JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

An Improved Method for the Quantitation of Flavonoids in Herba Leonuri by Capillary Electrophoresis

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A capillary electrophoresis system coupled with wall-jet amperometric detection was used to determine five flavonoids (kaempferol, rutin, hyperoside, quercitrin, quercetin) in Herba Leonuri. Several important effect factors, such as the pH and concentration of running buffer, separation voltage, injection time, and detection potential, were investigated to acquire the optimum conditions. With 50 mmoL/L $Na_2B_4O_7$−100 mmoL/L NaH_2PO_4 buffer (pH=7.50) as the running buffer, the five flavonoids were baseline separated within 15 min in a 60 cm length capillary at a separation voltage of 15 kV. The relationship between peak currents and analyte concentrations was linear over about 2 orders of magnitude, with detection limits (S/N = 3) ranging from 0.03 to 0.08 μg/mL for all analytes. The use of this method for the quantitation of the above flavonoids present in the real sample of Herba Leonuri was reported.

KEYWORDS: Flavonoids; capillary electrophoresis; amperometric detection; Herba leonuri

INTRODUCTION

Flavonoids constitute one of the largest groups of naturally occurring phenols commonly present in medicinal plants. Various studies reveal that flavonoids have antiinflammatory, antitumor, antivirus, antibacteria, and antioxidation functions (*1*). It is very significant to develop a simple and reliable method for quantitation of flavonoids in medicinal plants. Usually, high-performance liquid chromatography (HPLC) has occupied the leading position in the analysis of flavonoid-containing plants for identification and quality control purposes (*2*−*6*). However, due to the rather complex character of plant samples and the inherent structural similarity of most flavonoids, time-consuming sample pretreatment is frequently needed to remove the sample matrix before the chromatographic step and the gradient elution is also often required to provide sufficient HPLC separation power.

As a modern separation method, capillary electrophoresis (CE) has won increasing attraction for its extremely high efficiency, rapid separation, use of an ultrasmall sample, and ease of cleaning up contaminants. Until now, a few papers have reported the use of CE with ultraviolet (UV) detection for determination of flavonoids in medicinal plants (*7*−*12*), but the sensitivity of the UV detector is relatively low for the small diameter of the separation capillary. Compared with UV detection, amperometric detection (AD) is more sensitive and selective, which makes it very suitable for the analysis of trace electroactive compounds. Since most flavonoids are electroactive compounds, CE−AD has been employed for the determination of some flavonoids in medicinal plants (*13*−*16*).

Herba Leonuri, the dried aerial part of *Leonurus heterophyllus* Sweet (family Labiatae), has been used to activate blood flow and regulate menstrual discharge in the case of menstrual disorders, and also acts as a diuretic for the treatment of nephritic edema. Flavonoids, such as kaempferol, rutin, hyperoside, quercitrin, and quercetin (their molecular structures are shown in **Figure 1**), in Herba Leonuri are biologically active constituents. The spectrophotometry method for the determination of the total amount of flavonoids in Herba Leonuri has been reported (*17*). The aim of this study was to develop a simple and rapid method for simultaneous determination of individual flavonoids in Herba Leonuri. Therefore, a sensitive and selective CE−AD method for determination of the above five flavonoids was established, and the proposed method was then applied for determining flavonoids in ethanolic extracts from Herba Leonuri with relatively simple sample pretreatment.

MATERIALS AND METHODS

Chemicals. Kaempferol, rutin, hyperoside, quercitrin, quercetin, and Herba Leonuri were obtained from the Chinese Chemical and Biological Drugs Institute (Beijing, China). All aqueous solutions were made up in doubly distilled water. Other chemicals were of analytical grade. Standard stock solutions of five flavonoids at a concentration of 1.0×10^3 μg/mL were prepared in ethanol and diluted to the desired concentration with the running buffer just prior to use. All standard solutions were kept in a refrigerator and could be stable for 2 months. The borate−phosphate running buffer was prepared by mixing $Na_2B_4O_7$ solution (concentrations ranging from 20 to 70 mmol/L) with NaH_2PO_4 solution (corresponding concentrations ranging from 40 to 140 mmol/L), and the pH value of the running buffer was measured by a

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10.1021/jf0504216 CCC: $30.25
Published on Web 06/25/2005

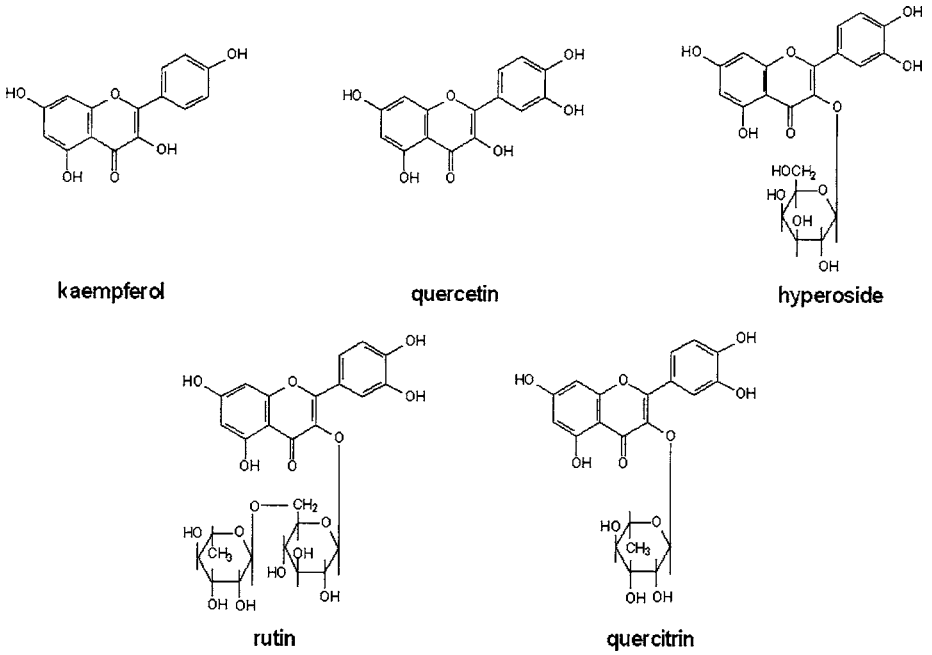

Figure 1. Molecular structures of flavonoids.

pH meter. Before use, all solutions were filtered through a 0.22 μm polypropylene filter film.

Apparatus. A laboratory-built capillary electrophoresis system equipped with a wall-jet amperometric detector was employed in the experiment. The details of this system have been described in previous works (*18*, *19*). A ±30 kV high-voltage dc power supply (Shanghai Institute of Nuclear Research, China) provided a separation voltage between the ends of the capillary. The inlet end of the capillary was held at a positive potential, and the outlet end of the capillary was kept at ground. The separation capillary was an untreated fused silica capillary of 60 cm × 25 μm i.d. × 370 μm o.d (Hebei Yongnian Optic Fiber Factory, China). A prealigned electrochemical cell (laboratory-built), consisting of three electrodes (a 500 μm diameter carbon disk working electrode, a platinum auxiliary electrode, and a Ag/AgCl reference electrode), was used in combination with a BAS LC-4C amperometric detector (Bioanalytical System, West lafayette, IN). The use of this prealigned electrochemical cell avoided the complicated operation of aiming the working electrode, saved experimental time, and improved the reproducibility of the experiments. The electropherograms were monitored using a chromatogram workstation (model HW-2000, Qianpu Software Co., Shanghai, China). A PHS-3C meter (Shanghai Dapu Instrument Co., Shanghai, China) was used to measure the pH value of the running buffer. A CHI660 electrochemical system (CH instruments, Austin, TX) was chosen to perform cyclic voltametry.

Sample Solution Preparation. After being air-dried and crushed into a powder, a 0.2006 g Herba Leonuri sample was extracted with 2 × 10 mL of ethanol by sonication for 30 min. The extracts were combined and concentrated to about 1.7 mL at 50−60 °C, and then diluted to 2.0 mL with ethanol. This extract was 13-fold diluted with 50 mmol/L $Na_2B_4O_7$−100 mmol/L NaH_2PO_4 (pH 7.50) running buffer just prior to analysis. Peak identification was performed by standard addition methods.

CE-AD Method. The carbon disk electrode was successively polished with sand emery paper and rinsed with doubly distilled water. The potential applied to the working electrode was 0.95 V (versus Ag/AgCl). The running buffer was 50 mmol/L $Na_2B_4O_7$−100 mmol/L NaH_2PO_4 (pH 7.50) solution, and the separation voltage was 15 kV. Samples were injected electrokinetically at 15 kV for 10 s.

RESULTS AND DISCUSSION

Electrochemistry. Since the standard stock solution of flavonoids was prepared in ethanol, 50 mmol/L $Na_2B_4O_7$−100 mmol/L NaH_2PO_4 (pH 7.50)−1% ethanol solution was selected as the blank solution to perform cyclic voltammetry for investigating the electroactivity of the five analytes at the glassy carbon electrode (GCE). The cyclic voltammograms are shown in **Figure 2**. **Figure 2** shows that in blank solution no peak was observed in the potential range of 0−600 mV, whereas all analytes exhibited an obvious anodic peak, which may be attributed to the oxidation of phenolic hydroxyl groups in analytes. In addition, the redox processes of flavonols and their glucosides were different. The anodic currents of kaempferol and quercetin were much higher than their corresponding cathodic currents due to the strong adsorption of the oxidation products of kaempferol and quercetin at the GCE (see **Figure 2D,F**). This indicates that the oxidation processes of these two flavonols were quasi-reversible. In the case of hyperoside, rutin, and quercitrin, the presence of the hydrophilic carbohydrate group seemed to inhibit the adsorption of the oxidation products at the GCE, so the anodic and corresponding cathodic currents of the above three flavonoids were almost equivalent (see **Figure 2B,C,E**), which indicates that the three flavonoids exhibited reversible oxidation processes. Thus, the five analytes are electroactive and can be determined by amperometric detection.

Selection of Detection Potential. To select an optimum detection potential, the hydrodynamic voltammetry experiment was performed in this work. As shown in **Figure 3**, the peak currents of all analytes were relatively small when the detection potential was 0.80 V (versus Ag/AgCl). In the potential range of 0.80−0.95 V, the oxidation peak currents increased with the applied potential. However, when the potential increased from 0.95 to 1.00 V, the current response almost remained the same, whereas the background current increased drastically from 49 to 80 nA; the baseline noise also increased greatly at the same time, which was obviously a disadvantage for obtaining a sensitive and stable detection. For a suitable compromise of high sensitivity and low background current, 0.95 V (versus Ag/AgCl) was selected as the detection potential in the subsequent experiments.

Effects of the pH and Concentration of the Running buffer. Three buffer solutions at pH 7.50 (phosphate, borate, and borate−phosphate) have been selected as the running buffer for testing their effects on the separation of analytes mentioned

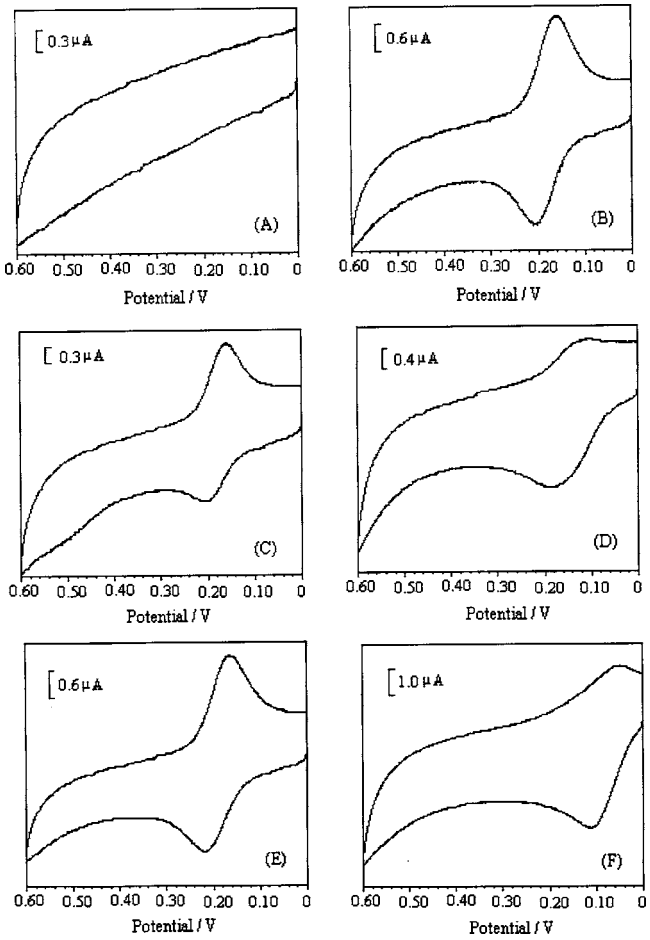

Figure 2. Cyclic voltammograms of the five analytes in 50 mmol/L $Na_2B_4O_7$–100 mmol/L NaH_2PO_4–1% ethanol solution (pH 7.50): working electrode, glassy carbon electrode; auxiliary electrode, platinum wire; reference electrode, Ag/AgCl; scan rate, 100 mV/s; (**A**) blank solution, (**B**) 9.9 μg/mL hyperoside, (**C**) 19.8 μg/mL rutin, (**D**) 1.0 μg/mL kaempferol, (**E**) 14.8 μg/mL quercitrin, (**F**) 21.8 μg/mL quercetin.

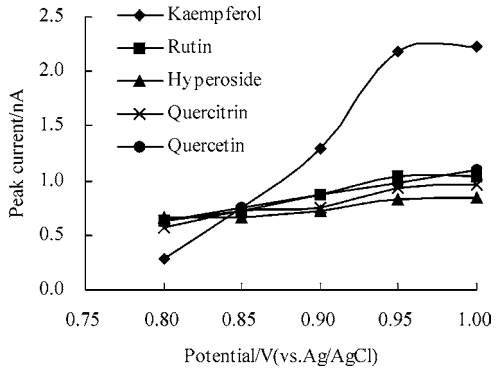

Figure 3. Hydrodynamic voltammograms of 8.0 μg/mL kaempferol, 8.0 μg/mL rutin, 2.4 μg/mL hyperoside, 2.0 μg/mL quercitrin, and 6.0 μg/mL quercetin in CE: fused-silica capillary, 60 cm × 25 μm i.d. × 370 μm o.d.; working electrode, 500 μm diameter carbon disk electrode; running buffer, 50 mmol/L $Na_2B_4O_7$–100 mmol/L NaH_2PO_4 (pH 7.50); separation voltage, 15 kV; electrokinetic injection, 10 s (at 15 kV).

above. The results showed that the five analytes could be baseline separated only in borate−phosphate running buffer under the same conditions, so $Na_2B_4O_7$−NaH_2PO_4 buffer was chosen as the running buffer. The pH of the running buffer drastically affects the resolution of the analytes. The dependence of the separation of the five analytes on pH was studied with

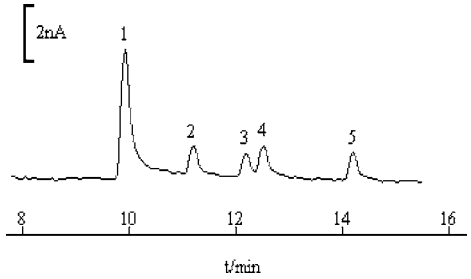

Figure 4. Electropherogram of the standard mixture solution of flavonoids: working potential, 0.95 V (vs Ag/AgCl); other conditions as in Figure 3; (1) kaempferol (16.0 μg/mL); (2) rutin (8.0 μg/mL); (3) hyperoside (2.4 μg/mL); (4) quercitrin (2.0 μg/mL); (5) quercetin (6.0 μg/mL).

50 mmol/L $Na_2B_4O_7$−100 mmol/L NaH_2PO_4 buffer in the pH range of 7.00−8.00. When the pH was at 7.00, kaempferol, rutin, and quercetin could be baseline separated, but hyperoside and quercitrin could not be separated completely. With increasing pH value, the migration times and resolution of the five analytes increased due to the dissociation of the hydroxyl groups for all analytes. At pH 7.50, the five analytes could be best separated within a relatively short time. Thus, pH 7.50 was found to be the optimum pH value for the running buffer.

The effect of the concentration of borate−phosphate running buffer (pH7.50) on separation was investigated. The experimental results indicate that hyperoside and quercitrin could not be resolved completely when the concentration of $Na_2B_4O_7$ was 20 mmol/L. With increasing running buffer concentration, the migration time and the resolution of all analytes increased, but the peak currents of all analytes decreased. Considering the separation efficiency, 50 mmol/L $Na_2B_4O_7$−100 mmol/L NaH_2PO_4 buffer with pH 7.50 was chosen as the running buffer in this work.

Effect of the Separation Voltage and Injection Time. The influence of the separation voltage on the migration time of the analytes was also studied. When the separation voltage was lower than 12 kV, the analysis time was too long and the peak was broadened owing to the diffusion of the sample in the capillary. The increasing separation voltage resulted in a shorter migration time for all analytes. However, it also produced more baseline noise, which resulted in higher detection limits. Hyperoside and quercitrin could not be separated completely when the separation voltage was 18 kV. Therefore, the optimum separation voltage is 15 kV, at which good separation could be obtained for all analytes within 15 min.

The injection time affects both the peak current and the peak shape. The effect of the injection time on the peak current was studied by varying the injection time from 5 to 15 s at 15 kV. Both the peak current and the peak width increased with sampling time. However, when the injection time was longer than 10 s, the peak currents increased slowly and peak broadening became more severe. In this experiment, 10 s (at 15 kV) was chosen as the optimum injection time in considering the resolution and sensitivity.

Under the above optimum conditions, the five flavonoids could be completely separated and detected within 15 min, and the typical electropherogram for a standard mixture solution is shown in **Figure 4**.

Repeatability, Linearity, and Detection Limits of the Five Analytes. The repeatability of the peak current and migration time for all analytes was estimated by making repetitive injections of a standard mixture solution under the optimum conditions. The results showed that the interday relative standard deviations (RSDs, $n = 6$) of the peak current and migration

Table 1. Results of Regression Analysis on Calibration Curves and Detection Limit[a]

compound	regression equation, $Y = a + bX$[b]	correlation coefficient	linear range (μg/mL)	detection limit[c] (μg/mL)
kaempferol	$Y = -0.023 + 0.2559X$	0.9999	0.40–16.0	0.08
rutin	$Y = 0.0552 + 0.1236X$	0.9991	0.20–28.2	0.05
hyperoside	$Y = 0.0287 + 0.3273X$	0.9974	0.20–16.0	0.04
quercitrin	$Y = 0.0326 + 0.4352X$	0.9993	0.20–16.0	0.03
quercetin	$Y = -0.0087 + 0.1662X$	0.9996	0.40–16.0	0.04

[a] Working potential 0.95 V (vs Ag/AgCl); other conditions as in Figure 3. [b] Y and X are the peak current (nA) and concentration of the analytes (μg/mL), respectively. [c] Detection limits corresponding to concentrations giving a signal-to-noise ratio of 3.

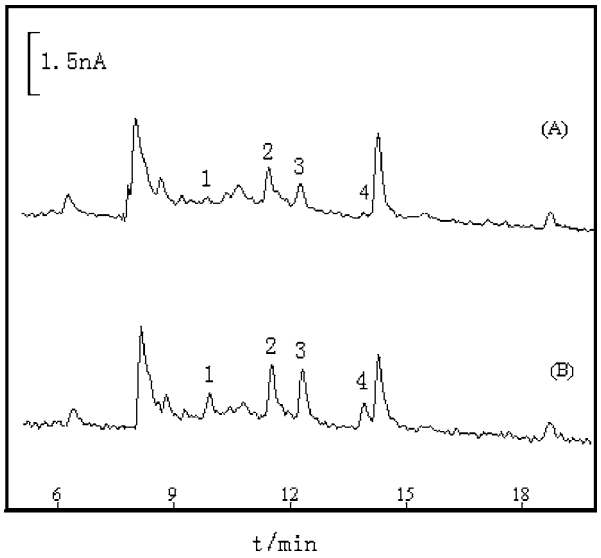

Figure 5. Electropherogram of (**A**) the diluted extract of Herba Leonuri and (**B**) the above diluted extract + the accurate amounts of kaempferol, rutin, hyperoside, and quercetin: working potential, 0.95 V (vs Ag/AgCl); other conditions as in Figure 3; (1) kaempferol; (2) rutin; (3) hyperoside; (4) quercetin.

time were 5.1% and 0.22% for kaempferol, 3.8% and 0.16% for rutin, 3.8% and 0.18% for hyperoside, 4.3% and 0.17% for quercitrin, and 5.7% and 0.17% for quercetin, respectively. A series of standard mixture solutions of the five analytes were tested to determine the linearity and detection limits of this method, and the results are presented in **Table 1**. The calibration curves show excellent linear behavior over the concentration range of about 2 orders of magnitude, with the detection limits ranging from 0.03 to 0.08 μg/mL for all analytes.

Sample Analysis and Recovery. Under the optimum conditions, kaempferol, rutin, hyperoside, quercitrin, and quercetin in Herba Leonuri were determined by CE−AD. The typical electropherograms of the diluted extract are shown in **Figure 5**. The contents of kaempferol, rutin, hyperoside, and quercetin were 100.2, 1105.1, 259.8, and 70.56 μg/g, respectively. The corresponding RSD values were 4.2%, 1.9%, 7.4%, and 4.7%, respectively ($n = 4$). Quercitrin could not be found in the sample.

Accurate amounts of the four analytes were added to the diluted extract of the sample, and the recovery values were obtained using their peak currents from the calibration curves under the same conditions. The average recoveries are listed in **Table 2**. The results demonstrate that the proposed CE-AD method is very suitable for the fast determination of flavonoids in Herba Leonuri. This work also shows that CE−AD is a powerful technique to study flavonoids in the complex extract of medicinal plants.

Table 2. Determination Results of the Recovery for This Method ($n = 4$)[a]

compound	added amt (μg/mL)	determined amt (μg/mL)	recovery (%)	RSD (%)
kaempferol	2.90	2.74	94.5	7.4
rutin	8.57	8.96	105	5.6
hyperoside	2.86	3.27	114	3.9
quercetin	2.90	3.01	104	6.6

[a] Working potential 0.95 V (vs Ag/AgCl); other conditions as in Figure 3.

ABBREVIATIONS USED

HPLC, high-performance liquid chromatography; CE, capillary electrophoresis; UV, ultraviolet; AD, amperometric detection; GCE, glassy carbon electrode; RSD, relative standard deviation.

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Received for review February 23, 2005. Revised manuscript received May 4, 2005. Accepted May 15, 2005. This project was financially supported by the National Nature Sciences Funding of China (Grant 20377007), the International Corporation Program of Science and Technology Department of Fujian Province, China (Grant 2004I015), and Fuzhou University (Grant 2004-XQ-06).

JF0504216