Analysis for Flavonoids in Bee Pollens by Capillary Electrophoresis

LU Ting[1], Edward P C Lai[2], Jorn C C Yu[2], HU Feng-zu[3]
(1.Qinghai Institute of Supervision and Inspection on Product Quality, Xining 810008, China; 2.Ottawa-Carleton Chemistry Institute, Department of Chemistry, Carleton University, Ottawa, ON K1S 5B6, Canada; 3.Northwest Plateau Institute of Biology, The Chinese Academy of Sciences, Xining 810001, China)

Abstract: A capillary electrophoresis (CE) method has been developed for the determination of six bioactive flavonoids that are commonly found in health foods: hesperidin, hyperin, isorhamnetin, kaempferol, quercetin and rutin. The effects of several parameters, such as pH, buffer concentration, separation voltage and UV detector wavelength, were investigated to find the optimal conditions. Using a H_3BO_3-$Na_2B_4O_7$ buffer (pH 9.2), the analytes can be separated within 8 min. The relative standard deviations of migration times in eight injections were between 0.77% and 0.93%, and those of the peak areas ranged from 3.8% to 8.6%. A high reproducibility and excellent linearity was observed over two orders of magnitude, with detection limits (S/N= 3) ranging from 0.34μg/ml to 2.9μg/ml for all the six analytes. Recoveries ranged from 80.4% to 113.9%. The new method is simple, reproducible and sensitive. No solid phase extraction for sample pretreatment is necessary. Analysis results are accurate in application to bee pollens.

Key words: analysis; flavonoids; bee pollen; capillary electrophoresis

毛细管电泳法分析蜂花粉中黄酮类化合物

卢 挺[1], Edward P C Lai [2], Jorn C C Yu [2], 胡风祖[3]
(1.青海省产品质量监督检验所, 青海 西宁 810008; 2.卡尔顿大学化学系, 加拿大 渥太华 ON K1S 5B6; 3.中国科学院西北高原生物研究所, 青海 西宁 810001)

摘 要：使用毛细管电泳法(CE)检测了健康食品中常见的六种黄酮类化合物：橙皮甙、海棠甙、异鼠李素、山奈酚、槲皮素、芦丁。研究了一系列的试验参数，如：pH、缓冲溶液浓度、分离电压以及UV检测器波长等，以确定出最佳条件。使用H_3BO_3-$Na_2B_4O_7$缓冲液(pH9.2)，各分析物可在8min内分出。相对标准偏差(RSD)：8次进样的迁移时间为0.77%～0.93%；峰面积为3.8%～8.6%；各检测限(S/N=3)范围为0.34μg/ml～2.9μg/ml，回收率为80.4%～113.9%。方法简单、灵敏，重现性高，线性好，无须固相萃取前处理，用于蜂花粉分析结果准确。

关键词：分析；黄酮类化合物；蜂花粉；毛细管电泳
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Bee pollen is a food for young bees. Worker bees collect flower pollens which are the male reproductive cells. They mix the pollens with nectar and put them in special baskets on their hind legs before returning them to the hive for storage[1]. Bee pollen is the most nutritionally rich of all products from the beehive. When compared to any other food, it contains a higher percentage of all necessary nutrients for human survival. It stimulates organs and glands, rejuvenates the human body, enhances vitality, and brings about a long life span. Bee pollen proves to be quite useful for activity enhancement and sports nutrition. It produces an accelerated rate of recovery, including a return to normal heart rate, breathing, and readiness for the next event. It provides energy, stamina, and strength to enhance performance levels[2].

Bee pollens also contain flavonoids[3]. Flavonoids have attracted considerable interest recently because of their beneficial effects on human health. They have anti-viral, anti-allergic, anti-platelet, anti-inflammatory, anti-tumor and anti-

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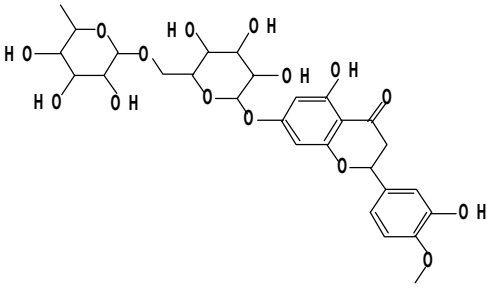
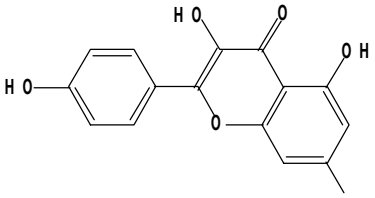
作者简介：卢挺(1954-)，男，研究员，研究方向为分析化学。

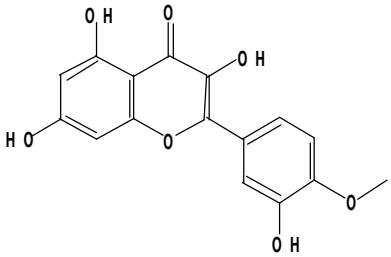
oxidant activities[4]. They are known to be excellent antioxidants and scavengers of oxygen free radicals[5,6]. They maintain the integrity of cells by trapping free radicals that would otherwise cause damage and hasten the aging process. They can help capillary walls become more resistant to harmful substances, they are blood lipid lower agents, and can control serum cholesterol. There are many kinds of flavonoids in bee pollens[3]. Among these compounds are six common bio-active ingredients: hesperidin, hyperin, isorhamnetin, kaempferol, quercetin and rutin. These six flavonoids were chosen in this study because of their proven pharmacological properties[7~9]. Their molecular structures are shown in Fig.1.



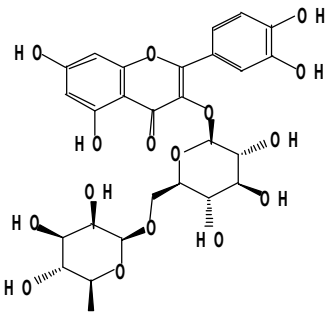
Hesperidin



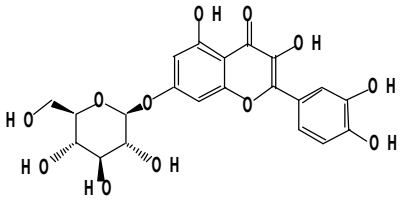
Kaempferol



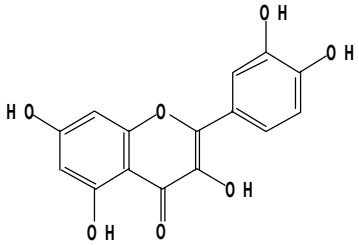
Isorhamnetin



Rutin



Hyperin



Quercetin

Fig.1 Molecular structures of hesperidin, kaempferol, isorhamnetin, rutin, hyperin and quercetin

Many methods have been applied to analyze for flavonoids in health foods. They include high performance liquid chromatography (HPLC)[10~12], thin layer chromatography[13], gas chromatography[14], UV spectrophotometry[15], EPR spectroscopy[16] and cyclic voltammetry[17]. Among these methods, HPLC coupled with UV, photodiode array or mass spectrometric detection is still the most popular analytical technique. However, HPLC has some shortcomings when used for the analysis of bee pollen crude, such as long analysis time, low resolution, and short life span of columns owing to easy contamination. Capillary electrophoresis (CE) is increasingly recognized as an important analytical separation technique because of its fast speed, high efficiency, small sample volume, low solvent consumption, and easy capillary cleaning. So far, CE has not been fully explored for the analysis of active ingredients in bee pollens. In this work, we successively develop a simple, dependable, and sensitive method for the determination of six flavonoids in bee pollens by CE.

1 Experimental

1.1 Instrumental

All CE experiments were performed on a laboratory-built system that included a Spellman CZE1000R (Hauppauge, NY) high voltage power supply, a Polymicro Technologies (Phoenix, AZ) fused silica capillary (50μm i.d., 375μm o.d., 58 cm total length, 50cm effective length), a Bischoff Lambda 1010 (Leonberg, Germany) UV detector, and a SRI 203 Peak Simple (Torrance, CA) chromatography data system. A wash

cycle with 0.1mol/L NaOH for 5min, distilled water for 2min, and running buffer for 5min was necessary to condition the capillary initially. The sample was injected at 15kV for 6s, and a separation voltage of 20kV was applied to generate a field strength of 345V/cm. The temperature was kept at 25℃, and the UV detector was set at a wavelength of 206nm. After each run, the capillary was flushed with running buffer for 2min to remove contaminants.

1.2 Reagents

All chemicals were of the analytical grade. Kaempferol, quercetin and rutin were purchased from Sigma (St. Louis, MO). Hesperidin, hyperin and isorhamnetin were purchased from the National Institute for Control of Pharmaceuticals and Biological Products (Beijing, China). Rape bee pollen was purchased from Qinghai Huabao Bee Product Co. Ltd. (Qinghai, China). The bee pollen mixture samples, #2 and #3, were purchased from local drugstores (Ottawa and Quebec, Canada).

Stock solutions of the six analytes were prepared in methanol (1000μg/ml each). They were diluted to the desired working concentrations with the running buffer (H_3BO_3-$Na_2B_4O_7$, pH9.2). Before use, all solutions were filtered through 0.45μm syringe filters.

1.3 Sample Preparation

About 2g of bee pollen was extracted with 15ml methanol for 10 min in an ultrasonic bath. After centrifugation, the supernatant solution was transferred into a 50ml volumetric flask. The extraction procedure was repeated three times. The total extract solution was diluted with methanol to 50ml, which was then stored in the refrigerator. Before analysis, 1.0ml of the extract solution was further diluted with the running buffer to 5.0ml. After passing through a 0.45μm syringe filter, the sample solution was injected electrokinetically for CE analysis.

1.4 Method validation

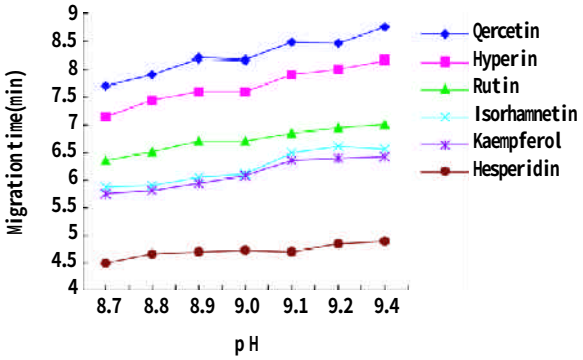
The optimized method was validated for flavonoids determination in bee pollens by analysis of a batch of triplicate samples. Each sample was spiked by addition of the six analytes at 10.0μg/ml. A calibration curve was drawn for each analyte using six standard solutions that were freshly prepared daily. Reproducibility was determined by running each flavonoid standard solution eight times, and each bee pollen extract three times. Recovery was estimated for every analyte by comparing standard addition (or spiking) results with the standard calibration curve.

2 Results and Discussion

2.1 Optimization of separation

The molecular structures of all six analytes suggested that they could be analyzed as anions. A borate running buffer was employed in this work because borate can chelate with the flavonoids to form more soluble complex anions[18]. Efficient separation and sensitive determination were achieved by optimizing the pH of borate buffer, borax concentration, running voltage, electrokinetic injection time, and UV detector wavelength.

The UV spectra revealed that the six flavonoids exhibited two absorption peaks, a strong one at 200～220nm and another one at 250～270nm. For the best detection sensitivity at a common wavelength, 206nm was chosen for UV detection in all CE analyses.



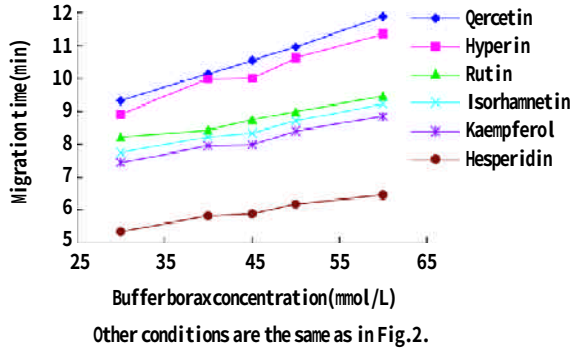
Fused capillary: 50μm i.d. ×58cm length (50cm effective); running buffer: 45mmol/L; injection time: 6s at 15kV; separation voltage: 20kV; concentration of six analytes: 20μg ml each; wavelength of UV detector: 206nm.

Fig.2 Effect of pH on migration times of hesperidin, kaempferol, isorhamnetin, rutin, hyperin and quercetin in CE

The pH dependence of migration times was investigated over the pH range from 8.7～9.4. As shown in Fig.2, the migration times of all six analyte anions increased with increasing pH. Separation of the analytes was best achieved at pH9.2. When the pH was 9.0, the isorhamnetin and kaempferol peaks could not be separated. When pH was 9.4, the isorhamnetin and kaempferol peaks were overlapping, and the hyperin and quercetin peaks nearly disappeared. Moreover, any higher pH would result in a longer analysis time and the analytes would be more susceptible to oxidation[19]. Therefore, pH9.2 was selected as the optimal pH of running buffer.

Besides pH, the concentration of running buffer is also an important parameter. The effect of the running buffer concentration on migration times was studied from 30mmol/L to

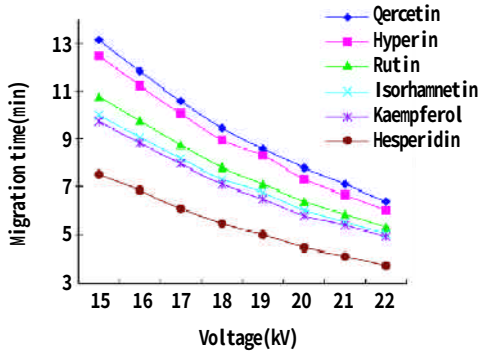
60mmol/L. As shown in Fig.3, the optimal concentration of borax was 45mmol/L. The EOF was 2.0mm/s, and the minimum resolution was 1.45 between kaempferol and isorhamnetin.



Other conditions are the same as in Fig.2.

Fig.3 Effect of buffer borax concentration on migration time

The influence of separation voltage on the migration times was next studied. Since electrophoretic mobility is proportional to applied voltage, a higher voltage gave shorter migration times for all six analytes (as shown in Fig.4). However, when the running voltage exceeded 20kV, separation of isorhamnetin, kaempferol and rutin could not be achieved. Besides, the baseline noise became more. The optimal separation voltage was therefore decided to be 20 kV because short migration time and reasonable good resolution was obtained for all analytes.

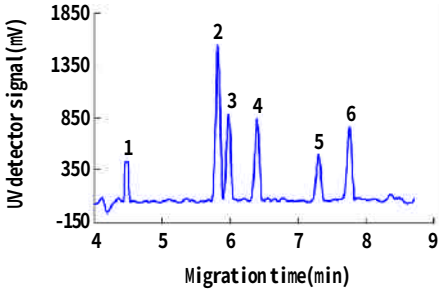


Other conditions are the same as in Fig.2.

Fig.4 Effect of separation voltage on migration time

The injection time determines the volume of sample solution analyzed by CE, which affects the area, height and shape of all analyte peaks. These effects were studied by varying the electrokinetic injection time from 3s to 12s, at a constant voltage of 20kV. The peak height increased with increasing injection time, and the peak width increased simultaneously. When the injection time was increased longer than 6s, peak broadening became severe, which was obviously unfavorable to the resolution. Eventually, 6s was selected as the optimal injection time.

Under the optimal conditions, a good separation of hesperidin, hyperin, isorhamnetin, kaempferol, quercetin and rutin was achieved within 8min. A typical electropherogram for a standard solution of the six analytes is shown in Fig.5. This elution order was in good agreement with the only one previous report for kaempferol and quercetin by Wang and Huang using 35mmol/L borax, pH8.9 and an applied field strength of 240V/cm on a fused capillary of 70cm (effective length: 45cm) × 75μm[20].



Peak identification: 1=hesperidin, 2=kaempferol, 3=isorhamnetin, 4=rutin, 5=hyperin and 6=quercetin. pH=9.2. Other conditions were the same as in Fig.2.

Fig.5 Electropherogram for a standard mixture of flavonoids (20μg/ml. each)

2.2 Regression Equations, Linear Ranges and Detection Limits of the Six Analytes

To determine the linear relationships between the peak area and the analyte concentration, a series of standard solutions from 1μg/ml to 100μg/ml were analyzed for each flavonoid by CE. The results of regression analysis on these calibration curves, linear ranges and detection limits, are summarized in Table 1. Each detection limit was evaluated on the basis of a signal-to-noise ratio of 3.

Table 1 Regression equations and detection limits

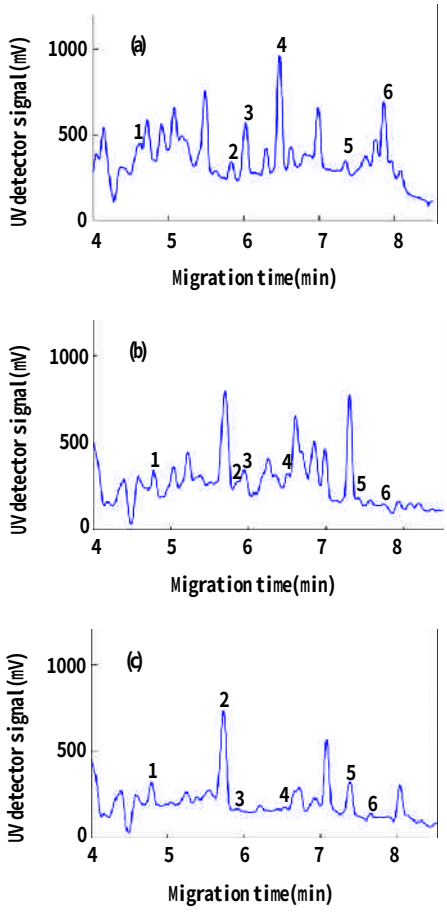
Compound	Linear range (g/ml)	Correlation coefficient	Detection limit (10^{-7}g/ml)
Hesperidin	1×10^{-6}～1×10^{-4}	0.9922	12
Kaempferol	1×10^{-5}～1×10^{-4}	0.9925	29
Isorhamnetin	1×10^{-6}～1×10^{-4}	0.9996	4.9
Rutin	1×10^{-6}～1×10^{-4}	0.9989	3.9
Hyperin	1×10^{-5}～1×10^{-4}	0.9929	27
Quercetin	1×10^{-6}～1×10^{-4}	0.9997	3.4

Note: CE-UV conditions are the same as in Fig.2.

2.3 System suitability test

The migration times of the six flavonoids were: 4.50±0.05 min for hesperidin, 5.83±0.08 min for kaempferol, 5.98±0.08 min for isorhamnetin, 6.40±0.09 min for rutin, 7.29±

0.08 min for hyperin, and 7.76±0.09 min for quercetin.



Peak identifications and determination conditions were the same as in Fig.5.

Fig.6 Electropherograms of (a) rape bee pollen extract, (b) bee pollen #2 extract, and (c) bee pollen #3 extract

Although sometimes unknown peaks were observed nearby the analyte peaks in the electropherograms (for instance, peak 1 in Fig.6(a), and peak 4 and peak 5 in Fig. 6(b)), they were readily identified by the method of standard addition (or spiking). The recovery results, ranging from 80.4% to 113.9%, also proved that the method has a reasonably good accuracy.

The CE method was validated with respect to reproducibility of the migration times and peak areas for the six analytes. The reproducibility was estimated by making eight replicate injections of a standard mixture solution (20 μg/ml for each analyte) under the optimal conditions. The relative standard derivations (RSDs) of migration time were 0.79%, 0.91%, 0.93%, 0.93%, 0.82% and 0.77%, and the RSDs of peak areas were 8.6%, 5.4%, 4.2%, 4.8%, 3.8% and 6.9% for hesperidin, kaempferol, isorhamnetin, rutin, hyperin and quercetin. Recoveries were also determined under the optimal conditions to evaluate the precision and accuracy of the method. Standard additions into a bee pollen extracts yielded results ranging from 80.4% to 113.9% recovery, as detailed in Table 2.

2.4 Analysis of bee pollens

Under the optimal conditions, hesperidin, kaempferol, isorhamnetin, rutin, hyperin and quercetin in bee pollen were determined by CE. Typical electropherograms of three different bee pollens are shown in Fig.6(a)～6(c). All of the common flavonoids were successfully separated on top of the complex sample matrix. Peaks were identified by adding pure compounds of the flavonoids. The selected ingredients in the

Table 2 Recoveries in CE analysis of three bee pollens

Sample	Ingredient	Original amount (g/ml)	Added amount (g/ml)	Found (g/ml)	Recovery (%)	RSD (%)
Bee pollen (Rape)	Hesperidin	4.51×10^{-5}	1.0×10^{-5}	5.68×10^{-5}	103.1	4.2
	Kaempferol	0.22×10^{-5}	1.0×10^{-5}	1.34×10^{-5}	110.3	2.6
	Isorhamnetin	0.96×10^{-5}	1.0×10^{-5}	1.66×10^{-5}	84.7	2.1
	Rutin	1.07×10^{-5}	1.0×10^{-5}	1.69×10^{-5}	81.7	2.3
	Hyperin	0.67×10^{-5}	1.0×10^{-5}	1.46×10^{-5}	87.7	1.7
	Quercetin	1.52×10^{-5}	1.0×10^{-5}	2.87×10^{-5}	113.9	3.2
Bee pollen (#2)	Hesperidin	0.85×10^{-5}	1.0×10^{-5}	1.53×10^{-5}	82.6	4.1
	Kaempferol	0.31×10^{-5}	1.0×10^{-5}	1.05×10^{-5}	80.4	4.9
	Isorhamnetin	0.73×10^{-5}	1.0×10^{-5}	1.64×10^{-5}	84.8	3.9
	Rutin	0.29×10^{-5}	1.0×10^{-5}	1.30×10^{-5}	100.1	2.1
	Hyperin	N.F.	–	–	–	
	Quercetin	N.F.	–	–	–	
Bee pollen (#3)	Hesperidin	0.74×10^{-5}	1.0×10^{-5}	1.51×10^{-5}	86.4	3.8
	Kaempferol	0.42×10^{-5}	1.0×10^{-5}	1.62×10^{-5}	113.9	4.8
	Isorhamnetin	0.37×10^{-5}	1.0×10^{-5}	1.46×10^{-5}	107.2	4.1
	Rutin	0.45×10^{-5}	1.0×10^{-5}	1.59×10^{-5}	109.8	2.9
	Hyperin	0.77×10^{-5}	1.0×10^{-5}	1.44×10^{-5}	81.1	3.4
	Quercetin	0.13×10^{-5}	1.0×10^{-5}	0.92×10^{-5}	81.4	3.5

bee pollen samples were identified as hesperidin (peak 1), kaempferol (peak 2), isorhamnetin (peak 3), rutin (peak 4), hyperin (peak 5) and quercetin (peak 6).

Quantitation of each flavonoid was achieved by comparison with the corresponding standard calibration curve. The quantitative analysis results are listed in Table 2. These results indicate that the CE method is simple, reproducible, sensitive and accurate for the analysis of bee pollens and their health food products without complicated sample pretreatment.

3 Conclusions

A CE method has been developed for the determination of six bioactive flavonoids in three kinds of bee pollen—hesperidin, kaempferol, isorhamnetin, rutin, hyperin and quercetin. Running a H_3BO_3-$Na_2B_4O_7$ buffer (pH9.2) at 20 kV, the analytes were separated rapidly within 8 min. Excellent linearity was observed over two orders of magnitude, with detection limits (S/N=3) ranging from 0.34μg/ml to 2.9μg/ml. The migration times of all six analytes were between 4.50 min and 7.76 min. The recovery results, ranging from 80.4% to 113.9%, also proved that the method has a reasonably good accuracy. The new method is simple, reproducible and sensitive. No solid phase extraction and sample pretreatment are necessary. All three bee pollens were found to consist of at least four of the six flavonoids. These flavonoids are well-known dietary antioxidants that exert significant anti-tumor, anti-allergic, anti-inflammatory and anti-viral effects. The CE method can potentially be used for evaluating the flavonoid compositions of other health foods.

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