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MATERIALS CHEMISTRY | RESEARCH ARTICLE

Rapid and sensitive determination of polyphenols composition of unifloral honey samples with their antioxidant capacities

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Abstract: A micellar electrokinetic chromatography method was developed to quantify the levels of four main polyphenols in various floral sources honeys. The effect of several parameters—such as pH, sodium dodecyl sulfate (SDS) of the buffer, separation voltage and injection time—were systematically investigated. The main polyphenols were successfully separated within 13 min of UV detection at 214 nm. In the tested concentration range, regression equations revealed good linear relationships between the peak areas and corresponding concentration (correlation coefficients ranged from 0.9984 to 0.9997). Moreover, the 1,1-diphenyl-2-picryl-hydrazyl (DPPH) radical-scavenging activities were also quantified. These compounds are not present in large amounts, but can reach up to 2758 μg in 100 g^{-1} of honey, which are the main flavonoid aglycones found in honey. The flavonoid glycosides are hydrolyzed by bee enzymes to render the aglycones. Therefore, the flavonoid aglycones should be served as a potential marker and capillary electrophoresis could be used as an effective way to look for in various floral sources.

Subjects: Analytical Chemistry; Food Chemistry; Food Science & Technology

Keywords: polyphenols; honey; capillary electrophoresis; DPPH; potential marker

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PUBLIC INTEREST STATEMENT

Recently, much attention has been paid to honey for its antioxidants and antimicrobial activities for eliminating free radical activities. The main compounds that have those activities are flavonoids of honey. Therefore, the flavonoid aglycones should be served as a potential marker and capillary electrophoresis could be used as an effective ways to look for in various floral sources honeys. As well as, a rapid and accurate method for detection is necessary.

1. Introduction

Honey is a traditional natural health food composed mainly of fructose and glucose. It also contains a wide range of minor constituents such as minerals, proteins, vitamins, organic acids, enzymes, and phenolic compounds (Alvarez-Suarez, Tulipani, Romandini, Bertoli, & Battino, 2010). Considerable attention has been paid to honey for its antioxidants and antimicrobial activities (Alvarez-Suarez, Tulipani, Romandini, Vidal, & Battino, 2009; Blasa, Candiracci, Accorsi, Piacentini, & Piatti, 2007; Kishore, Halim, Syazana, & Sirajudeen, 2011; Lachman, Orsák, Hejtmánková, & Kovářová, 2010), for eliminating free radical activities (Allen, Molan, & Reid, 1991; Boorn et al., 2010; Estevinho, Afonso, & Feás, 2011). The phenolic compounds in honey also exhibit potential activity as a useful adjuvant agent for the treatment of fungal infections (Candiracci, Citterio, & Piatti, 2012). The phytochemical composition of honey depends on the floral origin, the type of honeybee subspecies (Truchado, Ferreres, & Tomas-Barberan, 2009), and external factors such as climate and geographical origin (Gheldof, Wang, & Engeseth, 2002). Therefore, honey from various floral sources are appreciated by the consumers, for their characteristic sensory properties and protective effect on human beings (Arráz-Román, Gómez-Caravaca, Gómez-Romero, Segura-Carretero, & Fernández-Gutiérrez, 2006; Bravo, 1998; Hertog, Feskens, Hollman, Kromhout, & Karan, 1993). It was reported that nectar glycosides were hydrolyzed by bee enzymes to render the aglycones which were detected in honey (Ferreres, García-Viguera, Tomás-Lorente, & Tomás-Barberán, 1993; Gil, Ferreres, Ortiz, Subra, & Tomas-Barberan, 1995; Martos, Ferreres, & Tomás-Barberán, 2000; Truchado, Ferreres, Bortolotti, Sabatini, & Tomás-Barberán, 2008). Therefore, isolation of these minor constituents should be carried out in order to confirm the nature and quality of honey. This is a very difficult task due to the small amount of these markers in honey.

Several analytical methods were explored for determination of both the botanical and geographical origins of honey, including gas chromatography (Odeh et al., 2007), high-performance liquid chromatography (Inoue et al., 2005; Tomás-Barberán, Martos, Ferreres, Radovic, & Anklam, 2001), and capillary electrophoresis (CE) (Delgado, Tomás-Barberán, Talou, & Gaset, 1994; Domínguez-Álvarez, Rodríguez-Gonzalo, Hernández-Méndez, & Carabias-Martínez, 2012; Maria Rizelio et al., 2012). CE is advantageous in its good resolution, automation, simplicity, short analysis times, low consumption of chemicals, and reduced sample preparation (Rizelio et al., 2012; Sánchez-Hernández, Puchalska, García-Ruiz, Crego, & Marina, 2010). In addition, various modes of operation, for example capillary zone electrophoresis, micellar electrokinetic chromatography (MEKC), etc. are available to separate the diverse analytes present in food stuffs, thus making CE a truly powerful tool in food analysis.

The aim of the present study was to investigate concentration of polyphenols and the antioxidant activities of the various floral sources. The compounds of interest in this work were ferulic acid, kaempferol, apiolin, and luteolin. These polyphenols were selected because of their suggested health beneficial properties. A rapid and simple method for the simultaneous determination of aglycones and phenolic compounds in honey without the handicap of sugar interferences has been developed.

2. Materials and methods

2.1. Chemicals and apparatus

All the analytical standards (ferulic acid, kaempferol, apiolin, luteolin) were purchased from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Purified water (Wahaha Group Corporation, China), 1,1-diphenyl-2-picrylhydrazyl radical (DPPH, Sigma-Aldrich, Italy), sodium dodecyl sulfate (SDS), methanol, acetonitrile, n-butanol, sodium dihydrogen phosphate (NaH_2PO_4), and sodium borate ($\text{Na}_2\text{B}_4\text{O}_7$) (Tianjin Chemical Reagents Co., Ltd, China) were used for the study. All reagents were of analytical grade. Electropherograms were collected and plotted by the data acquisition system, Agilent Technologies Chem Station for CE (Agilent Technologies, 7100 Capillary Electrophoresis, USA). A Sartorius Basic pH Meter (PB-10, Sartorius AG, Germany) was used for adjusting the value of pH. A fused silica capillary (Yongnian Optical Fiber Factory, China) of 48.5 cm (effective length 40 cm) \times 75 μm i.d. was used for separation. UV absorbances of DPPH were recorded with Lambda 35 spectrophotometer (Perkin Elmer).

Table 1. The informations of honey samples

Samples	Floral origin	Location
Acacia honey-JT	<i>Flos Sophorae</i>	Jingtai-China
Acacia honey-SX	<i>Flos Sophorae</i>	Shanxi-China
Acacia honey-LZ	<i>Flos Sophorae</i>	Lanzhou-China
Jujube flower-LZ	<i>Fructus Jujubae</i>	Lanzhou-China
Litchi-GX	<i>Litchi chinensis</i>	Guangxi-China

2.2. Preparation of standard solution and samples

Stock solutions of ferulic acid, kaempferol, apiolin, and luteolin were prepared in methanol and stored at 4°C. Working standards were freshly prepared by appropriate dilution of the stock solutions to final concentrations in the ranges of 1.00–100 µg mL⁻¹ for ferulic acid, 1.50–150 µg mL⁻¹ for kaempferol, 1.13–227 µg mL⁻¹ for apiolin, and 1.05–210 µg mL⁻¹ for luteolin. About 10 g of the semi-solid honey samples was dissolved in 10 mL purified water, then simply extracted with n-butanol three times. The combined extract solution was concentrated to a final volume of 10 mL. The sample solution was filtered through a 0.45 µm membrane filter before analysis. The botanical origins were: jujube flower, litchi, and acacia honey (Table 1).

2.3. Separation conditions of CE

The new capillary was pretreated with methanol (5 min), purified water (5 min), 1 M NaOH (5 min), 1 M HCl (5 min), and purified water (5 min). Prior to daily use, the capillary was conditioned with 0.1 M NaOH for 5 min, purified water for 5 min, and running buffer for 5 min. Sample injection was injected at 50 mbar for 5 s. All standards and honey samples were injected in triplicate.

2.4. DPPH assay

Stock solution of 0.025 mg mL⁻¹ DPPH was prepared in methanol. The absorbance of the solutions was measured at 517 nm. The calibration curve was constructed by plotting the absorbance of DPPH against the corresponding different concentrations. About 2 mL honey or methanol was mixed with 2 mL of 0.025 mg mL⁻¹ DPPH. The mixtures were shaken vigorously and placed in the dark at room temperature for 30 min. The reduction of the DPPH radical was determined by measuring absorbance at 517 nm. The radical scavenging was expressed as inhibition percentage and calculated using the following equation: $RS (\%) = (1 - (A_i - A_j/A_c)) \times 100\%$, where A_c is the absorbance of DPPH, A_j is the absorbance in the presence of honey sample, and A_i is the absorbance of mixture of DPPH and honey samples. The IC_{50} was determined as the concentration of the tested honey sample causing 50% reduction of the initial DPPH concentration.

3. Result and discussion

3.1. Development of CE method

The effect of the concentration of the borate-phosphate mixture buffer was investigated. A series of 12.5, 15, 17.5, and 20 mM borates were mixed with 0, 5, 10, 15, and 20 mM phosphates, respectively. Better resolution was obtained in lower concentration of borate (Figure 1). The best separation was achieved in 10 mM NaH₂PO₄ (Figure 2). Consequently, 12.5 mM Na₂B₄O₇₋₁₀ mM NaH₂PO₄ buffer was employed for subsequent optimization.

The effects of pH of buffer on electrophoretic separation were evaluated in the range of 8.8–10.0. It can be seen that the migration time of all analytes increased with increasing pH of BGE (Figure 3). pH 9.3 was selected due to good resolution and short analysis time. The effect of SDS concentration on the separation was investigated in the range of 5–20 mM (Figure 4). Good separation was found when the SDS concentration was higher than 10 mM, whereas a further increase of the SDS

Figure 1. Effect of concentration of $\text{Na}_2\text{B}_4\text{O}_7$ on migration time.

Notes: pH of the buffer was 9.3 which contained 10 mM NaH_2PO_4 and 10% (v/v) methanol. Voltage 20 kV, 20°C, detection 214 nm, sample injection time 5 s with 50 mbar pressure.

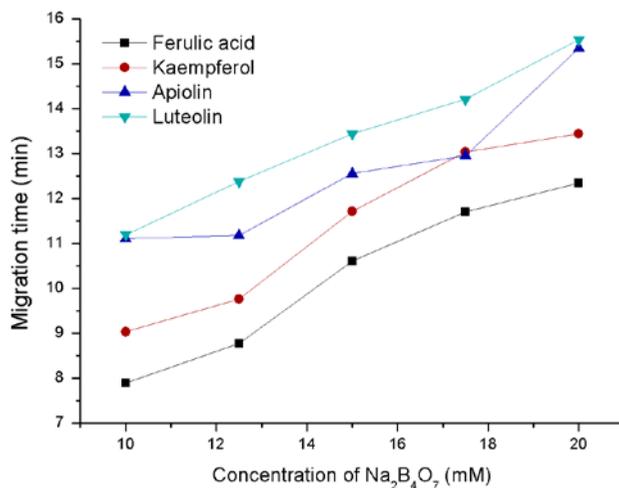


Figure 2. Effect of concentration of NaH_2PO_4 on migration time.

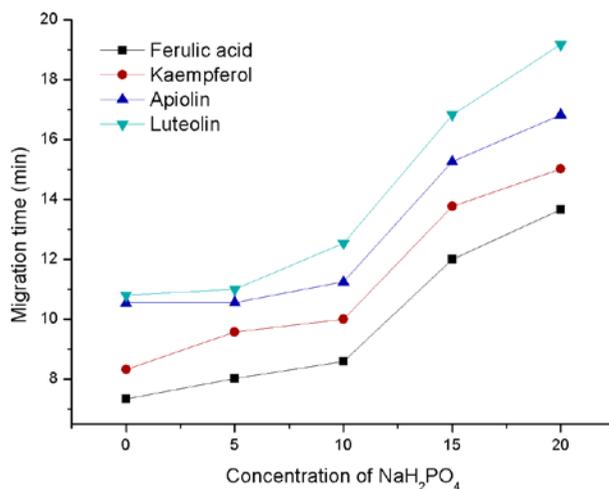


Figure 3. Effect of pH on migration time.

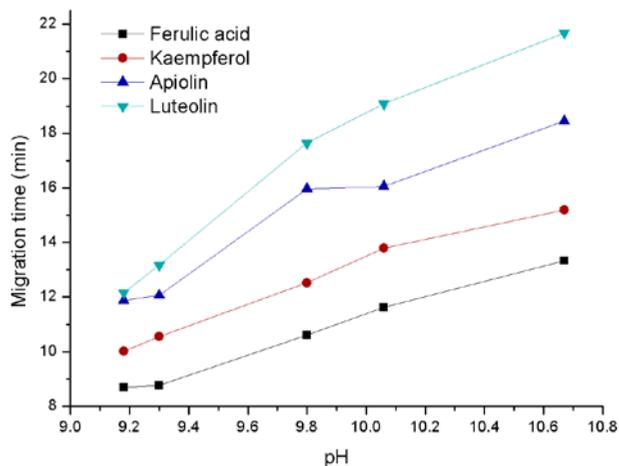
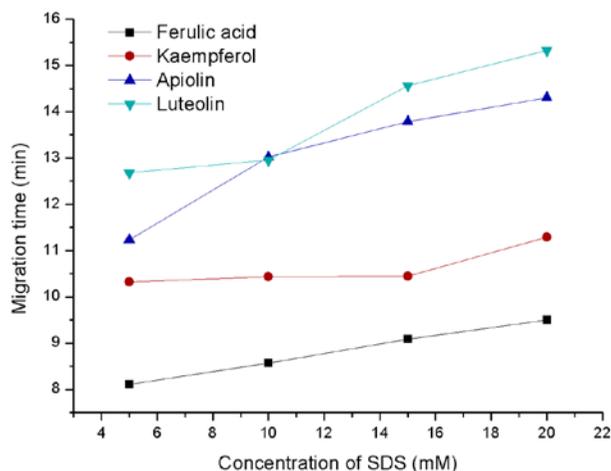


Figure 4. Effect of concentration of SDS on migration time.

Notes: Analytical conditions: pH of the buffer was 9.3 which contained 12.5 mM $\text{Na}_2\text{B}_4\text{O}_7$, 10 mM NaH_2PO_4 and 10% (v/v) methanol.



concentration resulted in a longer analysis time. Therefore, 15 mM SDS was chosen as the optimum concentration.

The addition of methanol or acetonitrile to the electrolyte (5–20%) was investigated, and the best separation was achieved in 10% (v/v) methanol. The effect of applied voltage (12–25 kV) was also investigated. The sharper peaks were obtained with good resolution with 20 kV under 100 mA system current.

3.2. DPPH radical-scavenging activity

There were significant differences among the honey samples in terms of their radical-scavenging abilities expressed as IC_{50} of the DPPH and radical-scavenging activities (RS) (Figure 5). Among the honey extracts tested, the lowest IC_{50} value was shown by jujube honey-Lanzhou (5.95 mg mL^{-1}), while the highest value was found in acacia honey-Jintai (15.05 mg mL^{-1}). The reasons behind the markedly higher RS capacity exhibited by different types of honey are probably due to their diverse botanical origins.

3.3. Method validation

Typical electropherogram of standards was shown in Figure 6. The proposed method was evaluated in terms of linearity, precision, apparent recovery, limit of detection (LOD), and limit of quantification (LOQ). The calibration curve was constructed by plotting the peak area of analytes against the concentrations of analytes. The analytical curves were constructed from five standard solutions in the concentration range of 1.0–227 $\mu\text{g mL}^{-1}$, with a triplicate injection at each concentration level. A

Figure 5. Scavenging abilities of honey samples expressed as IC_{50} of the DPPH and radical-scavenging activities.

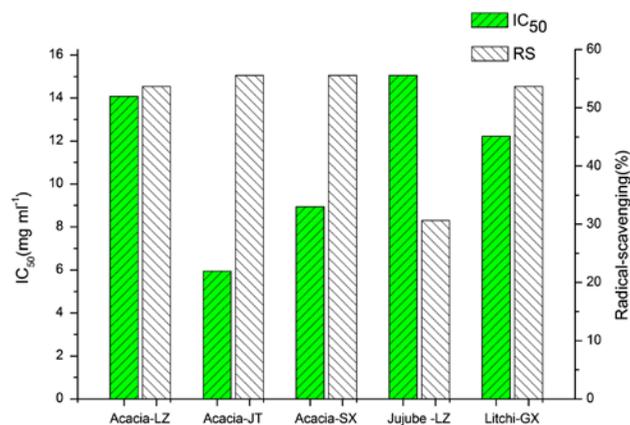
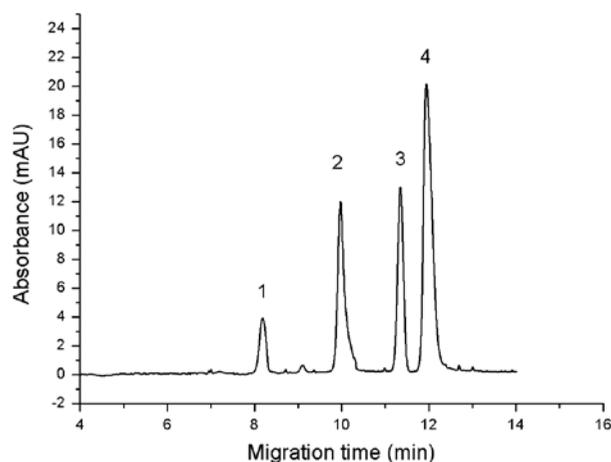


Figure 6. MEKC electrophoregram of standard mixture.

Notes: Concentration of standards were 20 $\mu\text{g mL}^{-1}$ for ferulic acid (1), 30 $\mu\text{g mL}^{-1}$ for kaempferol (2), 22.6 $\mu\text{g mL}^{-1}$ for apiolin (3), 21 $\mu\text{g mL}^{-1}$ for luteolin (4). Analytical conditions: Voltage 20 kV, 20°C, detection 214 nm, sample injection time 5 s with 50 mbar pressure. The buffer contained 12.5 mM $\text{Na}_2\text{B}_4\text{O}_7$, 10 mM NaH_2PO_4 , 15 mM SDS and 10% (v/v) methanol with pH 9.3.



linear relationship between the peak area and concentration of the analytes was obtained with a satisfactory coefficient of determination and intercepts close to the origin. The LODs ranged from 0.26 to 0.75 mg L^{-1} , which are sufficient to allow for quantification of low concentrations of flavonoids found in acacia honey and other honey samples (Martos et al., 2000; Truchado et al., 2008).

Table 2. Calibration plots, LOD, LOQ, and precision of the proposed method ($\mu\text{g mL}^{-1}$)

Analytes	Regression equation $y = ax + b$	r	Linear range	LOD	LOQ	RSD ₁ (%)	RSD ₂ (%)
Ferulic acid	$y = 1.206x + 0.52$	0.9984	1.00–100	0.26	0.81	0.51	2.51
Apiolin	$y = 7.337x - 11.02$	0.9991	1.13–227	0.75	2.24	0.92	1.02
Luteolin	$y = 9.741x - 7.109$	0.9997	1.05–210	0.47	1.41	0.71	2.33
Kaempferol	$y = 25.95x - 31.34$	0.9989	1.50–150	0.40	1.32	0.68	1.74

Notes: y and x are, respectively, the peak areas and concentration of the analytes. RSD₁ is the RSD of intra-day precision; RSD₂ is the RSD of inter-day precision.

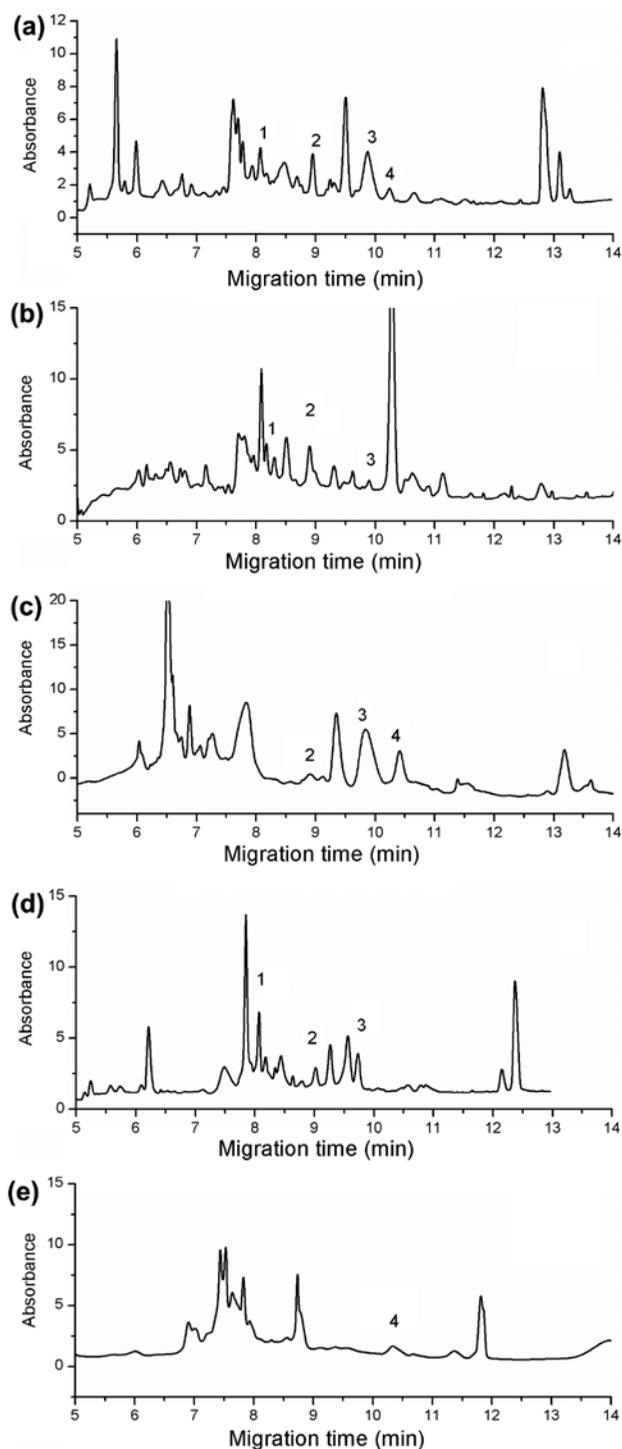
Table 3. Recovery of the proposed method ($\mu\text{g 100g}^{-1}$)

Analytes	Original	Added	Detected	Recovery (%)	RSD (%)
Ferulic acid	32.26	30.00	59.56	91.00	6.35
		32.00	63.09	96.34	6.22
		40.00	72.46	100.50	5.89
Apiolin	58.91	50.00	108.81	99.80	6.40
		60.00	120.00	101.82	5.33
		75.00	134.80	101.19	4.35
Luteolin	46.57	40.50	84.59	95.05	7.22
		54.00	100.35	99.59	6.01
		67.50	113.73	99.50	3.89
Kaempferol	24.03	21.00	43.37	92.10	10.21
		23.30	45.93	93.99	5.41
		31.50	56.03	101.59	6.44

Note: The recovery was tested in acacia-JT and jujube-LZ for luteolin.

Figure 7. Electropherograms of acacia-JT honey (a), acacia-SX honey (b), Litchi-GX honey (c), acacia-LZ honey (d), and jujube-LZ honey (e).

Notes: Concentration of standards were $20 \mu\text{g mL}^{-1}$ for ferulic acid (1), $30 \mu\text{g mL}^{-1}$ for kaempferol (2), $22.6 \mu\text{g mL}^{-1}$ for apiolin (3), $21 \mu\text{g mL}^{-1}$ for luteolin (4). Analytical conditions: Voltage 20 kV, 20°C , detection 214 nm, sample injection time 5 s with 50 mbar pressure. The buffer contained 12.5 mM $\text{Na}_2\text{B}_4\text{O}_7$, 10 mM NaH_2PO_4 , 15 mM SDS and 10% (v/v) methanol with pH 9.3.



The LODs ranged from 0.81 to 2.24 mg L^{-1} . The intra-day and inter-day precision of the assay method are also shown in Table 2. The RSDs of the inter-day and intra-day precision for all the four compounds were below 0.92 and 2.51%, respectively. The recoveries of the analytes at low, medium, and high spiked levels were shown in Table 3. The mean recoveries of the proposed method ranged between 91.00 and 101.82%.

Table 4. Content of the analytes in honey ($\mu\text{g } 100\text{g}^{-1}$)

Analytes	Acacia-SX	Acacia-JT	Acacia-LZ	Jujube-LZ	Litchi-GX
Ferulic acid	18.59	32.26	30.63	53.57	23.60
Apioin	25.52	46.57	31.05	ND	ND
Luteolin	ND	ND	ND	8.01	20.03
Kaempferol	59.57	58.91	75.40	ND	55.96

Note: ND denotes not detected.

3.4. Phenolic compounds contents of analyzed honey samples

The nectar is rich in flavonoid glycosides which can be readily hydrolyzed by the bee saliva glucosidases during the elaboration/ ripening process (Consonni & Cagliani, 2008). Honey samples were analyzed to detect the presence of the phenolic compounds. The electropherograms of honey samples were shown in Figure 7. Results were summarized in Table 4. The highest contents of flavonoids were found in acacia honey-JT honey, and high contents of ferulic acid were detected in acacia honey from Jingtai and Shanxi. The values depend on the floral and geographical origins of the honeys.

3.5. Linear correlation between polyphenols content and DPPH assay

Antioxidant capacity measured with DPPH assays was linearly correlated to total phenols measured with the CE method. The calibration curves were constructed by plotting the total content of four polyphenols components against their IC_{50} . The result of "r" was 0.910 and linear equation was $y = 7.560x + 21.41$. From the linear equation relationship, there were a good linear relationship between DPPH (IC_{50}) and contents of polyphenols.

4. Conclusion

The proposed method allows fast and simple separation of polyphenols in honey samples. Ferulic acid, kaempferol, apioin, and luteolin were selected as markers of honey. This work demonstrated acacia honey having high antioxidant and radical activities because of its richness in phenolic compounds. A good linear relationship exists between DPPH (IC_{50}) and polyphenols content. Unifloral honeys showed the different antioxidant capacities and contents of polyphenols. In conclusion, this method could apply the accurate quantification and fast separation time of honey samples. In addition, polyphenols analysis has proved to be potentially useful for unifloral honey characterization and quality.

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