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Analytical Methods

Development and validation of HPLC method for determination of salicin in poplar buds: Application for screening of counterfeit propolis

Cui-ping Zhang, Huo-qing Zheng, Gang Liu, Fu-liang Hu *

College of Animal Sciences, Zhejiang University, No.268 Kaixuan Road, Hangzhou 310029, China

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ABSTRACT

The main plant origins of propolis are the *populus* species and their hybrids, both located in China. Poplar tree gum, the extract of *populus* buds, has been widely used as counterfeit propolis, but no efficient method was known for detecting the counterfeit. Salicin is a characteristic marker of the genus *populus*, which may be hydrolysed by β-glucosidase during propolis collection and processing. A simple, sensitive and specific reversed-phase high-performance liquid chromatography (RP-HPLC) method was developed and validated for the rapid assay of salicin, which was aimed at distinguishing poplar tree gum from propolis. Isocratic elution at a flow rate of 1.0 mL/min was employed on a Sepax HP-C18 column (150 × 4.6 mm, 5 μm) and the column temperature was 30 °C. The mobile phase consisted of acetonitrile and 0.5% aqueous phosphoric acid (5:95, v/v). The UV detection wavelength was 213 nm. Following this method, salicin was detected in *populus* buds and leaves and 11 poplar tree gum samples, but not in any of the 40 propolis samples, which indicates that salicin was hydrolysed in propolis collection and processing but was stable in the production process of poplar tree gum. The proposed method could be an effective technique for routine analysis of salicin and monitoring the quality of propolis as possible counterfeit poplar tree gum.

1. Introduction

Propolis is a natural resinous substance collected by honeybees from parts of plants, buds and exudates. Propolis possesses many biological functions, including antibacterial, antifungal, antitumoral, antioxidative and immunomodulatory agent, as well as other beneficial activities (Ghisalberti, 1979; Marcucci, 1995). Propolis has been one of the most popular health food products and has had commercial availability all over the world (Castaldo & Capasso, 2002). The propolis yield can vary considerably depending on several factors, including honeybee species, season, climate, and the laborious production process that involves the manual harvest and extraction of crude propolis. The high demand for and limited availability of propolis have led to gross adulteration of the product.

The most important botanical sources of propolis are poplars, birches, willows, chestnut trees, elms, pine trees, oaks, spruces and others (Bankova et al., 1992; Bonvehí, Coll, & Jordà, 1994; Ghisalberti, 1979). It has been generally accepted and chemically demonstrated that the bud exudates of *Populus* species and their hybrids are the main sources of propolis in Europe and China (Bankova, de Castro, & Marcucci, 2000). Poplar tree gum, the extract of *populus* buds, has a similar chemical composition, colour and smell as the extract of the poplar-type propolis. Allegedly, the poplar tree gum has been adulterated into a counterfeit propolis. The gum is an attractive source for adulterated propolis because it costs much less and is difficult to discriminate from the beehive propolis due to the similar composition and appearance. Although the adulteration might not raise issues with safety, due to unfair trading practices, the adulteration with poplar tree gum is illegal. However, no official protocol has been developed for solving this problem. To guarantee the authenticity of propolis, it is necessary to develop a sensitive, selective, dependable and relatively simple assay to distinguish poplar tree gum from propolis.

There have been few attempts to investigate the chemical difference between propolis and poplar tree gum, with the exception of one study (Wu, Sun, Zhao, Li, & Zhou, 2008). They developed a method that combined the Fourier transform infrared spectra with the two-dimensional infrared correlation analysis and found that the differences in these two natural products were caused by the amounts of long-chain alkyl compounds.

The compounds identified in propolis originated from 3 sources: plant parts collected by bees; secreted substances from bee metabolism; and materials that are introduced during propolis elaboration (Ghisalberti, 1979; Marcucci, 1995). The salivary and enzymatic secretions can modify the original resin composition (Bonvehí et al., 1994; Burdock, 1998), which leads to the

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* Corresponding author. Tel./fax: +86 571 88982952.
E-mail address: flhu@zju.edu.cn (F.-l. Hu).

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differences of chemical constitutes between the propolis and its plant origins.

The genus *Populus* is characterised by the presence of phenolic glycosides, such as salicin and its derivatives (Pearl & Darling, 1971; Thieme, 1967). Although there were many publications associated with chemical compounds of propolis (e.g. Bankova, 2005; Bankova et al., 2000; Greenaway, Scaysbrook, & Whatley, 1987; Marcucci, 1995; Usia et al., 2002), none involved an analysis of the phenolic glycosides. Instead, salicylic acid and acetyl salicylic acid (the hydrolysates of salicin and its acetyl derivatives, respectively) were found in propolis (Krol et al., 1996; Marcucci, 1995). Phenolic glycosides are β-glycosides containing a D-glucose residue that can be cleaved by β-glycosidases, the activity that had been detected in propolis (Zhang, Zheng, & Hu, In press). We presumed that phenolic glycosides were hydrolysed by β-glucosidase during propolis collection and processing. In this case, the phenolic glucosides could be potential markers used to distinguish poplar tree gum from propolis.

The genus *populus* comprises more than 100 species, which are distributed in temperate and subtropical regions (Wang, Wang, Xu, & Xu, 1999). The characteristic phenolic glucoside of the whole genus *populus* is salicin, which was found in the barks, leaves and buds of all species (Clausen et al., 1989). Salicin has been the only commercially available phenolic glucoside. Therefore, it was suitable to be considered as the "marker compound" for screening poplar tree gum.

The aim of this work was to develop a reliable and sensitive HPLC method for determining amounts of salicin in poplar buds with a simple sample pretreatment method. Subsequently, the method was applied to distinguish poplar tree gum from propolis.

2. Experimental

2.1. Chemicals and reagents

HPLC-grade methanol and acetonitrile were obtained from Merck (Darmstadt, Germany). HPLC-grade water was purified by a Yjd-upws ultra-pure water system (China). Absolute alcohol and phosphoric acid were analytical grade. Salicin was purchased from Sigma–Aldrich (China).

2.2. Propolis and Populus samples

The poplar-type propolis samples were harvested in Tianjin of China, a region that was rich in *populus × beijingensis* and *populus × canadensis*, in June 2009. Samples of *populus* buds and leaves were collected for analysing and comparing with samples of propolis. Considering the degradation velocity of salicin in the process of propolis elaboration, different storing times for the propolis samples in the beehives were used. Seventy-five beehives were divided into 5 groups after propolis was scraped in all hives from two apiaries. Propolis samples were harvested from group A (5th day), group B (10th day), group C (15th day), group D (20th day) and group E (30th day) by scraping the frames of the beehives. Plant samples were frozen in liquid nitrogen and crushed to a fine powder. Propolis samples were frozen at −20 °C, ground and then homogenised. All samples were kept at −20 °C until they were analysed.

2.3. Optimisation of extraction conditions

An orthogonal experiment was performed to optimise the extraction conditions. Four factors were involved: (A) alcohol concentration; (B) sonication time; (C) solvent volume; and (D) extraction time. The experimental factors, corresponding levels and

Table 2
The geographical origins and harvest times of the Chinese propolis samples.

No.	Source	Date of collection
1	Yutian, Hebei	May 2009
2	Shijiazhuang, Hebei	August 2008
3	Mengcheng, Anhui	July 2009
4	Huludao, Liaoning	July 2009
5	Taiyuan, Shanxi	August 2008
6	Zaozhuang, Shandong	July 2008
7	Chengdu, Sichuan	July 2008
8	Yichun, Jiangxi	October 2008
9	Hangzhou, Zhejiang	November 2008
10	Fuzhou, Fujian	July 2009
11	Qingyuan, Anhui	October 2008
12	Jiangshan, Zhejiang	October 2008
13	Chifeng, Inner mongolia	August 2008
14	Hefei, Anhui	November 2008
15	Yangquan, Shanxi	July 2009
16	Pinghu, Zhejiang	October 2008
17	Shaoxing, Zhejiang	October 2008
18	Changxing, Zhejiang	July 2009
19	Xinxinag, Henan	June 2009
20	Taishan, Shandong	June 2009
21	Linyi, Shandong	June 2009
22	Yutian, Hebei	June 2009
23	Jixian, Tianjian	June 2009
24	Pinghu, Zhejiang	September 2008
25	Jiangshan, Zhejiang	November 2008
26	Yangquan, Shanxi	July 2008
27	Mengcheng, Anhui	October 2009
28	Zaozhuang, Shandong	May 2008

Table 1
The results and analysis of the orthogonal design for the optimisation of extraction conditions.

Run. no	A Alcohol concentration	B Sonication time (min)	C Solvent volume	D Extraction times (times)	Extraction percentage (%)
1	50	15	1:5	2	0.1437
2	50	30	1:10	3	0.1975
3	50	45	1:15	4	0.1765
4	75	15	1:10	4	0.1576
5	75	30	1:15	2	0.2078
6	75	45	1:5	3	0.1774
7	100	15	1:15	3	0.1776
8	100	30	1:5	4	0.1853
9	100	45	1:10	2	0.1806
K1	0.173	0.160	0.169	0.177	
K2	0.181	0.197	0.179	0.184	
K3	0.181	0.178	0.187	0.173	
R	0.008	0.037	0.018	0.011	
Optimal level	A2(3)	B2	C3	D2	

orthogonal designs L_9 (3^4), are presented in Table 1. The salicin content in *Populus* × *beijingensis* buds was used as a criterion for the selection of the optimal extraction conditions.

2.4. Liquid chromatographic conditions

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 (VWD) G1314B, and a Thermostatted Column Compartment G1316A. A Sepax HP-C18 column (150 × 4.6 mm, 5 μm) at 30 °C was applied for all analyses. The detection wavelength was set at 213 nm. The mobile phase consisted of (A) acetonitrile and (B) 0.5% aqueous phosphoric acid (v/v) (5:95) at a flow rate of 1 ml/min. The injection volume was 5 μl. All sample solutions were filtered through 0.45 μm membrane filters.

2.5. Method validation

Preliminary tests were performed with *Populus* × *beijingensis* buds. To select optimum conditions of separation, the following parameters were considered: detection wavelength, optimal mobile phase and their proportions, operating temperature and flow rate.

The method was validated for parameters including linearity, precision, accuracy, stability and limits of detection and quantification, following the international conference on harmonisation (ICH) guidelines (ICH, 1997).

2.5.1. Calibration curve

The stock solution of salicin was prepared in methanol to a final concentration of 1 mg/ml and was then diluted to the appropriate concentration ranges for establishing a calibration curve. The linearity of salicin was determined with three injections for each con-

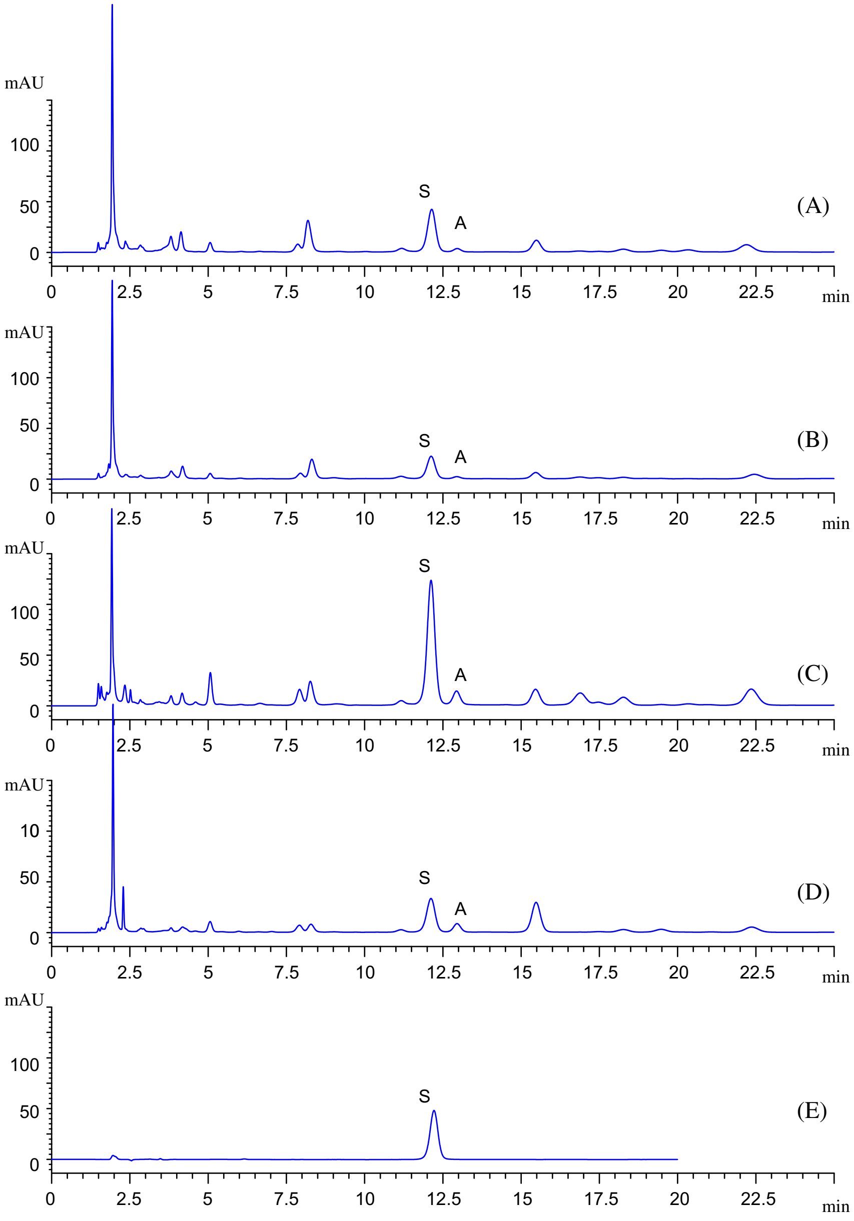

Fig. 1. HPLC profiles of *populus* × *beijingensis* buds (A), *populus* × *beijingensis* leaves (B), *populus* × *canadensis* buds (C), *populus* × *canadensis* leaves (D) and standard (E). Peaks represented: S, salicin; A, unknown.

centration and plotted using linear regression of the mean peak area versus concentration. The equation of linear regression was performed using a least-squares method.

2.5.2. Limits of detection (LOD) and quantification (LOQ)

The sensitivity of the method was evaluated by determining the LOD and LOQ. The standard stock solution was serially diluted with methanol. The limits of detection (LOD) and quantification (LOQ) were determined by measuring the signal-to-noise ratio by injecting a series of solutions until the S/N ratio was 3 for LOD and 10 for LOQ.

2.5.3. Precision

The precision of the method was estimated by the evaluation of the intra-batch precision and the inter-batch precision. The intra-batch precision was examined in six replicate analyses of the same sample solution in a single day, and inter-batch precision was determined for three independent days. The relative standard deviation (RSD) was taken as a measure of precision.

2.5.4. Extraction recovery

The accuracy of the method was evaluated by recovery experiments of salicin, at different concentrations, under analytical method conditions. Three different concentrations constituting low, medium and high contents of salicin were added to known amounts of poplar bud samples; then the resultant samples were extracted and analysed with the established HPLC method. Triplicate experiments were carried out at each level. The percentage recoveries were evaluated by calculating the ratio of detected amounts of salicin versus the added amounts.

2.6. Preparation of sample solutions

Two grams each of the powder of *populus* buds, leaves and propolis samples were extracted with 30 ml of 75% aqueous alcohol in an ultrasonic water bath for 30 min. The extraction procedure was repeated in triplicate for each sample. The extracted solution was filtrated through analytical filter paper and mixed; then the filtered solution was evaporated to dryness at 50 °C in a water bath. The dry extract was dissolved in 50 ml methanol and filtrated through a 0.45 μm membrane filter unit. Then, 5 μl of each sample solution was analysed by HPLC. The salicin content was determined from the corresponding calibration curves.

Poplar tree gum was extracted and assayed for salicin as described above. Considering that raw propolis is approximately composed of 50% resin (Marcucci, 1995), the amount of poplar tree gum used in each assay was halved (1 g) to guarantee a fair comparison.

Table 3
Content of salicin in different *populus* samples.

Samples	Part of plant	Content (%)
Populus × *beijingensis*	Bud	0.2078
	Leaf	0.1203
Populus × *canadensis*	Bud	0.4835
	Leaf	0.1501

2.7. Application to distinguish poplar trees' gum from propolis

Twenty-eight Chinese propolis samples were harvested by local beekeepers in 12 different provinces; Table 2 shows the geographical origins and harvest times of the propolis samples. Meanwhile, two Brazilian propolis (green propolis) samples were obtained from Hangzhou BEEWORDS Apiculture Co. Ltd. (Hangzhou, China). Eleven poplar tree gum samples were randomly purchased from different providers.

3. Results

3.1. Development of the method

3.1.1. Optimised extraction conditions

According to the statistical analysis shown in Table 1, the largest range of the four 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 second level of factor B had the largest average value (K_2 was 0.197) compared with the other two levels, which indicated that the second level was the best condition for factor B. The best conditions for the remaining factors were the second and third level of factor A, the third level of factor C and the second level of factor D. Therefore, the extraction conditions were optimised as follows: 2 g powder of the samples was extracted in triplicate with 30 ml 75% aqueous or absolute alcohol in an ultrasonic water bath for 30 min.

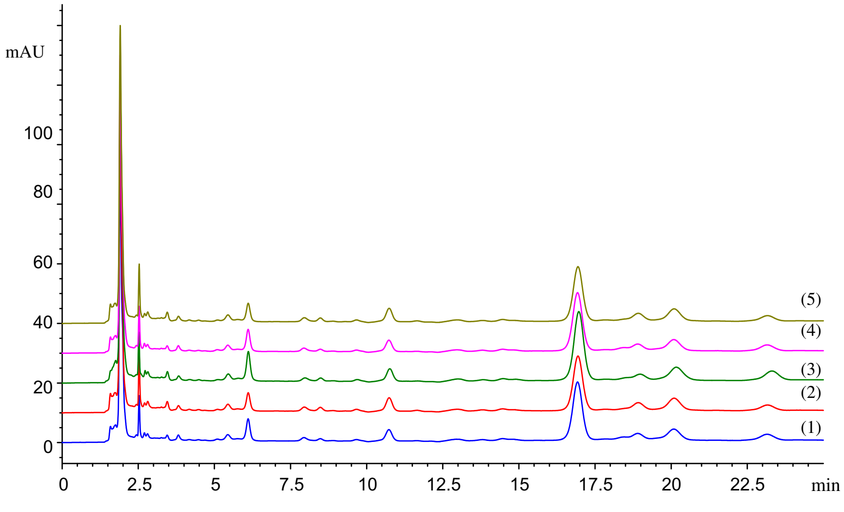

Fig. 2. HPLC chromatograms of propolis samples for different storing times in the hives. (1) group A; (2) group B; (3) group C; (4) group D; (5) group E.

3.1.2. Chromatographic conditions

An excellent separation of salicin was achieved under the following conditions: the mobile phase was composed of acetonitrile-0.5%, aqueous phosphoric acid (5:95, v/v) was isocratically eluted at a flow rate of 1.0 ml/min and the column temperature was set at 30 °C. The UV detection wavelength was 213 nm. Buffer and gradient of the mobile phase were not necessary. HPLC chromatograms of the *populus* extracts are shown in Fig. 1.

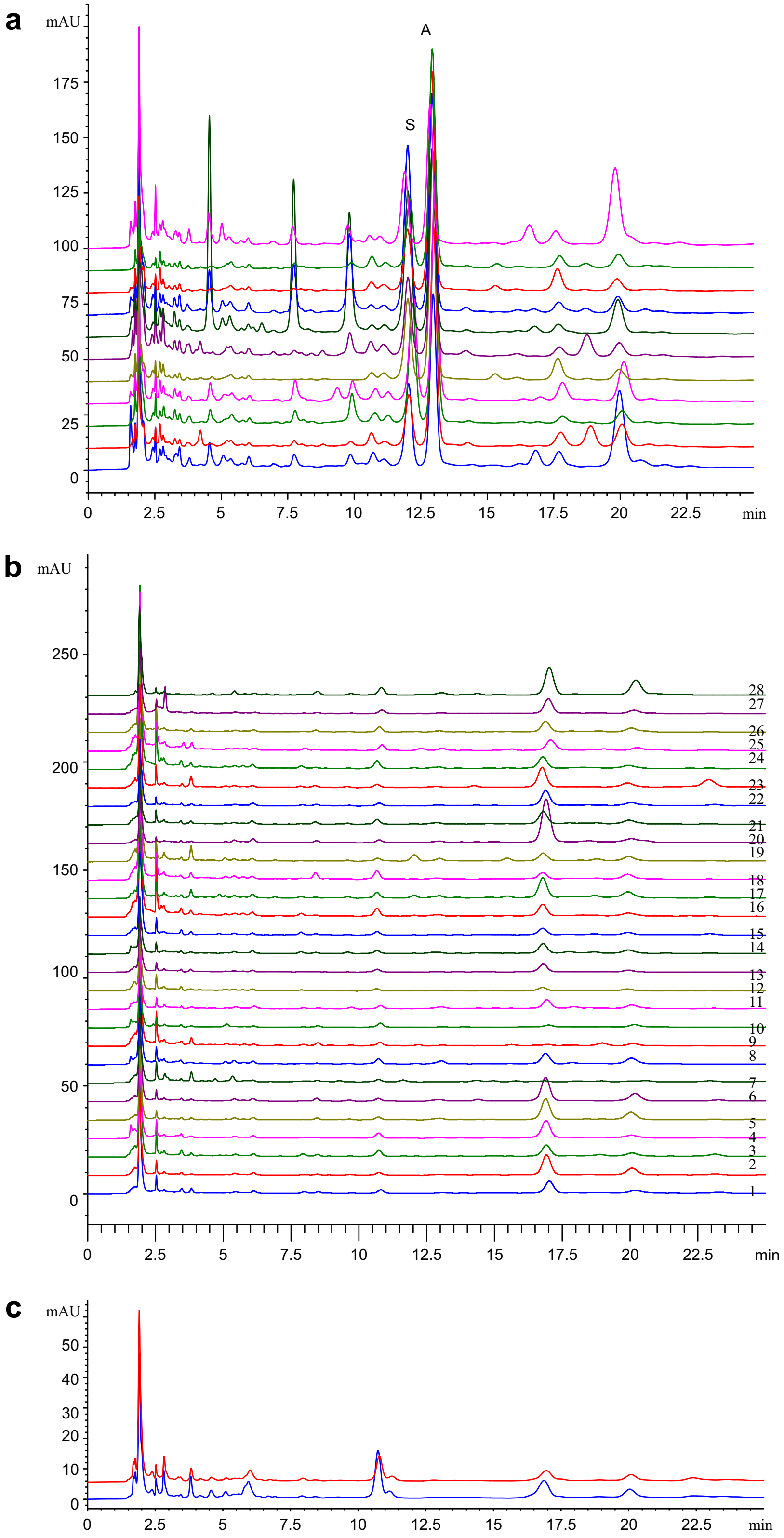

Fig. 3. HPLC chromatograms of (a) poplar trees' gum; (b) 28 Chinese propolis samples; (c) 2 Brazilian green propolis samples. Peaks represented: S, salicin; A, unknown.

3.2. Method validation

A linear detector response ($r^2 = 0.9998$) for salicin was obtained in the range of 10–200 μg/ml, which showed good linear relationships between the peak areas and the concentrations. The LOD and LOQ were 1.3 and 5.4 μg/ml, respectively. The obtained values for both LOD and LOQ were low, which indicated that the method was capable of detecting and quantifying trace amounts of salicin in samples. The inter- and intra-batch precisions were 0.97% and 2.94%, respectively, which showed good precision in the method. The results of the recovery experiments showed that the extraction method allowed a recovery of salicin of 92.56–98.14%. These results indicated that the analysis conditions for salicin were satisfactory.

3.3. Determination of salicin in different species and parts of populus samples

The developed HPLC assay method was successfully applied to the determination of salicin amounts in different *populus* samples (Fig. 1). The content of salicin was significantly different in different poplar species and different parts of the same species (Table 3).

3.4. Determination of salicin in different storing time propolis samples

The HPLC profiles of the different storing times for the propolis samples were nearly the same, and salicin was undetectable in all samples (Fig. 2).

3.5. Comparison of HPLC profiles of propolis and poplar's gum samples

Salicin was detected in all poplar tree gum samples (Fig. 3a), although its content was markedly different between the samples (0.0578–0.1424%). Salicin was undetectable in all Chinese propolis samples, whenever and wherever they were collected (Fig. 3b), and in the two Brazilian green propolis samples (Fig. 3c).

4. Discussion

A new HPLC method was developed to determine salicin amounts in poplar buds. A stable chromatographic pattern was developed. The mobile phase used in our method was sufficient to maintain the optimum pH for salicin stability and separation, and was also gentler on the instrumentation after long-term use. The method was successfully applied to analyse poplar leaves, propolis and poplar tree gum.

Salicin has been found in all species of the genus *Populus* (Palo, 1984). Our results also showed that it occurred in poplar buds and leaves of the two poplar species studied. Meanwhile, salicin occurred in all analysed poplar tree gum samples but was undetectable in all the propolis samples. These results supported our hypothesis that salicin was hydrolysed in the process of propolis collection and processing and indicated that the compound was stable in the production process of poplar tree gum. In our study, we harvested propolis samples from the beehives after different storing times. These samples actually contained fresh resin that was collected every day. However, salicin was undetectable in all the fresh propolis samples. A possible reason for this could be that the hydrolysis rate of salicin was so fast that it was completely hydrolysed before it reached the beehives. All of the above findings demonstrated that this was a reliable method to distinguish poplar tree gum from propolis.

The chromatographic patterns of 40 propolis samples were generally consistent, with differences in the absorption intensities of some peaks (Fig. 3b and c). In addition to the peak of salicin, there was another peak (peak A at 12.9 min) that occurred differentially between poplar tree gum and propolis samples. It was detected in each of the poplar tree gum samples (Fig. 3a) but not in any propolis samples (Fig. 2, Fig. 3b and c). Moreover, when compared with the peak of salicin, the area of peak A was found to be much higher for the poplar tree gum (Fig. 3a). Thus, it could be another potential marker to discriminate between propolis and poplar tree gum.

5. Conclusion

A HPLC method was developed to detect salicin in poplar buds, leaves, propolis and poplar tree gum. This method is rapid, highly specific and sensitive. It is an efficient method to screen poplar tree gum from propolis, providing a means to assess the authenticity of propolis.

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