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Total phenolic acids and flavonoid contents determination in Yemeni honey of various floral sources: Folin-Ciocalteu and spectrophotometric approach

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Abstract

Twenty-nine Yemeni honey samples were analyzed to determine total phenolic and flavonoid contents using Folin-Ciocalteu and spectrophotometric technique. These tested honey samples were collected from different regions corresponding to various floral species: jujube, cactus and multifloral plants. Folin-Ciocalteu method was adopted for the analysis of total phenolic and flavonoid contents. Gallic acid and quercetin were considered as the best standards as the spectrophotometric response of these compounds are equivalent to most other phenolic acids and flavonoid compounds, respectively. The obtained results of honey samples were in a wide range; the highest phenolic acid concentration was obtained for honey produced from cactus, while the lowest value corresponded to monofloral honey from jujube. On the other hand, a broad variation was also observed in total flavonoid content; the highest value was obtained for honey collected from cactus area (S_4) and the lowest value was found in honey produced from multifloral plants (A_1).

Keywords: honey; total flavonoids; total phenolic acids; botanical origin; determination.

Practical Application: Analysis of honey quality of various floral sources.

1 Introduction

An enchanting viscous sweetener usually made by honey bees is known as honey (Farooq Khan & Maqbool, 2008). From the earlier times, it has been used instead of sugar (Krell, 1996) and as food preservative (Cherbuliez & Domerego, 2001, 2003). Honey also has received much attention due to its consumption as healthy food and its physiological properties. Among its various benefits, honey has a marked antioxidant activity which is a main reason for its application as food preservative (Coskun & Karabulut Dirican, 2019; Khalil et al., 2010; Mohamed et al., 2009). The biological properties of honey including its antioxidant activity are mainly due to the presence of flavonoids and phenolic acids (Costa et al., 2019). The presence of such compounds in honey protects human health by reducing the damages that could be caused by various oxidizing agents (Ajani, 2009; Gheldof et al., 2002; Khalil et al., 2010; Lachman et al., 2010). The antioxidants found in natural honeys include organic acids, amino acids, proteins, polyphenols, carotenoids, etc (Khalil et al., 2010; Mohamed et al., 2009). Although these characteristic key constituents in honey are approximately the same, the specific chemical composition of natural honeys differs with respect to the plant species on which the bees collect the nectar (Atrouse et al., 2004; Duarte et al., 2018; Ebenezer & Olugbenga, 2010; Omafuvbe & Akanbi, 2009). On the other hand, the flavonoid composition, which plays an important role for evaluating the quality of honey can be affected by different factors such as the climatic conditions and the plant species.

The analytical methods mostly reported in the literature for analysis of flavonoids and other polyphenols are liquid chromatography (Ahmed et al., 2014, 2016; Milbury, 2001), liquid chromatography coupled with mass spectrometry (Wabaidur et al., 2015), gas chromatography hyphenated to mass spectrometry (Markham et al., 1996), as well as spectroscopic techniques (Bittar et al., 2018; Frausto-Reyes et al., 2017). The current study aimed to the determination of total polyphenol contents in several natural honeys produced from various plant species using UV-visible spectrophotometry. The developed method was simple and easy to apply for the routine analysis of honey; moreover, it could be potentially useful for measurement of polyphenols and flavonoids in many other biological samples.

2 Materials and methods

Quercetin (QUE), gallic acid (GAE) and lithium sulfate were purchased from Sigma Chemicals Co. (St Louis, MO, USA). Acetic acid, acetonitrile, diethylether, ethanol, formic acid, hydrochloric acid, methanol and anhydrous sodium carbonate were procured from BDH Chemicals Co. (U.K.). Sodium molybdate and sodium tungstate were supplied by Acros Organics (New Jersey, USA). Aluminium trichloride, phosphoric acid and potassium acetate were purchased from Riedel de Haen Co. (Seelze, Germany). Benzoic acid was obtained from Winlab and cinnamic acid from SAFC. Sodium sulfate was supplied by Koch-light Lab. Ltd. (Haverhill, Suffolk, UK). The multiwall carbon nanotubes

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(MWCNTs) were obtained from Timesnano (Chengdu Organic Chemicals Co. Ltd., China), and bromine from Parchem (New Rochelle, NY, USA). Folin-Ciocalteu reagent was supplied by Sigma-Aldrich Chemical Company (Steinheim, Germany).

2.1 Instrumentation

For measurement of total phenolic and flavonoid contents in honey samples, all spectrophotometric measurements were performed on a UV-visible spectrophotometer (Thermo Scientific Evolution 600, UK) at their respective maximum wavelengths (λ_{max}). Quartz UV cells with 1 cm path length were used for honey analysis and distilled water was used as blank for all spectrophotometric measurements.

2.2 Sample collection

All the analyzed honey samples were collected from regions covered with either jujube cactus or multifloral plants. These 29 kinds of honey samples were obtained from various Yemeni regions and in different harvesting seasons. The honey samples corresponding to monofloral species were from jujube (B1, B2, B3, B4, Bsh, M1, M2, Msh, Sh, CB, CW and CA) and cactus (S_1, S_2, S_3, S_4, S_{sh}, SL, L and SR); while the honey samples A_1, A_2, A_3, A_4, A_{sh}, Ab, F, MB and Cg corresponded to multifloral species. All these samples were preserved at a temperature below 0 °C. The production year and region of collection of these aforementioned honey samples are listed in Table 1.

2.3 Estimation of total polyphenol

For the determination of the total polyphenols content in the investigated honey samples, we used the Folin-Ciocalteu method (Singleton et al., 1999), which is a colorimetric *in vitro* assay measuring the total reducing capacity of a sample (Lachman et al., 2008; Singleton et al., 1999). An accurately weighed 5 g sample of each honey was put in a 50 mL volumetric flask, which was completed with Milli-Q water and filtered through Whatman No. 1 paper. 0.5 mL of this solution was then added with 5.0 mL Folin-Ciocalteu reagent (0.2 N), and mixed for 5 min followed by the addition of 4 mL of sodium carbonate (75 mg/L). Then the mixture solution was allowed

Table 1. The lists of production years and region of collection of honey samples.

Source	Sample	Species	Region (Yemen)	harvest (year)
Jujube/Mono-floral	B_1	*Ziziphus Spina-christs*	Hadramout	2008
	B_2	*Ziziphus Spina-christs*	Hadramout	2011
	B_3	*Ziziphus Spina-christs*	Hadramout	2010
	B_4	*Ziziphus Spina-christs*	Hadramout	2009
	B_{sh}	*Ziziphus Spina-christs*	Hadramout	2009
	M_1	*Ziziphus Spina-christs*	Hadramout	2009
	M_2	*Ziziphus Spina-christs*	Hadramout	2010
	M_{sh}	*Ziziphus Spina-christs*	Hadramout	2010
	Sh	*Ziziphus Spina-christs*	Shabwa	2009
	CB	*Ziziphus Spina-christs*	Hadja	2011
	CW	*Ziziphus Spina-christs*	Dhamar	2010
	CA	*Ziziphus Spina-christs*	Omran	2011
Cactus	S_1	*Acacia tortilis*	Hadramout	2009
	S_2	*Acacia tortilis*	Hadramout	2011
	S_3	*Acacia tortilis*	Hadramout	2010
	S_4	*Acacia tortilis*	Hadramout	2010
	S_{sh}	*Acacia tortilis*	Hadramout	2009
	SL	*Acacia ehrenbergiana*	Hadja	2011
	L	*Aloe vera barbadensis*	Mantuka	2010
	SR	*Aloe vera barbadensis*	Abh	2010
Multi-floral	A_1	*Various species*	Hadramout	2008
	A_2	*Various species*	Hadramout	2010
	A_3	*Various species*	Hadramout	2009
	A_4	*Various species*	Hadramout	2011
	A_{sh}	*Various species*	Hadramout	2010
	Ab	*Various species*	Abeen	2010
	F	*Wild plants*	Sanaa	2010
	MB	*Wild plants*	Hadja	2010
	Cg	*Wild plants*	Socotra	2010

for incubation at room temperature for 2 h and the absorbance was measured at 765 nm, while methanol was used as blank. Since this assay measures all phenolics, GAE is considered as the best standard, due to its availability and stability. In addition, the response to GAE has been shown to be equivalent to most other phenolic compounds.

A 250 mg/L stock solution was prepared by dissolving 25 mg of dry GAE in 100 mL of 70% methanol, using a volumetric flask. A series of GAE standard solutions with concentrations of 0, 5, 10, 20, 50 and 100 mg/L were prepared for constructing the standard calibration curve. The mean of three absorbance measurements was used for the calibration plot and the total phenolic content of the real samples was stated in mg of GAE equivalents/100 g of honey. On the other hand, the total flavonoid content of the target samples was obtained following the reported methods (Arvouet-Grand et al., 1994). In brief, 5 mL of 2% $AlCl_3$ in methanol was mixed with the same volume of a honey solution, and the absorbance was measured at 415 nm after 10 min; the blank solution was prepared by mixing 5 mL honey solution with 5 mL methanol without the addition of $AlCl_3$. The total flavonoid content was expressed as QUE equivalent. The standard calibration curve of QUE was established in the range of 0-100 mg/L and the values are calculated as mg of QUE equivalents/100 g of honey.

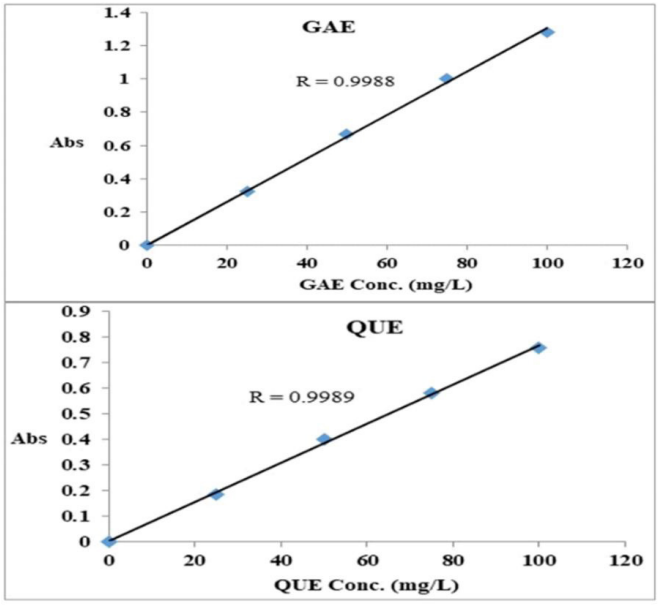

Figure 1. Calibration curve for GAE and QUE. Abs: Absorbance. QUE: Quercetin. GAE: Gallic acid.

3 Results

3.1 Calibration and linearity

A series of individual standard solutions of both GAE and QUE of 0, 5, 10, 20, 50 and 100 mg/L were prepared and their absorption was noted. The mean values of three absorbance measurements were used for the construction of calibration graph. This curve was found to be linear in the range of 0-100 mg/L for both GAE and QUE (Figure 1). All the absorbance determinations were performed in thrice at wavelengths 765 nm for GAE and 415 nm for QUE and the mean values of three measurements were used for constructing the calibration plot. The excellent correlation coefficient (r^2) values were found to be 0.9988 and 0.9989 for GAE and QUE, respectively. The total phenolic acids and flavonoid contents were determined as GAE and QUE equivalent using the two calibration curves.

3.2 Robustness and precision of the method

The robustness of the proposed method was checked by slightly changing the experimental conditions including absorption maxima of GAE and QUE. Negligible changes of the absorption wavelength values were noticed due to the slight shifting of lambda max values. This suggests the robustness of the method. For establishing the precision of the method the analyses were carried out in the same day as well as in three consecutive days for a set of six samples of different concentrations. The relative standard deviation (RSD) was found to be less than 3.50%. These low %RSD values suggest that the developed technique is precise and can be applied for routine and reliable analysis of honey. Table 2 summarizes accuracy and precision results including recovery for both intra-day and inter-day determinations of GAE and QUE, which were found to be in the range of 98.00 to 100.33 mg/L, and 97.03 to 99.60 mg/L, respectively.

3.3 Phenolic and flavonoid contents in honey sample

Using the calibration plot of GAE, the total phenolic content (mg GAE/100 g of honey) was determined for all the analyzed samples and found to be in the range of 10.74 to 86.80 mg GAE/100 g of honey. The maximum total phenolic content (86.80 mg GAE/100 g of honey) was found in the cactus honey (sample S_4) among the investigated samples, while the lowest quantity was found in jujube honey (sample M_1). The mean

Table 2. Summaries of the accuracy and precision experiments.

Precision	Amount added	GAE, mg/L			QUE, mg/L		
		30	45	95	30	45	95
Intra-day	Amount found ± SD	29.60 ± 0.89	45.15 ± 1.32	94.01 ± 0.65	29.15 ± 0.39	44.82 ± 0.62	93.25 ± 0.45
	Recovery ± RSD	98.66 ± 3.01	100.33 ± 2.92	98.95 ± 0.69	97.16 ± 1.33	99.60 ± 1.38	98.16 ± 0.48
Inter-day	Nominal ± SD	29.70 ± 1.01	44.10 ± 1.54	93.10 ± 0.99	29.11 ± 0.49	44.06 ± 0.84	93.16 ± 1.05
	Recovery ± RSD	99.00 ± 3.40	98.00 ± 3.49	98.00 ± 1.06	97.03 ± 1.68	97.91 ± 1.91	98.06 ± 1.13

SD: Standard deviation; RSD: Relative standard deviation; QUE: Quercetin; GAE: Gallic acid.

Table 3. The total phenolic and flavonoid contents in Yemeni honeys.

Sample	Abs[a]	Phenolic (mg GAE/100 g)	Abs[a]	Flavonoid (mg QUE/100 g)
B_1	0.663	50.84 ± 1.17	0.040	2.61 ± 0.11
B_2	1.026	78.67 ± 1.45	0.146	9.50 ± 0.24
B_3	0.726	55.67 ± 1.33	0.106	6.90 ± 0.18
B_4	0.670	51.38 ± 1.27	0.042	2.73 ± 0.17
B_{sh}	0.924	70.85 ± 2.07	0.126	8.20 ± 0.34
M_1	0.140	10.74 ± 0.57	0.051	3.32 ± 0.22
M_2	0.216	16.56 ± 0.77	0.069	4.49 ± 0.19
M_{sh}	0.362	27.76 ± 0.99	0.082	5.34 ± 0.21
Sh	0.628	48.16 ± 1.05	0.097	6.31 ± 0.24
CB	0.922	70.70 ± 1.85	0.106	6.90 ± 0.15
CW	0.599	45.93 ± 1.52	0.096	6.25 ± 0.17
CA	0.803	61.57 ± 1.88	0.092	5.99 ± 0.19
Mean		49.07 ± 1.07		5.71 ± 0.11
S_1	0.246	18.86 ± 0.52	0.082	5.34 ± 0.09
S_2	0.403	30.90 ± 0.86	0.096	6.25 ± 0.19
S_3	0.305	23.39 ± 0.55	0.089	5.79 ± 0.17
S_4	1.132	86.80 ± 1.99	0.808	52.58 ± 1.56
S_{sh}	0.376	28.83 ± 1.02	0.147	9.56 ± 0.25
SL	0.356	27.30 ± 0.86	0.046	2.99 ± 0.11
L	0.607	46.54 ± 0.96	0.101	6.57 ± 0.32
SR	0.483	37.04 ± 0.58	0.058	3.77 ± 0.07
Mean		37.46 ± 0.17		11.61 ± 0.33
A_1	0.265	20.32 ± 0.42	0.026	1.69 ± 0.12
A_2	0.316	24.23 ± 0.33	0.063	4.10 ± 0.27
A_3	0.309	23.70 ± 0.41	0.080	5.21 ± 0.31
A_4	0.559	42.86 ± 1.03	0.092	5.99 ± 0.35
A_{sh}	0.706	54.14 ± 1.23	0.103	6.70 ± 0.40
Ab	0.335	25.69 ± 0.53	0.043	2.90 ± 0.12
F	0.443	33.97 ± 0.84	0.112	7.29 ± 0.54
MB	0.459	35.20 ± 0.42	0.142	9.27 ± 0.60
Cg	0.397	30.44 ± 0.45	0.071	4.59 ± 0.37
Mean		32.28 ± 0.34		5.29 ± 0.44
[b]Average		39.6 ± 0.65	--	7.54 ± 0.59

Abs = Absorbance; [a]Abs = Average absorbance of three measurements; [b]Average phenolic and flavonoid components found in all 29 analyzed honey samples; QUE: Quercetin; GAE: Gallic acid.

values of total phenolic contents in jujube, cactus and multifloral honey was found to be 49.07, 37.46 and 32.28 mg GAE/100 g of honey, respectively (Table 3).

Similarly, the QUE calibration curve was employed for evaluation of the total flavonoid content of honey samples and the values were varied from 1.69 in a multifloral honey to 52.58 mg QUE/100 g of honey in cactus honey samples. The maximum amount of total flavonoid (52.58 mg QUE/100 g of honey) was found in the cactus honey (sample S_4), while the lowest amount (1.69 mg QUE/100 g of honey) was found in multifloral honey (sample A_1). The average quantity of total flavonoid contents was found to be 5.71, 11.61 and 5.29 mg QUE/100 g of honey in jujube, cactus and multifloral honey, respectively (Table 3).

4 Discussion

Several researchers have analyzed and reported the quantification of phenolic and flavonoid contents in honey (Amiot et al., 1989; Moniruzzaman et al., 2013; Pontis et al., 2014; Sant'Ana et al., 2014). In addition, the total phenolic and flavonoid content of various honeys have also been determined earlier (Amiot et al., 1989; Meda et al., 2005). The average value (39.6 ± 0.65 mg QUE/100 g of honey) of phenolic content of the 29 honey samples is similar to the reported average values of few French and Greek honeys (Amiot et al., 1989; Hussein et al., 2011), although *Acacia tortilis* (S_4) honeys showed the highest levels of phenolic compounds (86.80 mg GAE/100 g) among the analyzed samples.

The average quantity for total flavonoids contents in all analyzed honey samples were found to be 7.54 ± 0.59 mg of QUE/100 g and were relatively higher than in various European honeys. For example, some previous studies have found the following total flavonoid amounts in sunflower and rape honey (1.5-2.0 mg QUE/100 g), eucalyptus honey (2.0-2.5 mg QUE/100 g), arbutus and chestnut honey (less than 0.5 mg QUE/100 g), and lavender and acacia honey (0.5-1 mg QUE/100 g) (Amiot et al., 1989; Martos et al., 2000). The cactus honey from Hardramaout exhibited a total flavonoid content of 52.58 mg QUE/100 g, which is significantly greater than that of French honeys (less than 1 mg QUE/100 g). The phenolic and flavonoid contents of honey are usually been determined by HPLC with photodiode array detection or ultra-performance liquid chromatography with mass spectrometry, using amberlite XAD-2 columns for extraction. In the current study, we used a spectrophotometric quantification of total phenolic and flavonoids with aluminum chloride and it has been reported earlier for the analysis of such compounds in propolis extracts (Arvouet-Grand et al., 1994; Chang et al., 2002). In a previous study, Chang et al. (2002) have shown that the real content of total flavonoids must be the sum of flavonoid contents determined by the aluminum chloride method and that using the aluminum chloride method alone, it is possible to underestimate the content of total flavonoids. The correlation between the total flavonoids and the total amount of phenolic compounds could be affected by the presence of some amino-acids and proteins in honey that can react with Folin-Ciocalteu reagent.

5 Conclusion

The proposed study showed that all the analyzed honey samples contained phenolic and flavonoids compounds in good quantity. The individual highest levels of phenolic compounds (86.80 mg GAE/100 g) was found in cactus honey (S_4), whereas the highest phenolic contents of jujube honey and multifloral honey were found to be 78.67 mg GAE/100 g (B_2) and 54.14 mg GAE/100 g (A_{sh}), respectively. However, the average quantities of total phenolic compounds in the analyzed honeys were found in the order of jujube/mono-floral (49.07 ± 1.07) > cactus (37.04 ± 0.58) > multifloral (32.28 ± 0.34) honey (Table 3). On the other hand, the highest individual level of flavonoids (52.58 mg QUE/100 g of honey), was also found in the cactus honey (S_4), while the multifloral and jujube honeys had a flavonoid content of 9.27 mg QUE/100 g (MB) and 9.50 mg QUE/100 g (B_2), respectively. Similarly, the average values of total flavonoids in

the investigated honeys were found in the following order, cactus (11.61 ± 0.33) > jujube/mono-floral (5.71 ± 0.11) > multifloral (5.29 ± 0.44) honey (Table 3). The average amounts of phenolic and flavonoid compounds in all twenty-nine analyzed samples were found to be 39.60 mg GAE/100 g and 7.54 mg QUE/100 g, respectively. Compared to previously published results the obtained average values of phenolic compounds were slightly lower, while the average quantity of flavonoids was found to be three times higher [29].

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