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A modified Folin-Ciocalteu method for the microdetermination of total phenolic content in honey

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Abstract

Polyphenols are major antioxidant activity-associated bioactive substances in honey. Measurement of the total phenolic content in honey is of great importance for predicting antioxidant activity and characterising or selecting honey samples for their use as antioxidants or natural food preservatives. Considering that the original Folin-Ciocalteu (F-C) assay for total phenolic content determination is either time-consuming or high-cost, a microplate reader-based study was conducted to modify and validate the original F-C assay for the microdetermination of total phenolic content in honey samples. The linearity (R^2 = 0.9982), detection limit (8.61 ± 0.33 mg gallic acid equivalents (GAE)•kg^{-1} honey), quantitation limit (26.08 ± 0.99 mg GAE•kg^{-1} honey), and recovery rate (93.46 - 109.99%) were acceptable. The results suggested that the proposed F-C assay is fast, sensitive, precise, and repeatable. The method was successfully applied to analyse a total of 33 honey samples from 9 floral origins and 12 geographical regions. The modified F-C assay is low cost and easy to operate, and therefore could be applied to routine analysis.

Introduction

The Folin-Ciocalteu (F-C) assay is commonly used to evaluate the antioxidant activity of natural products by quantifying the total phenolic content (Magalhães *et al.,* 2006), which is based on the reaction of phenolic compounds with a colorimetric reagent (phosphotungstic-phosphomolybdic acid) and subject to spectrophotometric measurements. It has been reported that phenolic compounds reduce phosphomolybdate-phosphotungstic acid under alkaline conditions and form blue compounds, which can be spectroscopically determined at approximately 760 nm (Singleton and Rossi, 1965; Singleton *et al.,* 1999). This method was originally developed for the colorimetric determination of tyrosine and then improved to indirectly determine total protein concentration by measuring the content of tyrosine and tryptophan (Folin and Macallum, 1912a; 1912b; Folin and Denis, 1912; Folin and Ciocalteu, 1927). The F-C assay is sensitive and reproducible, and thereafter has been widely employed for total phenolic content quantification. Singleton and Rossi (1965) introduced a F-C assay that was applied to estimate the total phenolic content in plant products, grapes, and wines, and Kähkönen *et al.* (1999) and Gao *et al.* (2019) used this method to determine the total phenolic content of plant and food extract samples. Moreover, it was reported that the F-C assay can be used to determine the total phenolic content of various plant extracts and foods such as honey, medicinal plants, mushrooms, mulberries, red onions, spinach, *Limonium brasiliense*, and mixed fruit-based beverages (Meda *et al.,* 2005; Pourmorad *et al.,* 2006; Lin and Tang, 2007; Matanjun *et al.,* 2008; Silici *et al.,* 2010; Orhan and Üstün, 2011; Blainski *et al.,* 2013; López-Froilán *et al.,* 2018). However, the original F-C assay is time-consuming, uneconomical, and cannot be used for high-throughput measurements. Therefore, it is not suitable for quantifying the total phenolic content in a large number of samples. Accordingly, it is necessary to find a more efficient method to improve the original F-C assay.

The microplate method is a rapid, sensitive, accurate, and high-throughput method for microdetermination. Once introduced, it has rapidly developed and been used with new methods for the determination of certain compounds in food and biological samples (Nilubol *et al.,* 2012; Mathew *et al.,* 2013; O'Sullivan *et al.,* 2017; Li *et al.,* 2017). Ainsworth and Gillespie (2007) developed an improved F-C assay that utilised the F-C reagent and 96-well plates to determine the total phenolic content and other oxidation substrates in plant extracts (Ainsworth and Gillespie, 2007). However, this experiment was carried out in two steps, so

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it was time-consuming and could not be used for high-throughput measurements. In addition, the amounts of reagents used in the experiment were relatively large, so it was not economical. Therefore, we attempted to re-optimise the reaction conditions (absorption wavelength, reaction time, the amount of F-C reagent, and buffer) and operating procedures to establish an economical high-throughput F-C assay.

Honey is a kind of natural polyphenol-rich food with high nutritional and medicinal values (Ferreira *et al.,* 2009; Kuś *et al.,* 2014; Wang *et al.,* 2014; Zhao *et al.,* 2018). It has been reported that phenolic compounds, such as flavonoids and phenolic acids, are the major contributors to diverse biological activities of honey, including its anti-inflammatory, antibacterial, antitumor, and antioxidant effects (Schramm *et al.,* 2003; Bogdanov *et al.,* 2008; Tuksitha *et al.,* 2018; Ahmed *et al.,* 2018). Polyphenols can effectively scavenge peroxy radicals because their molecular structures include an aromatic ring with hydroxyl groups, which contains mobile hydrogens (Al-Mamary *et al.,* 2002). In addition, many studies have shown that the antioxidant activity and nutritional value of honey are positively correlated with the total phenolic content (Robards *et al.,* 1999; Bertoncelj *et al.,* 2007). Accordingly, the determination of total phenolic content is an important approach to evaluate the quality and antioxidant capacity of honey (Al-Mamary *et al.,* 2002; Krpan *et al.,* 2009).

In the present work, a modified F-C method for the microdetermination of total phenolic content in honey was established. It involved the synchronous determination of the total phenolic content in a large number of honey samples, and provided a more comprehensive and objective approach to the evaluation of the antioxidant activity of honey.

Materials and methods

Chemicals and reagents

The F-C reagent was purchased from Shanghai Zhanyun Chemical Co. Ltd. (Shanghai, China). Gallic acid (≥ 98% pure) was purchased from Sigma-Aldrich (Steinheim, Germany), and anhydrous sodium carbonate was purchased from Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China).

Honey samples

A total of 33 honey samples were directly obtained from honeycombs or through accompanying beekeepers from 12 geographical regions in China. Beeswax and other impurities were separated by an 80-mesh sieve before all samples were stored in the dark at 4°C in the refrigerator until further analysis.

Methods

The F-C assay was conducted in accordance with previously reported procedures with the following modifications from Cao and Suo (2003): a) reselection of the detection wavelength based on the absorbance spectrum; b) redetermination of the incubation time for the reaction to reach the platform period from 0 to 120 min; c) adjustment of the concentration ratio of the sample solution and sodium carbonate solution in the reaction system from 1:1 to 1:8; and d) adjustment of the volume ratio of the test sample and F-C reagent working solution from 1:1 to 1:7.

The final protocol for the assay was as follows: the F-C reagent was prepared and diluted 1:3 with ultrapure water (w/v), and the honey sample was diluted 1:10 with ultrapure water (w/v). Exactly 20 μL of the sample solution was added to a 96-well plate, and the F-C reagent and 1 M sodium carbonate solution were added at volumes of 100 and 40 μL, respectively. Afterwards, 40 μL of water was added to ensure that the total volume of the final mixture was 200 μL. The absorbance of the mixture was measured at 756 nm using an automated 96-well microplate reader after being well mixed and incubated in the dark for 80 min (Tecan Infinite M200Pro, Tecan Group Ltd. Switzerland).

Methodology validation

The modified F-C assay was validated based on the International Conference on Harmonisation (ICH) guidance entitled Q2B Validation of Analytical Procedures: Methodology (ICH, 1996). Honey samples S2 and S17 were randomly selected for methodology investigation. Linearity, sensitivity, stability, repeatability, precision, and recovery rate were investigated to evaluate this method.

Linearity

A stock solution of gallic acid standard was prepared with a concentration of 0.1 mg•mL^{-1}, and then diluted to a series of concentrations (0.01, 0.03, 0.05, 0.07, and 0.09 mg•mL^{-1}). The standard solution, F-C reagent and buffer were successively added to each well. After a complete reaction devoid of light, linear regression analysis of the concentration and absorbance of gallic acid standard solution was carried out. The concentration ranged from 0.01 to 0.1 mg•mL^{-1}, and the results were expressed as gallic acid equivalents (GAE)•kg^{-1} honey.

Sensitivity

The sensitivity of the modified F-C assay was evaluated by the detection limit (DL) and quantitation limit (QL). The DL and QL were calculated as

DL = 3.3σ/S, and QL = 10σ/S, respectively, where σ is the standard deviation of the response, and S is the slope of the calibration curve (ICH, 1996). In this experiment, 24 blank reagent samples were measured, standard deviations were calculated to determine σ, and 3 standard curves were drawn to determine the S. DL and QL were calculated based on these parameters.

Stability

The honey sample solution was prepared, and its absorbance was measured every 1 h continuously for 8 h. The stability of the sample solution was evaluated by calculating the relative standard deviation (RSD, %).

Repeatability

Six independently prepared honey solutions from the same homogeneous sample were measured under the same operating conditions in one day. The absorbance value was determined with the optimised conditions, and the repeatability was expressed as the relative standard deviation (RSD, %).

Precision

The precision of the modified F-C assay was evaluated by intraday and interday precision. In order to determine the intraday precision of honey, the honey solution was measured six times in one day under the same conditions. Interday precision was established similarly but measured for three consecutive days. All the data obtained were expressed as the relative standard deviation (RSD, %).

Recovery rate

Three known standard solutions of gallic acid with high, medium, and low concentrations were added to pre-analysed honey samples for re-analysis. The recovery rate per addition level was expressed as a percentage (%).

Application of the method

The modified F-C assay was used to determine the total phenolic content of 33 honey samples from 9 botanical origins and 12 geographical origins, and then the means and standard deviations were calculated based on the obtained data.

Statistical analysis

IBM SPSS Statistics software (version 24.0) was used for statistical analysis of the obtained data. Differences between the original F-C assay and modified F-C assay were tested using one-way ANOVA and considered to be significant when $p < 0.05$.

Results and discussion

Optimisation and modification of the F-C assay

Based on the original F-C assay (Cao and Suo, 2003), optimised protocols were carried out focusing on the absorption wavelength, incubation time, sodium carbonate solution, and F-C reagent volume.

The absorption spectrum of the reaction was measured between 600 - 800 nm. As shown in Figure 1(a), the maximum absorption peak appeared at 756 nm. It has been reported that phenolic compounds are determined spectroscopically at approximately 760 nm (Singleton and Rossi, 1965; Singleton *et al.,* 1999); 756 nm is close to 760 nm, and was chosen as the wavelength for subsequent experiments.

The absorbance at 756 nm was recorded every 20 min to select the optimum reaction time of the modified F-C assay. As shown in Figure 1(b), after incubation in the dark for 80 min, the reaction reached a plateau, and there was no significant difference in absorbance for approximately 80 min. To ensure completion of the reaction, the slightly longer incubation period (80 min) was selected as the reaction time. This time is longer than 60 min, which was chosen as the incubation time previously (Cao and Suo, 2003).

Sodium carbonate makes the solution system alkaline, which is conducive for the reaction. As shown in Figure 1(c), the maximum absorbance was recorded when the ratio of sample to sodium carbonate solution was 1:2; thus, this was considered the optimum ratio.

The F-C reagent is an indicator of the reaction. In the alkaline solution, the phenolic compounds are quantitatively oxidised by molybdate, and reduced to form a blue colour complex. The depth of the blue colour is proportional to the content of polyphenols, and a deeper colour indicates a higher absorbance and phenolic acid content (Folin and Denis, 1912; Singleton and Rossi, 1965; Singleton *et al.,* 1999). As shown in Figure 1(d), the maximum absorbance appeared when the sample and F-C reagent were added in a ratio of 1:5.

The final optimised procedure of the modified F-C method was as follows: the honey sample was diluted by 1:10 (w/v), then 20 μL of honey sample solution, 100 μL of F-C reagent, and 40 μL of 1 M sodium carbonate solution were added to a 96-well plate, and finally 40 μL of water was added. The absorbance of the mixture was measured at 756 nm using an automated microplate reader after being mixing well and 80 min of incubation in the dark.

Validation of the method

Validation of the analytical method is an important step to establish a new method. As many

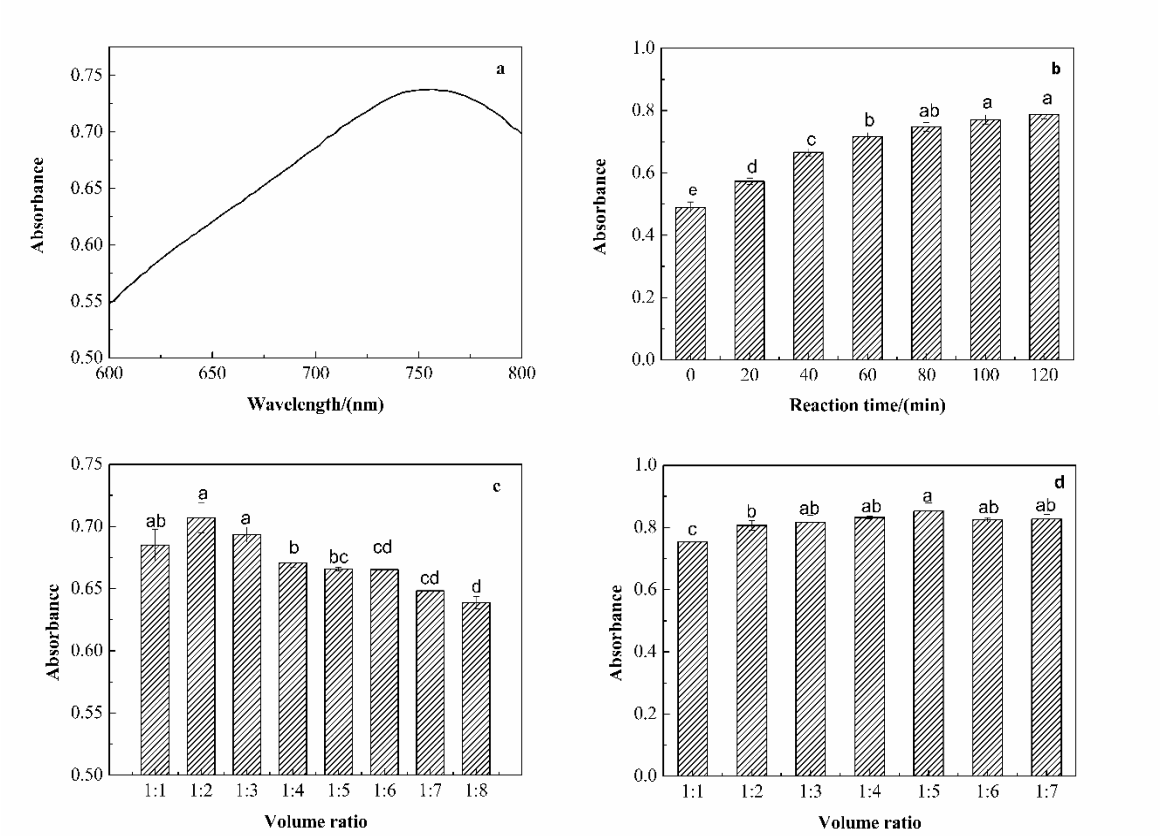

Figure 1. (a) Absorption spectrum from 600 - 800 nm, (b) the effect of reaction time on absorbance, (c) influence of sodium carbonate on absorbance, and (d) the effect of F-C reagent on absorbance.

validation methodologies have been issued by specific committees and regulatory bodies (Chandran and Singh, 2007), the relatively uniform criterion of ICH was followed.

Calibration and linearity

Before the quantitative analysis of the total phenolic content in honey, a linear relationship between the concentration of gallic acid and the absorbance of honey samples at 756 nm was established by the least squares method. The regression line fits equation $y = 6.8422x + 0.0949$, and the coefficient of determination (R^2) was 0.9982. This result shows a good linear relationship within the measured concentration range (0.01 - 0.1 mg•mL^{-1}).

Determination of the detection and quantitation limits

The DL and QL were calculated based on the standard deviation of the blank reaction to evaluate the sensitivity of the modified F-C assay. The DL and QL values of the modified method were found to be 8.61 ± 0.33 and 26.09 ± 0.99 mg GAE•kg^{-1} honey (0.86 and 2.61 μg•mL^{-1}, respectively). For the comparison of DL and QL values with other studies, the values obtained in the present work are similar to the F-C method reported by Bobo-García *et al.* (2015) (0.74 and 2.24 μg•mL^{-1} for DL and QL, respectively). This indicates the high sensitivity of the modified F-C method.

Stability, repeatability, and precision

Honey sample S17 was used to evaluate the stability of the method. The sample solution was analysed for 8 h within one day, and the results showed that the relative standard deviation was 3.98%, indicating that the sample solution was stable for 8 h.

Six solutions of the same honey sample were tested, and the repeatability of the method was expressed as the relative standard deviation. The results showed that the relative standard deviation was 2.24%, which was within acceptable limits considering that the food matrix is often complex.

The precision of the method was evaluated by intraday and interday precision. Honey sample S17 was used to evaluate the precision of the method. Six independently prepared honey solutions were analysed to evaluate the intraday precision, and the results showed an acceptable RSD (2.52% for the first day, 1.85% for the second day, and 4.14% for the third day), and the interday RSD was 2.90%, exhibiting the satisfactory precision of the modified F-C assay. All RSD data obtained were in compliance with the Food

and Drug Administration (FDA)'s Bioanalytical Method Validation, where an RSD value less than 15% is recommended for precision investigations (FDA, 2013).

Recovery

The recovery rate of the modified microdetermination method was evaluated by using randomly selected honey sample S17. Three known quantities of gallic acid standard solutions with different concentrations (0.02, 0.04, and 0.06 mg•mL^{-1}) were added to S17 and then re-analysed. As shown in Table 1, the average recoveries of the honey samples with different concentrations ranged from 94.05 to 107.32%. The relative standard deviations of all samples were low (less than 3.12%), and the results were acceptable. The above results indicate that the modified F-C method was reliable and can be applied to routine analysis.

Comparison between the modified F-C method and the original F-C method

The original F-C method followed the method described by Cao and Suo (2003). Briefly, the honey was diluted 10-fold (w/v) with ultrapure water. One millilitre of the diluted honey solution was transferred into the test tube, and 1 mL of Folin-Ciocalteu reagent and 5 mL of 1 mol L^{-1} sodium carbonate solution were added. Finally, 3 mL of ultrapure water was added to the tube to ensure that the total volume of the final mixture was 10 mL. The absorbance of the mixture was measured at 760 nm using a visible spectrophotometer (722G, INESA Analytical Instrument Co. Ltd., Shanghai, China) after being mixing well and incubated in the dark for 60 min. Four random samples, namely, S2, S6, S8, and S17 were selected, and their total phenolic content was measured by the original F-C assay and the current F-C assay. Data were analysed by SPSS (version 24.0). As shown in Table 2, there was no significant difference between these two methods in the determination of the four honey samples. This result indicated that the modified F-C method was suitable for total phenolic content measurements and could replace the original F-C assay. When compared with the original F-C method, the

Table 1. Analytical results of recovery rate.

Sample No.	Amount added (mg GAE·kg^{-1} honey)	Amount found (mg GAE·kg^{-1} honey)	Recovery (%)	Average (%)	RSD (%)
1	200	605.74	109.99		
2	200	592.87	103.55		3.13
3	200	602.63	108.43		
4	400	794.60	102.21		
5	400	788.94	100.79	100.80	1.07
6	400	786.11	100.08		
7	600	953.32	95.59		
8	600	950.35	94.10		0.60
9	600	946.53	93.46		

Table 2. The comparison between current F-C assay and original F-C assay.

No.	Floral origin	Total phenolic content/(mg/kg)	
		current assay	original assay
S8	*Lycium barbarum* L.	166.97 ± 4.95^{a}	166.01 ± 5.36^{a}
S2	*Tilia amurensis* Rupr	310.20 ± 6.06^{a}	331.74 ± 17.22^{a}
S17	*Robinia pseudoacacia* L.	414.70 ± 5.19^{a}	422.25 ± 8.80^{a}
S6	*Ziziphus jujuba* Mill	228.75 ± 10.02^{a}	242.54 ± 8.17^{a}

Values are means ± standard deviations of triplicates (n = 3). Values with different superscripts within a row are significantly ($p < 0.05$) different from each other.

Table 3. Total phenolic content in honey samples.

Assigned No.	Floral origin	Geographical origin	Total phenolic content (mg GAE·kg^{-1})	RSD (%)	Ranges (mg GAE·kg^{-1})
S1	*Lycium barbarum* L.	Ningxia	460.71 ± 9.49	2.84	
S2	*Lycium barbarum* L.	Ningxia	310.20 ± 4.32	0.97	310.20 - 467.11
S3	*Lycium barbarum* L.	Ningxia	467.11 ± 13.08	3.85	
S4	*Tilia amurensis* Rupr.	Heilongjiang Raohe	201.58 ± 9.03	4.48	
S5	*Tilia amurensis* Rupr.	Jilin Yanbian	279.29 ± 10.75	3.85	201.58 - 279.29
S6	*Tilia amurensis* Rupr.	Heilongjiang Hulin	228.75 ± 11.23	4.91	
S7	*Robinia pseudoacacia* L.	Gansu Qingshui	166.97 ± 6.81	4.08	
S8	*Robinia pseudoacacia* L.	Shaanxi Luochuan	166.45 ± 4.95	2.97	162.48 - 174.38
S9	*Robinia pseudoacacia* L.	Shaanxi Yongshou	174.38 ± 8.22	4.71	
S10	*Robinia pseudoacacia* L.	Shaanxi Longxian	162.48 ± 3.63	2.23	
S11	*Brassica campestris* L.	Sichuan Zigong	315.27 ± 8.78	2.79	
S12	*Brassica campestris* L.	Sichuan Meishan	275.00 ± 13.42	4.88	275.00 - 347.24
S13	*Brassica campestris* L.	Shaanxi Jiaxian	347.24 ± 8.28	2.38	
S14	*Ziziphus jujuba* Mill	Shaanxi Yulin	332.67 ± 8.51	2.56	
S15	*Ziziphus jujuba* Mill	Shaanxi Dali	569.95 ± 2.34	0.41	332.67 - 569.95
S16	*Ziziphus jujuba* Mill	Shaanxi Jiaxian	486.87 ± 21.28	4.37	
S17	*Ziziphus jujuba* Mill	Shaanxi Fugu	391.99 ± 4.94	1.26	
S18	*Dimocarpus longan* Lour.	Guangxi	553.63 ± 10.03	2.35	
S19	*Dimocarpus longan* Lour.	Fujian Zhangzhou	514.27 ± 5.66	1.46	474.91 - 553.63
S20	*Dimocarpus longan* Lour.	Guangxi Lingshan	474.91 ± 9.40	2.70	
S21	*Dimocarpus longan* Lour.	Guangxi Guiping	529.09 ± 10.09	2.51	
S22	*Fagopyrum esculentum* MoenCh.	Shaanxi Jingbian	817.15 ± 22.67	3.29	
S23	*Fagopyrum esculentum* MoenCh.	Sichuan Xichang	1717.71 ± 56.87	3.55	817.15 - 1740.13
S24	*Fagopyrum esculentum* MoenCh.	Ningxia Yinchuan	1553.66 ± 37.13	2.60	
S25	*Fagopyrum esculentum* MoenCh.	Gansu Tianshui	1740.13 ± 52.34	3.25	
S26	*Litchi chinensis* Sonn.	Hainan	517.19 ± 10.40	2.67	
S27	*Litchi chinensis* Sonn.	Hainan	475.74 ± 11.18	3.21	417.73 - 517.19
S28	*Litchi chinensis* Sonn.	Guangzhou	417.73 ± 10.04	3.46	
S29	*Litchi chinensis* Sonn.	Guangxi	430.19 ± 3.86	1.28	
S30	*Vitex negundo* L.	Shanxi Pingding	442.30 ± 2.94	0.93	
S31	*Vitex negundo* L.	Hebei Zanhuang	451.63 ± 11.65	3.59	415.40 - 451.63
S32	*Vitex negundo* L.	Shanxi Yangquan	416.72 ± 9.75	3.37	
S33	*Vitex negundo* L.	Henan Huixiann	415.40 ± 7.63	2.65	

Values are means ± standard deviations of triplicates (n = 3).

modified F-C method has the advantages of high throughput, small scale, simple operation, and low cost.

Applications of the method

The applicability of the current F-C assay was evaluated by the measurement and statistical analysis of total phenolic content in 33 honey samples from 9 kinds of monofloral honey (wolfberry, linden, acacia, rape, jujube, longan, buckwheat, lychee, and wattle honey) from 12 geographical regions. As shown in Table 3, the total phenolic content of honey samples from different floral and geographical origins significantly varied. Among all the samples tested, buckwheat honey recorded the highest total phenolic content (the range was 817.15 - 1740.13 mg GAE•kg^{-1} honey), while the acacia honey samples displayed the lowest total phenolic content (the range was 162.48 - 174.38 mg GAE•kg^{-1} honey).

The total phenolic content and antioxidant ability of honey mainly depend on botanical origin, which is primarily affected by environmental and climatic conditions (Dezmirean *et al.,* 2012; Oelschlaegel *et al.,* 2012). Particularly, areas characterised by hot and humid climates had a significant impact on the total phenolic content of the plants due to high exposure to sunlight. Therefore, the total phenolic content of plants exposed to sunlight is much higher than that of the same species grown in the shade (Tenore *et al.,* 2012). In addition, food processing, treatment, and storage also affected the total phenolic content in honey, thereby affecting the antioxidant capacity of honey (Wang *et al.,* 2004; Turkmen *et al.,* 2006; Saxena *et al.,* 2010; Escriche *et al.,* 2014). Accordingly, the total phenolic content of honey is an important index to evaluate the quality of honey and distinguish the botanical and geographical origins of honey (Bertoncelj *et al.,* 2007; Saxena *et al.,* 2010).

Conclusion

In the present work, a modified F-C assay for the microdetermination of total phenolic content in honey was established. The linearity (R^2 = 0.9982), DL (8.61 ± 0.33 mg GAE•kg^{-1} honey), QL (26.08 ± 0.99 mg GAE•kg^{-1} honey) and recovery rate (93.46 - 109.99%) were acceptable. The results proved that the modified method was fast, sensitive, accurate, precise, and repeatable. In addition, the modified F-C assay had no significant effect on the determination of total phenolic content in four randomly selected honey samples when compared with the original F-C assay. The newly optimised method was successfully applied to 33 honey samples from 9 botanical origins and 12 geographical origins. In conclusion, the proposed high-throughput F-C method presented in the present work is low-cost, easy to run, and could be used for routine analysis.

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