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Article

Detection of Chlorogenic Acid in Honeysuckle Using Infrared-Assisted Extraction Followed by Capillary Electrophoresis with UV Detector

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In this study, a novel infrared-assisted extraction method coupled capillary electrophoresis (CE) is employed to determine chlorogenic acid from a traditional Chinese medicine (TCM), honeysuckle. The effects of pH and the concentration of the running buffer, separation voltage, injection time, IR irradiation time, and anhydrous ethanol in the extraction concentration were investigated. The optimal conditions were as follows: extraction time, 30 min; extraction solvent, 80% (v/v) ethanol in water solution; and 50 mmol/L borate buffer (pH 8.7) was used as the running buffer at a separation voltage of 16 kV. The samples were injected electrokinetically at 16 kV for 8 s. Good linearity ($r^2 > 0.9996$) was observed over the concentration ranges investigated, and the stability of the solutions was high. Recoveries of the chlorogenic acid were from 95.53% to 106.62%, and the relative standard deviation was below 4.1%. By using this novel IR-assisted extraction method, a higher extraction efficiency than those extracted with conventional heat-reflux extraction was found. The developed IR-assisted extraction method is simple, low-cost, and efficient, offering a great promise for the quick determination of active compounds in TCM. The results indicated that IR-assisted extraction followed by CE is a reliable method for quantitative analysis of active ingredient in TCM.

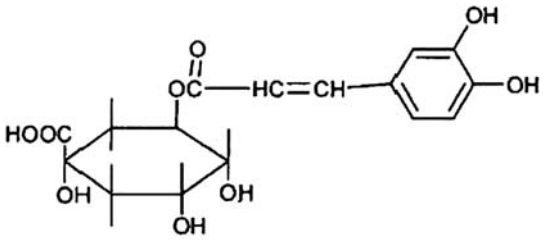
Introduction

Traditional Chinese medicines (TCM) have been extensively used to prevent and cure human disease for over a millennium in oriental countries. Because of their low toxicity and good therapeutical performance, TCM have attracted considerable attention in many fields (1). Honeysuckle, the dried flower of *Lonicera japonica Thunb.*, commonly known as "Jinyinhua" in TCM, has been used for the treatment of exopathogenic wind-heat or epidemic febrile disease in the early stage, as well as for sores, carbuncles, furuncles, and swellings for centuries (2). The plant has been reported to possess properties of detoxication, dispelling noxious heat from the blood, and arresting dysentery (3–4), and it can significantly increase blood neutrophil activity and promote the neutrophil phagocytosis (5). The constituents of this plant have been previously investigated and shown to contain iridoid glucosides (6) and polyphenolic compounds (7).

The main component in honeysuckle is chlorogenic acid (8). The molecular structure of the compound is shown in Figure 1. The quality control and evaluation of honeysuckle were generally concerned with chlorogenic acid considering its antipyretic property (9). In order to estimate the quality of honeysuckle, it is necessary to develop a method to assay the constituents mentioned earlier; however, it must be simple and reliable. Chlorogenic acid (5-O-caffeoyl-quinic acid), an ester of caffeic acid with quinic acid, has received considerable attentions for its wide distribution and potential biological effects (10). It is also an important bioactive compound and rich in some traditional Chinese medicine, such as flowers and buds of honeysuckle (*L. japonica*). It is also found in the leaves of Eucommia lmodies, which have been used for the treatment of exopathogenic wind-heat or epidemic febrile disease at the early stage, carbuncles, and swellings for centuries (11). A large number of studies revealed that chlorogenic acid has potential anti-inflammatory, analgesic, antipyretic (12), antimutagenic (13–14), and anti-carcinogenic activities (15–16). It can inhibit Bc–Abl tyrosine kinase and triggers p38 mitogen-activated protein kinase-dependent apoptosis in chronic myelogenous leukemic cells (17). Analysis of the chlorogenic acid in honeysuckle is a challenging task because of the diversity of the composition of the plant. The significant differences in the concentration of the active ingredients are the result of many factors, such as climates, regions of growth, and seasons of harvest. These all have an impact on the contents of active ingredients in medicinal herbs.

In the last decades, high-performance liquid chromatography (HPLC) has dominated the separation of Chinese herb and had been applied to analyze the chlorogenic acid in honeysuckle (18). Recently, capillary electrophoresis (CE) is becoming increasingly recognized as an important analytical separation technique due to its speed, efficiency, reproducibility, ultra-small sample volume, and ease of cleaning up the extracts. In 2000, U.S. Food and Drug Administration (FDA) published a draft of Guidance for Industry Botanical Drug Products. Before a plant drug can be marketed, its spectroscopic or chromatographic fingerprints must be recorded and a chemical assay of its characteristic markers is required. CE should find more applications in this area (19). CE has been used to determine chlorogenic acid; in this work, a lower limit of determination was found than in the previous method (2).

Extraction is the first step for preparation of medicine from raw plant materials and significantly affects the cost of the whole manufacturing process. Extraction of chlorogenic acid from the flower buds of *L. japonica* is conventionally performed by heat-reflux extraction. The traditional extraction process is time-consuming and laborious, and it involves lengthy operation techniques and large amounts of organic solvents. As an important form of electromagnetic wave, infrared



Chlorogenic acid

Figure 1. Structure of chlorogenic acid.

(IR) rays have wavelengths between ~ 750 nm and 1 mm and have found a wide range of applications. It has been widely employed as a heat resource due to its high penetration ability. Based on its wavelength, it can be divided into near-IR (0.75–1.5 μm), middle-IR (1.5–5.6 μm), and far-IR (5.6–1000 μm) rays. Recently, this method has been used to determine active compounds in *radix salviae miltiorrhizae* by HPLC (20). However, to our knowledge, IR-assisted extraction coupled with CE has not been fully explored, and its application to the analysis of chlorogenic acid from traditional Chinese medicines, such as honeysuckle, has not been conducted. It is of high interest to demonstrate the possibility of employing IR radiation as an energy source to enhance the efficiency of conventional reflux extraction. IR-assisted extraction is a process that uses infrared energy and solvents to extract target compounds from various matrices. Compared with conventional methods, IR-assisted extraction can considerably increase extraction efficiency.

In this work, a simple and rapid method was developed to determine chlorogenic acid in honeysuckle by CE, employing IR-assisted extraction as an efficient technique.

Experimental

Apparatus

In this work, a high-voltage (± 30 kV) power supply (Shanghai Institute of Nuclear Research, Shanghai, China) provided a voltage between the ends of the capillary. The separation was undertaken in a 50-cm length, 75-μm i.d. and 360-μm o.d. fused silica capillary (Hebei, China). The capillary was rinsed with 0.1 mol/L NaOH 30 min before use. The injector electrode was kept at a high positive voltage, detection of all the samples was performed by means of a UV detector positioned at the cathodic end of the capillary. Pressurized Capillary Electrochromatography System-2010GV (Unimcro Technology Company, Shanghai, China) was used as the UV detector, which was connected to a high-performance PC with the Windows XP operating system installed. Detection wavelength was 254 nm.

Reagents

Chlorogenic acid was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Stock solutions of chlorogenic acid (2.0×10^{-3} g/mL) were prepared in anhydrous ethanol (A.R. grade), stored in the dark at 4°C, and were diluted to the desired concentrations with the running buffer (50 mmol/L borate buffer, pH = 8.7). Before use, all solutions were filtered through 0.22-μm nylon filters.

Buffer preparation

A series of buffer solutions with pH from 7.00 to 10.00 were prepared by mixing boric acid and phosphoric acid stock solutions (0.1 mol/L). The pH of the buffer was measured at 25 ± 0.5 using a pHS-3C precise pH meter (Leici Instruments, Shanghai Precise Science Instrument Ltd. Co., Shanghai, China). The buffer solutions were filtered through a 0.22-μm syringe filter and degassed by ultrasonication prior to use.

Sample preparation

The herb, honeysuckle, was obtained from local drugstore in Shanghai. Five grams of dried honeysuckle was ground into powder in a mortar and accurately weighed. Each weighed sample was dissolved in 40 mL anhydrous ethanol (A.R. grade) and water (4:1). The IR-assisted extraction apparatus is shown in Figure 2. The distance between the top surface of the IR lamp and the bottom surface of the round-bottomed flask was 9 cm, because the solution can be heated by IR lamp to 70°C for 30 min at this distance. After cooling, the mixture was filtered through a paper filter, and the residues were washed with anhydrous ethanol. The extract and washings were combined and concentrated to approximately 45 mL under vacuum, and then diluted to 50 mL with anhydrous ethanol in a volumetric flask.

In the heat-solvent extraction experiment, five grams of dried herb honeysuckle was ground into powder in a mortar and accurately weighed. Each weighed sample was dissolved in 40 mL anhydrous ethanol (A.R. grade) and water (4:1). The solution was heated to 70°C for 30 min. After cooling, the mixture was filtered through paper a filter and the residues were washed with anhydrous ethanol. The extract and washings were combined and concentrated to approximately 45 mL under vacuum, and then diluted to 50 mL with anhydrous ethanol in a volumetric flask.

In the actual sample analysis, 0.50 mL of the sample solution was again diluted with the running buffer to 1 mL. After being filtered through a 0.22-μm syringe filter, all solutions can be injected directly to the CE system for analysis. Before use, all sample solutions were stored in the dark at 4°C

Results and Discussion

Effects of the pH value and the buffer concentration

In order to optimize the resolution and sensitivity of capillary zone electrophoresis (CZE), borate, phosphate, and borate–phosphate mixtures were employed as running buffers in the CZE separation. The experimental results showed that the best result was achieved using borate buffer. So the borate buffer was employed as the running buffer in this work.

The pH of the buffer is an important parameter that affects the electroosmotic flow (EOF), the overall charge, and the migration time of the analytes. Hence, the dependence of the migration time on buffer pH was investigated in the pH range of 7.0–10.0.

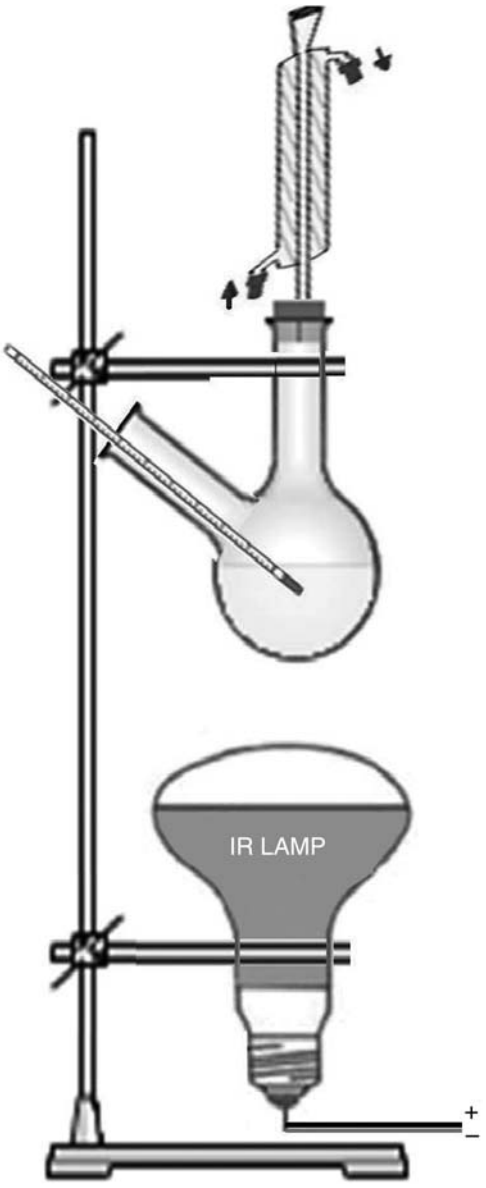


Figure 2. The IR-assisted extraction apparatus.

The migration time of the chlorogenic acid increases with the increasing pH value, and the baseline separation of the honeysuckle can be achieved from pH 8.7 to 10.0. When the pH is lower than 8.7, the herb cannot be separated very well. Moreover, higher pH values result in a long analysis time and easy oxidation of the analytes. Therefore, pH 8.7 was selected as the optimum pH value. Besides the pH value, the running buffer concentration, which affects peak height and theoretical plate number, is also an important parameter. The effect of the running buffer concentration on migration time was also studied, and the optimum running buffer concentration is 50 mmol/L.

Effects of separation voltage and injection time

For a given capillary length, the separation voltage determines the electric field strength, which affects both the velocity of electroosmotic flow and the migration velocity of the analytes,

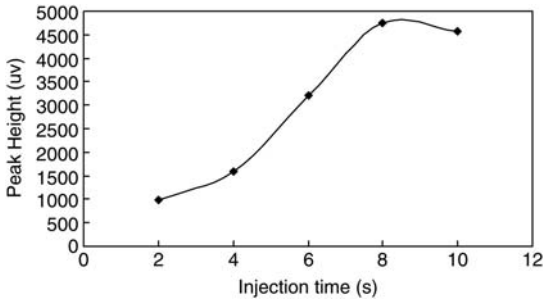


Figure 3. Effect of the injection time on the peak current of the chlorogenic acid.

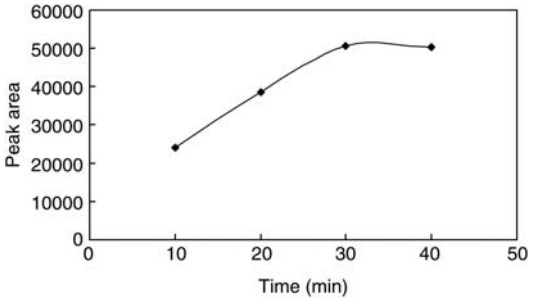


Figure 4. Effect of the irradiation time of IR-assisted extraction on the peak area of the chlorogenic acid in honeysuckle.

and in turn determines the migration time of the analytes. Higher separation voltage gives shorter migration time for all analytes. However, when the separation voltage exceeded 16 kV, the baseline noise increased. Therefore, the optimum separation voltage selected was 16 kV, at which good separation can be obtained for the analytes within 15 min. The injection time determines the amount of the sample and affects both the peak height and the peak shape. The effect of the injection time on the peak height was studied by varying the injection time from 2 s to 10 s at 16 kV. As seen from the data in Figure 3, it was found that the peak height increases with the increasing injection time. When the injection time is longer than 8 s, the peak height nearly levels off and peak broadening becomes more severe. In this experiment, 8 s (16 kV) is selected as the optimum injection time.

Effect of irradiation time

Figure 4 illustrates the effect of the irradiation time of IR-assisted extraction on the peak area of the chlorogenic acid in the extracts of a sample of honeysuckle. Upon increasing the irradiation time of IR-assisted extraction from 10 to 40 min, the peak area of the analytes increase to the maximum values at the time of 30 min, and they decrease gradually when the irradiation time is more than 30 min. This can be attributed to decomposition of the analytes. Hence, 30 min was selected as the optimum irradiation time.

Effect of anhydrous ethanol concentration on extraction efficiency of chlorogenic acid in honeysuckle

The influence of the anhydrous ethanol concentration on the extraction efficiency was studied. As seen from Figure 5,

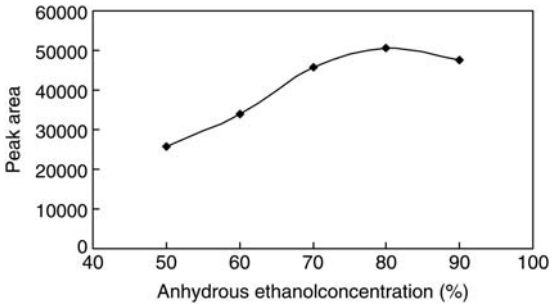


Figure 5. Effect of anhydrous ethanol concentration on the peak area of the chlorogenic acid in honeysuckle.

the maximum peak area of chlorogenic acid can be obtained at the anhydrous ethanol concentration of 80%.

Based on the results obtained here, the optimum conditions for chlorogenic acid were decided. A 50 mmol/L borate buffer (pH 8.7) was used as the running buffer at a separation voltage of 16 kV. Samples were injected electrokinetically at 16 kV for 8 s.

The typical electropherogram for a standard solution of the chlorogenic acid is shown in Figure 6A, and it is seen that good separation can be achieved within 15 min.

Method validation

Appropriate method validation information concerning new analytical techniques for analyzing pharmaceuticals is required by regulatory authorities. Validation of such methods included assessment of the stability of the solutions, linearity, reproducibility, detection limits, and quantification limits.

Stability of the solutions

The stability of the standard and sample solutions was determined by monitoring the peak area of standard chlorogenic acid solutions and sample solutions over a period of one day. The results showed that the peak area and migration time of chlorogenic acid was almost unchanged [relative standard deviation (RSD %) < 4.8] and that no significant degradation is observed within the given period, indicating the solutions are stable for at least one week.

Linearity repeatability, detection limits, and quantification limits

To determine the linearity of the peak area response on the concentration of chlorogenic acid, a series of standard chlorogenic acid solutions with concentrations from 0.2 to 400 μg/mL were tested. The calibration curve for quantifying chlorogenic acid was $y = 538546x + 348.44$. Where y is the chlorogenic acid peak area and X is the chlorogenic acid concentration (μg/mL), with a correlation coefficient (for R^2) of 0.9996; the linear range of the chlorogenic acid is 0.2–400 μg/mL.

The reproducibility of the peak area and migration time was estimated by making repetitive injections of a standard chlorogenic acid solution (40 μg/mL for chlorogenic acid) under the optimum conditions. The RSD of the peak area and the migration time were 3.5% and 3.8% for chlorogenic acid

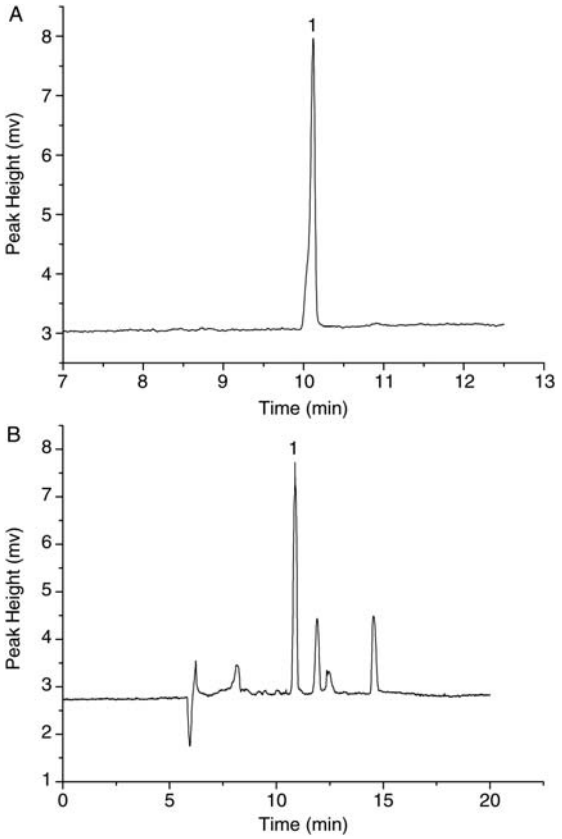


Figure 6. The electropherogram of a standard solution (4.0×10^{-5} g/mL for chlorogenic acid) (A), and the typical electropherogram of herb honeysuckle with IR-assisted extraction (B). Peak identification: 1 = chlorogenic acid.

($n = 7$). The detection limit of 0.05 μg/mL is based on a signal-to-noise ratio of 3. The calibration curves exhibit excellent linear behavior over the concentration range of about three orders of magnitude with the detection limit of 0.05 μg/mL for the analyte.

The LOQ is defined as the level at (or above) which the measurement precision is satisfactory for quantitative analysis. In this case, the LOQ was evaluated on the basis of a signal-to-noise ratio of 10. The LOQ was 0.15 μg/mL for chlorogenic acid. The RSD of the LOD and LOQ were 3.7% and 3.9% for chlorogenic acid ($n = 3$).

Practical sample analysis and recovery

Under the optimum conditions, CE was applied for the determination of chlorogenic acid in honeysuckle in combination with IR-assisted extraction. Typical electropherograms for a chlorogenic acid standard and for an IR-assisted extract of honeysuckle are shown in Figures 6A and 6B. The main compound in honeysuckle was identified as chlorogenic acid. The results show that chlorogenic acid in honeysuckle is 1.86 μg/mL, with a relative standard deviation of 3.5%. As seen in Figure 7, the peak area of the chlorogenic acid in the same herb sample obtained by using CE coupled with IR-assisted extraction was higher than that obtained with the heat-solvent extraction, indicating that an IR-assisted extraction approach is

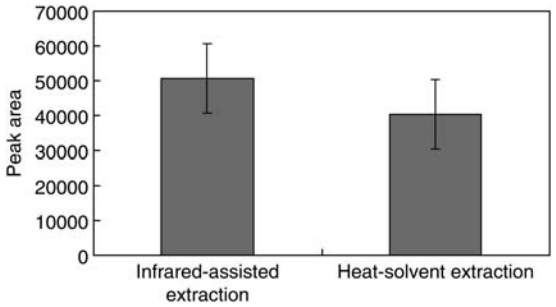


Figure 7. Comparison of peak height of chlorogenic acid in the extract of honeysuckle by IR-assisted extraction and heat-solvent extraction at 30 mins.

Table I
Determination Results of the Recovery for this Method with the Herb Honeysuckle Sample ($n = 3$)

Sample	Ingredient	Original amount (μg/mL)	Added amount (μg/mL)	Found amount (μg/mL)	Recovery (%)	RSD (%)
Herb Honeysuckle	chlorogenic acid	46.61	100	153.23	106.62	3.7
		93.22	100	188.75	95.53	4.1
		74.58	100	169.32	94.74	3.9

more efficient than the conventional heat-solvent extraction when the extraction times were the same.

Under the optimum conditions, the recovery and reproducibility experiments were also conducted to evaluate the precision and accuracy of the method. The recovery was determined by a standard addition method. The average recoveries and RSDs for the analytes are listed in Table I ($n = 3$).

Conclusion

This work presents the first application of CE for the qualitative and quantitative assay of chlorogenic acid in honeysuckle with the aid of IR-assisted extraction with higher extraction efficiency. The assay results mentioned herein indicate that this method is accurate, sensitive, and reproducible.

Acknowledgements

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