

Analysis of Flavonoids in Propolis by Capillary Electrophoresis

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ABSTRACT

Flavonoid contents of nine raw and nine commercial propolis samples were analyzed by capillary electrophoresis. At first, 15 commercially available flavonoid standards including apigenin, chrysin, luteolin, galangin, kaempferol, morin, myricetin, quercetin, quercitrin, rutin, hesperetin, naringin, naringenin, daidzein and genistein were used to develop the electrophoretic conditions. At a running temperature of 25°C and detection wavelength at 214 nm, the separation of these standards was most satisfactorily achieved by a combination of capillary zone electrophoresis (CZE), utilizing 0.1 M borate buffer (pH 9.5) containing 5% methanol under a voltage of 18 kV, and micellar electrokinetic capillary electrophoresis (MECC), utilizing 0.03 M sodium borate (pH 8.5) containing 0.05 M SDS under a voltage of -15 kV. We found 12 out of the 15 flavonoids were identified in raw propolis, but only 11 were detected in commercial propolis products. The total contents of the identified flavonoids in raw and commercial propolis were 254 to 19147 ppm and 1228 to 7985 ppm, respectively, suggesting that both the origin and processing are important factors affecting the flavonoid content in propolis.

Key words: propolis, flavonoids, capillary zone electrophoresis, micellar electrokinetic capillary electrophoresis

INTRODUCTION

Propolis, a viscous substance with complicated composition, is collected and modified by worker bees to construct and protect their hives⁽¹⁾. It has long been used by humans as a natural remedy, since it possesses various biological activities such as antibacterial, antiviral, anti-inflammatory and anaesthetic properties⁽¹⁻³⁾. Among over 150 identified components of propolis, flavonoids were suggested to be the key compounds contributing to the biological activities^(4,5). However, factors like the geographic and botanical origins of propolis greatly influence the species and contents of flavonoids⁽⁶⁻⁸⁾, which may hence affect the quality of propolis.

The flavonoid content of propolis can be determined by a variety of methods such as colorimetric reactions^(9,10), thin layer chromatography^(6,11), gas chromatography^(12,13), gas chromatography-mass spectrometry⁽¹⁴⁻¹⁶⁾, and high performance liquid chromatography^(7,15,17,18). Although the colorimetric methods targeting flavonoids with similar structures are convenient for routine analysis, none of the colorimetric methods can detect different kinds of flavonoids simultaneously⁽¹⁹⁾. Lately, capillary electrophoresis with superior resolution and economic operation has been extensively applied to flavonoid analysis⁽²⁰⁻²⁴⁾. Yet the flavonoid content in propolis has not been determined by this technique.

The objective of this work is to quantitatively analyze the flavonoid constituents of propolis by capillary electrophoresis. At first, we developed the most appropriate parameters of capillary electrophoresis using 15 commercially available flavonoid standards. Next, the developed methods were applied to determine the flavonoid contents of nine raw propolis and nine commercial products.

MATERIALS AND METHODS

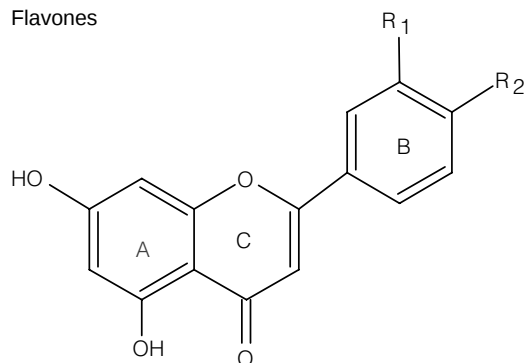
I. Materials

Fifteen flavonoid standards including apigenin, chrysin, luteolin, galangin, kaempferol, morin, myricetin, quercetin, quercitrin, rutin, hesperetin, (±)-naringenin, naringin, daidzein and genistein were purchased from Sigma-Aldrich (St. Louis, MO). The structures of these compounds are shown in Figure 1. Nine raw propolis samples termed Brazil-1, Brazil-2, Brazil-3, Brazil-4, China-1, China-2, China-3, Taiwan-1 and Taiwan-2 were obtained from the Miaoli District Agricultural Improvement Station (Miaoli, Taiwan), Chiafung Apiary (Taichung, Taiwan) and Yiwang Inc. (Taipei, Taiwan). Nine commercial propolis products, details of which are described in Table 1, were purchased from local retailers. All samples were stored at ambient temperature before analysis. The other reagents and solvents were all of analytical grade.

To prepare a standard sample solution, about 0.01 g

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Flavones

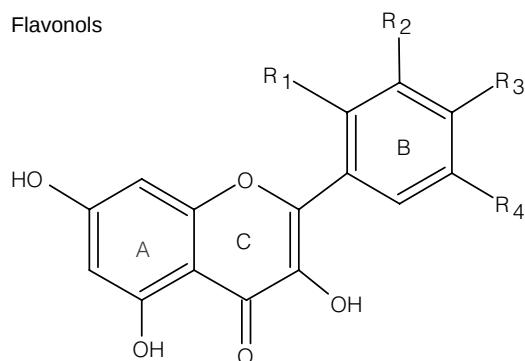


$R_1 = H, R_2 = OH$, apigenin

$R_1 = R_2 = H$, chrysin

$R_1 = R_2 = OH$, luteolin

Flavonols



$R_1 = R_2 = R_3 = R_4 = H$, galangin

$R_1 = R_2 = R_4 = H, R_3 = OH$, kaempferol

$R_1 = R_3 = OH, R_2 = R_4 = H$, morin

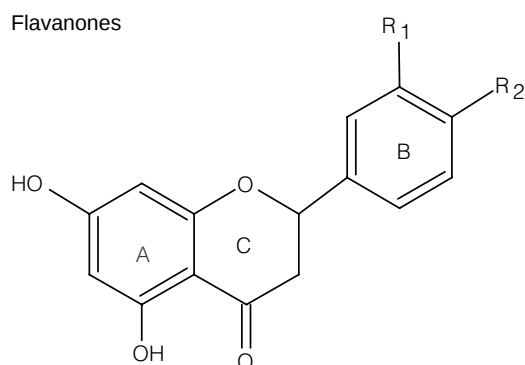
$R_1 = H, R_2 = R_3 = R_4 = OH$, myricetin

$R_1 = R_4 = H, R_2 = R_3 = OH$, quercetin

Quercetin-3-O-rhamnoside = quercitrin

Quercetin-3-O-rutinoside = rutin

Flavanones

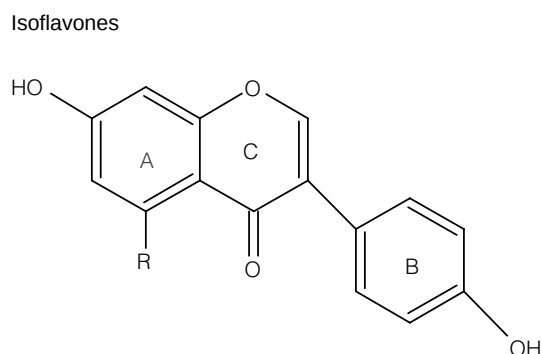


$R_1 = OH, R_2 = OCH_3$, hesperetin

$R_1 = H, R_2 = OH$, (±)naringenin

Naringenin-7-O-rhamnoglucoside = naringin

Isoflavones



$R = H$, daidzein

$R = OH$, genistein

Figure 1. Structures of the flavonoid standards.

(accurately weighed to 0.0001 g) of each authentic compound was weighed into one 10-mL volumetric flask and dissolved with methanol. The standard mixture containing 1000 ppm of each flavonoid standard was further diluted with methanol to 25, 50, 75, 100 and 125 ppm to

construct the calibration curves. During the development of analytical conditions, the flavonoid standards were individually injected, if necessary, to ensure no interference between peaks.

Table 1. The description of 9 commercial propolis products

Sample	Appearance	Color	Country of production
A	Liquid	Dark brown	Canada
B	Liquid	Reddish brown	New Zealand
C	Liquid	Yellowish brown	Brazil
D	Liquid	Dark brown	England
E	Liquid	Dark green	Australia
F	Liquid	Light brown	Brazil
G	Powder	Light brown	Brazil
H	Powder	Light brown	Brazil
I	Liquid	Light brown	Brazil

II. Extraction of Flavonoids from Propolis

About 1 g (accurately weighed to 0.0001 g) of pulverized raw propolis was extracted with 10 mL of 80% ethanol under 200 rpm shaking at ambient temperature for 24 hr. After filtration through a Toyo 5C filter paper (Toyo Roshi Kaisha, Ltd., Japan), the filtrate was made up to 25 mL with 80% ethanol and stored in an amber bottle until analysis.

For the commercial propolis powder, 0.1 to 1 g (accurately weighed to 0.0001 g) of the sample was extracted with 10 mL of 80% ethanol. After centrifugation at $1,000 \times g$ for 10 min, supernatant was collected and the precipitate was extracted again with another 5 mL of 80% ethanol twice. All the supernatants were combined and made up to 25 mL with 80% ethanol. Commercial propolis tinctures were diluted directly with 80% ethanol to the concentrations appropriate for analysis. For quantitation, those samples with peak areas beyond the range of calibration curve were further diluted before analysis.

Flavonoid contents were determined and calculated as the mean of three replicates.

III. Capillary Electrophoresis

(I) Capillary zone electrophoresis (CZE)

CZE was conducted using a Beckman P/ACE System 5000 apparatus equipped with a 57 cm (50 cm to the detector) $\times$ 75 μ m i.d. fused-silica capillary. Running buffer of 0.1 M borate (pH 9.5)-5% methanol was used. The voltage was 18 kV and the sample was injected electrokinetically for 4 sec. All runs were operated at 25°C and detected at 214 nm. The electrophoretograms were integrated with Beckman System Gold program (version 8.1).

To examine the resolution efficiency of flavonoid standards, theoretical plate number (N), relative standard deviation (RSD) and detection limit (DL) were calculated based on the following equations: $N = 16 (tm/w)^2$, where tm was the migration time and w was the peak width; $RSD = SD/mean \times 100\%$; $DL = 2 \times C \times S/R$, where C was the analyte concentration close to blank level, S was the standard deviation, and R was the average of analyte absorbance.

(II) Micellar electrokinetic capillary chromatography (MECC)

MECC was conducted using the same instrument as described above. The running buffer was 0.03 M sodium borate-0.05 M SDS (pH 8.5) and the voltage was -15 kV. The other parameters were identical to those used in CZE.

The capillary column was pre-equilibrated by 2 cycles of sequential washing with deionized water (2 min), 0.1N HCl (2 min), deionized water (2 min), 0.1N NaOH (2 min) and deionized water (2 min) daily before use. Between runs, the capillary was conditioned by rinsing with deionized water (1 min), 0.1N HCl (1 min), deionized water (1 min), 0.1N NaOH (1 min), deionized water (1 min) and running buffer (2 min).

RESULTS AND DISCUSSION

I. Separation of Flavonoid Standards by CZE and MECC

During method development, factors such as detection wavelength, electrophoretic buffer and pH value that would affect the separation conditions were examined. Based on the absorption spectra of flavonoid standards, 214, 254 and 280 nm were initially determined for optimal wavelength selection. Since at 214 nm the peak height was greatest and the absorbance of each compound was quite even (data not shown), this wavelength was utilized throughout the experiment.

In general, CZE separates compounds based on their differences in molecular weight and charge. However, with the use of Tris-HCl (pH 9.5) as running buffer all flavonoid standards could not be retained for more than 10 min and were grouped into 8 peaks (data not shown). It indicated that only the ionization of hydroxyl groups in flavonoid structure is not sufficient to result in significant difference in charge/mass ratio. Whereas the use of alkaline borate buffer as the running buffer significantly improved the separation of selected flavonoids (Figure 2). It is well known that borate anions reacted with polyols, preferentially at the *cis*-1, 2 hydroxyl groups, to form negatively charged borate complexes⁽²⁵⁾. Therefore, difference in ratios of borate-flavonoid complexes resulted in difference of electrophoretic mobility (EPM), which lead to the separation of flavonoid standards. In fact, the concentration of borate played an important role in the separation of flavonoids. At borate concentration of 0.05 M, electroosmotic flow (EOF) was much stronger than electrophoretic migration (EPM) and the migration time was short with poor resolution. As borate concentrations increased to higher than 0.1 M, quercitrin and quercetin co-migrated with naringenin and myricetin, respectively. Also, the increasing joule heat resulted in peak broadening of morin. Therefore, 0.1 M concentration was found most satisfactory to stabilize borate-flavonoid complexes, to avoid zone broadening caused by dispersion effect, and to separate the selected standards except for quercetin and myricetin.

The pH effect on the separation of flavonoids is controversial. Although the ionization of hydroxyl groups and

the stability of flavonoid-borate complexes increase as the pH value of the electrophoretic buffer increases, flavanones may be transformed to chalcones in alkaline media⁽²⁶⁾. By comparing the electrophoretograms obtained at pH 9.0, 9.2, 9.4, 9.5, 9.6, and 10, we found that the best separation of the selected flavonoid standards was achieved at pH 9.5 (Figure 3). At pH values lower than 9.5, increase in pH retarded the migration and facilitated the separation.

Whereas at pH values higher than 9.5, the excessive negative EPM of naringenin and galangin resulted in co-migration with quercitrin and luteolin, respectively.

To shorten the analytical time, the use of organic solvent was also considered. With the addition of 5% methanol to 0.1 M borate buffer (pH 9.5), we found that the migration time of the last eluted compound, morin, decreased from 29 min to 25.61 min (Figure 4A). Similarly,

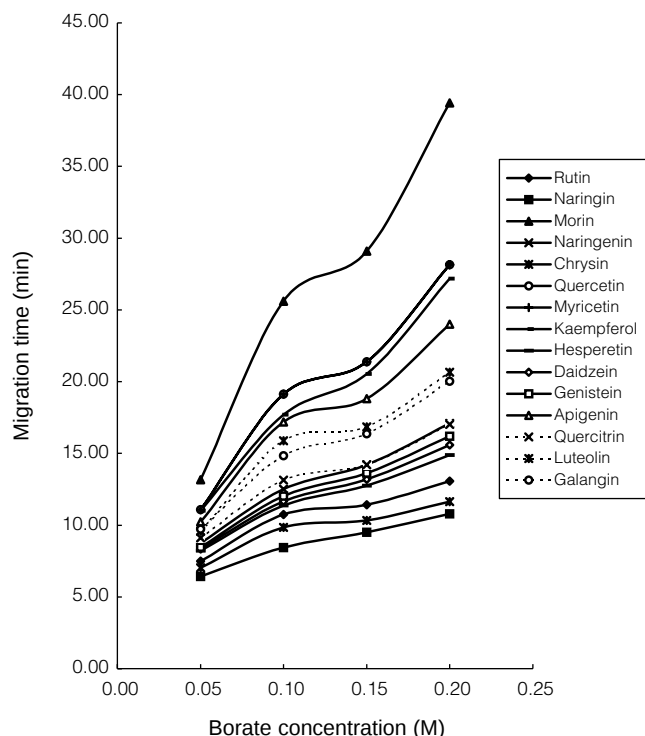


Figure 2. Influence of borate concentration on migration time of 15 flavonoid standards at pH 9.5. The concentration of each flavonoid standard was 50 ppm.

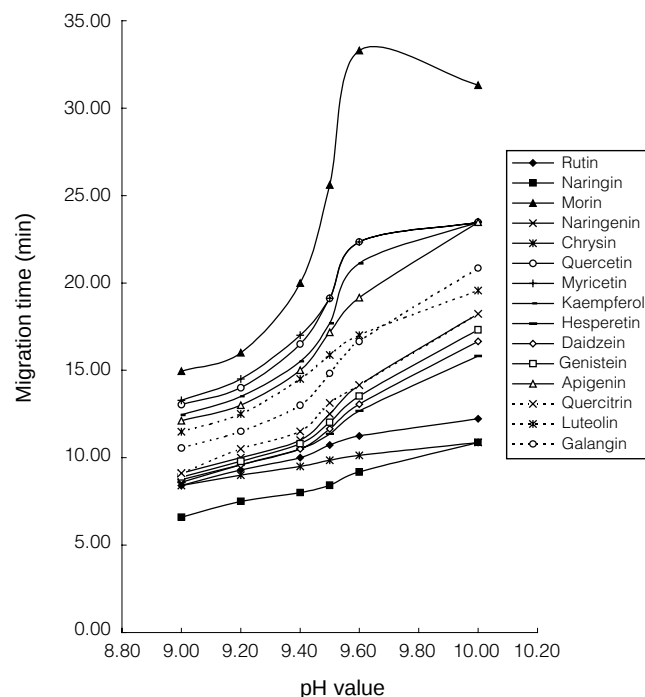


Figure 3. Influence of the pH value of 0.1 M H_3BO_3 buffer on the separation of 15 flavonoid standards. The concentration of each flavonoid standard was 50 ppm.

Table 2. The resolution efficiency of 15 flavonoid standards determined by CZE^a

Flavonoids	MT (min)	R^{2b}	N	RSD (%) (n = 4) ^c			DL (ppm)	Linear regression
				Height	Area	Time		
Apigenin	17.2	0.9901	169860	1.65	4.95	0.42	0.084	$y=0.6154x-0.051$
Chrysin	9.9	0.9921	84160	5.7	1.35	0.87	0.215	$y=0.3584x-0.035$
Luteolin	15.9	0.9901	142890	4.48	2.96	1.43	0.098	$y=0.5458x-0.0833$
Galangin	14.8	0.9944	126650	1.65	5.34	0.37	0.139	$y=0.5019x-0.0593$
Kaempferol	17.7	0.9937	183910	4.87	5.12	1.28	0.091	$y=0.4785x-0.1115$
Morin	25.6	0.9915	100130	4.82	2.77	0.67	0.333	$y=0.5811x-0.1061$
Myricetin+quercetin	19.1	0.9920	63310	2.44	4.60	0.37	0.271	$y=0.9088x-0.4767$
Quercitrin	13.1	0.9914	11610	5.06	1.08	1.15	0.135	$y=0.4776x+0.1236$
Rutin	10.7	0.9909	101530	0.01	2.12	1.11	0.111	$y=0.2239+0.0194$
Hesperetin	11.4	0.9939	115480	5.40	1.57	0.60	0.027	$y=0.5393x-0.0251$
Naringin	8.4	0.9909	161620	7.41	2.27	0.30	0.331	$y=0.1429x+0.036$
Naringenin	12.5	0.9928	359700	1.53	5.41	0.47	0.059	$y=0.5104x-0.0318$
Daidzein	11.7	0.9937	96040	1.29	6.35	0.42	0.006	$y=0.3953x-0.014$
Genistein	12.0	0.9932	129690	5.40	1.38	0.61	0.065	$y=0.4411x-0.0237$

^aMT, migration time; R^2 , coefficient of determination; N (theoretical plate number) = $16 (tm/w)^2$, where tm is the migration time and w is the peak width; RSD, relative standard deviation; DL (detection limit) = $2 \times C \times S/R$, where C is the analyte concentration close to blank level, S is the standard deviation, and R is the average of analyte absorbance.

^bCoefficients of determination were calculated from the peak area vs. the concentration of standard solutions at 25, 50, 75, 100 and 125 ppm.

^cRelative standard deviation was determined with standard solutions at 100 ppm in 4 replicates.

the aid of organic modifiers such as methanol and methylcellulose decreases EOF. McGhie (1993) manipulated pH and methanol effects to separate major flavones from sugarcane⁽²⁷⁾. The best separation was achieved with 25 mM borate buffer (pH 9.5) plus 20% methanol.

Figure 4A shows the typical electrophoretogram of flavonoid standards separated by CZE. Baseline separation was obviously achieved for flavonoids except for quercetin and myricetin. Based on the electrophoretograms, the coefficients of determination, R^2 , and relative standard deviation, RSD, were calculated for quantitative determination. Results show that the variation of peak area is closely related to that of concentration, since all R^2 are greater than 0.99 (Table 2). The RSDs of peak height, peak area and migration time are 0.01 to 7.41%, 1.08 to 6.35%, and 0.30 to 1.43%, respectively. Detection limits of selected flavonoid standards are also listed in Table 2.

Another mode of capillary electrophoresis, MECC, was supplemented to separate quercetin and myricetin. With the addition of anionic surfactant SDS at a concentration (0.05M) higher than the critical micelle concentration, 5.5–9.6 mM⁽²⁸⁾ or 8.1 mM⁽²⁹⁾, the smaller molecular polarity due to only one less hydroxyl group in the B ring of quercetin resulted in higher partition coefficient between

micellar and aqueous phases. Therefore, quercetin migrated slower than myricetin and the resolution was improved (Figure 4B). MECC conducted with a fused-silica column (75 cm $\times$ 75 μ m i.d.) and 0.2 M sodium borate buffer (pH 8.0)-50 mM SDS-10% methanol was utilized to separate honey flavonoids⁽²¹⁾. However, quercetin and luteolin did not reach the baseline-separation, neither did kaempferol and apigenin. The MECC conditions we developed were also unsatisfactory in the separation of some flavonoids (data not shown). Until the development of better MECC parameters, the current MECC method needs to be associated with the major CZE method.

II. Quantitative Determination of Flavonoids in Propolis

(I) Raw propolis

The electrophoretogram of raw propolis termed China 1 was shown in Figure 5. The peaks were identified by comparison of migration time and co-injection with the flavonoid standard. Although huge peaks between 7 to 10 min were observed, chrysin and rutin were not found in these samples. According to the electrophoretic profile of 15 flavonoid standards, it was suggested that those peaks before 10 min might be flavonoid glycosides, especially flavone glycosides, since naringin, chrysin and rutin were the first three eluted compounds.

Furthermore, the absolute amount of each identified flavonoid in nine samples was calculated and listed in Table 3. The total content of identified flavonoids in raw propolis

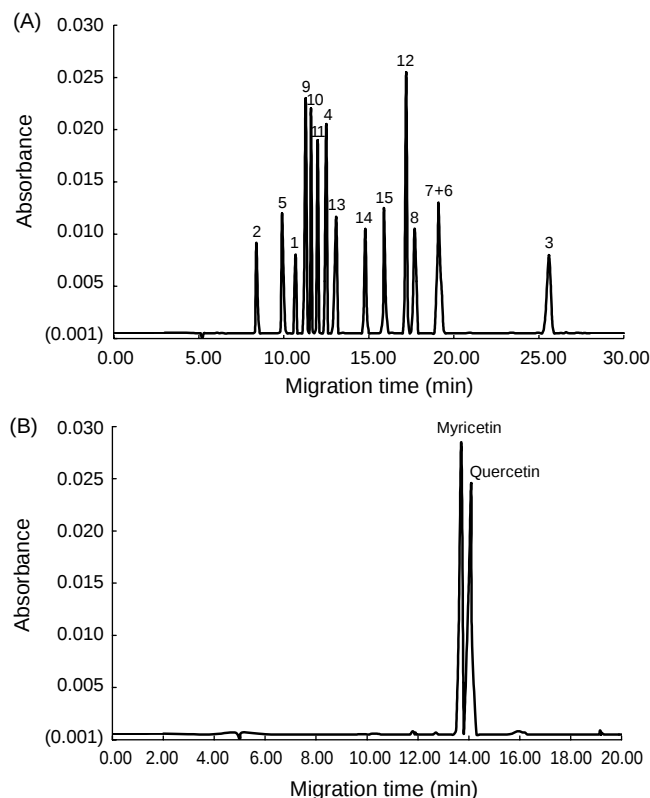


Figure 4. Typical electrophoretograms of flavonoid standards obtained by CZE (A) and MECC (B) with parameters described in Materials and Methods. The concentration of each flavonoid standard was 50 ppm. Peak identification: 1, rutin; 2, naringin; 3, morin; 4, naringenin; 5, chrysin; 6, quercetin; 7, myricetin; 8, kaempferol; 9, hesperetin; 10, daidzein; 11, genistein; 12, apigenin; 13, quercitrin; 14, luteolin; 15, galangin.

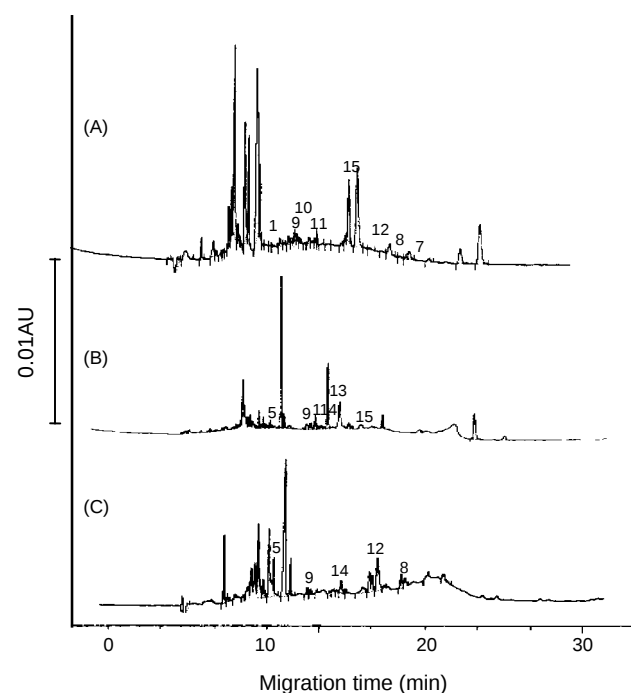


Figure 5. Electrophoretograms of raw propolis (A), commercial propolis tincture B (B) and commercial propolis powder H (C) analyzed by CZE with parameters described in Materials and Methods. Peak identification was given in Figure 4.

ranged from 254 to 19147 ppm. None of these samples contained luteolin, morin or quercitrin. Propolis produced in China contained the highest amount of flavonoids, including three major compounds, galangin, kaempferol and apigenin. However, the compositions of Brazil propolis and Taiwan propolis were inconsistent with that of China propolis. For instance, the most abundant flavonoid in Brazil 1, Brazil 2, Brazil 3 and Brazil 4 was naringin,

apigenin, hesperetin and galangin, respectively. Tomas-Barberan *et al.*⁽⁸⁾ and Markham *et al.*⁽¹⁶⁾ suggested flavonoid profiles as markers for the geographic origin of propolis. It is interesting that the collection of propolis varies with the races of honeybees⁽¹⁾. In contrast to Caucasian bees and dark forest bees, Italian, Ukrainian and tropical bees collect far less propoli.

Table 3. The flavonoid contents of 9 raw propolis samples

Flavonoids	Content (ppm) ^a								
	Brazil 1	Brazil 2	Brazil 3	Brazil 4	China 1	China 2	China 3	Taiwan 1	Taiwan 2
Flavones									
Apigenin	— ^b	1315	—	—	1592	994	811	—	—
Chrysin	—	308	1811	912	—	—	—	—	—
Luteolin	—	—	—	—	—	—	—	—	—
Flavonols									
Galangin	210	—	—	2918	13402	11355	8941	—	—
Kaempferol	120	699	—	829	1437	1863	1580	145	168
Morin	—	—	—	—	—	—	—	—	—
Myricetin	—	—	—	—	506	335	226	—	—
Quercetin	—	146	726	—	—	—	—	—	86
Quercitrin	—	—	—	—	—	—	—	—	—
Rutin	122	—	—	—	558	690	—	—	—
Flavanones									
Hesperetin	—	675	2753	558	103	325	296	—	—
Naringin	570	—	406	—	—	—	—	1231	—
Naringenin	—	252	—	—	—	—	—	—	—
Isoflavones									
Daidzein	31	704	—	—	1019	1207	434	—	—
Genistein	—	242	—	—	530	860	537	—	—
Total	1053	4341	5696	5217	19147	17629	12825	1376	254

^aFlavonoid content was calculated based on moist weight. Values are means of three replicates.

^b—: not detectable.

Table 4. The flavonoid contents of 9 commercial propolis samples

Flavonoids	Content (ppm, w/w) ^a								
	A (63.3%)	B (21.0%)	C (24.0%)	D (11.6%)	E (15.3%)	F (15.5%)	G (96.4%)	H (96.0%)	I (7.41%)
Flavones									
Apigenin	1067	— ^b	98	—	—	—	69	57	157
Chrysin	—	423	247	4782	66	314	1060	274	—
Luteolin	—	—	—	—	—	—	—	—	—
Flavonols									
Galangin	—	150	571	1369	—	—	369	393	608
Kaempferol	842	—	360	—	—	—	131	366	—
Morin	—	—	—	—	—	—	—	—	—
Myricetin	—	—	—	—	—	—	—	—	—
Quercetin	—	—	—	—	—	—	—	—	—
Quercitrin	1300	3311	—	—	—	649	—	—	358
Rutin	—	—	—	1259	179	—	—	—	—
Flavanones									
Hesperetin	510	559	104	—	77	84	—	138	—
Naringin	—	—	513	278	1075	2120	146	—	—
Naringenin	—	—	—	—	—	—	45	—	313
Isoflavones									
Daidzein	771	—	122	—	—	—	—	—	—
Genistein	912	235	263	297	111	—	133	—	—
Total	5402	4678	2278	7985	1508	3167	1953	1228	1436

^aData are means of three replicates. Numbers in parentheses indicate the solid content as weight percentage.

^b—: not detectable.

(II) Commercial propolis products

Figure 5 also shows the electrophoretograms of propolis tincture B and powder H to represent all commercial samples. In fact, luteolin, morin, myricetin and quercetin were not found in any commercial product.

The total content of identified flavonoids in nine propolis products ranged from 1228 to 7985 ppm (Table 4). Although the solid contents of samples G and H were several times higher than those of tinctures, the levels of known flavonoids in G and H were relatively lower, indicating the presence of adulterants.

CONCLUSIONS

The analytical methods for 15 commercially available flavonoid standards using capillary zone electrophoresis and micellar electrokinetic capillary electrophoresis were developed. Moreover, the developed methods were applied to investigate the absolute amount of each flavonoid in raw and processed propolis samples. Since many peaks of propolis products in the electrophoretograms have not been identified, our understanding about the composition of propolis will be extended as more standard compounds become available.

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