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Journal of Pharmaceutical and Biomedical Analysis 41 (2006) 493–499
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
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Determination of flavonoids in *Portulaca oleracea* L. by capillary electrophoresis with electrochemical detection

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Received 12 October 2005; received in revised form 29 December 2005; accepted 5 January 2006
Available online 3 March 2006

Abstract

A general method based on capillary electrophoresis with electrochemical detection (CE–ED) was developed for identification and determination of five flavonoids (kaempferol, apigenin, myricetin, quercetin and luteolin) in plant species. Running buffer, pH and concentration, separation voltage, injection time and detection potential were investigated to acquire the optimum conditions. The working electrode was a 500 μm diameter carbon disc electrode positioned opposite the outlet of capillary. At room temperature, the five flavonoids could be well separated within 21 min in a 60 cm length capillary at a separation voltage of 19.5 kV with 50 mM $Na_2B_4O_7$–100 mM NaH_2PO_4 (pH 8.50) as the running buffer. The relationship between peak currents and analyte concentrations was linear over about two orders of magnitude, and the detection limits (S/N = 3) were ranging from 0.12 to 0.21 μg/ml for all analytes. The optimized CE–ED method was employed to analyze the above flavonoids in different parts of *Portulaca oleracea* L.

Keywords: *Portulaca oleracea* L.; Flavonoids; Capillary electrophoresis; Electrochemical detection

1. Introduction

Known as "vegetable for long life" in Chinese folklore, *Portulaca oleracea* L. (*P. oleracea* L.) is widely used not only as an edible plant, but also as a traditional Chinese herbal medicine. It has been used for the treating dysentery with bloody stools, and externally for boils and sores, eczema, erysipelas, and snake- and insect-bite [1]. Recent studies show that the consumption of *P. oleracea* L. may help to reduce the occurrence of cancer and heart diseases [2]. The flavonoids in *P. oleracea* L. are the biologically active constituents, which have been reported to have the functions of anti-oxidation, anti-bacteria, anti-virus, anti-ulcerogenic, anti-inflammatory, checking cough and dispelling phlegm [3]. Kaempferol, apigenin, myricetin, quercetin and luteolin (their molecular structures are shown in Fig. 1) are five major flavonoids in *P. oleracea* L., so identification and determination of the above five flavonoids in *P. oleracea* L. will play important role to control its quality and safety for clinical applications.

Methods for the determination of flavonoids in medicinal plants have been reported, including ultraviolet spectrophotometry, thin-layer chromatography [4], high performance liquid chromatography [5–7]. Ultraviolet spectrophotometry method for the determination of the total amount of the flavonoids in *P. oleracea* L. has also been developed [8,9]. However, due to the inherent structural similarity of most flavonoids and the complex characteristics of the sample matrices, none of the above methods has been proved to be adequate for simultaneous determination of the individual flavonoids in medicinal plants.

As a modern separation method, capillary electrophoresis (CE) has won increasing acclaim for its extremely high efficiency, ultra-small sample and reagent volume used, rapid analysis, and ease of cleaning up contaminants. Previously, CE has been reported to be applied for the determination of flavonoids based on ultraviolet (UV) detection [10–13]. However, the UV detection lacks sensitivity due to the small diameter of the separation capillaries. Electrochemical detection (ED), based on the electrochemical reaction of analytes on electrode surface,

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doi:10.1016/j.jpba.2006.01.013

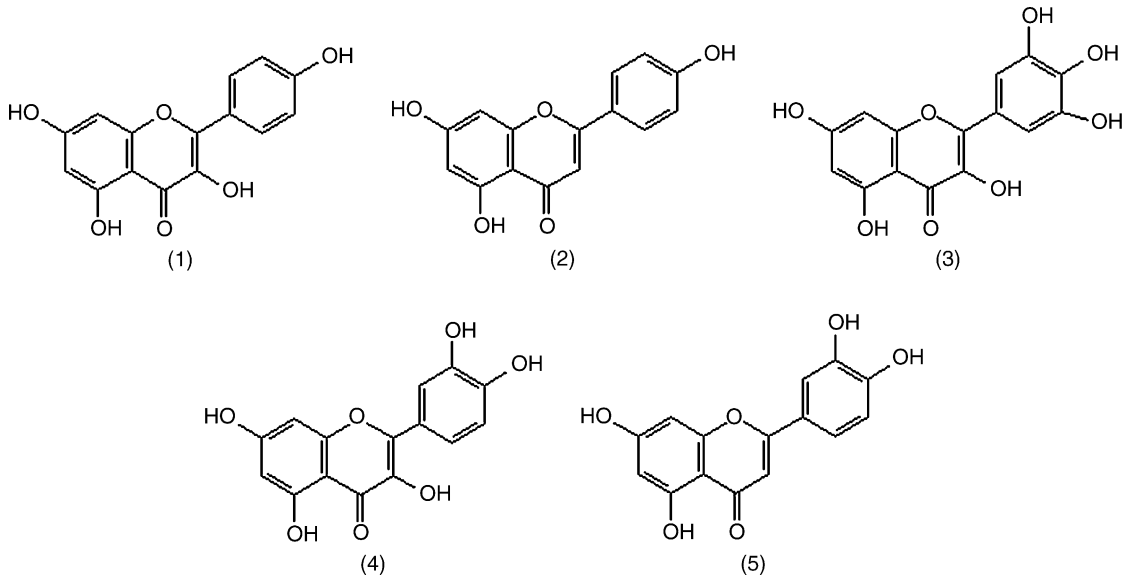

Fig. 1. Molecular structures of the analytes: (1) kaempferol; (2) apigenin; (3) myricetin; (4) quercetin; (5) luteolin.

provides one of the most sensitive and selective detection methods for CE. Most flavonoids are electroactive compounds, so CE–ED has also been employed for the determination of some flavonoids in plants [14–16]. In this paper, a simple CE–ED method is described to simultaneously identify and determine five flavonoids (kaempferol, apigenin, myricetin, quercetin and luteolin), and it has been successfully employed to analyze the above five flavonoids in ethanolic extracts from different parts of *P. oleracea* L.

2. Experimental

2.1. Chemicals

Quercetin, kaempferol and luteolin were obtained from Chinese Chemical and Biological Drugs Institute (Beijing, China), apigenin and myricetin were obtained from Sigma (St. Louis, MO, USA). *P. oleracea* L. was purchased from Laoyaogong Drugstore, Fuzhou, Fujian Province, China. All aqueous solutions were made up in doubly distilled water. Other chemicals were of analytical grade.

Standard stock solutions of five flavonoids at concentration of 1.00×10^3 μg/ml were prepared in ethanol and diluted to the desired concentration with the running buffer just prior to use. The borate-phosphate running buffer was prepared by mixing $Na_2B_4O_7$ solution (concentrations ranging from 30 to 80 mM) with NaH_2PO_4 solution (corresponding concentrations ranging from 60 to 160 mM).

2.2. Apparatus

A laboratory-built capillary electrophoresis system equipped with wall-jet amperometric detector was employed in the experiment. The details of this system have been described in the previous work [17,18]. A pre-aligned electrochemical cell, consisting of three electrodes, a 500 μm diameter carbon disc working electrode (its preparation was reported by Wang et al. [17]), 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, USA). Separation capillary was an untreated fused silica capillary with 60 cm × 25 μm i.d. × 370 μm o.d. (Hebei Yongnian Optic Fiber Factory, China). The inlet end of the capillary was held at a positive potential and outlet end of capillary was kept at ground. 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 electropherograms were monitored using a Chromatographic workstation (Model HW-2000, Qianpu Software Company, Shanghai, China). A pHS-3C meter (Shanghai Dapu Instrument Company, Shanghai, China) was used to measure the pH value of the running buffer. A CHI660 electrochemical system (CH instruments, Austin, TX, USA) was chosen to perform cyclic voltametry.

2.3. Sample solution preparation

After being air-dried and crushed into powder, 0.9465 g leaves of *P. oleracea* L. were extracted with 2× 30 ml ethanol by sonication for 30 min. The extracts were combined and concentrated to 2.0 ml. This extract was 6.7-fold diluted with the running buffer prior to the analysis. 0.4096 g stems of *P. oleracea* L. were also extracted with 2× 12.5 ml ethanol by sonication for 30 min. The extracts were combined and concentrated to 1.0 ml. This extract was 3.7-fold diluted with the running buffer prior to the analysis.

The total of air-dried *P. oleracea* L. powder (10.0 g) was extracted with 130 ml ethanol in Soxhlet extractor for 10 h. A 40 ml ethanol extract was mixed with 0.5 g polyamide and evaporated to nearly dryness in an evaporating dish, then the

residue was transferred into a polyamide column (containing 5 g polyamide). Impurities such as chlorophyll, amino acid and saccharides were eluted with 50 ml $CHCl_3$ and 10 ml H_2O, respectively. Finally, flavonoids were eluted with ethanol. A total of 32 ml ethanol eluted solution was concentrated and made up to a final volume of 3.0 ml with ethanol. This extract was 4.3-fold diluted with the running buffer prior to the analysis. Peak identification was performed by standard addition methods.

2.4. Procedures

The carbon disc electrode was successively polished with sand emery paper and sonicated in doubly distilled water. CE was performed at the separation voltage of 19.5 kV with a 50 mM $Na_2B_4O_7$–100 mM NaH_2PO_4 (pH 8.50) solution as running buffer at room temperature. The potential applied to the working electrode was +0.95 V (versus Ag/AgCl). Sample was injected electrokinetically at 19.5 kV for 10 s. Sample solution, standard

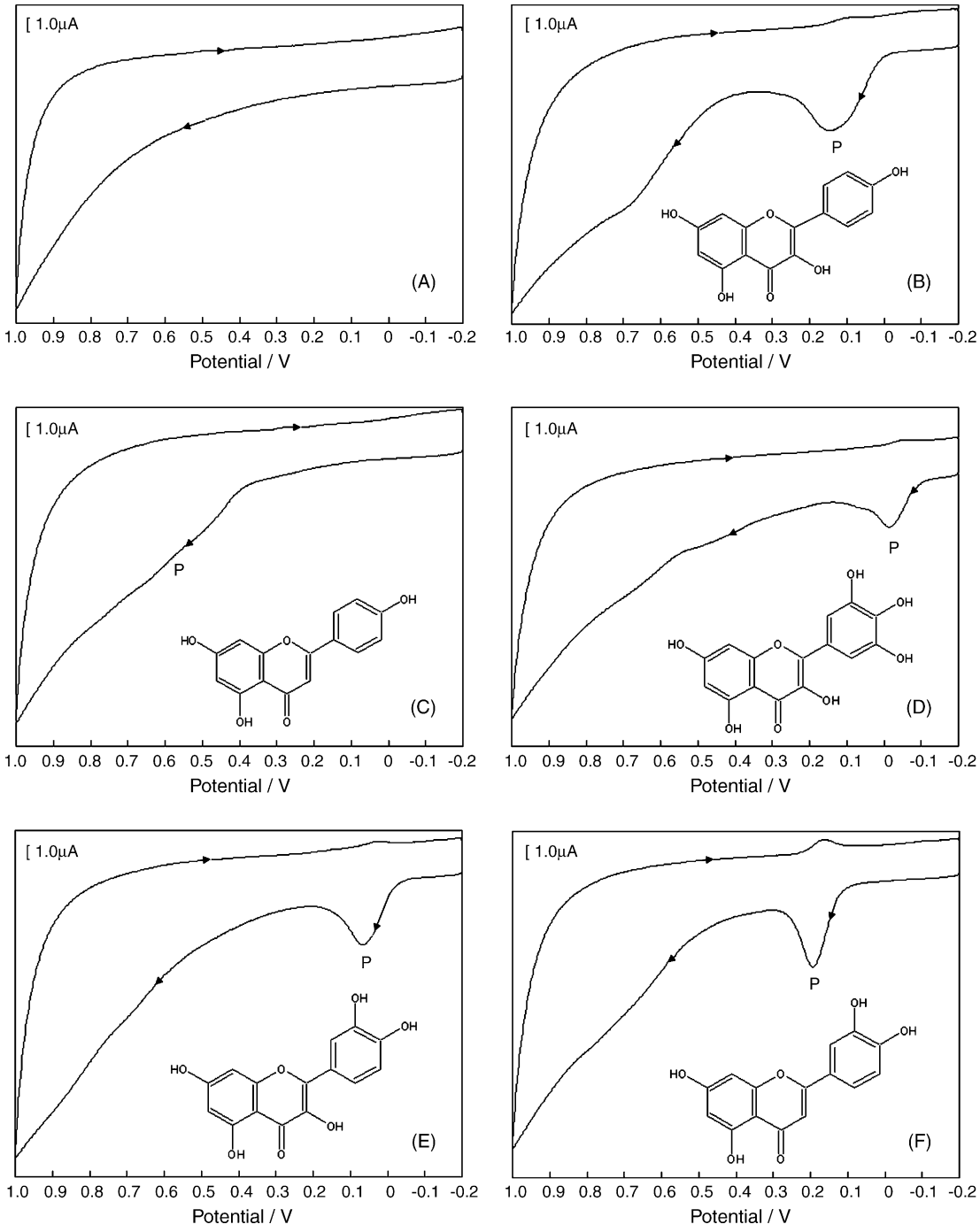

Fig. 2. Cyclic voltammograms of the five analytes in 50 mM $Na_2B_4O_7$–100 mM NaH_2PO_4–0.5% ethanol solution (pH 8.50). Working electrode: glassy carbon electrode; auxiliary electrode: platinum wire; reference electrode: SCE; scan rate: 100 mV/s; (A) blank solution, (B) 1.7×10^{-2} mM kaempferol, (C) 1.7×10^{-2} mM apigenin, (D) 1.7×10^{-2} mM myricetin, (E) 1.7×10^{-2} mM quercetin, (F) 1.7×10^{-2} mM luteolin.

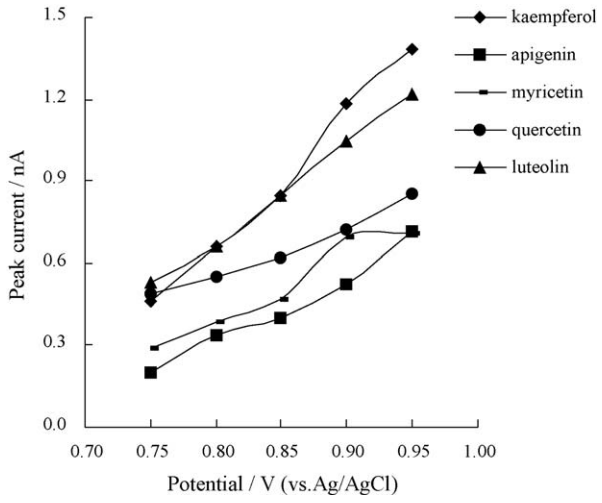

Fig. 3. HDVs of 8.14 μg/ml kaempferol, 5.82 μg/ml apigenin, 6.95 μg/ml myricetin, 13.8 μg/ml quercetin and 8.82 μg/ml luteolin. Conditions: fused-silica capillary: 60 cm × 25 μm i.d. ×370 μm o.d.; working electrode: 500 μm diameter carbon disc electrode; running buffer: 50 mM $Na_2B_4O_7$–100 mM NaH_2PO_4 (pH 8.50); separation voltage: 19.5 kV; electrokinetic injection: 10 s (at 19.5 kV); room temperature.

solution and running buffer were all filtered through a syringe cellulose acetate filter (0.22 μm) prior to use.

3. Results and discussion

3.1. Electrochemistry

Since the standard stock solutions of flavonoids were prepared in ethanol, 50 mM $Na_2B_4O_7$–100 mM NaH_2PO_4 (pH 8.50) in 0.5% 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 Fig. 2. Fig. 2 shows that in blank solution no peak was observed in the potential range of 0–1000 mV, whereas all analytes exhibited an obvious anodic peak, respectively, and their redox processes were irreversible, which may be due to the oxidation of phenolic hydroxyl groups in the analytes and the strong adsorption of their oxidation products at GCE. It should be noted that the anodic potential of apigenin was higher than that of kaempferol, myricetin, quercetin and luteolin. This phenomenon may be attributed to that the oxidation of *m*-hydroxyl group is harder than that of *o*-hydroxyl group and apigenin has no hydroxyl group in position 3. To sum up, the five analytes are electroactive and can be determined by amperometric detection.

3.2. Effect of the potential applied to the working electrode

Hydrodynamic voltammograms (HDVs) of 8.14 μg/ml kaempferol, 5.82 μg/ml apigenin, 6.95 μg/ml myricetin, 13.8 μg/ml quercetin and 8.82 μg/ml luteolin are illustrated in Fig. 3. The peak currents of all analytes increased with the applied potential. However, the experimental results showed that an applied potential higher than +0.95 V (versus Ag/AgCl) would produce larger oxidation currents for all analytes, both the baseline noise and the background current would also increase greatly, which was a disadvantage for sensitive and stable detection. For a suitable compromise of high sensitivity and low background current, a value of +0.95 V (versus Ag/AgCl) was selected for the subsequent experiments.

3.3. Effect of the running buffer

In order to improve the resolution, three running buffer at pH 8.50 (50 mM $Na_2B_4O_7$–100 mM NaH_2PO_4, 100 mM NaH_2PO_4–100 mM NaOH and 50 mM $Na_2B_4O_7$–100 mM H_3BO_3) have been selected as the running buffer for testing their effects on the separation of the analytes at the separation voltage of 19.5 kV. The experimental results showed that $Na_2B_4O_7$–NaH_2PO_4 buffer gave not only the best resolution but also the highest sensitivity under the same conditions. So $Na_2B_4O_7$–NaH_2PO_4 buffer was chosen as the running buffer and the optimum pH and concentration of the running buffer were selected as below.

3.4. Effects of the pH and concentration of the running buffer

To verify the effect of running buffer pH on migration behavior, experiments were performed using 50 mM $Na_2B_4O_7$–100 mM NaH_2PO_4 buffer with different pH. The results are shown in Fig. 4. The five analytes could be baseline separated from pH 8.00 to 8.50, but apigenin was unstable when pH was lower than 8.25. At pH 8.75 quercetin and luteolin could not be separated. Meanwhile, the peak currents were low and the peak shapes became poor at pH value above 8.75. At pH 8.50, the five analytes could be well separated within a relatively short time. In this paper, 50 mM $Na_2B_4O_7$–100 mM NaH_2PO_4 buffer with pH 8.50 was chosen as the running buffer considering the sensitivity, resolution and analysis time.

Besides the pH value, the running buffer concentration is also an important parameter. The experiments indicated that the migration time and the resolution increased due to the increasing degree of chelation between flavonoids and borate with the concentration of running buffer. However, high running buffer concentration had a negative effect on the detection limits because the peak currents of all analytes decreased and the effect of Joule heat became more obvious. So 50 mM $Na_2B_4O_7$–100 mM NaH_2PO_4 buffer with pH 8.50 was chosen as the running buffer.

3.5. Effects of separation voltage and injection time

The influence of separation voltage on the migration time of the analyte was also studied. The results indicated that increasing the separation voltage gave shorter migration time for all analytes, but also increased the baseline noise and decreased the resolution of the analytes. However, too low separation voltages would increase the analysis time considerably and cause peak broadening. Based on the experiments, 19.5 kV was selected as the optimum separation voltage to accomplish a good compromise.

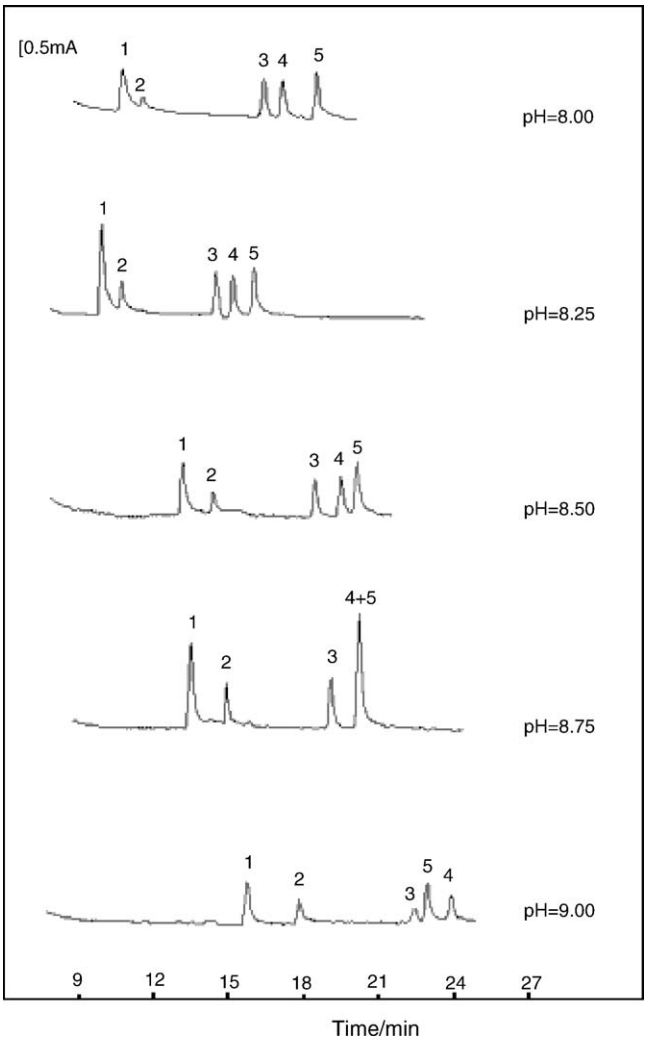

Fig. 4. Recordings at different pH values. Working potential: +0.95 V (vs. Ag/AgCl); other conditions as in Fig. 3: (1) kaempferol (5.82 μg/ml); (2) apigenin (2.86 μg/ml); (3) myricetin (6.73 μg/ml); (4) quercetin (10.3 μg/ml); (5) luteolin (6.75 μg/ml).

The effect of injection time on peak current was investigated by varying injection time from 2 to 25 s at 19.5 kV. Both the peak current and peak width increased with sampling time. However, when the injection time was longer than 10 s, the peak current increased slowly and peak broadening became more severe. In this experiment, 10 s at 19.5 kV was chosen as the optimum injection time.

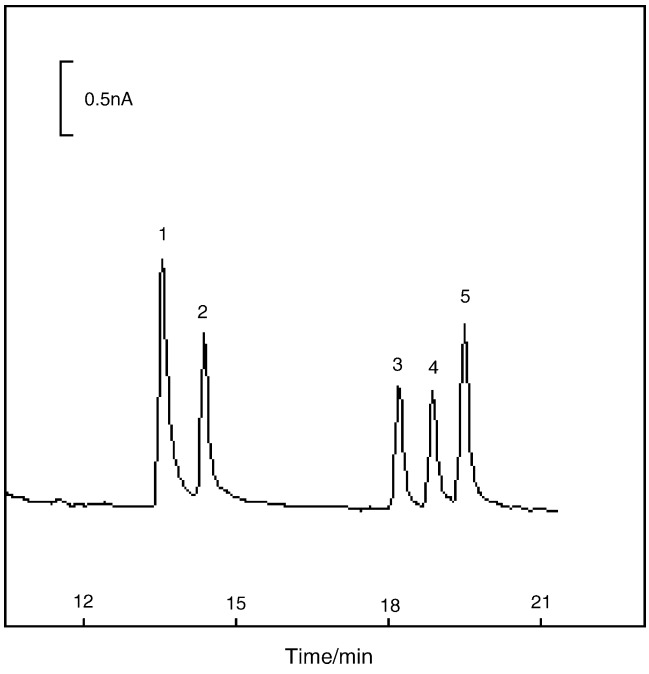

Fig. 5. Electropherogram of the standard mixture solution of flavonoids. Working potential: +0.95 V (vs. Ag/AgCl); other conditions as in Fig. 3: (1) kaempferol (10.7 μg/ml); (2) apigenin (9.86 μg/ml); (3) myricetin (8.65 μg/ml); (4) quercetin (13.4 μg/ml); (5) luteolin (9.36 μg/ml).

Under the optimum conditions, 10.7 μg/ml kaempferol, 9.86 μg/ml apigenin, 8.65 μg/ml myricetin, 13.4 μg/ml quercetin and 9.36 μg/ml luteolin could be completely separated and detected within 21 min. A typical electropherogram for a standard mixture solution is shown in Fig. 5.

3.6. Repeatability, linearity and detection limits

The intra-day relative standard deviations (R.S.D.s) of peak current for kaempferol, apigenin, myricetin, quercetin and luteolin were 1.1%, 2.4%, 4.1%, 3.6% and 1.2%, respectively, and those of migration times were 0.52%, 0.45%, 0.40%, 0.42% and 0.34%, respectively ($n = 5$).

A series of the standard mixture solutions of the five analytes were tested to determine the linearity and detection limits. The results are presented in Table 1. The calibration curves exhibit excellent linear behavior over the concentration range of about two orders of magnitude and the detection limits (on the basis of

Table 1
The results of regression analysis on calibration curves and detection limit[a]

Analytes	Regression equation, $Y = a + bX$[b]	Correlation coefficient	Linear range (μg/ml)	Detection limit[c] (μg/ml)
Kaempferol	$Y = -0.058 + 0.162X$	0.9904	0.37–21.4	0.13
Apigenin	$Y = -0.0052 + 0.1165X$	0.9930	0.53–20.3	0.12
Myricetin	$Y = -0.0028 + 0.0968X$	0.9998	0.62–18.2	0.17
Quercetin	$Y = 0.088 + 0.0524X$	0.9962	0.47–29.1	0.18
Luteolin	$Y = 0.1321 + 0.1131X$	0.9950	0.26–16.3	0.21

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

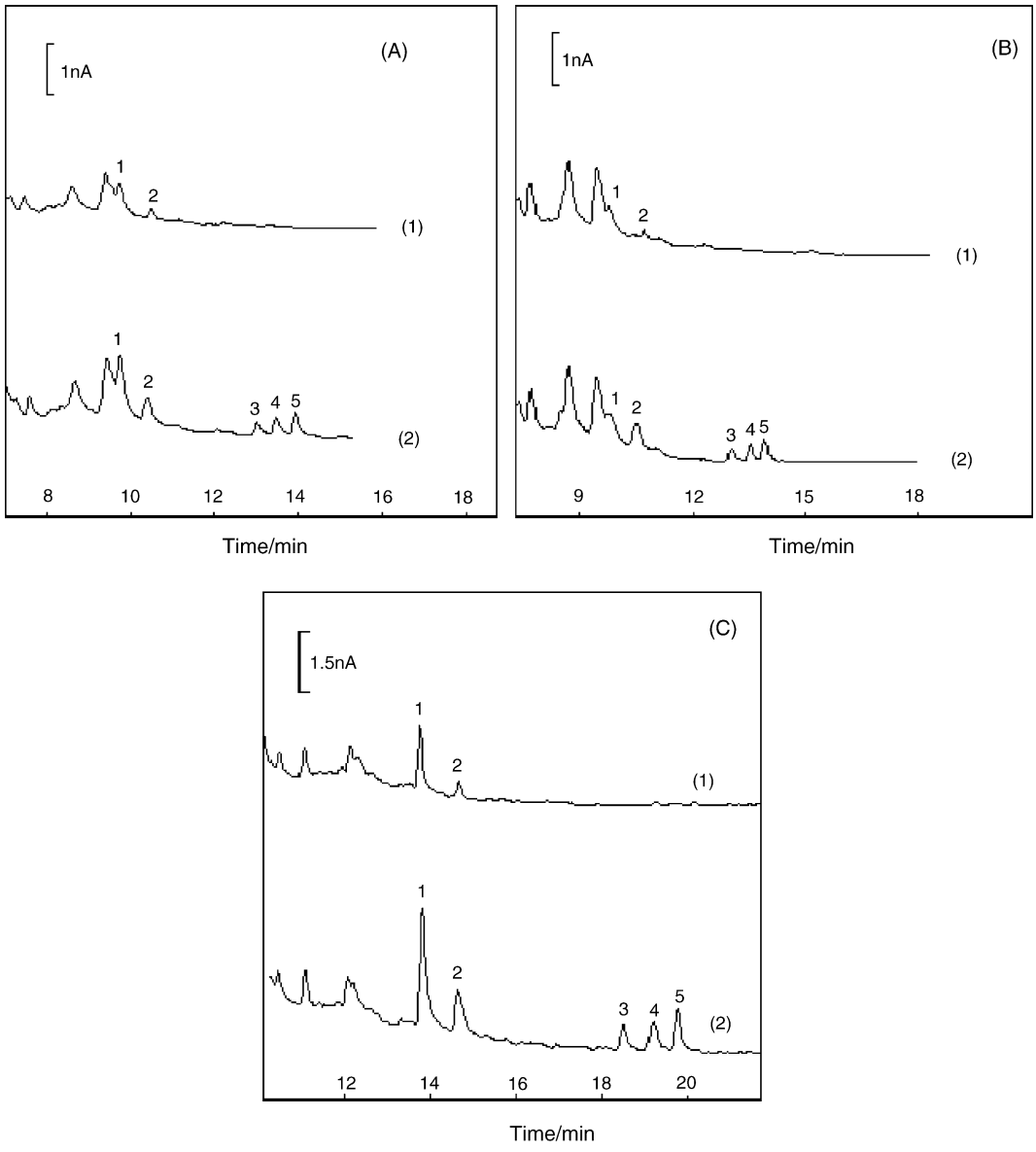

Fig. 6. Electropherograms of (A) (1) the diluted extract of *P. oleracea* L. leaves and (2) the above diluted extract + the accurate amounts of analytes. (B) (1) the diluted extract of *P. oleracea* L. stems and (2) the above diluted extract + the accurate amounts of analytes. (C) (1) the diluted extract of the total *P. oleracea* L. and (2) the above diluted extract + the accurate amounts of analytes. Working potential: +0.95 V (vs. Ag/AgCl); the room temperatures were quite different between (A)–(B) and (C); for peak identification, see Fig. 4; other conditions as in Fig. 3.

signal-to-noise ratio of 3) for kaempferol, apigenin, myricetin, quercetin and luteolin are 0.13, 0.12, 0.17, 0.18 and 0.21 μg/ml, respectively.

3.7. Sample analysis and recovery

Kaempferol, apigenin, myricetin, quercetin and luteolin in the different parts of *P. oleracea* L. were determined by CE–ED under the optimum conditions. Typical electropherograms are shown in Fig. 6(A)–(C). It should be noted that kaempferol and the other coexisting components could not be baseline separated without sample pre-treatment (namely solid-phase extraction) (see Fig. 6(A)–(B)). The contents of analytes in samples were calculated using their peak currents from the calibration curve under the same conditions. The comparisons of the above analytes in the different parts of the herbal plant, respectively, leaves, stems and the total plant are shown in Table 2. As shown in there, only kaempferol and apigenin could be found and the contents of these two analytes in these three different parts were quite dif-

Table 2
Assay results of the analytes in the different parts of *P. oleracea* L. ($n = 4$, μg/g)[a]

Parts	Kaempferol	Apigenin	Myricetin	Quercetin	Luteolin
Leaves	NS[b]	50.1 (2.1)[c]	NF[d]	NF	NF
Stems	NS	16.6 (5.1)	NF	NF	NF
The total plant	29.2 (2.2)	17.7 (4.7)	NF	NF	NF

[a] Working potential: +0.95 V (vs. Ag/AgCl); other conditions as in Fig. 3.
[b] NS refers to not separated.
[c] The data in the parentheses refer to the intra-day relative standard deviation.
[d] NF refers to not found.

Table 3
Determination results of the recovery for this method ($n = 3$)

Samples	Analytes	Added amount (μg/ml)	Determined amount (μg/ml)	Recovery (%)
P. oleracea L. leaves	Kaempferol	–	–	–
	Apigenin	4.09	3.65	89.2
	Myricetin	5.51	4.87	88.4
	Quercetin	4.15	3.86	93.0
	Luteolin	5.40	5.38	99.6
P. oleracea L. stems	Kaempferol	–	–	–
	Apigenin	4.57	4.75	104
	Myricetin	4.82	4.48	92.9
	Quercetin	3.72	3.56	95.7
	Luteolin	4.33	4.89	113
The total of *P. oleracea* L.	Kaempferol	4.08	3.81	93.4
	Apigenin	3.97	3.48	87.7
	Myricetin	4.08	3.63	89.0
	Quercetin	6.68	6.05	90.6
	Luteolin	3.67	2.97	80.9

Working potential: +0.95 V (vs. Ag/AgCl); other conditions as in Fig. 3.

ferent. The contents of kaempferol and apigenin in leaves were higher than those in stems.

Accurate amounts of the five analytes were added to the diluted extracts of samples, and the recovery values were obtained using their peak currents from the calibration curve under the same conditions. The average recoveries are listed in Table 3. The results indicated that this method was suitable for the real sample analysis.

4. Conclusion

Although ultraviolet spectrophotometry method has been reported for determination of the total amount flavonoids in *P. oleracea* L., to the best of our knowledge, there are no methods yet for simultaneous determination of the five above-mentioned flavonoids from the extracts of the herbal plant. In this paper CE–ED method was successfully applied to simultaneously determine flavonoids in different parts of *P. oleracea* L. The electrochemical detection can provide a high selectivity since only electroactive substances can be detected. The proposed method promises to be applicable to the identification and determination of the five flavonoids (kaempferol, apigenin, myricetin, quercetin and luteolin) in other plant species as well.

Acknowledgements

This project was financially supported by the National Nature Sciences Funding of China (20377007), the International Corporation Program of Science and Technology Department of Fujian Province, China (2004I015) and the Department of Education of Fujian Province (JB02063).

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