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Determination of components in propolis by capillary electrophoresis and photodiode array detection

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Abstract

The capillary electrophoretic separation of components in propolis, a commonly used natural medicine, was investigated. Optimum conditions for the separation were established. Photodiode-array detection permitted the rapid identification of the components in the samples analysed. The determination of these components, including caffeic acid, dimethylcaffeic acid, isoferulic acid and quercetin, was performed on a commercial propolis sample.

1. Introduction

Propolis is a resinous hive product collected by bees from tree buds. It contains exudate from many tree buds. Propolis has been long used in folk medicine [1] and has been reported to possess many biological activities such as antibacterial, antiviral, fungicidal, antiulceral, hypotensive and cytostatic properties [2,3]. More than 160 compounds, mainly phenolics, have been identified in propolis collected from different regions [4]. Many of the phenolic compounds present in propolis have antimicrobial and other biological effects. For examples, caffeic acid plays a major part both in the antimicrobial activities of propolis and its allergenic properties, and isoferulic acid was found to have an inhibitive effect against *Staphylococcus aureus* [5]. Quercetin has spasmolytic activity [3].

The concentrations of phenolic compounds in propolis vary substantially according to the origin of the samples, and such differences in propolis composition are likely to have considerable effects on its antimicrobial properties.

Thin-layer chromatography (TLC), high-performance liquid chromatography (HPLC) [6] and gas chromatography (GC) [7] have been used for the determination of phenolic compounds. TLC and HPLC are limited in separation power. Capillary GC has been used for the quantitative analysis of phenolic mixtures owing to its sensitivity and resolving power [8], but some compounds, such as flavonoids [1] and caffeic acid [4], are liable to break down under the conditions used. Usually a time-consuming procedure is needed to prepare derivatives of phenolic compounds before GC analysis.

Capillary electrophoresis (CE) is a highly efficient and fast technique, the application of which in the biological and pharmaceutical fields has developed rapidly in recent years [9,10]. Usually, the separation of the analytes by CE is

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performed at mild temperatures, so during the analysis the thermal degradation of the analytes is much reduced compared with GC methods. The application of CE to propolis analysis has not hitherto been reported. For HPLC analysis, usually clean samples are preferred, whereas with CE, very complicated samples can be analysed directly without much pretreatment, because any contaminants in the CE tube can be rinsed away with a suitable solvent after each analysis. In this work, propolis samples were analysed directly after ethanolic extraction without further purification. In the case of HPLC, such samples will contaminate columns quickly. This work also showed that CE combined with photodiode-array detection permits the rapid identification of the components in the samples.

2. Experimental

2.1. Instrumentation

Experiments were conducted on a laboratory-built CE system. A Spellman (Plainview, NY, USA) Model RM15P10KD power supply was used. Fused-silica capillary tubing of 45 cm × 50 μm I.D. was obtained from Polymicro Technologies (Phoenix, AZ, USA). A Shimadzu (Kyoto, Japan) Model SPD-M6A photodiode-array UV–Vis detector and a MicroUVIS 20 UV detector (Carlo Erba, Milan, Italy) were used for detection. Chromatographic data were collected and analysed using a Shimadzu Chromatopac CR6A data processor. For quantitative analysis, a UV wavelength of 215 nm was used for the detection. The peak heights of related compounds were used for quantitative calculations.

2.2. Materials and reagents

A propolis sample was collected from Jiangsu, China. Isovanillin (Isova), vanillin (Va), caffeic acid (3,4-dihydroxycinnamic acid) (34H), dimethyl caffeic acid (3,4-dimethoxycinnamic acid) (34M), isoferulic acid (3-hydroxy-4-methoxycinnamic acid) (3H4M), ferulic acid (4-hydroxy-3-methoxycinnamic acid) (4H3M), cinnamic acid (Cin) and chrysin (Chr) were purchased from Aldrich. Quercetin [2-(3,4-dihydroyphenyl)-3,5,7-trihydroxy-4*H*-1-benzopyran-4-one) (Que), naringenin (Nar), vanillic acid (Vaa) and hesperetin (Hes) were purchased from Sigma. β-Cyclodextrin was purchased from Fluka.

Stock standard solutions equivalent to 5 mg/ml of drugs in 70% ethanol [ethanol–water (70:30, v/v)] were freshly prepared. These solutions were diluted with 70% ethanol to provide standards in the concentration range 5–400 μg/ml. The buffer solutions used in the CE system were obtained by mixing appropriate portions of 25 m*M* sodium dihydrogenphosphate, 25 m*M* sodium tetraborate and 1 *M* sodium hydroxide solutions to the desired pH. β-Cyclodextrin was used as a modifier at the concentration range of 0–10 m*M* in the electrophoretic medium.

A mixed standard solution was prepared by dissolving the following compounds in 70% ethanol: Nar, Hes and Chr, 25 μg/ml; Va, Isova, 4H3M and 34H, 130 μg/ml; Vaa, 3H4M and 34M, 60 μg/ml; and Que, 190 μg/ml.

2.3. Analytical procedure

The propolis sample (100 mg) was sonicated in 2 ml of 70% ethanol for 20 min and the solution was left overnight. The solution was then passed through a 0.45-μm filter (Whatman, Maidstone, UK). The filtrate was introduced directly into the CE system for the determination of caffeic acid and quercetin. Subsequently, the sample solution was diluted eightfold with 70% ethanol for the determination of 3,4-dimethylcaffeic acid and isoferulic acid. Sample solutions were introduced into the CE system manually: one end of the capillary was placed in a sample vial containing the sample solution and the sample was introduced by siphoning from the sample solution at a level 9 cm higher than the electrophoretic solution in which the other end of the tube was immersed. The injection time was 10.0 s; 15 kV was used for the determination of Que and 34H and 8 kV for 34M and 3H4M. After each analysis, the capillary was washed with

water for 1 min, then with a cleansing solution [containing methanol and concentrated hydrochloric acid (10:2, v/v)] for 8 min and water for 1 min. Because the propolis samples were analysed directly without precleaning, it was necessary to use this rinsing procedure after each analysis to remove the impurities adsorbed on the CE column.

3. Results and discussion

3.1. Effect of pH

Some compounds that have been reported to occur in propolis were tested for separation by CE. The pH of the buffer solution was varied by mixing appropriate portions of 25 m*M* sodium dihydrogenphosphate, 25 m*M* sodium tetraborate and 1 *M* sodium hydroxide solutions. The relationship between the pH of the buffer and the migration times is shown in Fig. 1. At lower pH, poor separation was obtained, as shown in Fig. 1, whereas when the pH was increased to 10.1 most components were well separated, except for Cin and Va. The pH of the buffer was not further increased in order to avoid possible degradation of the silica tubing.

3.2. Effect of β-cyclodextrin

In order to obtain a better separation, some additives to the eluent were tried. Sodium dodecyl sulphate (SDS) was added to the eluent at different concentrations (0–30 m*M*), but no improvement of the separation was observed. Addition of β-cyclodextrin to the eluent improved the separation, as shown in Fig. 2. The best separation was obtained when 0.7 m*M* of β-cyclodextrin was added to the buffer, and this concentration was used in subsequent experiments. An electropherogram of a standard mixture is shown in Fig. 3. All the compounds were well separated under these separation conditions.

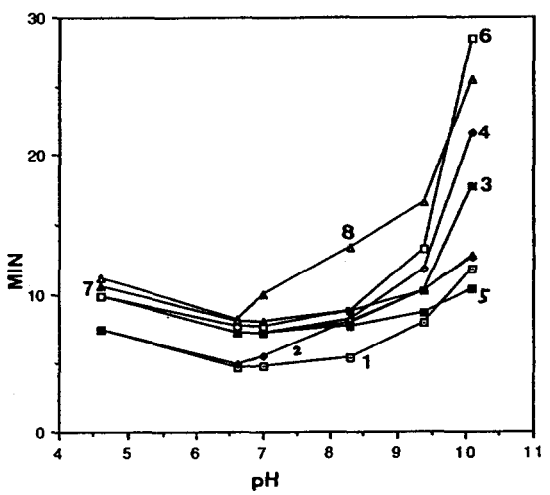

Fig. 1. Effect of pH of the buffer on the migration times of some phenolic compounds. The buffer solution used in the CE system was obtained by mixing appropriate portions of 25 m*M* sodium dihydrogenphosphate, 25 m*M* sodium tetraborate and 1 *M* sodium hydroxide solutions to give the desired pH. 15 kV was used for the separation. Curves: 1 = Isova; 2 = Va; 3 = 3H4M; 4 = 4H3M; 5 = 34M; 6 = Vaa; 7 = Cin; 8 = 34H. Detector response: 0.05 AUFS.

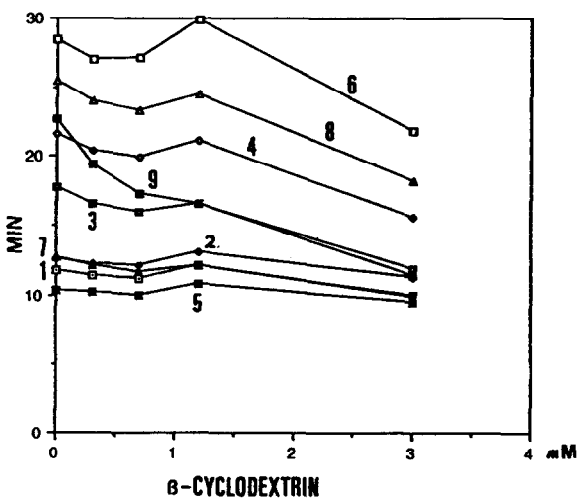

Fig. 2. Effect of β-cyclodextrin concentration on the separation of some phenolic compounds. The buffer solution used in the CE system was obtained by mixing appropriate portions of 25 m*M* sodium tetraborate and 1 *M* sodium hydroxide solutions to give a pH of 10.1. 0–4 m*M* β-cyclodextrin was added to the buffer solution. 15 kV was used for the separation. Curves: 1 = Isova; 2 = Va; 3 = 3H4M; 4 = 4H3M; 5 = 34M; 6 = Vaa; 7 = Cin; 8 = 34H; 9 = Que. Detector response: 0.05 AUFS.

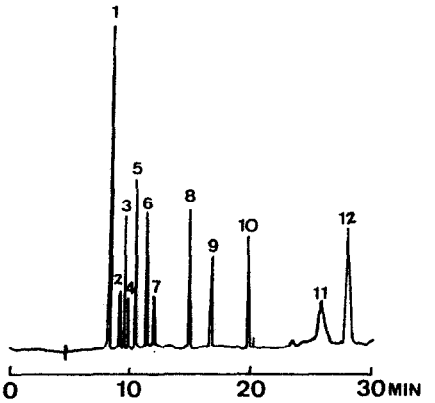

Fig. 3. Electropherogram of some phenolic compounds obtained by injection of a standard mixture. Buffer composition as in Fig. 2; 0.7 m*M* β-cyclodextrin was added to the buffer solution. 15 kV was used for the separation. Peaks: 1 = Chr; 2 = Hes; 3 = 34M; 4 = Nar; 5 = Isova; 6 = Cin; 7 = Va; 8 = 3H4M; 9 = Que; 10 = 4H3M; 11 = 34H; 12 = Vaa.

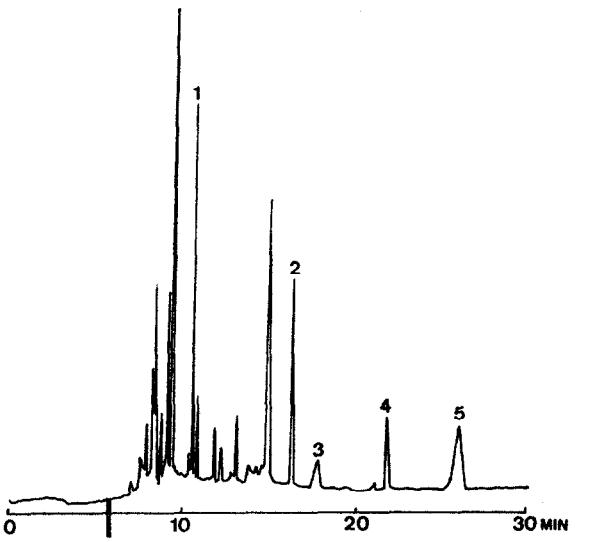

Fig. 4. Electropherogram of a propolis sample from Jiangsu, China. Buffer composition as in Fig. 3. 15 kV was used for the separation. Peaks: 1 = 34M; 2 = 3H4M; 3 = Que; 4 = unknown; 5 = 34H.

3.3. *Identification of some compounds in propolis*

The components in propolis samples were identified by comparing their migration times with those of standards. Pure standards were also added to the samples so that the peak heights of related compounds were increased in order to improve their detectability. Those peaks with the same migration times as those of standards were further studied with a photodiode-array detector connected to the CE system. The UV spectra of the compounds were taken and compared with those of standards.

Peaks 1, 2, 3 and 5 in the electropherogram of the propolis sample (Fig. 4) have the same migration times as those of 34M, 3H4M, 34H and Que standards. The UV spectra of these peaks obtained with the photodiode-array detector were identical with those of the standards.

3.4. *Ferulic acid*

Peak 4 in the electropherogram (Fig. 4) has the same migration time as that of ferulic acid. It was not possible to obtain a clear UV spectrum directly from peak 4 because it was too small. The UV spectrum of the compound was masked by that of the background. An alternative way is to plot the peak heights of peak 4 at different wavelengths (the peak heights were taken at 5-nm intervals between 200 and 400 nm) against wavelength. Such peak heights were stored in the memory of the diode-array detector during the analysis. In this way the UV spectrum of peak 4 was obtained, and was found to be different from that of ferulic acid.

3.5. *Selection of detection wavelength*

The UV wavelength for the detection of 34M, 3H4M, Que and 34H was selected according to the UV spectra obtained with the photodiode-array detector. At 215 nm, all four compounds

Table 1
Linear ranges and detection limits for the compounds

Compound	Linear range (μg/ml)	Detection limit (μg/ml)
34M	0–200	1.0
3H4M	0–200	1.3
Que	0–100	4.8
34H	0–400	3.8

Table 2
Results of analyses of a real propolis sample from China and a spiked sample

Sample	Amount	Compound			
		34M	3H4M	Que	34H
Original	Found	3.84 ± 0.39 mg/g ($n = 5$)	3.33 ± 0.28 mg/g ($n = 5$)	0.25 ± 0.19 mg/g ($n = 3$)	2.34 ± 0.72 mg/g ($n = 3$)
Spiked	Added	4 mg/g	4 mg/g	2.4 mg/g	4 mg/g
	Recovered	100.6 ± 10.95% ($n = 5$)	100.6 ± 17.6% ($n = 5$)	100.1 ± 22.7% ($n = 3$)	108.5 ± 16.7% ($n = 3$)

have relatively strong UV adsorbances and this wavelength was used for detection in subsequent experiments.

3.6. Extraction

Methanol, acetone, 95% ethanol and 70% ethanol were used to dissolve propolis samples. The resulting peak heights of the four compounds analysed were the same. The solutions obtained by dissolving the samples in 70% ethanol contained minimum wax, which is in agreement with the finding reported by Bankova et al. [4]. Therefore, 70% ethanol was used in subsequent experiments.

3.7. Linear ranges and detection limits

Linear ranges of the analysis were studied by series injections of standard mixtures containing different concentrations of 3H4M, 34M, Que and 34H into the CE system. The peak heights of the compounds were measured. The linear ranges for the compounds are shown in Table 1. The correlation coefficients were 0.99 for all the four compounds. The detection limits, based on a signal:noise ratio of 2:1, were obtained as shown in Table 1.

3.8. Sample analysis

The propolis sample was analysed using the CE method developed. The sample was also spiked with 34M, 3H4M, Que and 34H standards at levels of 4.0, 4.0, 2.4 and 4.0 mg/g respectively. The results are shown in Table 2.

4. Conclusions

Propolis samples can be analysed directly using the proposed method without any precleaning. As the CE separation process is performed at room temperature, the degradation of the components in the sample, which may occur in GC chromatographic separations owing to the high temperatures used, is avoided. The combination of photodiode-array detection with CE permitted the rapid identification of the components in the samples analysed.

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