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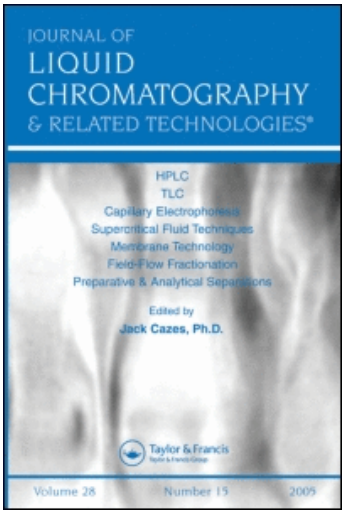

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ANALYSIS OF ACTIVE CONSTITUENTS IN RHIZOMA PICRORHIZAE BY CAPILLARY ZONE ELECTROPHORESIS

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ANALYSIS OF ACTIVE CONSTITUENTS IN *RHIZOMA PICRORHIZAE* BY CAPILLARY ZONE ELECTROPHORESIS

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ABSTRACT

An effective capillary zone electrophoresis (CZE) method has been developed for the determination of picroside II, cinnamic acid, ferulic acid, and vanillic acid in *Rhizoma picrorhizae* for the first time. After a series of optimization experiments, 100 mM borate buffer (pH 9.5), 30 kV applied voltage and 25°C temperature were selected. The contents of the four active constituents in crude drug, extracts, and capsule preparation of *Rhizoma picrorhizae* were successfully determined within 12 min, with gallic acid as internal standard, and the repeatability and recovery are satisfactory. In all samples, the content of picroside II is the highest, and can be regarded as the representative compound. The contents of picroside II were also determined by high performance liquid chromatography (HPLC), showing that the results by CE and by HPLC are comparable. The technological process of extract preparation containing different amounts of picroside II was investigated by CZE.

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INTRODUCTION

Rhizoma picrorhizae, a small herb with tuberous root, which grows in the Himalayan area at altitudes of 3000–5000 m, is a well-known Ayurvedic drug. The plant is mainly used in therapy for liver and lung diseases, but also for the treatment of chronic dysentery and other complaints.[1,2] Previous phytochemical investigations[1,2] resulted in the isolation of three iridoid glycosides, picroside I, II, and III, which were regarded to be responsible for the antihepatotoxic activity of this plant. Among these iridoid glycosides, phytochemical work shows that picroside II is the main active compound due to its high content in *Rhizoma picrorhizae*. Cinnamic acid, ferulic acid, and vanillic acid have been reported to show strong antibacterial activity.[1,2]

Up to now, the analysis of four active constituents in *Rhizoma picrorhiza* has not been reported, and all of the research work about *Rhizoma picrorhiza* has focused on phytochemistry and pharmacology. The development of capillary electrophoresis (CE) has attracted tremendous attention in past two decades,[3–6] and it continues to be a very active research area in separation science, since this technique often provides higher resolution, shorter analysis time, and lower operation cost than conventional methods. Now there are many research groups that are devoting themselves to the analysis of traditional medicines by CE.[7–16] In this work, we describe an effective capillary zone electrophoresis (CZE) method for the determination of picroside II, cinnamic acid, ferulic acid, and vanillic acid in *Rhizoma picrorhizae* for the first time, and their chemical structures are shown in Fig. 1. After the systematical optimization of such parameters as pH, concentration of running buffer, applied voltage, and column temperature, the contents of the four active constituents in crude drugs, extracts, and capsule preparation of *Rhizoma picrorhizae* were successfully determined. In all samples, the content of picroside II is the highest, and can be regarded as the representative compound. The content of picroside II were also determined by high performance liquid chromatography (HPLC), showing that the results by CE and by HPLC are comparable. The technological process of extracts preparation containing different amount picroside II was investigated by CZE.

EXPERIMENTAL

Apparatus and Conditions

All experiments were conducted on an Agilent 3D CE system with air-cooling and a diode array detector (Agilent Technologies, Waldbronn, Germany). A 58.5 cm × 50 μm I.D. uncoated fused silica capillary (Ruifeng Inc., Hebei, China) was utilized with an effective length of 50 cm, and the temperature was

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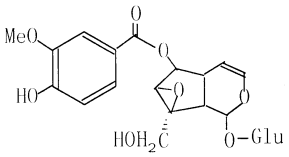
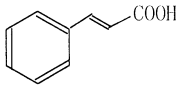
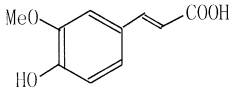


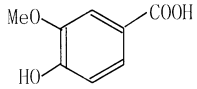
1. Picroside II



2. Cinnamic acid



3. Ferulic acid



4. vanillic acid

Glu = Glucose

Figure 1. The chemical structures of four studied constituents.

maintained at 25°C. The other conditions are as follows: applied voltage 30 kV, UV detection at 210 nm, samples injection at 50 mbar for 10 seconds.

The electrolyte solution consists of 100 mM boric acid (pH 9.5), which was filtered through a 0.45 μm membrane filter and degassed by ultrasonication for approximately 10 min before use. The capillary was conditioned daily by washing first with 0.5 M sodium hydroxide (10 min), then with water (10 min), and finally with the running buffer (15 min). Between consecutive analyses, the capillary was flushed with 0.5 M sodium hydroxide (1 min), then with water (2 min), and finally

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the running buffer (3 min), in order to improve the migration time and peak area repeatability.

High perfomance liquid chromatorgraphic analysis was carried out using a JASCO HPLC 1500 instrument and Zorbax ODS (150 × 4.6 mm I.D., 5 μm) column with UV detection at 265 nm, under room temperature. The mobile phase was composed of methanol–water–acetic acid (40 : 60 : 0.3) at the flow-rate of 1.0 mL/min under isocratic mode.

Reagents and Materials

The standards of picroside II, cinnamic acid, ferulic acid, and vanillic acid, crude drug, extract, and capsule preparation of *Rhizoma picrorhiza* were provided by the Institute of Medicine Plant Development (Beijing, P.R. China). All chemicals were of analytical-reagent grade: boric acid, hydroxide sodium, methanol, from Beijing Chemical Factory (Beijing, P.R. China); pure water prepared by Milli-Q system (Millipore, Bedford, MA, USA) was used for all the buffer solutions.

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Sample Preparation

Pulverized dried crude drug (0.08 g), extract (0.10 g), and the internal content of capsule preparation (0.2 g) of *Rhizoma picrorhizae*, respectively, were extracted with methanol (7 mL) by ultrasonicating at room temperature for 30 min, then centrifuged at 1500 rpm for 10 min. After three repeated extractions, the extracts were combined and diluted to 100 mL with methanol, as the sample stock solution. For CZE analysis, 0.5 mL of internal standard solution (2.54 mg mL^{-1} gallic acid solution) was diluted to 10 mL with the stock sample solution, which was then passed through a 0.45 μm membrane filter.

Solutions for Calibration Curve and Recovery Testing

Five calibration solutions containing picroside II, cinnamic acid, ferulic acid, and vanillic acid were prepared in methanol in the concentration range from 10.9 to 432.0 μg mL^{-1} depending on different components, and each of the solution contains 0.127 mg L^{-1} internal standard gallic acid.

Known amounts of picroside II and vanillic acid were added to the sample of the extract of *Rhizoma picrorhizae* in order to evaluate the recovery. The mixtures were extracted and analyzed by the same procedures as used for other samples described above.

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RESULTS AND DISCUSSION

Optimization of Analytical Conditions

The effect of buffer pH on migration behavior of these analytes was investigated by using 100 mM borate at pH 8–10.5, under applied voltage 20 kV and 25°C temperature. The results showed that the migration times of four constituents increased with the increase of pH within the studied range (see Fig. 2), due to greater ionization of the phenolic hydroxyl groups at higher pH, resulting in greater mobilities of the constituents in the opposite direction to the electro-osmotic flow (EOF). At pH 9.5, four constituents can be completely separated with moderate analysis time, and, therefore, pH 9.5 was selected for further optimization of other conditions.

The borate concentration, from 50 mM to 200 mM in the buffer at pH 9.5, was used with 20 kV applied voltage and 25°C temperature, to study the effect of buffer concentration on the separation, indicating that, with the increase in the borate concentration, the migration times of the constituents increased because of the reduced EOF resulting from the increase of ionic strength (see Fig. 3). Borate, 100 mM, was shown to be the optimum concentration when taking the better peak shape and good resolution into account.

The applied voltage (15–30 kV) and temperature (25–40°C) were finally optimized with the running buffer of 100 mM borate (pH 9.5), and the optimum

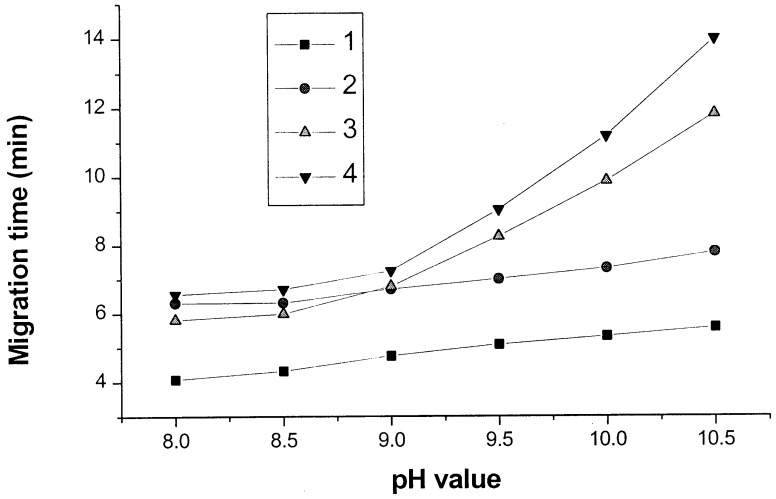

Figure 2. The plot of pH vs. migration time of the analytes. See Experimental for conditions. The curve label stands for the same compounds as in Fig. 1.

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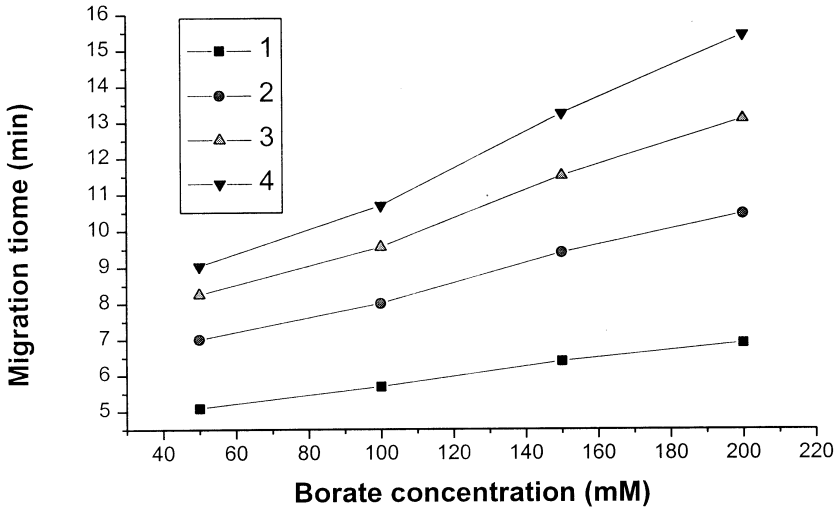

Figure 3. The plot of borate concentration vs. migration time of analytes. See Experimental for conditions. The curve label stands for the same compounds as in Fig. 1.

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voltage and temperature were found to be 30 kV and 25°C, respectively, which combined sufficient resolution with a moderate analysis time (see Fig. 4).

Therefore, the 100 mM borate buffer (pH 9.5), under 30 kV applied voltage and 25°C capillary temperature were proven to be the optimized conditions for this separation. Figure 5(A) shows a typical electropherogram obtained from a mixture of four standard constituents.

Calibration Curves

Calibration curves were constructed based on an internal standard method in the concentration ranges 72.0–432.0 $\mu g\,mL^{-1}$ for picroside II, and 51.2–128.0 $\mu g\,mL^{-1}$ for cinnamic acid, 44.0–176.0 $\mu g\,mL^{-1}$ for ferulic acid, and 4.36–109.0 $\mu g\,mL^{-1}$ for vanillic acid. The linear regression equations and correlation coefficients were:

$$\text{Picroside II: } Y = 1215.7X + 9.8535 \ (r = 0.9997)$$
$$\text{Cinnamic acid: } Y = 204.34X - 1.4230 \ (r = 0.9927)$$
$$\text{Ferulic acid: } Y = 250.87X + 4.0674 \ (r = 0.9973)$$
$$\text{Vanillic acid: } Y = 166.88X - 5.9629 \ (r = 0.9947)$$

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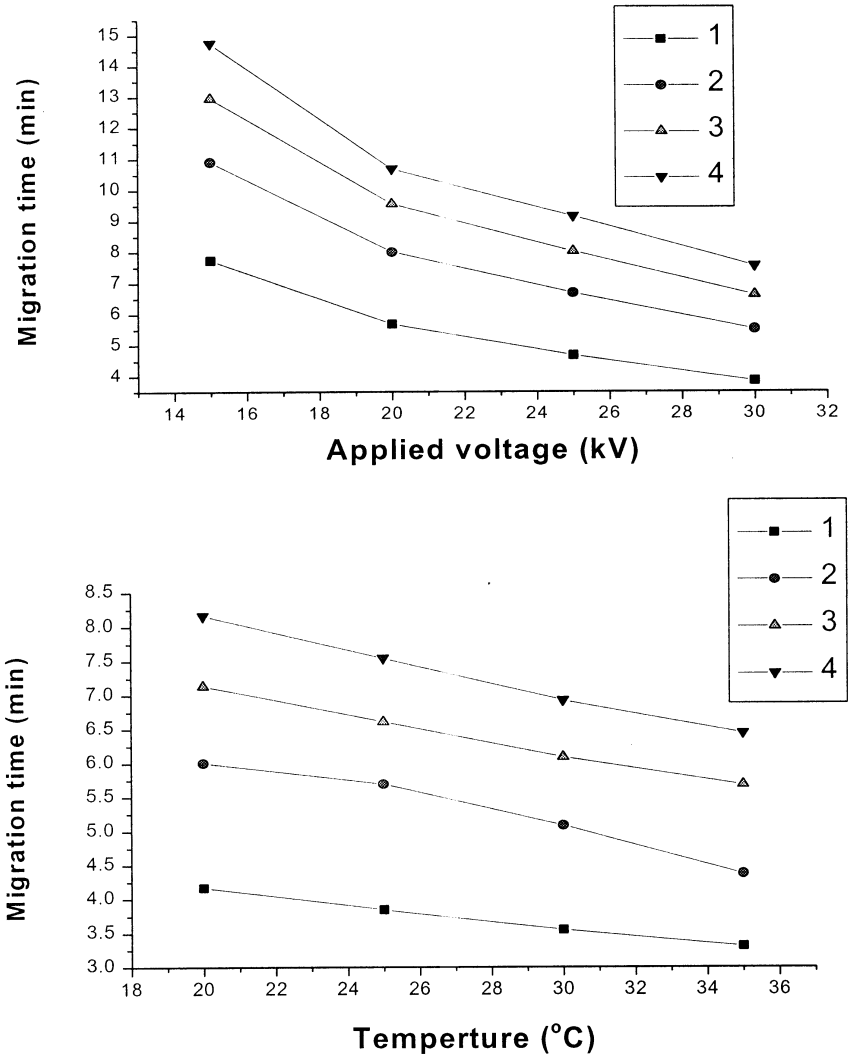

Figure 4. The effects of applied voltage and temperture on migration time of the flavonoids. Conditions see the Experimental. The curve label stands for the same compounds as in Fig. 1.

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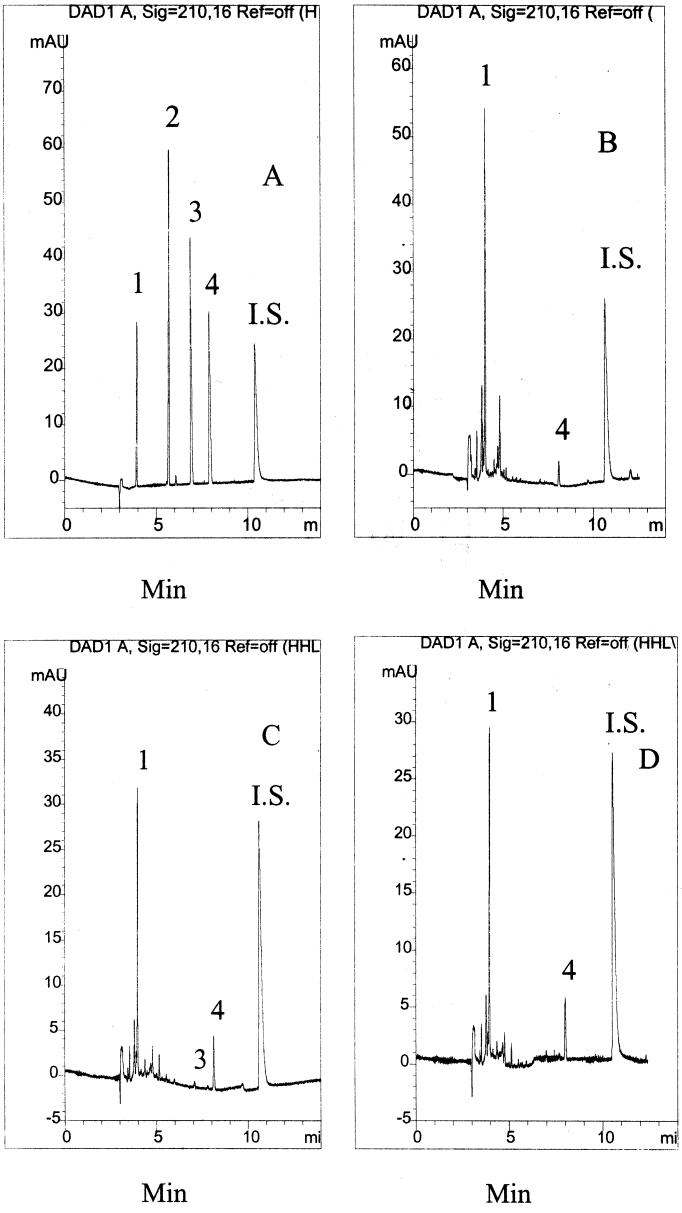

Figure 5. Electropherograms obtained from the four constituents standards and the samples of *Rhizoma picrorhizae*. (A) standards mixture, (B) crude drug, (C) extract, (D) capsule preparation. See Experimental for conditions. See Fig. 1 for peak identification.

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where X is the ratio of the peak area of individual active constituents to gallic acid, Y is the concentration ($\mu g\,mL^{-1}$), and r is the correlation coefficient, demonstrating that the linearity method is satisfactory with all the $r > 0.99$.

System Suitability Test

The repeatabilities (relative standard deviation, RSD), recoveries, and detection limits of the method for these analytes are listed in Table 1, showing that the RSD ($n = 5$) for peak area ranges from 0.64% to 0.98%, and for migration time from 0.11% to 0.44%, respectively. The detection limit, based on 3 S/N, is varied from 2.8 to 18.0 $\mu g\,mL^{-1}$ depending on different analytes. The recovery of 99.2% for picroside II and 95.8% for vanillic acid were obtained, respectively. The recovery of cinnamic acid and ferulic acid was not measured, because they were not detected or had only a trace amount in all samples.

Determination of Four Constituents in *Rhizoma picrorhiza*

The amounts of picroside II, cinnamic acid, ferulic acid, and vanillic acid in the crude drug, extract, and capsule preparation of *Rhizoma picrorhiza* have been quantified by use of the regression line or equation, and the results are listed in Table 2. Figure 5(B)–(D) illustrates the typical electropherograms obtained from the crude drugs, extracts, and capsule preparation of *Rhizoma picrorhiza*. It can be concluded, that the content of picroside II in all samples is very high, so it can be regarded as the characteristic constituent of *Rhizoma picrorhiza*. What is more, for investigating the accuracy of the CZE method, the content of picroside II in the samples were also determined by HPLC, as shown in Table 3, showing that

Table 1. Repeatability (RSD), Recovery, and Detection Limit of the Active Constituents in *Rhizoma picrorhizae* Determined by CZE

	Repeatability (RSD, $n = 5$) (%)		Recovery ($n = 5$)		
Component	Peak Area	Migration Time	Recovery (%)	RSD (%)	Detection Limit ($\mu g\,mL^{-1}$)
Picroside	0.76	0.22	99.2	2.38	18.0
Cinnamic acid	0.98	0.44	—	—	3.2
Ferulic acid	0.77	0.14	—	—	3.0
Vanillic acid	0.64	0.11	95.8	3.37	2.75

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Table 2. Contents of the Four Constituents in Crude Drug, Extract, and Capsule Preparation of *Rhizoma picrorhiza* Determined by CZE ($n = 5$)

Sample	Picroside II	Cinnamic Acid	Ferulic Acid	Vanillic Acid
Crude drug	9.13%	Not found	Trace	Trace
RSD	4.21%	—	—	—
Extract	28.89%	Not found	Not found	0.77%
RSD	2.12%	—	—	0.72%
Capsule	24.45 mg/ granule	Not found	Not found	0.55 mg/ granule
RSD	1.11%	—	—	1.12%

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the results agree with each other. The retention time of picroside II in HPLC was 14.86 min and a typical chromatogram is shown in Fig. 6. For the HPLC method, its linear regression equation was $Y = 0.077169X + 3.4909$ ($r = 0.9999$), where X is the peak area, Y is the concentration ($\mu g\,mL^{-1}$) of picroside II, and r is the correlation coefficient; the RSD ($n = 5$) was 0.93% for picroside II, and the recovery is 98.0% with a RSD ($n = 5$) of 0.75%, showing that HPLC results are comparable with those of CZE. Therefore, CZE should be an effective method, with shorter analytical time, for the determination of the active constituents in *Rhizoma picrorhizae*, although, the linearity of HPLC is better than that of CZE.

The Technological Process of Extract Preparation

Picroside II is the representative constituent of *Rhizoma picrorhizae*, and the different extracts containing different amount picroside II show variable pharmacological effects, so extracts preparation with different amounts of picroside II play a very important role in pharmacological study. What is more, the extract containing higher picroside II can reduce dosage in the clinic treatment. Here, the technological process of extracts preparation, by means of

Table 3. Contents of Picroside II in Crude Drug, Extract, and Capsule Preparation of *Rhizoma picrorhiza* Determined by HPLC ($n = 5$)

Sample	Crude Drug	Extract	Capsule
Picroside II	8.84%	28.54%	25.28 mg/unit
RSD	1.33%	1.39%	1.94%

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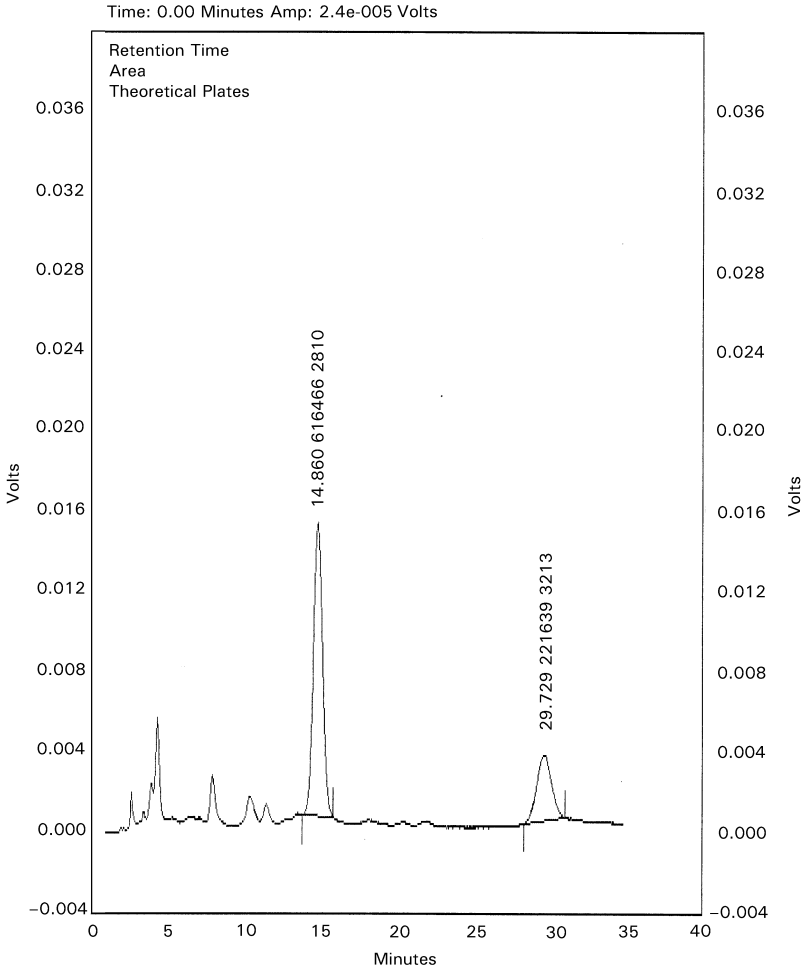

Figure 6. HPLC chromatogram of extract of *Rhizoma picrorhizae*. The number (14.860) on peak indicates retention time of picroside II in minutes. See Experimental for conditions.

macroporous resin was investigated, and the picroside II contents of extracts is monitored by CZE as described above. The technological process of extracts preparation is shown in Fig. 7, and the picroside II contents in different extracts are indicated in Table 4. The experiment shows that this technique is an effective and simple method for monitoring the process for preparing extracts containing different picroside II contents.

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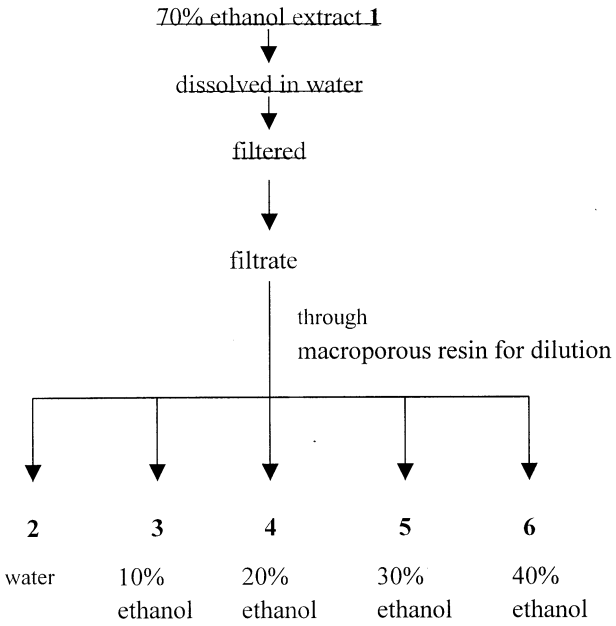

Figure 7. The technological process of extracts preparation.

Table 4. The Contents of Picroside II in Different Extracts Determined by CZE ($n = 5$)

Extract	1	2	3	4	5	6
Picroside II (%)	14.01	11.87	28.89	49.50	61.05	70.75
RSD (%)	2.97	1.53	2.12	3.05	2.44	1.57

See Fig. 7 for the number of extract.

CONCLUSIONS

The contents of picroside II, cinnamic acid, ferulic acid, and vanillic acid in *Rhizoma picrorhizae* were determined by the described CZE method within 12 min under the optimized conditions, with satisfactory repeatability and recovery. The result by CZE was comparable with that by HPLC. This CZE method could be effective for quality control of crude drug and its preparation of *Rhizoma picrorhizae*. Moreover, the technological process of extracts preparation containing different amounts of picroside II was investigated by CZE.

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