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Application of Capillary Electrophoresis To Study Phenolic Profiles of Honeybee-Collected Pollen

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Honeybee-collected pollen is promoted as a health food with a wide range of nutritional and therapeutic properties. A high-performance capillary electrophoresis with amperometric detection method has been developed for the simultaneous determination of bioactive ingredients in 10 samples of honeybee-collected pollen in this work. Under the optimum conditions, 13 phenolic components can be well-separated or nearly baseline-separated (apigenin and vanillic acid peaks) within 29 min at the separation voltage of 14 kV in a 50 mM borax running buffer (pH 9.0), and adequate extraction was obtained with ethanol for the determination of the above 13 compounds. Recovery (94.1–104.0%), repeatability of the peak current (<5.4%), and detection limits (6.9×10^{-7}–6.4×10^{-9} g mL^{-1}) for the method were evaluated. This procedure was successfully used for the analysis and comparison of the phenolic content of honeybee-collected pollen samples originating from different floral origins based on their electropherograms or "phenolic profiles".

KEYWORDS: Capillary electrophoresis; amperometric detection; phenolic profiles; electromigration profiles; bee pollen

INTRODUCTION

Honeybee-collected pollen ("bee pollen"), that is, floral pollen collected by the honeybee for its abundance of nutrimental constituents and bioactive compounds, has won a favorable reputation as a "natural mininutrition treasury". Bee pollen has been used as a "perfect health food" for many centuries, and its benefits have been widely lauded (*1–5*). Modern research has also shown that bee pollen mainly possesses the therapeutic effects of improving the cardiovascular system, enhancing body immunity, delaying consenescence, maintaining the digestive system, and preventing prostate degeneration (*1, 6, 7*). The Pharmacopoeia Committee of the People's Republic of China (*8*) and the German Federal Board of Health (*2*) have officially recognized pollen as a medicine.

More specifically, the ingestion of bee pollen by rats has also been shown to decrease the level of the lipid oxidation products, malondialdehyde and conjugated dienes, in the erythrocytes (*9*), and the key components providing such activities are likely to be the known dietary antioxidants, phenolic compounds (*10*). Practically, considerable worldwide attention has been given to natural phenolic antioxidants for their potential protective effects against the damage from biological oxidants in the last decade (*11–16*). However, at present, only a minimum quantity of 4 mg/100 g of vitamin C and 15% of protein has been recommended as the national standard of commercial honeybee-collected pollen in the Chinese market. Therefore, it is necessary to develop simple, economical, and efficient methods for the analysis of phenolic profiles in honeybee-collected pollen to supplement and consummate the quality of the product.

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However, so far, only a few reports can be found for the analysis of phenolic ingredients in bee pollen, including UV spectrophotometry approaches (*17, 18*), colorimetry (*19*), thin-layer chromatography (*20*), conductometric titration (*21*), high-performance liquid chromatography (HPLC) (*22–27*), and capillary electrophoresis (CE)-UV (*28, 29*). CE is becoming increasingly recognized as an important analytical separation technique due to its speed, efficiency, reproducibility, ultrasmall sample volume, and ease of clearing the contaminants. In combination with amperometric detection (AD), CE-AD can offer high sensitivity and good selectivity for electroactive species (*30, 31*). In comparison with HPLC, CE is a more efficient separation method without complicated operations and high costs. However, so far, this technique has not been explored for the analysis of bee pollen.

The major objectives of our investigation were to develop a sensitive and reliable method for the separation and quantitation of 13 bioactive ingredients (namely, hesperidin, chrysin, naringenin, rutin, baicalein, kaempferol, apigenin, vanillic acid, luteolin, quercetin, morin, gallic acid, and protocatechuic acid) in honeybee-collected pollen using CE-AD (rosmarinic acid and caffeic acid were not found in real pollen samples); most of these compounds have similar molecular structures as shown in **Figure 1**. Subsequently, this procedure was successfully used for the analysis of the phenolic content difference of bee pollen samples collected from different plant resources based on their electromigration profiles or "electrochemical characteristic chromatograms".

MATERIALS AND METHODS

Instrumentation and CE Procedures. The laboratory-built CE-AD system used in this work was described previously (*30*).

Chemical and Reagents. Hesperidin, chrysin, naringenin, baicalein, kaempferol, apigenin, morin, caffeic acid, and luteolin were purchased from

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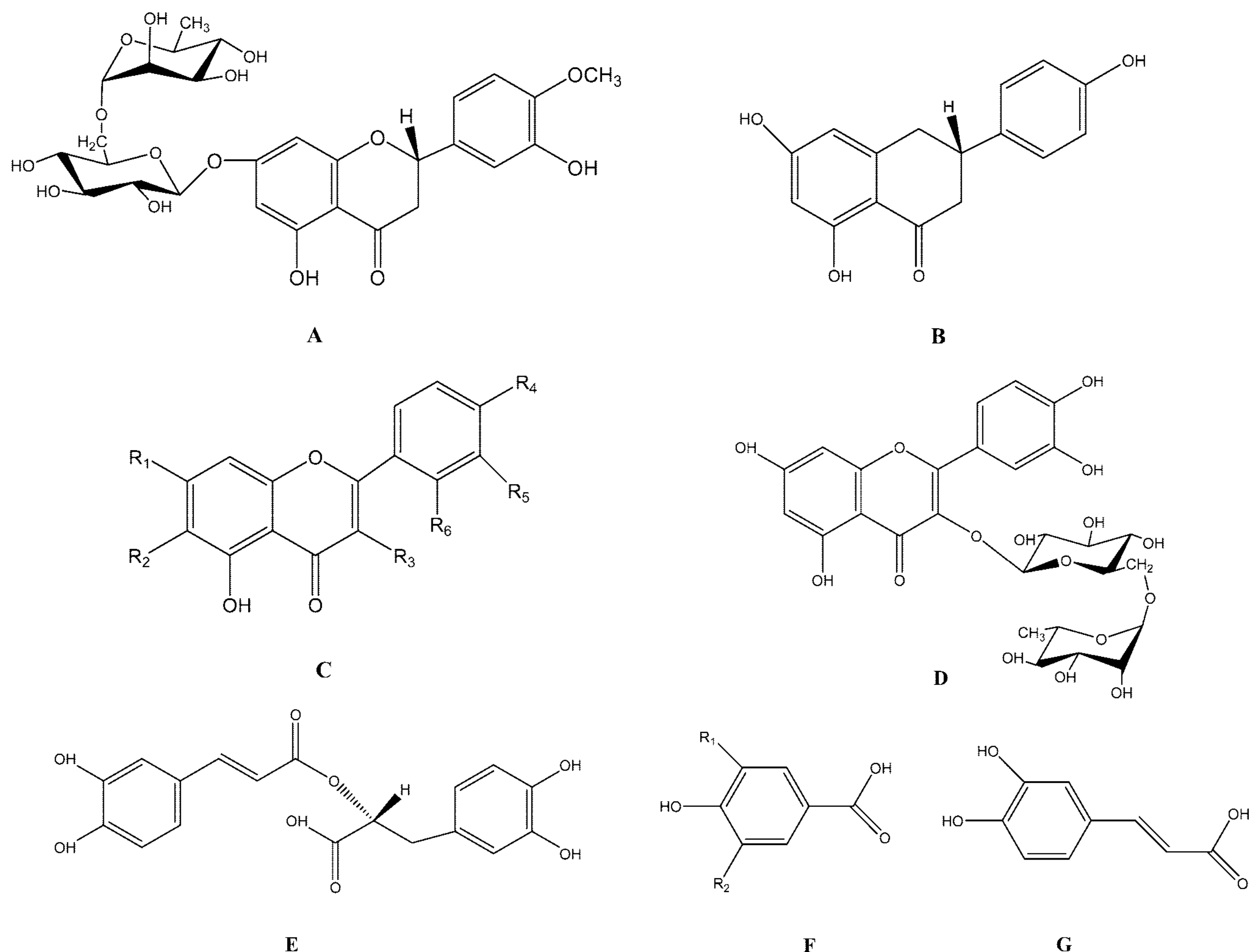

Figure 1. Molecular structures of (**A**) hespetidin; (**B**) narigenin; (**C**) chrysin (R_1= OH and R_2, R_3, R_4, R_5, and R_6 = H), baicalein (R_1 and R_2 = OH and R_3, R_4, R_5, and R_6 = H), kaempferol (R_1, R_3, and R_4 = OH and R_2, R_5, and R_6 = H), apigenin (R_1 and R_4 = OH and R_2, R_3, R_5, and R_6 = H), luteolin (R_1, R_4, and R_5 = OH and R_2, R_3, and R_6 = H), quercetin (R_1, R_3, R_4, and R_5 = OH and R_2 and R_6 = H), and morin (R_1, R_3, R_4, and R_6 = OH and R_2 and R_5 = H); (**D**) rutin; (**E**) rosmarinic acid; (**F**) vanillic acid (R_1 = H and R_2 = CH_3O), gallic acid (R_1 and R_2 = OH), and protocatechuic acid (R_1 = OH and R_2 = H); and (**G**) caffeic acid.

Sigma (St. Louis, MO); rutin and rosmarinic acid were purchased from Aldrich (Milwaukee, WI); quercetin, gallic acid, vanillic acid, and protocatechuic acid were obtained from Shanghai Reagent Factory (Shanghai, China); and they were all used as received. Bee pollen samples were purchased from Shanghai Senfeng Yuan Beekeep Co. Ltd. (Shanghai, China), and the plant name was the floral origin of the pollen samples.

Stock solutions of 15 analytes (1.0×10^{-3} g mL^{-1} each) were prepared in anhydrous ethanol (A.R. grade), were stored in the dark at 4 °C, and were diluted to the desired concentration with the running buffer (H_3BO_3–$Na_2B_4O_7$ buffer with a pH value from 8.4 to 9.2). Before use, all solutions were filtered through 0.22 μm nylon filters.

Sample Preparation. Bee pollen samples were ground into powder and accurately weighed. Each sample was extracted with 10 mL of anhydrous ethanol (A. R. grade) and water (4:1) for 30 min in an ultrasonic bath. Then, each of the samples was filtered through filter paper first, followed by a 0.22 μm syringe filter. After filtration, the solutions were injected directly to the CE-AD system for analysis. Before use, all sample solutions were stored in the dark.

RESULTS AND DISCUSSION

Optimum of Analytical Procedure. Because the phenolic hydroxy groups of the 15 analytes could be readily oxidized electrochemically at a relatively moderate potential (*32*), AD was used in this work. In AD, the potential applied to the working electrode directly affects the sensitivity, detection limit, and stability of this method. Therefore, a hydrodynamic voltammetry experiment was investigated to obtain optimum detection results. As shown in **Figure 2**, the applied potential was maintained at +950 mV (vs SCE) where the background current was not too high and the S/N ratio was the highest. Moreover, the working electrode exhibited good stability and high reproducibility at this optimum potential.

The effect of the running buffer pH on the migration time and resolution of the analytes was investigated in the pH range of 8.4–9.2, as shown in **Figure 3**. When the pH was lower than 8.7, satisfactory separation of most of the analytes could not be achieved. When the pH was higher than 9.2, naringenin could not be separated from rutin; meanwhile, the peak current (AD signal) was low, and the peak shape became poor. At pH 9.0, all 15 analytes could be well-separated or nearly baseline-separated (apigenin and vanillic acid peaks) within a relatively short time.

Besides the pH value, the running buffer concentration was also an important parameter. The effect of the running buffer concentration on migration time and resolution was also studied ranging from 20 to 100 mM, and 50 mM borax buffer (pH 9.0) was chosen as the running buffer concentration in consideration of the peak current, resolution, analytical time, and buffer capacity.

For a given capillary length, the separation voltage determines the electric field strength, which affects both the velocity of the electro-osmotic flow (EOF) and the migration velocity of the

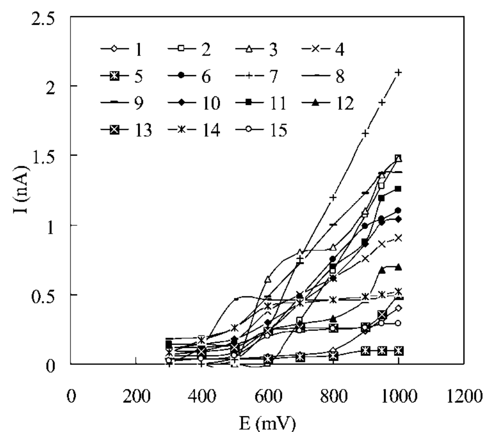

Figure 2. Hydrodynamic voltammograms (HDVs) of hesperidin (1), chrysin (2), naringenin (3), rutin (4), baicalein (5), kaempferol (6), apigenin (7), vanillic acid (8), luteolin (9), quercetin (10), morin (11), rosmarinic acid (12), caffeic acid (13), gallic acid (14), and protocatechuic acid (15) in CE-AD. Fused silica capillary, 25 μm i.d. $\times$ 75 cm; working electrode, 300 μm diameter carbon disk electrode; running buffer, 50 mM (pH 9.0); separation voltage, 14 kV; injection time, 6 s/14 kV; and concentration of analytes, 5.0 $\times$ 10^{-6} g mL^{-1} each.

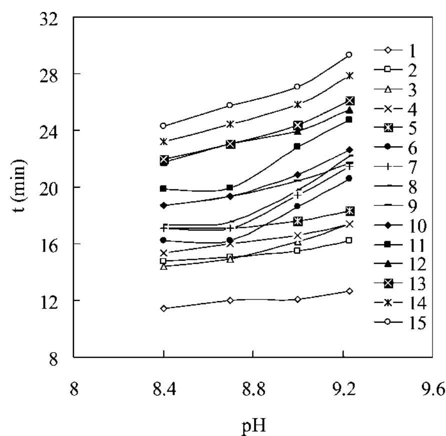

Figure 3. Effects of the running buffer pH on the migration time of the analytes. The working electrode potential is +950 mV (vs SCE), and other conditions and peak identifications were the same as in **Figure 2**.

analytes, which in turn determines the migration time of the analytes. As expected, a higher separation voltage gave a shorter migration time for all analytes. However, when the separation voltage exceeded 16 kV, the baseline noise became larger. Therefore, the optimum separation voltage selected was 14 kV, at which good separation could be obtained for all analytes within 29 min.

In our work, samples were all injected electrokinetically, which is a commonly used and widely recognized injection method in CE work. The injection time determining the amount of sampling affects both peak current and peak shape. The effect of injection time on peak current was studied by varying the injection time from 2 to 8 s at 14 kV. It was found that the peak current increased with an increase in the sampling time. When the injection time was longer than 6 s, the peak current nearly leveled off and peak broadening became more severe. In this experiment, 6 s (14 kV) was selected as the optimum injection time.

Through the experiments above, the optimum separation and detection conditions for 15 phenolic compounds were decided, and the typical electropherogram for a standard mixture solution of the 15 analytes under optimum conditions is shown in **Figure 4A**.

Method Validations. The repeatability of the peak current was estimated by making repetitive injections of a standard mixture solution (5.0 $\times$ 10^{-6} g mL^{-1} for each analyte) under the selected optimum conditions ($n = 7$). The relative standard deviations (RSDs) of the peak currents were between 2.2 and 5.4% for 15 phenolic compounds, and the details are presented in **Table 1**. The repeatability data exhibited in the present study showed that it was feasible to determine the above analytes by the developed CE-AD method.

To determine the linearity of the 15 analytes, a series of standard solutions from 2.0 $\times$ 10^{-7} to 1.0 $\times$ 10^{-4} g mL^{-1} were tested (the results of regression analysis on calibration curves are summarized in **Table 1**). The peak current and concentration of each analyte were subjected to regression analysis to obtain the calibration equations and correlation coefficients, and the results showed that within the concentration range there was an excellent correlation between the peak current and the concentration of each analyte. The limit of detection (LOD) was established based on a signal-to-noise ratio of 3, and the LODs of 15 analytes ranged from 6.9 $\times$ 10^{-7} to 6.4 $\times$ 10^{-9} g mL^{-1} as shown in **Table 1**.

To evaluate the accuracy of the method, the recovery experiments under the optimum conditions were also conducted with camellia pollen sample ($n = 3$). Recovery was determined by a standard addition method, and the results are listed in **Table 2**. The results indicated that the method was sufficiently accurate for the simultaneous determination of the above analytes.

Comparing the above assay results obtained using the developed CE-AD method with HPLC–diode array detection (*26*), the same analytes such as rutin and quercetin can be obtained with much lower LODs (5.6 $\times$ 10^{-8}–7.1 $\times$ 10^{-8} g mL^{-1} vs 1.4–1.9 mg kg^{-1}), better repeatabilities (3.5–3.7 vs 6.20–6.67%), and/or more acceptable recovery ranges (101.7–102.6 vs 83–86%). Because the analytes (typhaneoside and isorhamnetin-3-*O*-neohesperidoside) reported in ref *29* based on CE-UV were different from those in our work, the comparison was not done here.

Sample Analysis and Discussion. Under the optimum conditions, the proposed procedure was followed for the determination of bioactive ingredients in real-world pollen samples based on CE-AD. Typical electropherograms of different floral origin of pollen samples are shown in **Figure 4B–K**, respectively. By a standard addition method and using the migration time of each analyte as compared with the electropherogram of the standard mixture solution (**Figure 4A**), 13 bioactive ingredients [namely, hesperidin (1), chrysin (2), naringenin (3), rutin (4), baicalein (5), kaempferol (6), apigenin (7), vanillic acid (8), luteolin (9), quercetin (10), morin (11), gallic acid (14), and protocatechuic acid (15)] in different pollen samples were determined. The analytes of rosmarinic acid (12) and caffeic acid (13) were not found in the tested samples. Because of the matrix effect of real samples, the baselines of sample electropherograms were not as smooth as those of standard mixture solutions. Furthermore, although some analyte peaks in real samples could not be well-separated as shown in sample electropherograms, they were nearly baseline-separated, so the quantitative data of the analytes could still be calculated based on the "effective" peak height from which the baseline drifting was subtracted. The assay results are listed in **Table 3**, and the data showed that the pollen samples contained an abundance of polyphenols, particularly chrysin, rutin, baicalein, kaempferol, apigenin, vanillic acid, and luteolin. Furthermore, the above assay results showed that the overall amount of 13 bioactive constituents in rape pollen sample (1 g) was about

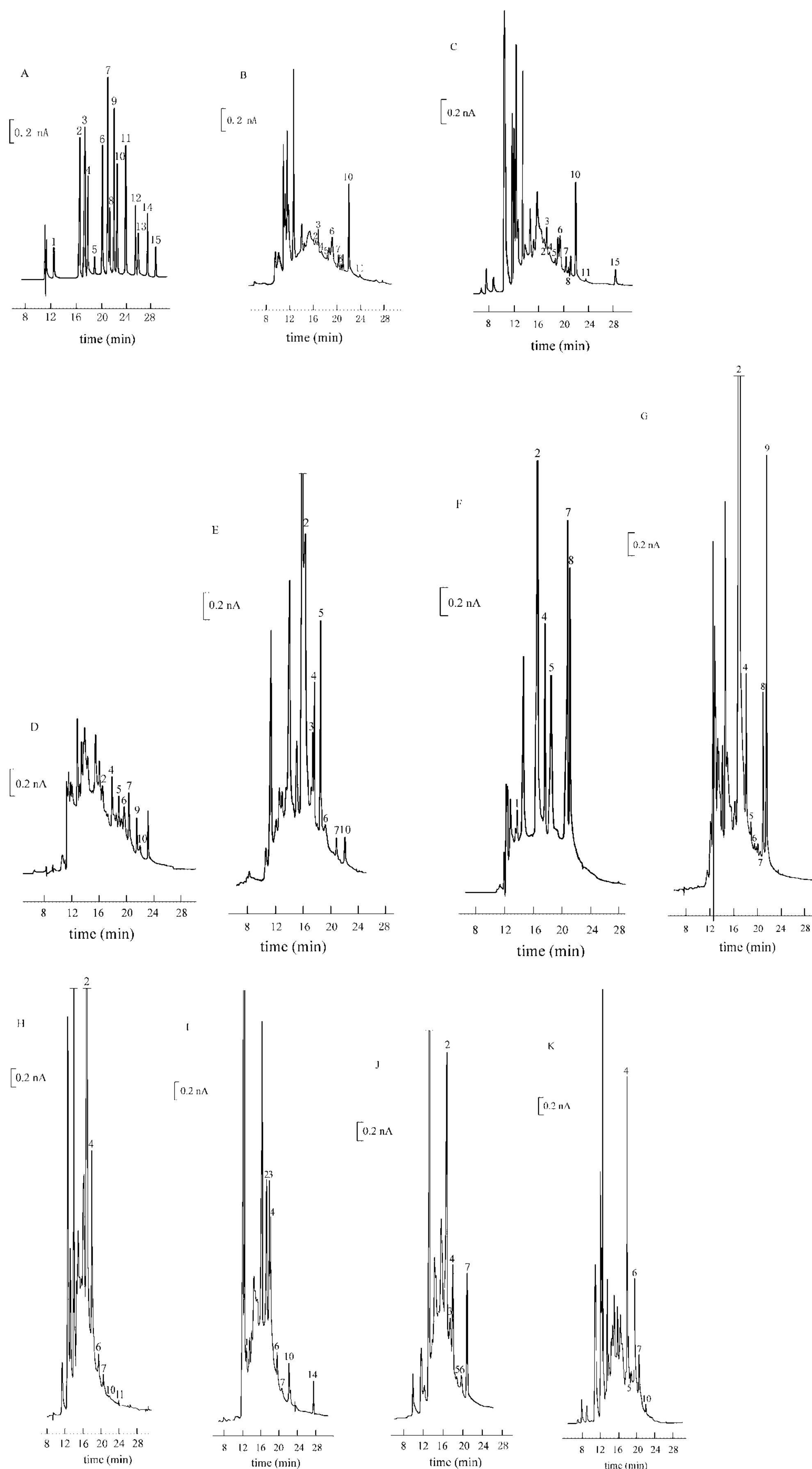

Figure 4. Electropherograms of a standard mixture solution (5.0×10^{-6} g mL^{-1} each) (**A**) and sample solutions of natural pine pollen (**B**), broken pine pollen (**C**), buckwheat pollen (**D**), corn pollen (**E**), rape pollen (**F**), papaver pollen (**G**), mixed pollen (**H**), camellia pollen (**I**), basswood pollen (**J**), and Chinese gooseberry pollen (**K**). Other experiment conditions and peak identification are the same as in **Figure 3**.

Table 1. Regression Equations and Detection Limits of 15 Analytes[a]

compound	regression equation[b]	correlation coefficient	linear range (g mL^{-1})	detection limit (10^{-8} g mL^{-1})	RSD (%)
hesperidin	$y = 4.35 \times 10^4x + 0.01$	0.9996	1×10^{-6}–1×10^{-4}	39	3.6
chrysin	$y = 2.30 \times 10^5x + 0.07$	0.9996	5×10^{-7}–5×10^{-5}	3.5	2.4
naringenin	$y = 2.43 \times 10^5x - 0.07$	0.9995	5×10^{-7}–1×10^{-4}	3.8	3.3
rutin	$y = 1.33 \times 10^5x - 0.03$	0.9992	5×10^{-7}–1×10^{-4}	5.6	3.5
baicalein	$y = 1.98 \times 10^4x + 0.05$	0.9990	2×10^{-6}–1×10^{-4}	69	5.4
kaempferol	$y = 1.48 \times 10^5x - 0.01$	0.9991	5×10^{-7}–1×10^{-4}	1.4	2.3
apigenin	$y = 2.91 \times 10^5x - 0.12$	0.9998	5×10^{-7}–1×10^{-4}	0.64	2.2
vanillic acid	$y = 6.94 \times 10^4x + 0.08$	0.9989	1×10^{-6}–1×10^{-4}	12	3.9
luteolin	$y = 2.70 \times 10^5x - 0.02$	0.9998	5×10^{-7}–5×10^{-5}	3.4	2.5
quercetin	$y = 1.19 \times 10^5x + 0.08$	0.9998	5×10^{-7}–5×10^{-5}	7.1	3.7
morin	$y = 1.57 \times 10^5x - 0.00$	0.9988	5×10^{-7}–5×10^{-5}	5.6	3.4
rosmarinic acid	$y = 6.81 \times 10^4x + 0.06$	0.9989	1×10^{-6}–1×10^{-4}	1.3	4.5
caffeic acid	$y = 4.33 \times 10^4x + 0.10$	0.9991	1×10^{-6}–1×10^{-4}	2.9	5.1
gallic acid	$y = 7.44 \times 10^4x - 0.01$	0.9997	1×10^{-6}–1×10^{-4}	1.1	4.4
protocatechuic acid	$y = 3.34 \times 10^4x + 0.06$	0.9990	2×10^{-6}–1×10^{-4}	3.0	4.6

[a] CE-AD conditions were the same as in **Figure 3**. [b] In the regression equation, the *x* value was the concentration of analytes (g mL^{-1}), and the *y* value was the peak current (nA).

Table 2. Assay Results of Recovery with Camellia Pollen Sample in This Method ($n = 3$)[a]

	g mL^{-1}			%	
ingredient	original amount	added amount	found	recovery	RSD
hesperidin	2.9×10^{-6}	5.0×10^{-6}	8.03×10^{-6}	102.6	2.9
chrysin	6.6×10^{-6}	5.0×10^{-6}	1.18×10^{-5}	104.0	2.2
naringenin	5.8×10^{-6}	5.0×10^{-6}	1.07×10^{-5}	98.0	2.5
rutin	4.6×10^{-6}	5.0×10^{-6}	9.68×10^{-6}	101.7	3.0
baicalein	N.F.[b]	5.0×10^{-6}	4.70×10^{-6}	94.1	4.7
kaempferol	1.1×10^{-6}	5.0×10^{-6}	6.21×10^{-6}	102.2	3.2
apigenin	0.1×10^{-6}	5.0×10^{-6}	5.02×10^{-6}	98.4	3.7
vanillic acid	N.F.	5.0×10^{-6}	5.08×10^{-6}	101.6	4.0
luteolin	N.F.	5.0×10^{-6}	5.12×10^{-6}	102.4	3.6
quercetin	2.2×10^{-6}	5.0×10^{-6}	7.33×10^{-6}	102.6	2.8
morin	N.F.	5.0×10^{-6}	5.16×10^{-6}	103.2	3.5
rosmarinic acid	N.F.	5.0×10^{-6}	4.85×10^{-6}	97.0	4.2
caffeic acid	N.F.	5.0×10^{-6}	4.82×10^{-6}	96.4	4.4
gallic acid	3.3×10^{-6}	5.0×10^{-6}	8.45×10^{-6}	103.0	2.7
protocatechuic acid	N.F.	5.0×10^{-6}	4.78×10^{-6}	95.6	5.2

[a] CE-AD conditions were the same as **Figure 3**. [b] N.F. means that the relevant analyte has not been found in the mentioned samples.

Table 3. Assay Results for 10 Bee Pollen Samples ($n = 3$)[a]

	content of bee pollen samples: μg/g (RSD %)									
ingredients	natural pine	broken pine	buckwheat	corn	rape	papaver	mixed	camellia	basswood	Chinese gooseberry
hesperidin	N.F.[b]	N.F.	N.F.	N.F.	819.0 (1.6)	N.F.	N.F.	400 (3.5)	N.F.	N.F.
chrysin	10.9 (4.1)	23.1 (4.9)	34.2 (3.3)	[c]	2936.2 (0.7)	4125.7 (0.5)	3295.1 (0.8)	887.1 (0.8)	2773.2 (1.5)	N.F.
naringenin	36.9 (3.0)	93.3 (2.2)	N.F.	113.6 (1.8)	N.F.	N.F.	N.F.	791.2 (1.3)	217.8 (3.5)	N.F.
rutin	8.1 (4.4)	15.7 (4.6)	143.2 (1.6)	306.8 (1.0)	2151.5 (1.1)	1359.8 (1.4)	2303.0 (1.2)	626.3 (2.1)	1311.6 (2.2)	9924.2 (0.7)
baicalein	88.2 (4.9)	179.2 (5.0)	513.9 (1.9)	3229.2 (0.5)	9333.3 (1.5)	791.7 (3.7)	N.F.	N.F.	338.5 (5.8)	1697.9 (6.1)
kaempferol	45.2 (2.7)	98.2 (3.5)	53.6 (2.6)	15.2 (5.4)	N.F.	47.4 (5.2)	184.2 (3.9)	152.1 (3.1)	138.2 (3.9)	2580.4 (1.4)
apigenin	24.4 (3.3)	53.1 (3.0)	71.6 (2.4)	26.3 (5.2)	1798.7 (1.1)	25.3 (5.0)	86.3 (3.2)	17.0 (5.8)	958.3 (1.6)	671.6 (2.7)
vanillic acid	46.4 (5.1)	91.8 (3.9)	N.F.	N.F.	4985.5 (0.9)	3087.0 (1.1)	N.F.	N.F.	N.F.	N.F.
luteolin	N.F.	N.F.	85.6 (2.2)	N.F.	N.F.	2372.7 (0.6)	N.F.	N.F.	N.F.	N.F.
quercetin	269.7 (1.6)	548.6 (1.0)	14.8 (4.4)	72.9 (3.9)	N.F.	N.F.	22.4 (5.2)	300.9 (2.9)	N.F.	281.3 (5.5)
morin	6.4 (5.8)	12.8 (4.9)	N.F.	N.F.	N.F.	N.F.	46.1 (4.6)	N.F.	N.F.	N.F.
gallic acid	N.F.	N.F.	N.F.	N.F.	N.F.	N.F.	N.F.	460.9 (3.3)	N.F.	N.F.
protocatechuic acid	N.F.	309.5 (3.3)	N.F.	N.F.	N.F.	N.F.	N.F.	N.F.	N.F.	N.F.

[a] CE-AD conditions were the same as in **Figure 3**. [b] N.F. means that the relevant analyte has not been found in the mentioned samples. [c] Because the analyte of chrysin was not separated well from its neighbor peak, it was not quantified but differentiated qualitatively.

41.1 times as that of natural pine pollen sample (1 g) as shown in **Table 3**. In particular, the rape pollen, Chinese gooseberry pollen, and papaver pollen contained the highest levels of the assayed phenolics.

As is evident from **Figure 4**, significant differences among electropherograms for each sample could be found; in other words, the peak structure including peak number and peak height of these electropherograms was noticeably different. For example, peak 2 in **Figure 4J** (basswood pollen sample) was about 66.5 times higher than that of **Figure 4B** (natural pine pollen sample). Therefore, these "electrochemical electropherograms" or phenolic profiles could provide an alternative method

for the comparison of component diversity of pollen samples from different floral origins. However, as limited by the number of standard phenolic compounds and bee pollen samples, all of the constituents and within-species pollen samples were not completely assayed in the present work. Besides, the assay data further validated that the method of "dilapidating walls" of pollen benefits the full release of bioactive ingredients. For instance, the total amount of 13 phenolics in broken pine pollen sample (1 g) was about 2.7 times as that of natural (or unbroken) pine sample (1 g) as shown in **Table 3**.

At present, there is no commonly recognized research standard, although various investigation data have been reported about chemical constituents of bee pollen. Therefore, it is an important research direction to establish the fingerprint of bee pollen by using its proper chemical constituents. The above assay results indicated that the CE-AD method was accurate, sensitive, and reproducible, providing a useful quantitative method for the analysis of bee pollen phenolics, and every kind of floral pollen possessed its own distinct phenolic CE-AD profile. Of course, the preliminary data presented here were insufficient to identify an unknown sample based on the phenolic profile; more additional samples need to be analyzed, and statistical methods should be employed. Furthermore, this product of bee pollen can be considered as a potential source of polyphenols and nutrients for human consumption, and the differences in the nature and levels of phenolic compounds would suggest that the effectiveness of various floral pollens (and therefore of the bee pollen mixtures) as antioxidants/free radical scavengers may vary widely.

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