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Determination of active components in rosemary by capillary electrophoresis with electrochemical detection

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Abstract

Rosemary (*Rosmarinus officinalis* L.) is a spice and medicinal herb widely used around the world. Among natural antioxidants, rosemary has been widely accepted as one of the spices with the highest antioxidant activity. A capillary electrophoresis method for the determination of its active components using electrochemical detection was developed. Effects of several important factors were investigated to acquire the optimum conditions. The detection electrode was a 300 μm carbon disc electrode at a working potential of +0.90 V (versus SCE). The analytes can be well separated with 25 min in a 75 cm length fused-silica capillary at a separation voltage of 16 kV in an 80 mmol/l borate buffer (pH 9.0). The current response was linear over about three orders of magnitude with detection limits (S/N = 3) ranging from 2×10^{-7} to 1×10^{-6} g/ml for all the analytes. The method was successfully used in the analysis of rosemary with relatively simple extraction procedures, and the assay results were satisfactory.

Keywords: Capillary electrophoresis; Electrochemical detection; Active components; Rosemary

1. Introduction

Lipid oxidation may reduce the flavor and nutritive value of fats, oils and lipid-containing products. Unsaturated fatty acids are sensitive to oxidation because of their chemical structure [1]. Protein cross-linking, denaturation, polypeptide chain scission, enzyme inactivation and amino acid destruction in the presence of oxidizing lipids have been reported [2]. To overcome this problem, synthetic antioxidants such as butylated hydroxy toluene (BHT) and butylated hydroxy anisole (BHA) are incorporated into fats and oils; The use of synthetic antioxidants in the food industry is severely restricted as to both application and level.

It has been known for some time that addition of certain aromatic herbs or spices to lipid-containing materials will delay the oxidation process [3,4]. Therefore, in recent years a lot of interest has been devoted to preparing antioxidants from natural sources [5,6]. Rosemary (*Rosmarinus officinalis* L.) is a popular Labiatae herb used as spice and folk medicine around the world, and the extract of rosemary is now widely used commercially to increase the shelf life of foods [7]. Among natural antioxidants, rosemary has been widely accepted as one of the spices with the highest antioxidant activity [4]. The compounds mainly responsible for the antioxidant properties of rosemary and the major phenolic diterpenes present in fresh rosemary leaves have been found to be carnosic acid [8,9] and rosmarinic acid [10]. There are also flavoids such as chlorogenic acid and caffeic acid in rosemary [11].

Several analytical methods including high-performance liquid chromatography (HPLC) [11–13], liquid chromatographic–mass spectrometry (LC–MS) [14] and GC–MS [15] have been employed for the determination of phenolic deterpenes in rosemary. Capillary zone electrophoresis (CZE) with UV detection has also been employed [16],

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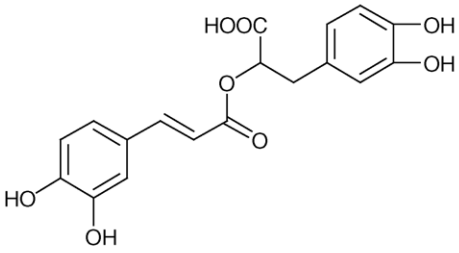
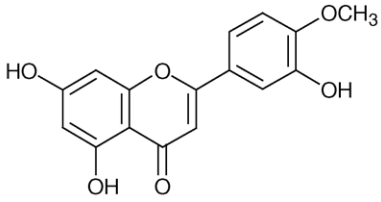
the drawback of this approach being its low sensitivity. Capillary electrophoresis (CE) coupled with UV-diode array detector are also introduced for the determination of the phenolic acids from rosemary [17–19]. HPLC, regarded as a prime separation method, has good reproducibility and can provide the structural information of the analytes if combined with MS, has some shortcoming in analysis of plants, including time-consuming sample pre-treatment [20], short column lifetime owing to numerous co-existent interferences, and the apparatus is expensive, which is not accessible in common laboratories.

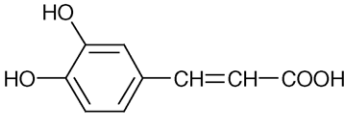
Capillary electrophoresis has emerged as a powerful analytical technique complementary in many features to HPLC [21,22], such as simple extraction procedure and ease of



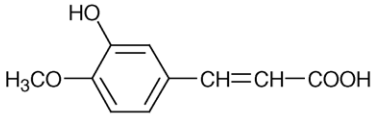
Rosmarinic acid



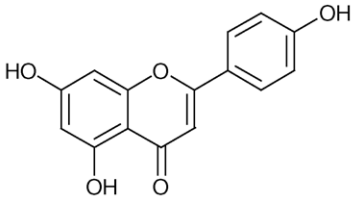
Diosmetin



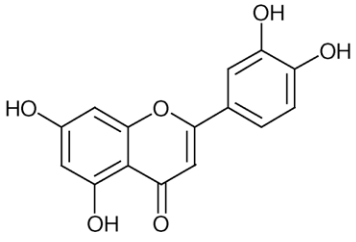
Caffeic acid



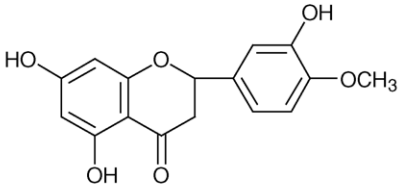
Ferulic acid



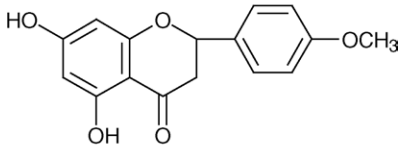
Apigenin



Luteolin



Hesperitin



Acacetin

Fig. 1. The molecular structures of the analytes.

clearing up the contaminants. In addition, with electrochemical detection (ED), CE–ED offers high sensitivity and good selectivity for electroactive analytes. Therefore, it has been proposed as a complementary technique to HPLC for the separation of phenolic compounds present in plants such as phenolic components and flavonoids [23,24]. To our knowledge, there is no method developed for the simultaneous determination of the active components such as hesperetin, acacetin, diosmetin, apigenin, luteolin, ferulic acid, rosmarinic acid and caffeic acid in rosemary till now. In this work, the potential of CE–ED as an alternative to conventional HPLC methods for the determination of active components of rosemary is exemplarily shown, which has been proven to be simple and convenient, as well as sensitive and selective Fig. 1.

2. Experimental

2.1. Apparatus

In this work, a CE–ED system has been constructed and is similar to that described [25] previously. A ±30 kV high-voltage dc power supply (Shanghai Institute of Nuclear Research, Shanghai, China) provided a voltage between the ends of the capillary. The inlet of the capillary was held at a positive potential and the outlet end of the capillary was maintained at ground. The separations were undertaken in a 75 cm length, 25 μm i.d. and 360 μm o.d. fused silica capillary (Polymicro Technologies, Phoenix, AZ, USA).

A three-electrode electrochemical cell consisting of a 300 μm diameter carbon disc working electrode, a platinum auxiliary electrode, and a saturated calomel electrode (SCE) as the reference electrode was used in combination with a BAS LC-3D amperometric detector (Biochemical System, West Lafayette, IN, USA). Before use, the carbon disc electrode was polished with emery paper and sonicated in doubly distilled water, and finally carefully positioned opposite the outlet of the capillary with the aid of a micro-manipulator (Correct, Tokyo, Japan) and arranged in a wall-jet configuration [26]. The distance between the tip of the working electrode and the capillary outlet was as close as possible so that the CE effluent directly impinged upon the electrode surface. The electropherograms were recorded using a chart recorder (Shanghai Dahua Instrument Factory, China). CE was performed in a 80 mmol/l borate buffer (pH 9.0) used as the running buffer at a separation voltage of 16 kV. The potential applied to the working electrode was +0.90 V (versus SCE). Samples were injected electrokinetically at 16 kV for 8 s.

2.2. Reagents and solutions

Hesperetin, acatetin, apigenin, luteolin, diosmetin, and caffeic acid were purchased from Sigma (St. Louis, USA), rosmarinic acid was purchased from Aldrich (Milwaukee, WI, USA) and ferulic acid was obtained from Shanghai Reagent Factory (Shanghai, China). Rosemary was purchased from a drug store in Shanghai. Stock solutions of the analytes (1.00×10^{-3} g/ml each) were prepared in anhydrous ethanol (A.R. grade), stored in the dark at 4 °C, and diluted to the desired concentrations with the running buffer (80 mmol/l borate buffer, pH 9.0), in which carbon working electrode shows excellent response to all the compounds. Before use, all solutions were filtered through 0.22 μm nylon filters.

2.3. Sample preparation

Rosemary was ground into powder and accurately weighed. The weighed sample (about 2 g) was extracted with 10 ml 70% ethanol for 30 min in an ultrasonic bath. Next the sample solution was filtered through filter paper first, then through a 0.22 μm syringer filter. Sample solution was stored at 4 °C in the dark and could be injected electrokinetically without pre-concentration.

3. Results and discussion

3.1. Hydrodynamic voltammograms (HDVs)

Since the phenolic hydroxy group of the analytes can be readily oxidized electrochemically, electrochemical detection was based on this feature. In amperometric detection the potential applied to the working electrode directly affects the sensitivity, detection limit and stability of this method. Therefore, the effect of working electrode potential on the peak current (calculated by measuring the peak height) of the analytes was investigated to obtain optimum detection. Fig. 2 illustrates the hydrodynamic voltammograms of the analytes. When the applied potential reaches +0.60 V (versus SCE), the peak currents increase rapidly. However, when the potential exceeds +0.90 V (versus SCE), the current levels off. Although applied potential greater than +0.90 V (versus SCE) results in larger peak current, the background current of the working electrode increases sharply. Hence, the applied potential of the working electrode was maintained at +0.90 V (versus SCE), where the background current is not too high and the S/N ratio is the highest.

3.2. Effects of pH and concentration of the buffer

The acidity and concentration of the running buffer play an important role in CE for their effect on zeta potential (ζ) and the overall charge of all the analytes, which affect the migration time and the separation of the analytes. Therefore, it is important to study their influences on CE in order to obtain optimum separations. The effect of the running buffer pH on the migration time of the investigated analytes is shown in Fig. 3. The running buffer was 80 mmol/l borate buffer at five different pH values (8.4, 8.7, 9.0, 9.2 and 9.5). As shown in Fig. 3A, the resolution of the analytes is poor at pH 8.4.

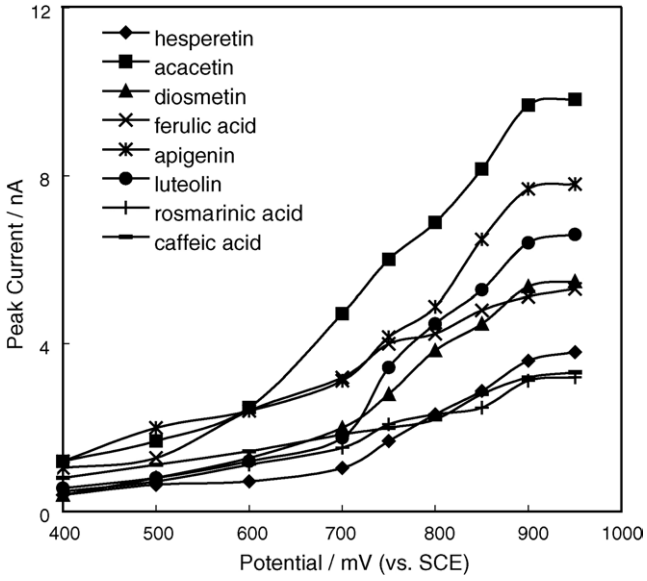


Fig. 2. Hydrodynamic voltammograms (HDVs) for the analytes in CE (for hesperetin, acacetin, diosmetin, apigenin, luteolin, ferulic acid, the concentration is 2.0×10^{-5} g/ml; and for rosmarinic acid and caffeic acid, the concentration is 4.0×10^{-5} g/ml). Working conditions: fused-silica capillary, 25 μm i.d. × 75 cm; working electrode, 300 μm diameter carbon disk electrode; running buffer, 80 mmol/l borate buffer (pH 9.0); separation voltage, 16 kV; electrokinetic injection, 8 s (16 kV).

When the running buffer pH increases, the resolution of all compounds is improved with increase in migration time. It is also found that the peak current is low and the peak shape became poor when the pH value is larger than 9.0. The analytes were migrated in the dissociated forms, the electrostatic force was unchanged with pH but electro-osmotic flow was decreased as pH was increased, which results in the gradual increase of retention time with increasing pH values for all the analytes. When pH is greater than 9.0, both the carboxyl and phenolic hydroxyl groups of the analytes are dissociated to form the carboxylate–phenolate divalent anions, which were more strongly pulled back by electrostatic force. And pH is not an isolated parameter; it also influences the ionic strength of the solution and the velocity of EOF was decreased with increasing concentration of electrolyte. So, it is observed that the peak shape became poor and the current was decreased when pH is large than 9.0. Therefore, 80 mmol/l borate buffer with pH 9.0 was chosen as the running buffer in considering the peak current, resolution and the analytical time.

As the buffer concentration influences the viscosity coefficient of the solution, the diffusion coefficient of analytes and the ζ-potential of the inner surface of capillary tube as well, it affects not only the resolution and migration time of the analytes but also the peak current. The migration time and the resolution increase with increasing buffer concentration as shown in Fig. 3B. However, higher buffer concentrations (>80 mmol/l) also have a negative effect on the detection limits because the peak currents of all analytes decrease and the effect of Joule heat becomes more pronounced, so 80 mmol/l borate buffer (pH 9.0) was chosen as the running buffer in considering the peak current, resolution and the analytical time and buffer capacity.

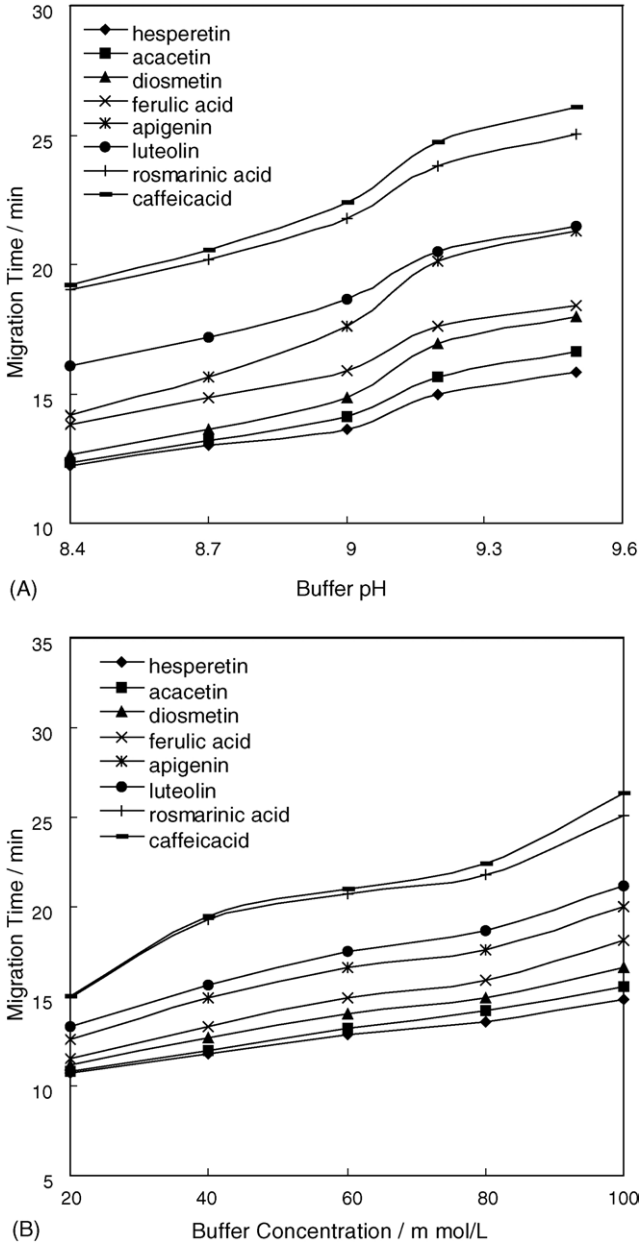


Fig. 3. Effect of buffer pH (A) and concentration (B) on the migration time of the analytes. Working potential: +0.90 V (vs. SCE); other conditions as in Fig. 2.

3.3. Effect of separation voltage and injection time

The separation voltage affects the electric field strength, which in turn affects the electro-osmotic velocity and the electrophoretic velocity of charged particles, which determine the migration time of analytes. Moreover, higher separation voltage may result in higher Joule heating. The effect of separation voltage on the migration time of the analytes is shown in Fig. 4A: increasing the voltage not

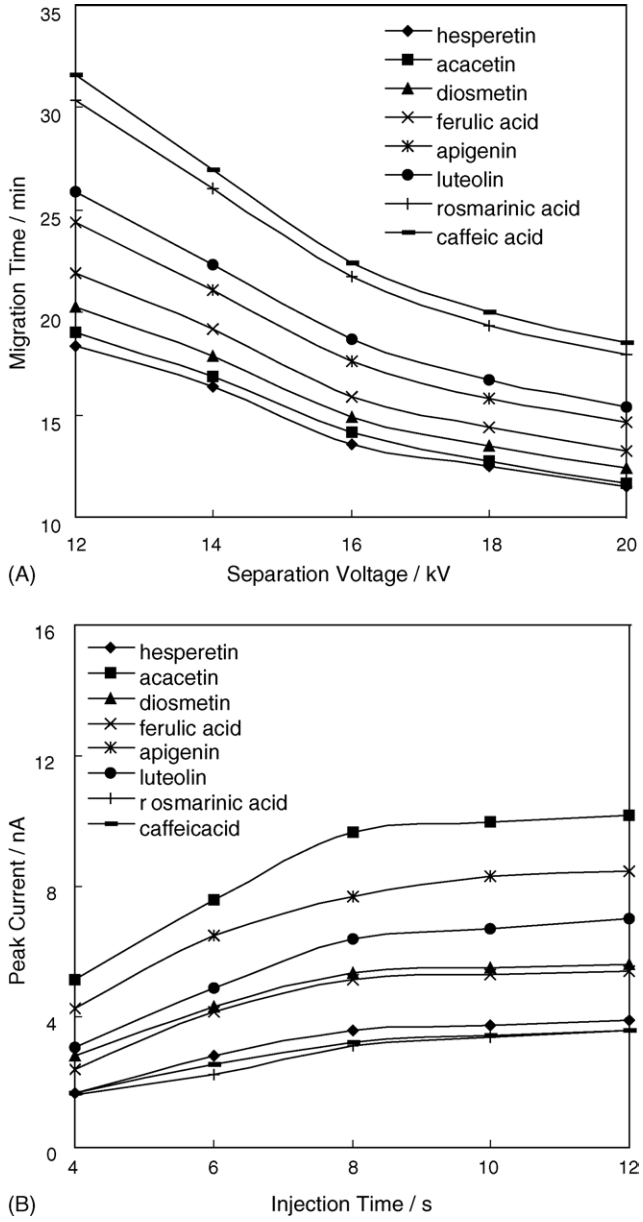


Fig. 4. Effect of separation voltage on the migration time of the analytes (A) and effect of injection time on the analytes peak current (B). Working potential: +0.90 V (vs. SCE); other conditions as in Fig. 2.

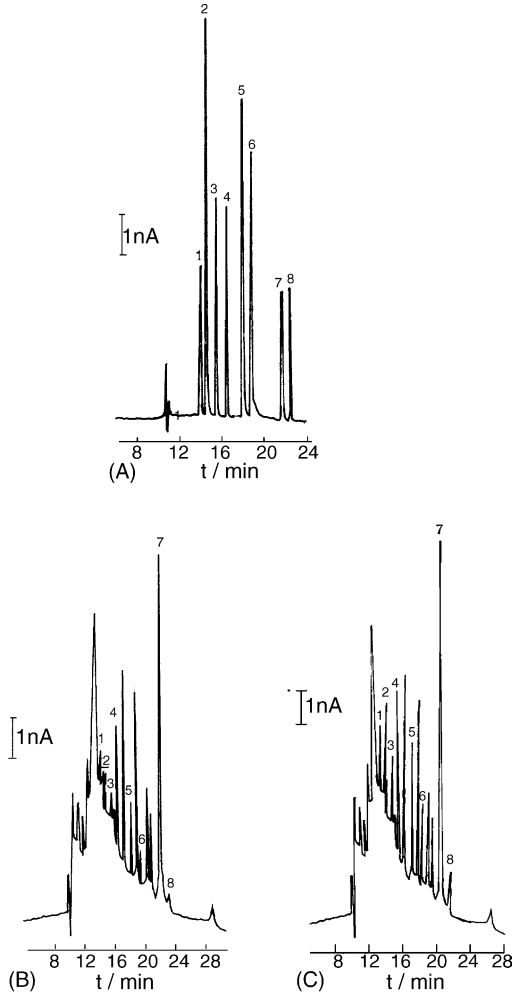


Fig. 5. The electropherogram of a standard mixture solution (for hesperetin, acacetin, diosmetin, apigenin, luteolin, ferulic acid, the concentration is 2.0×10^{-5} g/ml; and for rosmarinic acid and caffeic acid, the concentration is 4.0×10^{-5} g/ml) (A), and the typical electropherogram of the rosemary (B) (dilution: 1:100). (7) Rosmarinic acid; (8) caffeic acid. (C) The electropherogram of the real sample after adding the standards (for hesperetin, acacetin, diosmetin, apigenin, luteolin, ferulic acid, the concentration added is 5.0×10^{-6} g/ml; for rosmarinic acid and caffeic acid, the concentration added is 4.0×10^{-5} g/ml; and for caffeic acid, the concentration added is 1.0×10^{-5} g/ml). Working potential: +0.90 V (vs. SCE); other conditions as in Fig. 2. Peak identification: (1) hesperetin; (2) acacetin; (3) diosmetin; (4) ferulic acid; (5) apigenin; (6) luteolin; (7) rosmarinic acid; (8) caffeic acid.

only gives shorter migration time but also increases the background noise, resulting in a higher detection limit. Although the resolution of analytes can be improved to some extent, too low a separation voltage will increase the analytical time considerably, which in turn causes severe peak broadening. Based on experiments, 16 kV was chosen as the optimum voltage to accomplish a good compromise.

Injection time determines the amount of sample and affects both the peak current and peak shape. The effect of injection time on separation was investigated by changing the sampling time (4, 6, 8, 10 and 12 s at a voltage of 16 kV, as shown in Fig. 4B). The peak current increases with the increase in sampling time, and it is found the peak width increases simultaneously. When the injection time is more than 8 s, the peak current levels off and peak broadening becomes more severe. Eight second (16 kV) was therefore selected as the optimum injection time.

A typical electropherogram for the standard mixture solution under the optimum conditions is shown in Fig. 5A. Baseline separation for all analytes can be achieved within 25 min.

3.4. Reproducibility, linearity,and detection limit of the analytes

A standard mixture solution of 2.0×10^{-5} g/ml hesperetin, acacetin, diosmetin, apigenin, luteolin, ferulic acid

Table 1
Results of regression analysis on calibration and the detection limits[a]

Compound	Regression equation $y = a + bx$[b]	Correlation coefficient	Linear range (μg/ml)	Detection limit[c] (g/ml)
Hesperetin	$y = 186714x - 0.155$	$R = 0.9993$	5–1000	1×10^{-6}
Acacetin	$y = 502857x - 0.34$	$R = 0.9994$	1–1000	2×10^{-7}
Diosmetin	$y = 266286x + 0.06$	$R = 0.9994$	2–1000	5×10^{-7}
Ferulic acid	$y = 262857x - 0.16$	$R = 0.9996$	2–1000	5×10^{-7}
Apigenin	$y = 377714x + 0.1$	$R = 0.9997$	1–1000	2×10^{-7}
Luteolin	$y = 316857x + 0.03$	$R = 0.9993$	2–1000	5×10^{-7}
Roamarinic acid	$y = 77571x + 0.03$	$R = 0.9996$	5–500	1×10^{-6}
Caffeic acid	$y = 78571x + 0.04$	$R = 0.9993$	5–500	1×10^{-6}

[a] Working potential is +0.90 V (vs. SCE). Other conditions as in Fig. 2.
[b] Where the y and x are the peak current (nA) and concentration of the analytes (g/ml), respectively.
[c] The detection limits corresponding to concentrations giving signal to noise ratio of 3.

and 4.0×10^{-5} g/ml rosmarinic acid and caffeic acid was analyzed seven times to determine the reproducibility of the peak current and migration time for all analytes under the optimum conditions in this experiment. The relative standard deviations (R.S.D.) of peak current and migration time are 3.8% and 1.2% for hesperetin, 3.0% and 0.9% for acacetin, 3.5% and 1.3% for diosmetin, 3.2% and 0.8% for apigenin, 3.5% and 1.2% for luteolin, 2.5% and 0.8% for ferulic acid, 2.8% and 1.0% for rosmarinic acid, 3.0% and 1.3% for caffeic acid, respectively.

A series of the standard mixture solutions of hesperetin, acacetin, diosmetin, apigenin, luteolin, ferulic acid, rosmarinic acid and caffeic acid with a concentration range of 5.0×10^{-7} to 1.0×10^{-3} g/ml were tested to determine the linearity for all analytes at the carbon disc electrode in this method. The results of regression analysis on calibration curves and detection limits are presented in Table 1. The determination limits are evaluated on the basis of 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 limits ranging from 2×10^{-7} to 1×10^{-6} g/ml for all the analytes.

3.5. Sample analysis and recovery

Under optimum conditions, the determination of hesperetin, acacetin, diosmetin, apigenin, luteolin, ferulic acid, rosmarinic acid and caffeic acid in rosemary was carried out according to the procedures described earlier. Typical electropherogram of rosemary is shown in Fig. 5B. The analysis of natural herbs is a challenging task as the components are often very complicated. In this work, we use a routine method for the quantitative analysis, that is to compare the peak height of the analytes in standard solution and that in real samples, and the content of the active ingredients could be determined. Further identification of the peaks is confirmed by spiking experiments (Fig. 5C). The assay results are listed in Table 2, which agree with those obtained by HPLC [11,27]. Although there are many other phenolic components in rosemary, such as hesperetin, acacetin, diosmetin, apigenin, luteolin [16,28,29], most research work emphasizes particularly on the analysis of phenolic acids in rosemary. In fact, synergistic effects between various constituents in rosemary may exist. It is the first time that the contents of the flavonoids in rosemary are determined. Fig. 5B contains many other peaks. Further confirmation work has not been conducted because of lack of standards which are not available here and such confirmation could be achieved if combined with LC–MS.

Accurate amounts of the analytes were added to the diluted extract of rosemary, and the recovery values were obtained using their peak currents from the calibration curve under the same conditions. The results are listed in Table 2. The above assay results indicate that this method is accurate, sensitive and reproducible, providing a useful quantitative method for the analyses of active ingredients in rosemary.

Table 2
Results of the recovery of this method ($n = 3$, mg/g)[a]

Compound	Original amount	Added amount	Found amount	Recovery (%)	R.S.D. (%)
Hesperetin	0.36	0.50	0.84	96	2.8
Acacetin	0.57	0.50	1.03	92	3.7
Diosmetin	0.21	0.50	0.68	94	2.6
Ferulic acid	1.2	0.50	1.69	98	3.0
Apigenin	0.45	0.50	0.96	102	3.8
Luteolin	0.26	0.50	0.84	96	3.5
Roamarinic acid	10.7	10.0	20.2	95	3.2
Caffeic acid	0.23	0.50	0.71	96	3.0

[a] Working potential is 0.90 V (vs. SCE). Other conditions as in Fig. 2.

4. Conclusion

This paper presents the first application of CE–ED for qualitative and quantitative assay of hesperetin, acacetin, diosmetin, apigenin, luteolin, ferulic acid, rosmarinic acid and caffeic acid in rosemary. The realization of such analysis is more economical in comparison to HPLC, since the consumption of electrolytes is negligible and the use of organic solvents is practically avoided. The reproducibility of quantitative analysis is satisfactory. ED coupled with CE enabled selective and sensitive detection of the electroactive constituents in the crude herbs, and simplification of the electropherograms. It is concluded that CE–ED is a powerful technique for study of the constituents of natural plants and has become an alternative, competitive and supplementary method for HPLC, because of its special attributes.

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