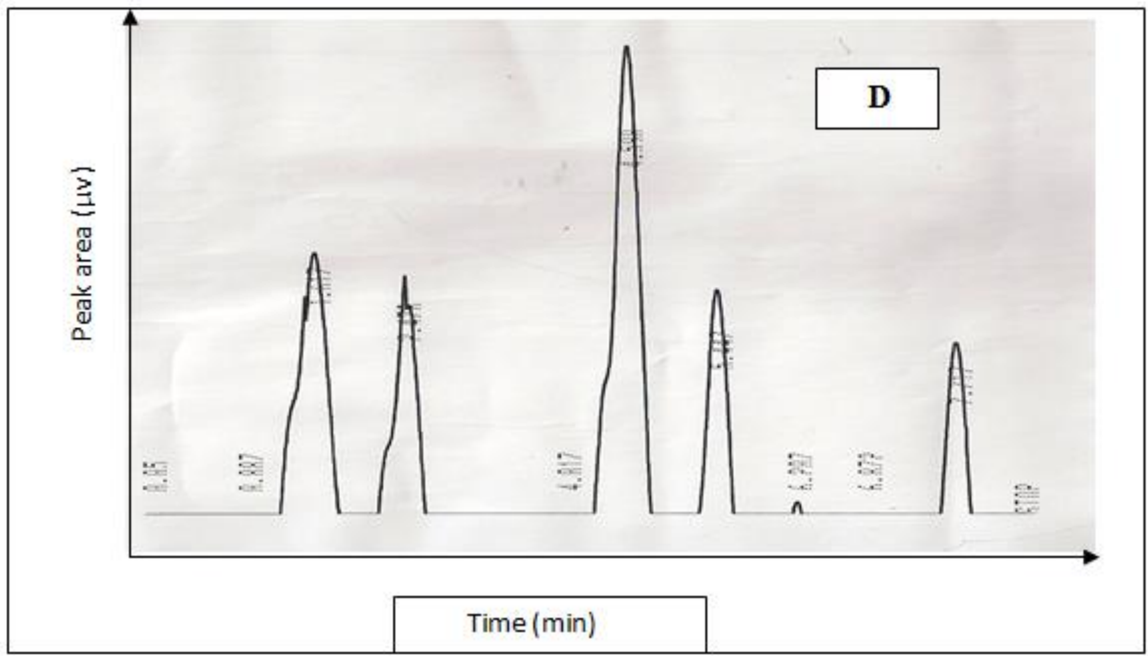

Figure 6: Separation of mixture Flavonids in honey samples on reversed phase column (50×4.6mm.Id) flow 1.0 ml/min UV detector 280 nm(A=Sider(2) ,B=Citrus ,C= Sider (3),D= Trefoil

Table 9: Concentration of Flavonoids compound in Honey samples bee under study values are expressed in mg/kg as mean ±SD

Sample honey	Myricetin	Quercetin	Hesperdin	Naringenin	Apigenin	Kaempferol	Chrisin
Flower	18.50±1.000	9.20±1.000	8.60±1.000	20.397±0.577	15.61±1.000	4.31±1.528	6.20±1.000
Trefoil	10.30±1.762	3.31±1.000	7.31±1.000	8.707±0.577	10.507±0.577	4.403±1.528	4.60±1.000
Seder(1)	---------	13.91±1.732	8.44±1.000	12.63±0.577	4.713±1.527	38.13±1.155	7.00±2.082
Seder(2)	1.903±1.528	15.01±1.155	9.22±1.000	3.21±1.000	9.72±2.000	--------	19.0±1.000
Eucalyptuses (1)	6.61±1.000	8.09±1000	5.90±1.000	6.03±1.000	13.60±1.000	11.317±2.082	28.0±1.000
Nigella sativa	--------	16.21±1.000	16.20±1.000	8.51±1.000	8.20±1.527	3.54±56.871	16.80±5.225
Mountain	15.1±1.000	22.01±1.000	14.00±1.000	24.68±1.528	28.81±1.000	15.79±1.000	3.80±1.526
Eucalyptuses (2)	6.09±1.000	18.513±0.577	9.20±.5.774	7.41±1.000	20.41±1.000	15.60±0.577	4.30±1.000
Citrus	14.31±1.000	11.31±1.000	5.41±1.1547	5.90±0.577	14.98±2.646	7.297±1.528	4.40±1.000
Eucalyptuses (3)	8.203±0.577	12.10±0.577	8.607±1.000	15.407±0.577	18.71±1.000	11.81±1.000	2.50±1.000
Olive	6.20±1.000	5.09±1.000	10.09±1.000	7.10±1.528	9.29±1.000	3.31±1000	5.13±1.000
Sunflower	11.49±1.000	---	----	5.81±1.000	6.79±1.000	----	7.61±1.000
Germany	-------	12.11±1.000	8.61±1.000	4.40±1.000	17.80±1000	11.84±1.971	2.00±1.000
American	331±1000	1031±1.000	455±1.000	531±1.000	741±1.000	411±1.528	261±1.000
India	5.80	9.30	8.00	6.60	8.33	4.70±1.000	3.50±1.000

Table 10: Concentration of Flavonoids compound in different location of Honey bee mg/kg

Type of homey	Myricetin	Quercetin	Hesperdin	Apigenin	Kaempferol	Chrisin	Ref
China	---------	554-643	-----------	-----------	-------------	--------	1
Brazilian	---------	21.69-29.42	-----------	----------	---------	--------	19
Sudan	--------	0.018-3.21	3.91-46.41	0.383-0.526	0.399-5.353	--------	20
Bangladesh	1.99	------- ---	-----------	-----------	3.01	--------	21
Brazil	3.284	--------	-----------	7.24	95.590	--------	22
Malaysian	--------	6.219-17.01	4.941-17.01	---------	---------	5.42-15.98	23
Finland	--------	----------	---------	Trace	Trace	36	24
Czech Republic	--------	6.60	---------------	--------------	--------------	0.6	25

Lithuanian	----------	115.50-376	55.80-115.00		39.90-496.90	-----------	26
Australian and New Zealand	0.61-150	0.40-170	-----------	---------	-------	0.10-130	27
Italy	----------	---------	----------	-------	0.58-1.088	1.43-3.140	28
Spain	3.22	--------	--------	----------	1.167-4.356	0.750-6.99	29

Table (9) show the obtained results of concentration of Flavonoids compound in Honey samples bee under study values are expressed in mg/kg as mean ±SD and Table (10) shows that the different value of Flavonoids concentrations in various types of honey under study. Highest concentration of (Myrsten, Quercetin, Hesperetin, Narngen , Apigenin, Kaempferol, Chrysin) in honey samples (flower 18.50 m/kg, Mountain 22.00 m/kg, Nigella sativa 16.20 m/kg, Mountain 24.68 mg/kg, Mountain28.80 mg/kg, Eucalyptuses(2) 15.60 mg/kg, Nigella sativa 16.80 mg/kg) lowest value in(Mountain 1.50 mg/kg , Trefoil 3.3 mg/kg , American 4.55 m/kg, Seder(2) 3.20 mg/kg, Seder(1) 4.71 mg/kg, Olive 3.30 mg/kg, Seder(2) 1.90 mg/kg) respectively. While the table (11) shows concentration of Flaovonids in different kind of sample the highest value for (Myrsten, Quercetin, Hesperetin, Apigenin, Kaempferol, Chrysin) in honey samples (Brazil 3.282 mg/kg, China 643 mg/kg, Lithuanian 115.0 mg/kg, Brazil 7.24 mg/kgm, Lithuanian 496.6mg/km, Malaysian 5.98mg/kg) respectively and lowest value in (Australian and New Zealand 0.61mg/kg, Sudan 0.018mg/kg, Finland Trace, Finland Trace, Australian and New Zealand 0.1mg/kg).

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