Fast Separation and Determination of Flavonoids in Honey Samples by Capillary Zone Electrophoresis

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Abstract

Flavonoids have crucial applications in the biological and physiological fields. Honey, as an important sweet food made by bees, is rich in flavonoids. In this paper, the analytical method for flavonoids determination in different sorts of honey from different geographical locations was developed by capillary zone electrophoresis with direct ultraviolet detection. With a running buffer (borate, 20 mmol l^{-1}) at pH of 8.4, four typical flavonoids, rutin, quercetin, kaempferol and myricetin, were separated in five minutes under a applied potential of 25 kV. A linear relationship within the range of 2.0–500 mg l^{-1} was found for these four kinds of flavonoids. Moreover, the detection limits ranged from 1.17 to 1.76 mg l^{-1}. The recoveries lie in the range between 80 %–107 %. The developed method was then used in the separation and determination of flavonoids in real honey samples collected from 12 geographical locations in the Henan Province of China. Rutin was detected in six, and quercetin in eight honey samples, which may be the markers for the identification of honey from different geographical origins.

Keywords

Capillary zone electrophoresis, flavonoids, honey, UV detection

1 Introduction

Honey is an important secondary metabolism product of plants produced by honeybees. It is made up of more than one hundred substances with high nutritional and prophylactic-medicinal value.[1] In ancient times, Egyptians and Greeks usually used honey as a medicine to treat ailments (*e.g.*, stomach ulcers and skin wounds).[2] Moreover, it is one of the most complex foodstuffs produced by nature, and certainly the only sweetening agent that can be used by humans without processing.[3] Due to its sweetness, colour, and flavour, honey was always used as a substitute for sugar, an ingredient or a natural preservative in many manufactured foods. Honey possesses valuable nourishing, healing and prophylactic properties, which mainly originate from its special chemical composition. The content of polyphenolic compounds (*e.g.*, flavonoids and phenolic acids) in honey is strongly affected by floral and geographical origin, as well as by climate characteristics of the site.[2,4] These antioxidants reportedly have a beneficial effect on human health. In addition, honey can be considered a biomarker for environmental pollution, and can accumulatively indicate the level of air, water, plant and soil contamination over the forage area of the bees.[5,6,7] Because of the importance of active ingredients, including natural polyphenols, minerals, amino acids, vitamins *etc*., interest in their identification and quantification in honey samples has significantly increased in recent years.[8,9,10]

Honey is rich in flavonoids. Flavonoids are popular natural antioxidants, which exhibit a wide range of biological effects. Epidemiological investigations have found that they have great potential applications in preventing cardiovascular diseases and even cancer. The dietary significance of honey is mainly due to its ingredients such as flavonol, flavone, anthocyanidin, and organic acid.[1] They can be used as antioxidants with some functions, such as trapping reactive oxygen species, inhibition of enzymes responsible for producing superoxide anions, prevention of the peroxidation process by reducing alkoxyl and peroxyl radicals, and chelation of transition metals involved in processes forming radicals.[1,11,12]

In general, the determination of flavonoid compounds in a sample involves some basic steps, which include isolation from a sample matrix, analytical separation, identification and quantification. The recovery step usually involves solid-phase extraction (SPE), solid phase micro-extraction (SPME) or liquid-liquid micro-extraction (LLME).[13,14,15] Separation is commonly achieved by high performance liquid chromatography (HPLC) or gas chromatography (GC).[16,17] The reversed-phase systems, typically with a C_{18} column and various mobile phases, were usually employed as the common mode of separation. Detection is routinely achieved by ultraviolet (UV) absorption, often involving a photodiode detector and various mass-spectral methods.

Compared to the above analytical methods, CE analysis is much faster with less sample consumption and high efficiency.[18,19,20] The geometric structures of flavonoids are composed of two phenyl rings and a heterocyclic ring, which is the basic structure. There are often different substituents on the basic structure, such as hydroxyl. Herein, typical flavonoids in honey, including rutin, quercetin, kaempferol, and myricetin (shown in Fig. 1) were rapidly separated by capillary zone electrophoresis (CZE) with ultraviolet absorption.

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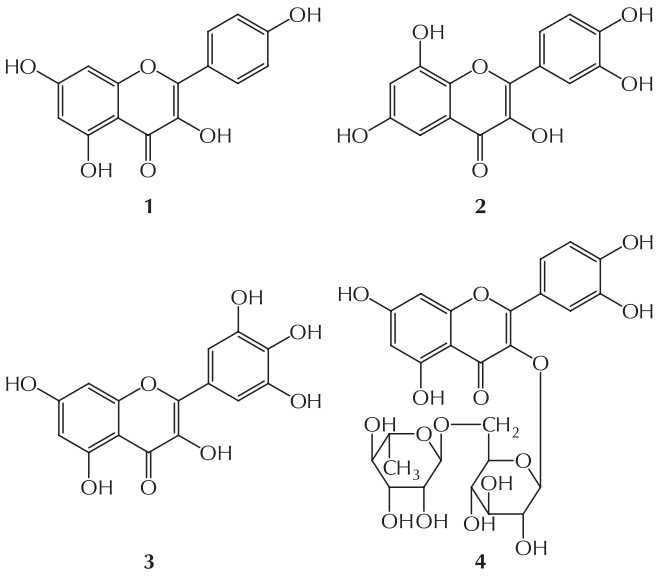

Fig. 1 – Structures of four flavonoids (**1** kaempferol, **2** quercetin, **3** myricetin, **4** rutin

Baseline separation was quickly obtained for the target flavonoids in several minutes using the optimal experimental conditions. In addition, the flavonoid components in honey samples, which were collected in twelve counties on-spot from their local beekeepers in the Henan Province of China were successfully determined using the proposed method.

2 Experimental

2.1 Experimental methods

CE separation was performed with a HP^{3D} CE system with a photodiode array detector (Agilent Technologies, Inc., Walbronn, Germany). An uncoated fused-silica capillary of 50 mm I.D. and 41.5 cm total length (32 cm effective length) was used for the experiment. The capillary was conditioned before each analysis by flushing successively with H_2O, and buffer solution for 3 min, respectively. Samples were injected with pressure at 50 mbar for 15 s, and separated at 7–25 kV. Data in the range of 190–600 nm was acquired and processed with the matching ^{3D}CE ChemStation software. Other instruments were used in the experiments, including ultrasonic cleaning device (KQ5200DE CNC, Kunshan Ultrasonic Instrument Co., Ltd.), electronic analytical balance (BS-124S, Beijing Sartorius Scientific Instruments Co., Ltd.), pH meter (PHSJ-3F, Shanghai Instrument Scientific Instrument Co., Ltd. China), RE-5203 rotary evaporator (Shanghai YaRong Instrument Factory, China), centrifuge (TGL-20B, Shanghai Anting Scientific Instrument Factory, China) *etc*.

2.2 Reagent and materials

Rutin, quercetin, kaempferol, and myricetin were purchased from Aladdin Company (Shanghai, China). Borate and NaOH were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). The methanol was purchased from Kermel Chemical Reagent Co., Ltd. (Tianjin, China). The running buffer solutions were prepared by adding 5 mol l^{-1} NaOH into borate to obtain the desired solution with different pH values. All these solutions were filtered through a 0.22 μm polypropylene filter before use. All reagents were of analytical grade and the water used in this experiment was Milli-Q water.

2.3 Preparation of standard solutions and real samples

A stock solution (1000 mg l^{-1} in ultrapure water) of each flavonoid was prepared daily, stored at 4 °C, and diluted with ultrapure water to give the concentration required for the CE experiments. The concentrations of the mixed standards used for the external calibration ranged between 2.0 and 500 mg l^{-1}. It should be noted that these honey samples were obtained from local beekeepers. The collection of these honeys was organized and carried out by our group members at different blossoming times. The collected honey samples were harvested in April and June 2016, from different locations across the Henan Province of China: Luyi, Tuocheng, Yuanyang, Huaxian, Lingbao, Xiquan, Jiyuan, Huixian, Xicun (Table 1), and stored at 4 °C in a refrigerator prior to analysis.

Table 1 – Geographical distribution of honey samples

Sample	Area	Longitude	Latitude	Species
1	HuaishuliCun	115.49° E	33.95° N	Tong nectar
2	LianglouCun	115.25° E	34.05° N	Acacia honey
3	ZhuzhuangCun	114.19° E	34.97° N	Tong nectar
4	HelinCun	114.49° E	35.27° N	Acacia honey
5	ChenjiapoCun	110.51° E	34.32° N	Tong nectar
6	YuanpingCun	111.47° E	33.14° N	Tong nectar
7	WeiheCun	112.25° E	35.06° N	Rape nectar
8	HuangshuiCun	142.20° E	41.46° N	Vitex honey
9	Su Cun	115.25° E	37.05° N	Acacia honey
10	DongjiangCun	114.32° E	34.67° N	Acacia honey
11	ChenlingCun	110.35° E	32.08° N	Acacia honey
12	XiaoyouheCun	112.45° E	35.04° N	Acacia honey

The pretreatment of real honey samples was performed as follows: The sample was extracted with hydrochloric acid (pH = 2) and centrifuged, then enriched for use. Briefly, take 10.00 g of honey into 100 ml volumetric flask, then add 30 ml HCl (pH = 2), followed by extraction with shaking for about 1 hour. The excessive precipitate was rinsed twice using 5 ml acidulated water. The combined solution was then centrifuged 20 min with a speed of 5000 revolutions *per* minute, followed by the concentration using a rotary evaporator at 40 °C. The final volume was 50 ml by adding methanol into the flask. The resulted solution should be filtered before determination.

2.4 Electrophoretic conditions

The capillary was rinsed with 0.1 mol l^{-1} NaOH, H_2O and running buffer for three minutes, respectively, before the separation operation. The capillary was then filled with buffer solution. The running buffer was driven by an inner pump using 990 mbar gas pressure, and operated by high voltage power supply until the stable baseline was obtained. The injection was performed hydrodynamically at 50 mbar for 15 s. The electro-chromatograms were recorded by the chromatography workstation in real time.

3 Results and discussion

3.1 Optimization of analysis conditions

Fast separation and determination were carried out by optimizing the pH of borate buffer, concentration, applied potential of separation, and temperature of column. As the different pK_b values of the studied flavonoids were not available, their ionization states at different pH values were not known exactly. The previous reports suggested they could be separated as anions. In this work, a borate running buffer was employed. Under CZE mode, flavonoids with a diol group in ring B can yield negatively charged complexes with borate, which can change the migration characters of flavonoids. Even if the flavonoids were charged, the closely similar structures caused difficulties for their separation. In general, flavonoids with multiple hydroxyl groups in ring B yielded negatively charged borate complexes with higher migration times than those with less hydroxyl group in ring B. Therefore, the their migration characters were mainly attributed to the interaction between the concentration of borate solution and the pH value.

The increased pH value can increase the EOF to improve the separation speed. However, it also affects the ionization degree of the hydroxyl groups in the flavonoids that prolongs the migration time. The dependence of the migration times of the four flavonoids on pH was studied with borate buffer. As shown in Fig. 2, the migration time was greatly prolonged with the increase of pH value from 6.0 to 9.0. In the range of pH value from 6.0 to 6.8, myricetin migrated together with quercetin (3 and 4 in Fig. 2). At pH = 9.0, their peaks still partly overlapped. Therefore, pH = 8.4 was chosen as the optimal value for the running buffer in order to obtain baseline separation.

The effect of running buffer concentration on separation was investigated in the range of 5–25 mmol l^{-1} (see Fig. 3). Four kinds of flavonoid were separated completely when the concentrations of running buffer were higher than 15 mmol l^{-1}. However, too high concentration resulted in the decrease of sensitivity, thus, 20 mmol l^{-1} borate was selected as the optimal concentration in the experiment.

Applied potential mainly affected the migration time and peak resolution during separation. The effect of applied potential on separation was studied between 7 kV and 25 kV, and the result is shown in Fig. 4. The migration times were shortened with the increase in applied separation potential from 38 to 5 min. To obtain higher separation resolution and reduce the analysis time, we selected 25 kV as the optimal separation potential.

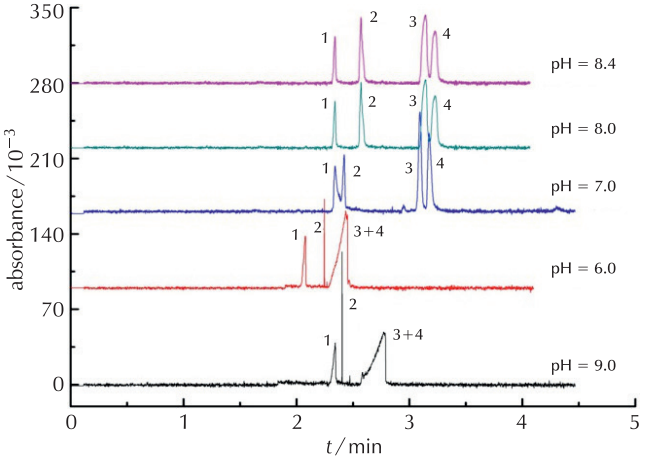

Fig. 2 – Effect of pH value on separation. Peak order: 1 – rutin; 2 – kaempferide; 3 – myricetin; 4 – quercetin. Experimental conditions: the concentrations of four flavonoids were all 250 mg l^{-1}; buffer: 20 mmol l^{-1} borate; applied separation potential 25 kV; injection pressure 50 mbar; injection time 15 s; temperature 25 °C.

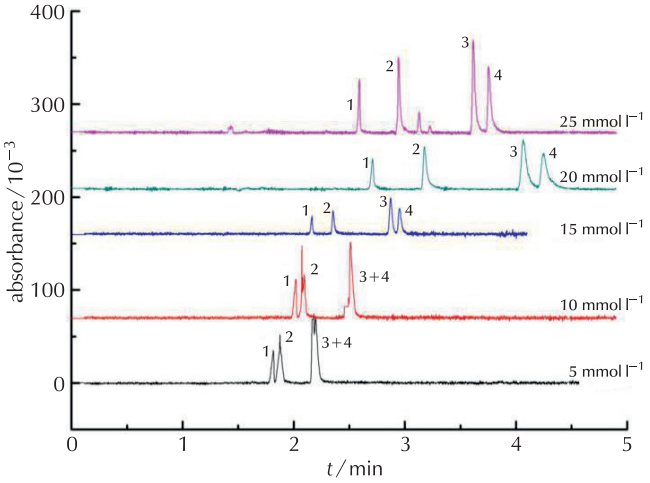

Fig. 3 – Effect of running buffer concentration on separation. Peak order and other experimental conditions are the same as in Fig. 2.

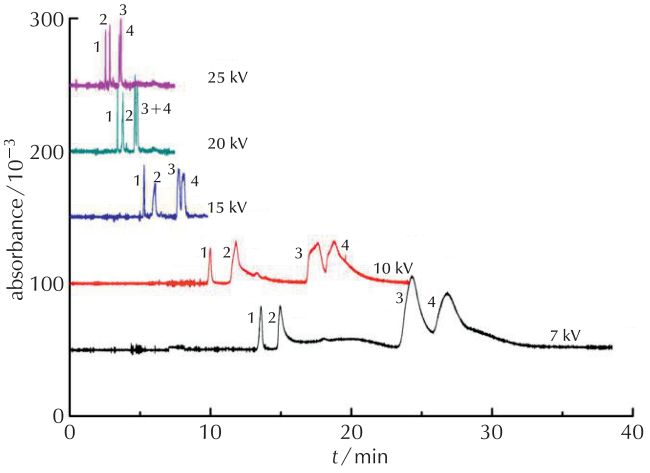

Fig. 4 – Effect of applied separation potential on separation. Peak order and other experimental conditions are the same as in Fig. 2.

Table 2 – Regression equation, correlation coefficient (r^2), linear range, and detection limit

Compound	Regression equation	r^2	Linear range / mg l^{-1}	Detection limit / mg l^{-1}
rutin	$Y = 1.238X + 2.665$	0.9998	2.0–500	1.17
myricetin	$Y = 2.851X + 21.19$	0.9976	2.0–500	1.48
kaempferol	$Y = 2.797X - 14.54$	0.9972	2.0–500	1.76
quercetin	$Y = 4.562X - 10.15$	0.9992	2.0–500	1.28

Y is the peak area; X is the concentration of analytes in mg l^{-1}

It is well-known that viscosity of water decreases with the increase in temperature. The temperature rise results in an increase in some variables, including the EOF, the current and the linear velocity of components. Again, an increasing temperature has a positive effect on column efficiency with the decrease of migration time for each flavonoid. If the temperature is too high, the current is significantly increased, the electrophoretic medium might change. Therefore, the optimal temperature of 25 °C was chosen in the experiments (see Fig. 5).

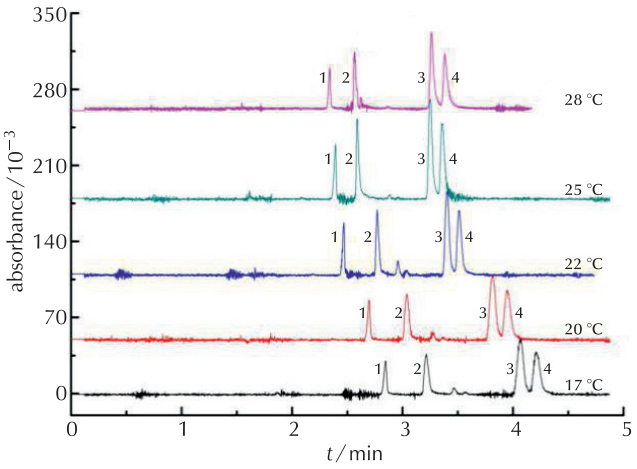

Fig. 5 – Effect of temperature on separation. Peak order and other experimental conditions are the same as in Fig. 2.

Using the optimal conditions, the studied flavonoids could be completely separated within 5 min with the largest theoretic plate number more than 128000/m for myricetin.

3.2 Linear relationship and detection limit

To determine the linear relationship of the four flavonoids, a series of concentrations of mixed standards were tested under the optimized conditions. The detection limits were obtained on the basis of an S/N ratio of 3, and the results are listed in Table 2. All of the concentrations and peak heights showed good linear relationships.

3.3 Application to real honey samples

According to the procedure described above, flavonoids from honey samples 1–12 were extracted, and then separated, respectively, under the optimal conditions.

Table 3 – Content of flavonoids in real honey samples

Sample	Rutin	Myricetin	Kaempferol	Quercetin
	mass fraction / (mg/100 g)			
1	1.64	–	–	1.27
2	–	–	–	1.35
3	1.58	–	–	1.20
4	–	–	–	1.32
5	1.58	–	–	1.26
6	1.59	–	–	1.24
7	1.58	–	–	1.24
8	1.85	–	–	1.42
9	–	–	–	–
10	–	–	–	–
11	–	–	–	–
12	–	–	–	–

– not found

As shown in Table 3, the flavonoid contents in samples of acacia honey 9–12 was found less than the detection limits, only quercetin in samples 2 and 4 was detected with a concentration of 1.35 mg/100 g and 1.32 mg/100 g, respectively. Furthermore, both rutin and myricetin were discovered and determined in the samples 1, 3, 5–8 with concentrations in the range of 1.58 to 1.85 mg/100 g, 1.20 to 1.35 mg/100 g, respectively. It should be noted that the flavonoids composition and content were different due to different honey origins. Both rutin and myricetin were found in real samples of rape nectar, *Vitex* honey and Tong nectar. Flavonoids were detected in methanol abstracts of four types of Sabah wild honey produced by bee species native to Sabah, where the total flavonoid content was found higher in one of the four samples.[21] This is the result

of different botanical resources of the geographical origins, which is similar to the current results.

To verify the reliability of the measuring method, the recovery experiment was performed under the optimal conditions. The recoveries for four flavonoids lied within the range of 80 to 107 %, which suggests that the currently developed method is reliable.

4 Conclusion

A method to separate and determine four flavonoids was established by capillary zone electrophoresis. Baseline separation was obtained within 5 min using simple and cheap buffer solution. It was successfully used to analyse the flavonoids in real honey samples from different geographic origins. Rutin (in six samples) and quercetin (in eight samples) were detected in some of the samples, but myricetin and kaempferol did not exist in all twelve samples. The developed method is simple, sensitive and reliable in the detection of flavonoids. It has potential for application in the analysis of flavonoids in unifloral honey characterization.

List of abbreviations and symbols

CE – capillary electrophoresis

CZE – capillary zone electrophoresis

EOF – electroosmotic flow

t – time, min

ACKNOWLEDGEMENTS

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SAŽETAK

Brzo odvajanje i određivanje flavonoida kapilarnom zonskom elektroforezom u uzorcima meda

Jian-Qiu Tu, Zhong-Yin Zhang, Cheng-Xing Cui, Meng Yang, Ying Li i *Yu-Ping Zhang**

Flavonoidi nalaze važnu primjenu u biološkim i fiziološkim područjima. Med je važno slatko jelo bogato flavonoidima. U ovom radu razvijena je metoda za određivanje flavonoida u medu s različitih geografskih položaja kapilarnom zonskom elektroforezom uz UV detekciju.

Četiri tipična flavonoida (rutin, kvercetin, kempferol, miricetin) odijeljena su za pet minuta u boratnom puferu (20 mmol l^{-1}) pri pH = 8,4 uz primjenu potencijala 25 kV. Za ta četiri flavonoida linearna ovisnost vrijedila je u rasponu od 2,0 do 500 mg l^{-1}. Granica detekcije je između 1,17 i 1,76 mg l^{-1}. Iscrpak je u rasponu 80 – 107 %. Razvijena metoda upotrijebljena je za odvajanje i određivanje flavonoida u uzorcima meda s 12 mjesta u kineskoj provinciji Henan. Rutin je nađen u šest, a kvercetin u osam uzoraka pa bi ti flavonoidi mogli poslužiti za utvrđivanje geografskog porijekla meda.

Ključne riječi
Kapilarna zonska elektroforeza, flavonoidi, med, UV detekcija

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