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

Zhang Cui-ping, Huang Shuai, Wei Wen-ting, Ping Shun, Shen Xiao-ge, Li Ya-jing, and Hu Fu-liang

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 (Hu and others 2005; Sforcin 2007; Sforcin and Bankova 2011). More than 500 compounds have been identified in propolis from different sources (Zhang and Hu 2009, 2012a; Huang and others 2013; Zhang and others 2013), and due to the modern analytical methods new compounds are being added.

The composition of propolis and its properties depend on the local floral and climatic conditions of the collection of resin and secretions of bees. The bud exudates of *populus* species and their hybrids are considered the main sources of "poplar type" propolis in Europe and China (Bankova and others 2000). It is the most studied and the best known type of propolis, both from chemical and pharmacological point of view. Until now, poplar-type propolis has been used frequently in health food, medicine, and daily chemical industry all over the world. As commercialization of propolis is going on, phytochemical data obtained are important for quality control and efficacy of propolis products.

Despite the extensive use of propolis either in alternative medicine or as health food, there are few reports regarding the analysis of individual compounds present in Chinese propolis samples (Jiang and others 2008; Zhou and others 2008; Luo and others 2011). Rapid spectrophotometric methods are widely used for the determination of total flavonoids and total phenolics, total flavanones and dihydroflavonols and total flavones and flavonols (Popova and others 2004). While high-performance liquid chromatography (HPLC) coupled with mass spectrometry (MS), ultraviolet (UV), or diode array detection (DAD) represents the most popular and reliable analytical technique for the characterization of phenolic acids and flavonoids. Total flavonoid content (GB/T 24283–2009) and the total content of 8 flavonoids for rutin, myricetin, quercetin, kaempferol, apigenin, pinocembrin, chrysin, and galangin (GB/T 19427–2003) were adopted by the Chinese national standard for the quality control of Chinese propolis. In Chinese Pharmacopeia (2010), standardization of Chinese propolis is by calculation of chrysin (≥2%) and galangin content (≥1%) in raw propolis materials. However, these methods are inefficient to evaluate poplar-type propolis comprehensively. In particular, they are often incapable of detecting the existence of poplar tree gum (the extract of *populus* buds) or artificially added flavonoids, such as rutin, quercertin, and other plant extracts rich in flavonoids. So it is necessary to develop a standardization technique to represent authenticity, medical stability, and efficacy of poplar-type propolis.

Many experiments have shown the relationship between propolis and *populus*, which was reflected in similarities and differences in their chemical compounds (Greenaway and others 1987; Bankova 2005; Vardar-Ünlüand others 2008; Zhang and others 2011a). The characteristic constituents of poplar-type propolis and *populus* buds are flavonoids without B-ring substituents, such as pinocembrin, pinobanksin, galangin, and chrysin. Other major constituents are phenylpropanoid acids and their esters, for example, caffeic acid phenethyl ester (CAPE; Salatino and others 2011). Chemical analysis and pharmacological studies on poplar-type propolis have further linked flavonoids and phenylpropanoid acids (Ahn and others 2007; Guo and others 2011). Flavonoids and phenylpropanoids acid in poplar-type propolis should be from resin secreted by *populus*. In our previous work, the differences between propolis and *populus* were identified to be phenolic glycosides, which are hydrolyzed by β-glucosidase secreted by

MS 20140241 Submitted 2/12/2014, Accepted 4/24/2014. *Authors Cui-ping, Shuai, Wen-ting, Shun, Xiao-ge, and Fu-liang, are with College of Animal Sciences, Zhejiang Univ., Nr. 866 Yuhangtang Road, Hangzhou 310058, China. Author Ya-jing is with Zhejiang Economic and Trade Polytechnic, Hangzhou 310018, China. Direct inquiries to author Hu Fu-Liang (E-mail: flhu@zju.edu.cn).*

doi: 10.1111/1750-3841.12510

Journal of Food Science A Publication of the Institute of Food Technologists

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 dissolved in methanol. The mixed standard stock solution was prepared and a series of working standard solutions were prepared by dilution of the stock solution according to the level of these reference standards expected in samples. All stock and working standard solutions were stored in brown bottles at 4 °C until analysis.

Optimization of extraction conditions. An orthogonal experiment was performed to test the extraction efficiency. Four factors were involved: (A) alcohol concentration (70%, 85%, 95%); (B) sonication time (15, 30, 45 min); (C) solvent volume (solid–liquid ratio: 1:15, 1:30, 1:45); and (D) extraction times (2, 3, 4 times). The optimal condition was determined by the content of extracted pinocembrin in the ethanol extract of propolis.

Sample preparation. The raw samples (10.0 g) were 1st grounded, homogenized, then mixed with 150 mL 95% ethanol and agitated in an ultrasonic water bath for 45 min. The resulted mixture was filtered and the residue was re-extracted twice under the same conditions. After the 3rd extraction, the filtered solution was combined and transferred into a flask and then evaporated to dryness. The dry residue powder of propolis or poplar tree gum (0.2 g) was extracted with methanol (25 mL) for 15 min in ultrasonic water bath and filtered through a 0.45 μm membrane filters. The peaks were assigned by comparing their retention times with that of each reference compound. The content of each compound was determined from the corresponding calibration curve.

Method validation. In this study, 20 major components belonging to 2 types (flavonoids and phenolic acids) were chosen as chemical markers to simultaneously evaluate the quality of Chinese propolis. These compounds have very broad range of polarity, different chromatographic conditions such as column, gradient, mobile phase, temperature, and flow rate were investigated, aiming to achieve good peak separation of major compounds in a suitable time range.

The method was validated for parameters including linearity, precision, accuracy, repeatability, and limits of detection and quantification.

Calibration curve. Calibration curves were plotted as the peak area versus the amount of each analyte. The linearity was evaluated with 3 injections for each concentration by appropriate dilution of the stock solutions to yield 6 concentrations and plotted using linear regression of the mean peak area versus concentration by the least squares regression method. The limit of detection (LOD) and limit of quantification (LOQ) under the present chromatographic conditions were determined on the basis of response at a signal-to-noise ratio (S/N) of 3 or 10, respectively.

Precision and repeatability. The precision of the method was estimated by the evaluation of the intra-batch precision and the interbatch precision. The intrabatch precision was examined by a set of 6 replicate analyses of a given sample solution in a single day, and interbatch precision was determined by a set of 6 replicate analyses of the same samples in 3 consecutive days. The relative standard deviation (RSD) was taken as a measure of precision.

The repeatability of the method was evaluated by the injection of 6 different samples prepared by the same samples preparation procedure. The RSD of retention time and component content for the 20 phenolic compounds was used to estimate the repeatability.

Extraction recovery. Accuracy was evaluated by the recovery experiments. However, it is difficult to get a good estimation of the true recovery due to lack of blank matrix. Three different concentrations constituting low, medium and high contents of the reference compounds were respectively added to the known amounts of raw propolis samples, using the procedure described for sample preparation and the extracts were analyzed by the use of the HPLC method described as above. The quantity of each component was subsequently obtained by use of the corresponding calibration plots. Each set of addition was repeated 3 times. The percentage recoveries were evaluated by calculating the ratio of detected amounts versus the added amounts.

Results

Optimization of Extraction Procedure

The extraction of flavonoids and phenolic acids in raw propolis was performed by using aqueous ethanol with ultrasound-assisted extraction. To obtain optimal extraction efficiency, variables involved in the extraction method were optimized. According to the statistical analysis, the largest range of the 4 factors was observed for factor B and the smallest for factor A, which meant that factor B was the primary factor in the extraction conditions. The 2nd level of factor B had the largest average value (K2 was 2.513) compared with the other 2 levels, which indicated that the 2nd level

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

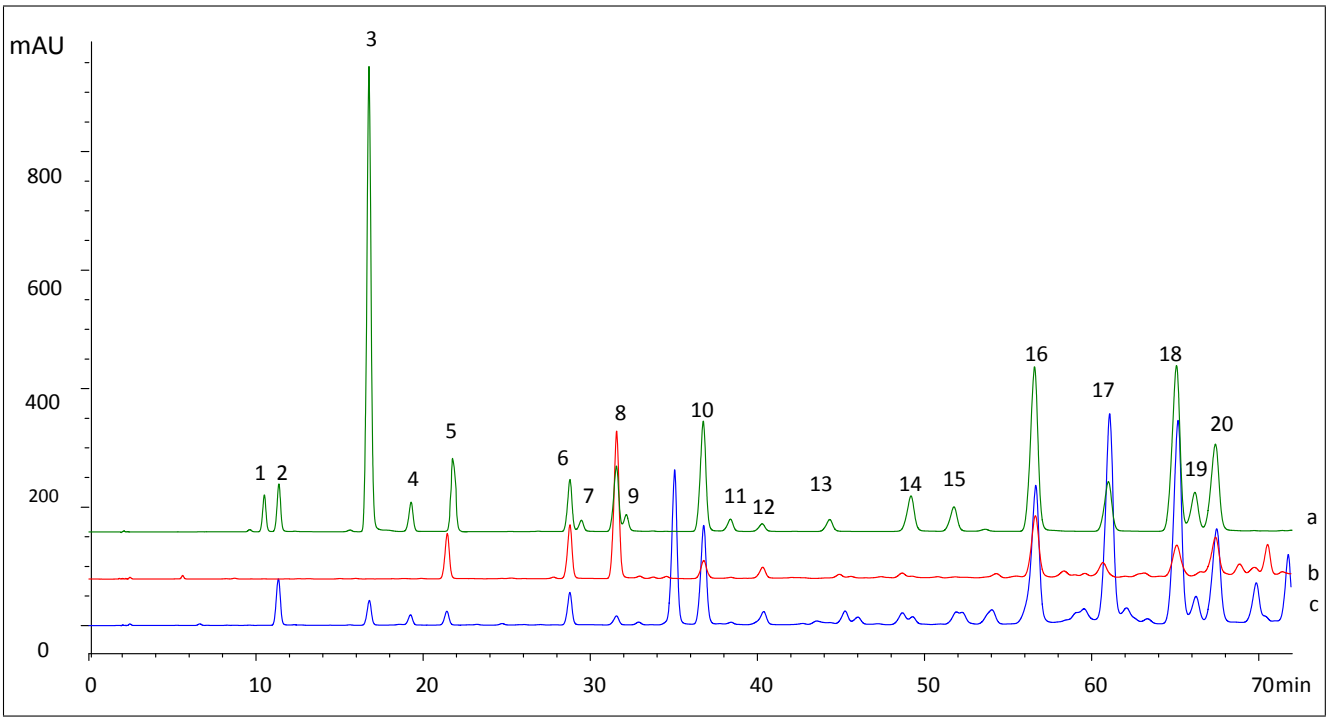

Figure 2–The HPLC chromatograms of the standard solution (A), poplar tree gum (B), and propolis (C) at a wavelength of 280 nm: 1. Vanillic; 2. Caffeic acid; 3. p-Coumaric acid; 4. Ferulic acid; 5. Isoferulic acid; 6. 3,4-Dimethoxycinnamic acid; 7. Rutin; 8. Cinnamic acid; 9. Myricetin. 10. Pinobanksin; 11. Naringenin; 12. Quercetin; 13. Luteolin; 14. Kaempferol. 15. Apigenin; 16. Pinocembrin; 17. 3-O-acetylpinobanksin; 18. Chrysin; 19. CAPE; 20. Galangin.

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

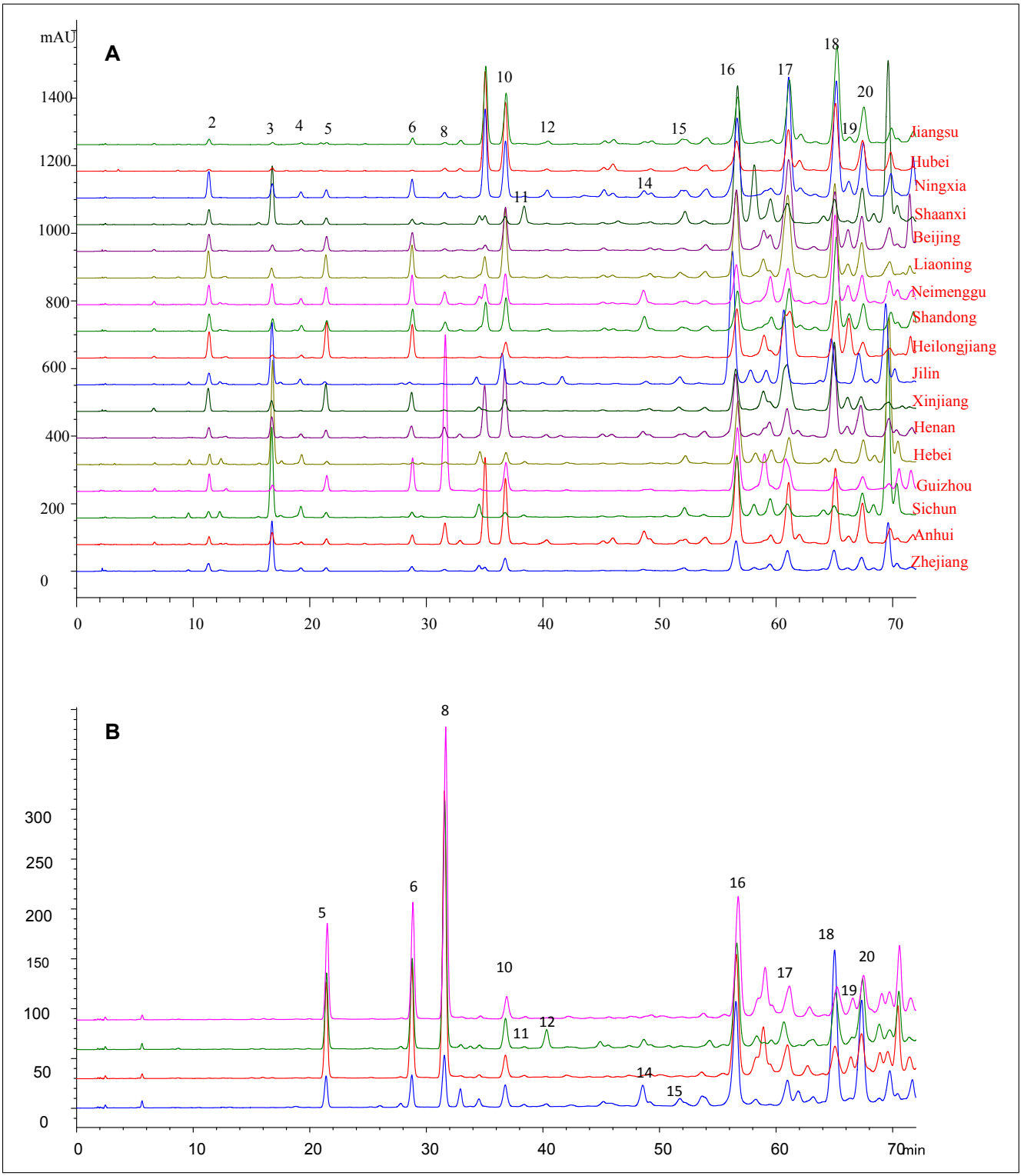

Figure 3–The typical HPLC chromatograms of propolis from different geographies (A) and poplar tree gum (B) samples.

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 ± 26.111	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 ± 24.75	39.72 ± 11.35	14.95 ± 6.42	15.95 ± 3.44
Poplar tree gum	6.97 ± 3.63	12.75 ± 6.71	11.73 ± 7.59	6.24 ± 2.23	30.50 ± 3.85	15.47 ± 7.28	19.90 ± 16.23	7.47 ± 2.80	13.08 ± 4.07

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.

Acknowledgments

We thank Hu Yuan-qiang from Apicultural Technological Association of Jinyun County for supplying propolis samples and Wu Xiao-dan and Zhu Ya-er from Center of Analysis & Measurement of Zhejiang Univ. for technical support and assistance.

This work was supported by the grant from the Natl. Natural Science Foundation of China (Project Nr. 31272512), the earmarked fund for Modern Agro-industry Technology Research System form the Ministry of Agriculture of China (CARS-45), and Dept. of Science and Technology of Zhejiang Province (2012C22050).

References

Ahn MR, Kumazawa S, Usui Y, Nakamura J, Matsuka M, Zhu F, Nakayama T. 2007. Antioxidant activity and constituents of propolis collected in various areas of China. Food Chem 101(4):1383–92.

Bankova V. 2005. Chemical diversity of propolis and the problem of standardization. J Ethnopharmacol 100(1–2):114–7.

Bankova VS, de Castro SL, Marcucci MC. 2000. Propolis: recent advances in chemistry and plant origin. Apidologie 31:3–15.

Cai R, Wang SS, Meng Y, Meng QG, Zhao WJ. 2012. Rapid quantification of flavonoids in propolis and previous study for classification of propolis from different origins by using near infrared spectroscopy. Anal Methods 4:2388–95.

Chinese Pharmacopoeia Commission. 2010. Pharmacopoeia of the People's Republic of China. Beijing, China: China Medical Science and Technology Press, I:336–7.

Greenaway W, Scaysbrook T, Whatley FR. 1987. The analysis of bud exudate of populus $\times $ euramericana, and of propolis, by gas chromatography–mass spectrometry. Proc R Soc Lond B232:249–72.

Guo XL, Chen B, Luo LP, Zhang X, Dai XM, Gong SJ. 2011. Chemical compositions and antioxidant activities of water extracts of Chinese propolis. J Agric Food Chem 59:12610–16.

Hu FL, Hepburn HR, Li YH, Chen ML, Radloff S, Daya S. 2005. Effects of ethanol and water extracts of propolis (bee glue) on acute inflammatory animal models. J Ethnopharmacol 100(3):276–83.

Huang S, Zhang CP, Hu FL. 2013. Research progress in chemical composition of propolis between 2008 and 2012. Nat Prod Res Dev 25:1146–53, 1165.

Jiang L, Fang GZ, Zhang Y, Cao GJ, Wang S. 2008. Analysis of flavonoids in propolis and ginkgo biloba by micellar electrokinetic capillary chromatography. J Agric Food Chem 56 (24):11571–7.

Luo CY, Zou XL, Li YQ, Sun CG, Jiang Y, Wu ZY. 2011. Determination of flavonoids in propolis-rich functional foods by reversed phase high performance liquid chromatography with diode array detection. Food Chem 127(1):314–20.

Popova M, Bankova V, Butovska D, Petkov V, Damyanova BN, Sabstini AG, Marcazzan GL, Bogdanov S. 2004. Validated methods for the quantification of biologically active constituents of poplar-type propolis. Phytochem analysis 15(4):235–40.

Salatino A, Fernandes-Silva CC, Righi AA, Salatino MLF. 2011. Propolis research and the chemistry of plant products. Nat Prod Rep 28:925–36.

Sforcin JM. 2007. Propolis and the immune system: a review. J Ethnopharmacol 113(1):1–14.

Sforcin JM, Bankova V. 2011. Propolis: is there a potential for the development of new drugs?. J Ethnopharmacol 133(2):253–60.

Truchado P, Tourn E, Gallez LM, Moreno DA, Ferreres F, Tomas-Barberan FA. 2010. Identification of botanical biomarkers in Argentinean Diplotaxis honeys: flavonoids and glucosinolates. J Agric Food Chem 58(24):12678–85.

Vardar-Ünlü G, Silici S, Ünlü M. 2008. Composition and in vitro antimicrobial activity of Populus buds and poplar-type propolis. World J Microb Biot 24:1011–1017.

Zhang CP, Hu FL. 2012a. Terpenoids in propolis. Nat Prod Res Dev 24:976–84.

Zhang CP, Hu FL. 2009. Flavonoids in propolis. Nat Prod Res Dev 21(6):1084–90.

Zhang CP, Liu G, Hu FL. 2012b. The hydrolysis of flavonoid glycosides by propolis β-glucosidase activity. Nat Prod Res 26(3):270–3.

Zhang CP, Wang K, Hu FL. 2013. Phenolic acid in propolis. Chin JMAP 30(1):102–5.

Zhang CP, Zheng HQ, Hu FL. 2011b. Extraction, Partial characterization and storage stability of β-glucosidase from propolis. J Food Sci 76(1):75–9.

Zhang CP, Zheng HQ, Liu G, Hu FL. 2011a. Development and validation of HPLC method for determination of salicin in poplar buds: application for screening of counterfeit propolis. Food Chem 127:345–50.

Zhou JH, Li Y, Zhao J, Xue XF, Wu LM, Chen F. 2008. Geographical traceability of propolis by high-performance liquid-chromatography fingerprints. Food Chem 108:749–59.