Development of High-Performance Liquid Chromatographic for Quality and Authenticity Control of Chinese Propolis

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Abstract: A RP-high-performance liquid chromatography (HPLC) method was developed for quality control of Chinese propolis by simultaneous analysis of 12 flavonoids and 8 phenolic acids. The results showed that vanillic acid, rutin, myricetin, and luteolin were not detected in all of the analyzed propolis and poplar tree gum samples. The caffeic acid, ferulic acid and p-coumaric acid were not detected in poplar tree gum but were detected in propolis, which suggest that they are practical indexes of distinguishing propolis from poplar tree gum. The flavonoid profiles of poplar tree gum were found to be similar to those of propolis, which are dominated by pinobanksin, pinocembrin, 3-O-acetylpinobanksin, chrysin, and galangin. Therefore, the proposed method could be applied to exclude poplar tree gum from propolis with cafferic acid, ferulic acid, and p-coumaric acid as qualitative markers, and distinguish poplar source resin from other illegal substances, and evaluate the quality grading of poplar-type propolis with pinobanksin, pinocembrin, 3-O-acetylpinobanksin, chrysin, and galangin as qualitative and quantitative markers.

Keywords: flavonoids, HPLC, phenolic acid, poplar tree gum, quality control

Introduction

Propolis is a resinous substance collected by honeybees from buds and exudates of different plants which mixed with beeswax and salivary enzymes (Bankova and others 2000). Propolis is used for coating hive parts and sealing cracks and crevices with a low incidence of bacteria and moulds in the hive. It is also a traditional remedy in folk medicine and has been reported to possess various biological activities, such as antibacterial, antifungal, antiviral, anti-inflammatory, antioxidant, and immunostimulating activities

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honeybees (Zhang and others 2011a, 2011b; Zhang and others 2012b).

Consequently, the present work was intended to establish an accurate and practical analysis method for the simultaneous determination of individual flavonoids and phenolic acids so as to provide technical support for quality control and authenticity assessment of Chinese propolis. Concerning more than 100 flavonoids and 100 phenolic acids have been found in propolis of different origins (2009; Zhang and others 2013), while most of them are not commercially available. Therefore, 12 flavonoids and 8 phenolic acids were selected as markers for qualitative and quantitative determination.

Materials and Methods

Chemicals and reagents. HPLC-grade methanol was obtained from Merck (Darmstadt, Germany). HPLC-grade water was purified by Yjd-upws ultra-pure water system (China). Absolute alcohol and acetic acid were analytical grade.

Vanillic acid, caffeic acid, ferulic acid, isoferulic acid, p-coumaric acid, cinnamic acid, 3,4-dimethoxycinnamic acid, CAPE, rutin, myricetin, apigenin, galangin, chrysin, pinocembrin, quercetin, kaempferol, luteolin, naringenin were purchased from Sigma-Aldrich (St. Louis, Mo., U.S.A.), pinobanksin, 3-O-acetylpinobanksin were purchased from Ningbo Haishu Apexocean Biochemicals Co., Ltd. (Ningbo, China).

Propolis and poplar tree gum samples. In total, 66 propolis samples were harvested by local beekeepers in 17 provinces between July 2011 and August 2013, Figure 1. Eight poplar tree gum samples were randomly purchased from different providers. These samples were frozen at −20 °C until analysis.

Liquid chromatographic procedure. The chromatographic system consisted of an Agilent 1200 series, equipped with a vacuum degasser G 1322A, a quaternary pump G1311A, an autosampler G1329A, a programmable variable wavelength detector G1314B, and a Thermostatted Column Compartment G1316A (Agilent Technologies, Inc., Santa Clara, Calif., U.S.A.). A Sepax HP-C18 column (150 mm × 4.6 mm, 5 μm; Sepax Technologies, Inc., Newark, Del., U.S.A.) was applied for all analyses. The mobile phase was 1.0% aqueous acetic acid (v/v) (A) and methanol (B) in the gradient mode at 33 °C as follows: 15% to 40% (B) at 0 to 30 min, 40% to 55% (B) at 30 to 65 min, 55% to 62% (B) at 65 to 70 min, 100% (B) at 70 to 85 min at a flow rate of 1.0 mL/min. The effluent was monitored at 280 nm. The injection volume was 5 μL.

Preparation of standard stock solutions. The reference standards of the twenty compounds were accurately weighed and

was the best condition for factor B. The best conditions for the remaining factors were the 3rd level of factor A (K3 was 2.457), the 1st level of factor C (K1 was 2.500) and the 3rd level of factor D (K3 was 2.503). Therefore, the extraction conditions were optimized as follows: 10 g powder of the samples was extracted in triplicate with 150 ml 95% aqueous ethanol in an ultrasonic water bath for 45 min.

Chromatography

The best separation and resolution of peaks, allowing the quantification of the 20 phenolic compounds in propolis samples were achieved with the parameters described in Section 2.3, in a 70 min analysis.

The chromatogram of a standard mixture of the evaluated phenolic compounds is shown in Figure 2A. Base line separation was obtained for all the compounds. The identity of each peak from samples was confirmed by comparing their retention time and UV spectrum with that of reference compound. In addition, spiking samples with the reference compounds further confirmed the identities of the peaks. Vanillic acid, rutin, myricetin, and luteolin were not detected in analyzed propolis and poplar tree gum samples as well as caffeic acid, ferulic acid, and p-coumaric acid were not detected in poplar tree gum samples, as shown in Figure 2B–C.

Method validation

Linearity. The working standard solutions were freshly prepared in methanol by appropriate dilution of the stock solutions to yield 6 concentrations. Table 2 listed linear calibration curve with R^2, linear range, LOD, and LOQ of each compound determined. As a result, the obtained linear range was adequate for all the compounds. The correlation coefficient for the standard compounds was higher than 0.999, with the exception of myricetin for 0.9981, which gave a good linearity response for the developed method. The obtained values for both LOD and LOQ were low ranged from 0.10 to 3.38 μg/mL and from 0.95 to 11.25 μg/mL, respectively, which meant that the method is capable of not only quantifying all the used standards, but also detecting traces of these phenolic compounds.

Recovery. As shown in Table 1, recovery of the components ranged from 89.9% to 112.6% and the RSDs were all less than 4%.

Repeatability, precision, and accuracy. The results of precision showed that the RSD of the intra- and interday for retention times was 0.12% to 0.23% and 0.20% to 0.32%, and for peak areas was 0.99% to 2.15% and 0.94% to 2.58%, respectively (Table 2).

Figure 1–Distribution of sampling locations. The numbers in parentheses indicate the sample size of each province.

Table 1–Regression data, LODs, LOQs, and recovery for 20 analytes.

Compound	Regression equation[a]	r^2	Linear range (μg/mL)	LOQ (μg/mL)	LOQ (μg/mL)	Revovery (percentage)
Vanillic acid	$y = 7.36x + 3.3435$	0.9995	10 to 120	3.31	0.94	101.8 to 103.9
Caffeic acid	$y = 17.111x + 4.8313$	0.9995	6 to 72	1.35	0.41	98.6 to 101.4
p-Coumaric acid	$y = 23.054x + 86.406$	0.9994	50 to 600	2.16	0.39	102.7 to 106.4
Ferulic acid	$y = 14.766x + 4.1992$	0.9994	5 to 60	4.0	1.2	103.5 to 106.7
Isoferulic acid	$y = 20.84x - 3.26$	0.9996	10 to 50	2.12	0.64	99.9 to 101.1
3,4-dimethoxycinnamic acid	$y = 13.73x + 11.921$	0.9994	10 to 120	2.32	0.70	101.7 to 112.2
Rutin	$y = 3.0759x + 3.8067$	0.9991	20 to 120	11.25	3.38	100.4 to 110.1
Cinnamic acid	$y = 39.96x + 11.055$	0.9994	5 to 60	0.95	0.28	98.8 to 102.4
Myricetin	$y = 4.8536x + 10.804$	0.9981	10 to 120	9.0	2.7	100.0 to 108.3
Pinobanksin	$y = 12.289x + 23.356$	0.9994	30 to 360	0.98	0.29	98.3 to 106.5
Naringenin	$y = 7.9146x + 4.9733$	0.9992	10 to 60	5.14	1.54	98.2 to 104.3
Quercetin	$y = 5.604x + 0.8267$	0.9992	10 to 60	8.57	2.57	94.6 to 103.5
Luteotin	$y = 7.6679x + 3.1333$	0.9992	12 to 72	6.35	1.91	94.4 to 111.8
Kaempferol	$y = 15.177x - 0.5384$	0.9994	10 to 120	3.79	1.14	101.3 to 107.1
Apigenin	$y = 10.269x + 4.4761$	0.9993	10 to 120	5.14	1.54	91.7 to 97.8
Pinocembrin	$y = 14.659x + 25.562$	0.9991	50 to 600	3.69	1.11	93.7 to 109.4
3-O-Acetyl pinobanksin	$y = 10.818x + 15.734$	0.9994	20 to 240	4.97	1.49	92.6 to 112.6
Chrysin	$y = 19.334x + 46.601$	0.9994	40 to 480	2.88	0.86	89.9 to 98.3
CAPE	$y = 8.6529x + 1.8164$	0.9992	20 to 240	6.55	1.96	96.1 to 105.2
Galangin	$y = 21.131x - 8.1069$	0.9990	20 to 240	3.03	0.91	97.2 to 111.8

[a] y is the peak area in UV chromatograms monitored at 280 nm, x the compound concentration injected.

Although the repeatability of retention time and component content for every phenolic compound exhibited a difference, RSD values for component content were all less than 4.2% and retention time were all less than 0.3%, which could meet the need of quantitative analysis (Table 2).

Above results demonstrated that the HPLC method is precise, accurate, and sensitive for the quantitative determination of flavonoids and phenolic acids in propolis and poplar tree gum samples.

Simultaneous quantification of phenolic compounds in propolis and poplar tree gum. Considering the chemical composition of propolis samples may vary depending on the chemical composition of the plants visited by honeybees, 66 Chinese propolis samples from 17 provinces and 8 poplar tree gum samples were analyzed using established extraction method under the above HPLC conditions. Phenolic compounds quantification was achieved by the absorbance recorded in the chromatograms relative to standards of phenolic compounds. Each sample was analyzed

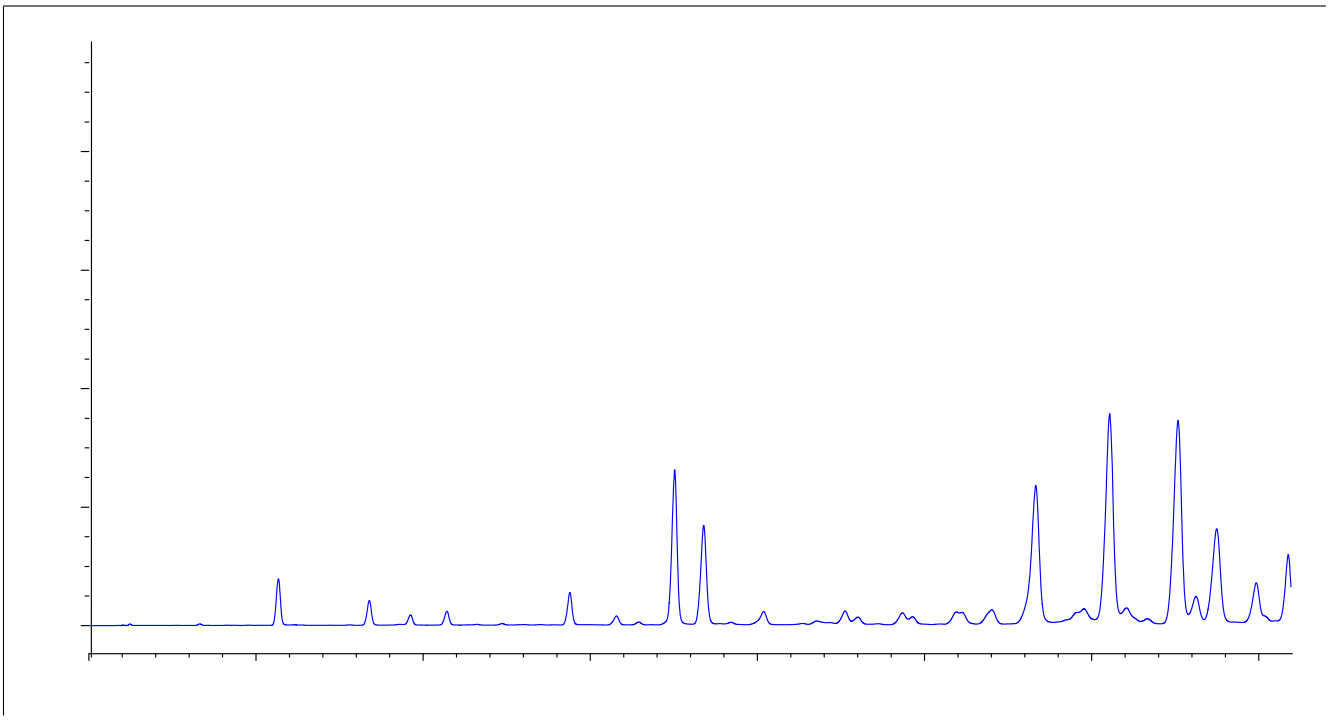

Table 2–Precision and repeatability data of 9 flavonoids and 7 phenolic acids in propolis ($n = 6$).

	Precision (RSD percentage)				Repeatability (RSD percentage)	
	Interday		Intraday			
Compound	Retention time	Peak area	Retention time	Peak area	Retention time	Content
Caffeic acid	0.23	1.18	0.32	1.64	0.24	3.68
p-Coumaric acid	0.16	1.18	0.24	1.60	0.22	1.90
Ferulic acid	0.14	1.29	0.22	1.60	0.18	4.18
Isoferulic acid	0.13	1.28	0.21	1.82	0.15	2.16
3,4-Dimethoxycinnamic acid	0.13	1.25	0.23	1.65	0.10	2.05
Cinnamic acid	0.12	0.99	0.21	1.70	0.09	1.24
Pinobanksin	0.13	1.06	0.20	1.50	0.11	2.07
Naringenin	0.15	1.06	0.21	1.48	0.10	1.78
Quercetin	0.18	1.34	0.26	2.17	0.16	1.62
Kaempferol	0.17	1.21	0.23	2.34	0.14	1.87
Apigenin	0.23	1.07	0.32	1.33	0.15	1.29
Pinocembrin	0.16	1.15	0.23	1.96	0.10	1.97
3-O-Acetyl pinobanksin	0.15	1.27	0.22	2.58	0.10	1.29
Chrysin	0.17	1.01	0.23	0.94	0.11	3.51
CAPE	0.14	2.15	0.20	2.92	0.09	2.03
Galangin	0.17	1.28	0.23	2.15	0.10	2.16

in triplicate to determine the mean contents of each phenolic compound in ethanol extract of propolis and poplar tree gum.

The typical HPLC chromatograms of the various propolis from different geographical origins and poplar tree gum samples are shown in Figure 3A and 3B, respectively. Among all the peaks observed in propolis samples, they were generally consistent although the quantity and absorption intensity of peaks were different. Caffeic acid, p-coumaric acid, ferulic acid, isoferulic acid, 3,4-dimethoxycinnamic acid, cinnamic acid, pinobanksin, pinocembrin, 3-O-acetylpinobanksin, chrysin, CAPE and galangin were defined as common peaks because they showed up in all propolis samples with exception of isoferulic acid in 2 samples and CAPE in 3 samples were not detected. For poplar tree gum, isoferulic acid, 3,4-dimethoxycinnamic acid, cinnamic acid, pinobanksin, pinocembrin, 3-O-acetylpinobanksin, chrysin, CAPE, and galangin were detected in all samples. Naringenin, quercetin, kaempferol, and apigenin were detected in some propolis or poplar tree gum samples, whose content varied from 0.68 to 21, 0.99 to 12.08, 0.1 to 3.87, and 0.77 to 9.55 mg/g for propolis samples and varied from 0.59 to 0.82, 1.75 to 8.30, 0.38 to 1.25, and 0.42 to 5.44 mg/g for poplar tree gum samples, respectively.

Table 3 shows the content of the common compounds in different propolis and poplar tree gum samples, the content of each analyte varied greatly among the propolis samples from different provinces (RSD percentage varied from 21.59 to 263.88), even within the same province (RSD percentage varied from 2.18 to 131.34) and poplar tree gum samples (RSD percentage varied from 10.89 to 81.56). The flavonoid profiles of poplar tree gum samples were found to be similar to those of propolis, which are dominated by pinobanksin, pinocembrin, 3-O-acetylpinobanksin, chrysin, and galangin, whose total content varied from 62.12 to 315.35 mg/g for propolis samples and varied from 59.76 to 141.21 mg/g for poplar tree gum samples, which accounts for more than 88 percent and 86 percent of all analyzed flavonoids in propolis and poplar tree gum samples, respectively. Moreover, the average content of isoferulic acid, 3,4-dimethoxy cinnamic acid, and cinnamic acid in poplar tree gum was higher while the pinobanksin, pinocembrin, 3-O-acetyl pinobanksin, chrysin, CAPE, and galangin was lower than that in propolis samples.

Discussion

In this study, the developed RP-HPLC method proved to be sensitive and reliable for the analyses of phenolic compounds in propolis. Parameters of method validation such as linearity, precision, and accuracy gave satisfactory results, allowing its use in quality control of propolis.

According to our results, rutin and myricetin were detected neither in propolis nor poplar tree gum, caffeic acid, ferulic acid, and p-coumaric acid were observed in all propolis but not detected in poplar tree gum. In addition, pinobanksin, pinocembrin, 3-O-acetylpinobanksin, chrysin, and galangin are the most common flavonoids in all analyzed propolis and poplar tree gum samples. Therefore, this developed method could be applied to exclude poplar tree gum and the artificially added rutin with caffeic acid, ferulic acid, p-coumaric acid and rutin as qualitative markers. Meanwhile, we may distinguish poplar source resin from other illegal substances, and evaluate the quality grading of poplar-type propolis with pinobanksin, pinocembrin, 3-O-acetylpinobanksin, chrysin, and galangin as qualitative and quantitative markers. This study will provide useful information for formulating quality control criteria for Chinese propolis.

Propolis has a common flavonoid profile with poplar tree gum, which hints that *populus* are the main plant origin of Chinese propolis. However, caffeic acid, ferulic acid, and p-coumaric acid were not detected in poplar tree gum. Considering the average content of isoferulic acid, 3,4-dimethoxy cinnamic acid, and cinnamic acid in poplar tree gum was higher than those in propolis samples, cafferic acid, ferulic acid, and p-coumaric acid may be enzymatic hydrolysate of cinnamic acid and its derivatives. The hydrolytic enzymatic activities of honey bees have been recognized to contribute to the differences between the phenolic profiles of mature honey and freshly deposited honey (Truchado and others 2010). We have demonstrated that flavonoids monoglycosides such as quercetin 3-O-glucoside were rapidly hydrolyzed by beta-Glycosidase from honeybee (Zhang and others 2011a, 2012b). Therefore, honeybees collect substances from local plants available to them as raw material and metabolize them to produce the characteristic propolis using their enzymes. On the other hand, the process-cycle of poplar tree gum by water boiling and condensing of *populus* buds may give rise to possible degradations or

oxidation. However, these theoretical predictions require a considerable further study.

Rutin and myricetin, the markers which have been used previously to evaluate the quality of propolis, could not be detected in this study. They may coelute with other analytes and disturb the quantitation in previous study. Moreover, relative low levels of free quercetin, kaempferol, apigenin are present in Chinese propolis samples and poplar tree gum. These results are inconsistent with previous reports (Zhou and others 2008; Cai and others 2012) and national standard (GB/T 19427–2003). As a result, it is necessary and rational to improve the current quality control of propolis.

Although the results of the analyses on the 66 propolis samples suggested that the content of phenolic compounds varied significantly in samples from geographical origins, the content of

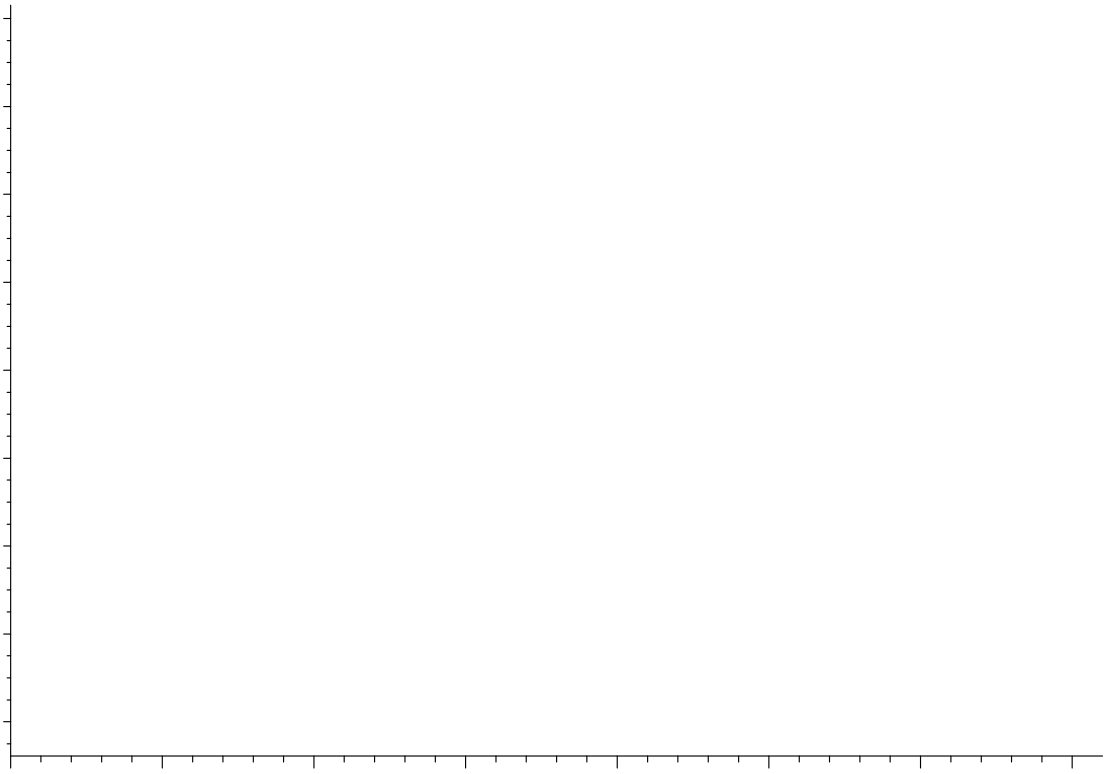

Table 3–The content of common compounds in different propolis and poplar tree gum samples.

	Amount (mg/g, mean ± SD)								
Sample location	**Isoferulic acid**	**3,4-Dimethoxy cinnamic acid**	**Cinnamic acid**	**Pinobanksin**	**Pinocembrin**	**3-O-acetylpinobanksin**	**Chrysin**	**CAPE**	**Galangin**
Zhejiang	2.77 ± 1.47	8.14 ± 6.38	0.42 ± 0.17	18.19 ± 10.31	23.91 ± 21.74	62.21 ± 43.89	37.03 ± 29.85	11.15 ± 7.21	14.92 ± 7.82
Anhui	3.74 ± 2.09	11.45 ± 6.29	2.25 ± 1.98	28.0 ± 13.51	43.7 ± 18.85	53.4 ± 20.95	44.82 ± 6.74	15.35 ± 2.43	16.53 ± 5.48
Sichuan	3.35 ± 1.35	10.31 ± 5.84	2.47 ± 3.09	36.63 ± 3.07	62.41 ± 3.06	109.46 ± 26.76	37.23 ± 0.81	12.88 ± 10.28	20.14 ± 4.14
Guizhou	4.85	16.66	30.49	20.23	48.94	48.02	8.73	2.16	8.90
Hebei	3.85 ± 2.82	8.56 ± 5.69	1.77 ± 1.87	14.95 ± 8.32	39.02 ± 9.96	50.36 ± 16.11	34.79 ± 19.82	14.95 ± 9.38	14.08 ± 2.22
Henan	2.09 ± 0.65	6.13 ± 2.33	2.04 ± 1.65	31.69 ± 16.96	49.17 ± 9.12	57.41 ± 31.49	46.88 ± 22.45	16.60 ± 9.28	16.74 ± 4.71
Xinjiang	9.92 ± 2.09	12.50 ± 5.06	0.22 ± 0.047	18.17 ± 12.96	36.08 ± 7.71	108.79 ± 39.64	42.21 ± 12.58	14.67 ± 11.66	12.33 ± 5.35
Jilin	2.11 ± 1.54	3.83 ± 3.55	0.44 ± 0.24	20.30 ± 12.32	68.00 ± 36.79	76.18 ± 23.24	33.92 ± 14.63	9.72 ± 6.54	17.27 ± 5.78
Heilongjiang	6.59 ± 4.04	9.66 ± 6.13	0.27 ± 0.20	14.79 ± 8.05	46.29 ± 7.14	83.46 ± 48.23	36.37 ± 19.20	26.07 ± 19.04	13.79 ± 4.81
Shandong	4.35 ± 2.16	9.81 ± 4.65	1.33 ± 0.83	26.39 ± 11.56	36.97 ± 7.14	61.96 ± 19.59	51.28 ± 11.89	23.05 ± 10.02	15.93 ± 3.98
Neimenggu	4.13 ± 2.49	7.44 ± 6.62	0.33 ± 0.18	9.58 ± 10.13	58.99 ± 36.67	64.66 ± 55.51	28.86 ± 26.67	9.28 ± 7.29	16.03 ± 6.74
Liaoning	5.81 ± 2.90	11.44 ± 5.49	0.36 ± 0.10	21.43 ± 16.04	47.45 ± 11.01	100.95 ± 39.72	38.16 ± 6.51	20.59 ± 7.76	15.38 ± 3.87
Beijing	7.65 ± 0.22	17.32 ± 0.57	0.82 ± 0.01	13.25 ± 2.56	36.44 ± 3.64	50.74 ± 14.90	54.32 ± 7.83	24.16 ± 2.95	12.84 ± 2.90
Shaanxi	5.21 ± 0.50	14.55 ± 0.01	1.28 ± 1.57	23.87 ± 3.83	27.46 ± 4.16	94.21 ± 69.86	38.78 ± 19.88	21.33 ± 3.68	15.87 ± 2.65
Ningxia	2.70 ± 1.73	11.48 ± 5.36	0.42 ± 0.32	38.81 ± 1.74	47.19 ± 17.90	119.95 ± 20.11	56.13 ± 12.88	13.85 ± 2.82	22.10 ± 3.90
Hubei	1.99 ± 1.85	3.35 ± 4.40	0.90 ± 0.83	35.44 ± 32.96	37.83 ± 15.59	41.41 ± 34.17	33.52 ± 19.78	8.42 ± 10.48	15.33 ± 5.29
Jiangsu	0.78 ± 0.25	3.30 ± 0.56	0.53 ± 0.25	39.89 ± 6.98	53.50 ± 23.13	64.53 ± 4.62	52.26 ± 7.20	9.95 ± 1.30	22.97 ± 3.01
Average	4.23 ± 2.31	9.76 ± 4.22	2.73 ± 7.19	24.21 ± 9.47	44.90 ± 11.74	73.39 ± 24.7T0Tc04-24033TJETBT/F81Tf07.9701-7.97010339.31797551.42877Tm()TJET			

pinobanksin, pinocembrin, 3-O-acetylpinobanksin, chrysin, and galangin were relatively high in all analyzed propolis which suggested that they are appropriate quality markers to evaluate the Chinese propolis.

Conclusions

In this study, a RP-HPLC method was developed to accurately determine twenty phenolic compounds for quality control of Chinese propolis. This method proved to be a simple, accurate, highly specific and sensitive analytical technique. Qualitative and quantitative analysis of caffeic acid, ferulic acid, p-coumaric acid, rutin, pinobanksin, pinocembrin, 3-O-acetylpinobanksin, chrysin, and galangin for quality control of Chinese propolis is definitely an improvement over the old methodology. This method has predominance in showing the authenticity and quality consistency of propolis. It could be readily utilized as a suitable quality control method for Chinese propolis.

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