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Quantitative and qualitative analysis of flavonoid markers in *Frucus aurantii* of different geographical origin by capillary electrophoresis with electrochemical detection

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Abstract

A capillary electrophoresis with electrochemical detection (CE-ED) method was developed for the simultaneous determination of four major flavonoid markers (synephrine, naringin, hesperidin and naringenin) in *Frucus aurantii* of different geographical origin. Operated in a wall-jet configuration, a 300 μm diameter carbon disc electrode was used as the working electrode, which exhibits a good response at +0.85 V (versus saturated calomel electrode) for the analytes. Under the optimum conditions, the analytes were baseline separated within 20 min in a 80 mmol/L borax buffer (pH 8.45). The intra-day relative standard deviations (R.S.D.) and inter-day R.S.D.s were based on the analysis of the standard solution on the same day and on the following 6 consecutive days. The intra-day R.S.D.s ranged from 0.8% (naringin) to 3.6% (hesperidin). The inter-day R.S.D.s ranged from 1.2% (hesperidin) to 4.6% (naringenin). Calibration curves were linear in ranges between 0.05 and 1000 μg/mL for the markers. Limits of detection ranged from a low of 1×10^{-8} g/mL (hesperidin) to a high of 5×10^{-7} g/mL (naringin). The method was successfully used in the analysis of *F. aurantii* of different geographical origin with relatively simple extraction procedures, and the assay results were satisfactory.

Keywords: *Frucus aurantii*; Capillary electrophoresis; Electrochemical detection; Flavonoids

1. Introduction

Flavonoids are an important group of secondary metabolites, which are synthesized by plants as a result of plant adaptation to biotic and abiotic stress conditions (infection, wounding, water stress, cold stress, high visible light). Protective phenylpropanoid metabolism in plants has been well documented [1–4]. In recent years flavonoids have attracted the interest of researchers because they show promise of being powerful antioxidants that can protect the human body from free radicals for their hydrogen radical donating abilities [5,6]. Many epidemiological studies have shown that consumption of edible plants rich in phenolic compounds is associated with a lowered risk of degenerative diseases such cancers [7], cardiovascular diseases [8], and immune dysfunctions [9]. These epidemiological results are corroborated by many in vitro and in vivo studies demonstrating the impact of flavonoids on mammalian biology [10] and displaying the remarkable scope of biochemical and pharmacological actions of these compounds, among others their antiviral [11], antiinflammatory [12], and antiallergic [13] properties.

Frucus aurantii, referred as 'Zhi-Ke' in China, is the dried ripen fruit of *Citrus aurantium* L. As a common traditional Chinese medicine that has been used for more than 2000 years in China, the use of *F. aurantii* was first recorded in *Shen Nong Bencao Jing* in ∼100 b.c., the described functions were reducing temperature, soothing asthma, stimulating the appetite, and enhancing the function of the immune system. Recent studies show that citrus plants may have beneficial human health effects, such as antioxidant, antiallergic, and anti-carcinogenic benefits and can protect against high blood pressure or cholesterol increase [14,15].

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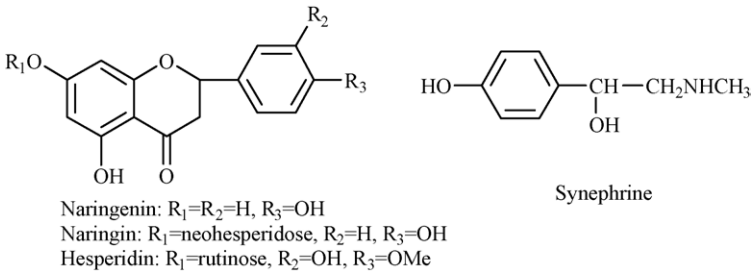

Fig. 1. The molecular structures of synephrine, naringin, hesperidin and naringenin.

The constituents most often associated with the pharmacological activities of *F. aurantii* are the flavonoids such as synephrine, naringin, hesperidin and naringenin. As a synonym of phenylephrine, synephrine is a sympathomimetic agent given as the tartrate for the treatment of hypotensive states [16], it is also well known that synephrine have effects on the cardiovascular system through adrenergic stimulation [17]. Naringin, hesperidin and naringenin possess antioxidant [18], blood lipid lowering [19] and anti-carcinogenic activities [20]. Naringin and hesperidin could improve venous tone, enhance microcirculation, assist healing of venous ulcers and they are used for the treatment of chronic venous insufficiency [21] and have protective effect on glycerol-induced acute renal failure [22].

Although the biological activity of *F. aurantii* as well as its superior safety is well documented, very few studies have been published on the qualitative and quantitative presence of flavonoids in *F. aurantii* [23,24]. The amounts of flavonoids in *F. aurantii* could vary significantly according to their geographical sources. *F. aurantii* is mainly cultivated in Jiangxi province and partly in Jiangsu, Sichuan, and Fujian provinces of China. Li Shi Zhen (1596 a.d.) in *Ben Cao Gang Mu* reported that *F. aurantii* produced in Jiangxi, China, was the authentic source and had the superior quality. Since the physical appearance of *F. aurantii* of different origin is similar and many commercial *F. aurantii* products are sold in various formulations, therefore, methods for the quantitative and qualitative analysis of flavonoid markers in *F. aurantii* from different geographical origin are desirable. Flavonoids are usually used as chemical markers for the quality control of natural plants and fruits because of their ubiquity and multiplicity in fruits, and specificity to different varieties and even cultivars.

Many analytical approaches have been used to identify flavonoids in citrus fruits. Kanaze et al. [25] has determined diosmin, hesperidin and naringin in different citrus fruit juices by using reversed-phase high-performance liquid chromatographic method. Agabbio and co-workers [26] has reported a liquid chromatography method coupled with a photodiode array detector (DAD) to assess the changes of flavonoids and antioxidant capacity in citrus juices during storage. Among these methods, the use of high-performance liquid chromatography (HPLC) in conjunction with mass spectrometry (MS) [27,28] appears to be most promising. However, HPLC has some shortcoming in analysis of natural products, including time-consuming sample pre-treatment [29,30], short column lifetime owing to numerous co-existent interferences, and the apparatus that HPLC-MS needed is expensive, which is not accessible in common laboratories. Now, capillary electrophoresis (CE) is becoming increasingly recognized as an important analytical separation technique for charged compounds and partially charged organics such as phenolic compounds at a high pH. It provides faster analysis time and better separation efficiency than HPLC, and consumes only small amounts of aqueous solvents. CE has environmental and economic advantages, however, HPLC is better in terms of accuracy, sensitivity and precision. Therefore, CE has been proposed as a complementary technique to HPLC for the separation of phenolic compounds present in herbs such as phenolic acids and flavones. In combination with electrochemical detection (ED), CE-ED offers high sensitivity and good selectivity for electroactive species.

In 2000, U.S. Food and Drug Administration (FDA) published a draft of Guidance for Industry Botanical Drug Products. Before a plant drug becomes legally marketed, its spectroscopic or chromatographic fingerprints and chemical assay of characteristic markers are required. CE should find more applications in this area. In this work, CE-ED was proposed for the determination of synephrine, naringin, hesperidin and naringenin (Fig. 1) in *F. aurantii* from different geographical origin. The method is simple, sensitive, reliable, and efficient, providing not only a way for evaluating the quality of *F. aurantii* from different origin, but also an excellent method for quality control in medicinal factories and constituent investigation of other plants. To our best knowledge, there are no reports published on the simultaneous determination of synephrine, naringin, hesperidin and naringenin in *F. aurantii*. The optimization, detailed characterization, and advantages of CE-ED approach are reported in the following sections.

2. Experimental

2.1. Apparatus

In this work, a CE-ED system has been constructed and is similar to that described previously [31]. 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 potential. 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). The carbon disc electrode was made of polymer leads (Sakura, Japan). 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 [32]. The distance between the tip of the working electrode and the capillary outlet was 30 μm 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 borax buffer (pH 8.45) at a separation voltage of 12 kV. The potential applied to the working electrode was +0.85 V (versus SCE). Samples were injected electrokinetically at 12 kV for 6 s.

2.2. Reagents and solutions

Synephrine was purchased from Sigma (St. Louis, MO, USA), naringin, hesperidin and naringenin were obtained from Aldrich (Milwaukee, WI, USA). Stock solutions of synephrine, naringin, and naringenin (1×10^{-3} g/mL each) and hesperidin (1×10^{-4} g/mL) were prepared in anhydrous ethanol (A.R. grade), stored in the dark at 4 °C, and was diluted to the desired concentrations with the running buffer (80 mmol/L borax buffer, pH 8.45), in which carbon working electrode shows excellent response to phenolic compounds. Before use, all solutions were filtered through 0.22 μm nylon filters.

2.3. Sample preparation

The ripen fruit of *Citrus aurantium* L. from different geographical origin (Zhangshu of Jiangxi, Suzhou of Jiangsu, Yibin of Sichuan, and Fuzhou of Fujian) were collected by ourselves. All of the fruits were collected in August. The botanical origins of all the fruits were identified morphologically by ourselves during the field collection in different regions of China.

The ripen fruits were collected and dried under vacuum. About 10 batches of individual species having similar but not identical geographical properties of the same region were tested. Individual samples were prepared from about 500 g of powder that was ground from the fruits of the same population. These grinding processes were done during the field collection before they were delivered to the laboratory. The collected powder was stored with silica gel, which could stabilize the chemical constituents.

Each weighed sample (about 2 g) was extracted with 10 mL 80% ethanol for 2 h in an ultrasonic bath. Next each of the samples was filtered through filter paper first, then through a 0.22 μm syringer filter. Sample solutions were stored at 4 °C in the dark. The samples could be injected electrokinetically without preconcentration.

3. Results and discussion

3.1. Effect of the potential applied to the working electrode

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 synephrine, naringin, hesperidin and naringenin. When the applied potential reaches +0.60 V (versus SCE), the peak currents increase rapidly, however, when the potential exceeds +0.85 V (versus SCE), the peak currents of all the analytes level off. Although applied potential greater than +0.85 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 +0.85 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 buffer concentration

The acidity and concentration of the running buffer plays an important role in CE for its effect on zeta potential (ζ), the electroosmotic flow (EOF), as well as 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 running buffer was 80 mmol/L borate buffer at five different pH values (8.0, 8.2, 8.45, 8.7 and 9.0). The resolution

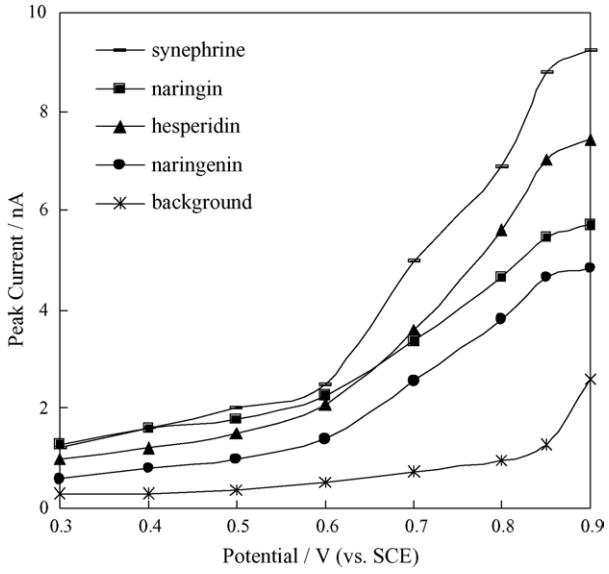

Fig. 2. Hydrodynamic voltammograms (HDVs) for synephrine, naringin, hesperidin and naringenin in CE. Working conditions: fused-silica capillary, 25 μm i.d. × 75 cm; working electrode, 300 μm diameter carbon disc electrode; running buffer, 80 mmol/L borate buffer (pH 8.45); separation voltage, 12 kV; electrokinetic injection: 6 s (12 kV); concentrations, 1.0×10^{-6} g/mL for hesperidin, 1.0×10^{-5} g/mL for synephrine and naringenin and 2.0×10^{-5} g/mL for naringin.

of hesperidin and naringenin is poor at pH 8.0. For hesperidin and naringenin, the resolution (*R*s) is 0.8, 1.3, 1.7, 1.9 and 2.0 respectively, corresponding to the five pH values (8.0, 8.2, 8.45, 8.7 and 9.0). The resolution index was obtained likewise as $2(t_{R2} - t_{R1})(\omega_1 + \omega_2)^{-1}$, where t_R and ω are migration time and peak width, respectively; the suffix represents the neighboring peaks. When the running buffer pH increases, the resolution of all compounds is improved with migration time increased. At pH 8.45 the analytes can be well separated, it is also found that the peak current is low and the peak shape became poor when pH value exceeds 8.45 (for example, the peak current for synephrine is 9.6, 9.3, 8.8, 7.2 and 5.8 nA, respectively, corresponding to the five pH values, i.e. 8.0, 8.2, 8.45, 8.7 and 9.0, there is a significantly decrease in the peak current when pH value is higher than 8.45). Therefore, 80 mmol/L borate buffer with pH 8.45 was chosen as the running buffer in considering the peak current, resolution and the analytical time.

Increasing the running buffer concentrations (ionic strength) generally decreases the EOF, thus lengthens the migration times. Results show that higher buffer concentrations led to longer migration times and decreasing of electrophoretic mobility. 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. For example, the peak current of synephrine is 10.4, 9.8, 9.3, 8.8 and 6.8 nA, respectively, corresponding to the five buffer concentration values, i.e. 20, 40, 60, 80 and 100 mmol/L, in accordance with the report by Tsuda et al. [33] describing that velocity of electroosmotic flow was decreased with increasing concentration of electrolyte, which results in the decrease of peak currents. At 80 mmol/L, all compounds were well separated with an *R*s of 1.7 (calculated from hesperidin and naringenin) with the total migration time of 20 min and the migration order of synephrine, naringin, hesperidin, naringenin, respectively. Consequently, 80 mmol/L borate buffer (pH 8.45) was selected as a compromise between resolution, efficiency, and analysis time and employed for subsequent optimization.

3.3. Effect of separation voltage and injection time

The separation voltage affects the electric field strength, which in turn affects the EOF and the migration velocity of charged particles, which determine the migration time of the analytes. Moreover, higher separation voltage may result in higher Joule heating. Increasing the voltage gives shorter migration times 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, 12 kV was chosen as the optimum voltage to accomplish a good compromise.

The effect of injection time on CE separation was investigated by varying the sampling time (2, 4, 6, 8 and 10 s at a voltage of 12 kV). It was found that both the peak current and the peak width increase with increasing sampling time. When injection time is longer than 6 s, peak current levels off and peak broadening becomes severe. In this experiment, 6 s (12 kV) is selected as the optimum injection time.

Through the experiments above, the optimum conditions for the determination of synephrine, naringin, hesperidin and naringenin were decided. The typical electropherogram for a standard solution of the analytes is shown in Fig. 3A, as we can see baseline separation could be achieved within 20 min.

3.4. Precision, linearity and detection limits

Precision of the method was determined by measuring the repeatability of injection ($n = 7$), intra-day ($n = 10$), and inter-day ($n = 7$) analyses in the standard mixture solution of 1.0×10^{-6} g/mL for hesperidin, 1.0×10^{-5} g/mL for synephrine and naringenin and 2.0×10^{-5} g/mL for naringin under the optimum conditions in this experiment. The results are listed in Table 1. The relative standard deviations (R.S.D.s) of peak current varied from 1.0 to 4.6%, and the migration time varied from 0.5 to 1.6% for the analytes.

A series of standard solutions of synephrine, naringin, hesperidin and naringenin ranging from 1.0×10^{-7} g/mL to 1.0×10^{-3} g/mL in concentration were tested to determine the linearity of the determination. Results from regression analysis of both calibration curves and detection limits are listed in Table 2. The detection limits were evaluated on the basis of a signal-to-noise ratio of 3.

3.5. Sample analysis and recovery

Under optimum conditions, the determination of synephrine, naringin, hesperidin and naringenin in real samples was carried out according to the procedures described earlier. Typical electropherograms of *F. aurantii* from different geographical origin are shown in Fig. 3B. The migration times in CZE in fused-silica capillary tubes can be varied about 1–2% due to the nonrepeatable EOF, which is caused by the unstable surface condition of the inner wall of the tubes and the change in the effective electric field strength [34]. Additionally, migration times are highly sensitive to slight changes in buffer pH and ionic strength. It is observed in the experiment that the migration time of the phenolic compounds in different samples (Fig. 3B) shifted from those of the standard solutions (Fig. 3A), which made the identification by migration time unreliable, further identification of the peaks are confirmed by spiking experiments, The assay results are listed in Table 3.

By comparing with the electropherogram of the standard solution and spiking experiments, it was found that all samples has identical profiles on the basis of relative peak heights and migration time. The active ingredients namely synephrine, naringin, hesperidin and naringenin were found presented in all the samples, so the four flavonoids can be defined as common peaks in the fingerprint of *F. aurantii* under the selected conditions. However, the content of individual analytes varied greatly in different samples, indicating the ecological environment had great impact on the contents of the constituents investigated. The content of synephrine in *F. aurantii* of Jiangxi province is about two times as that of *F. aurantii* in other provinces, and

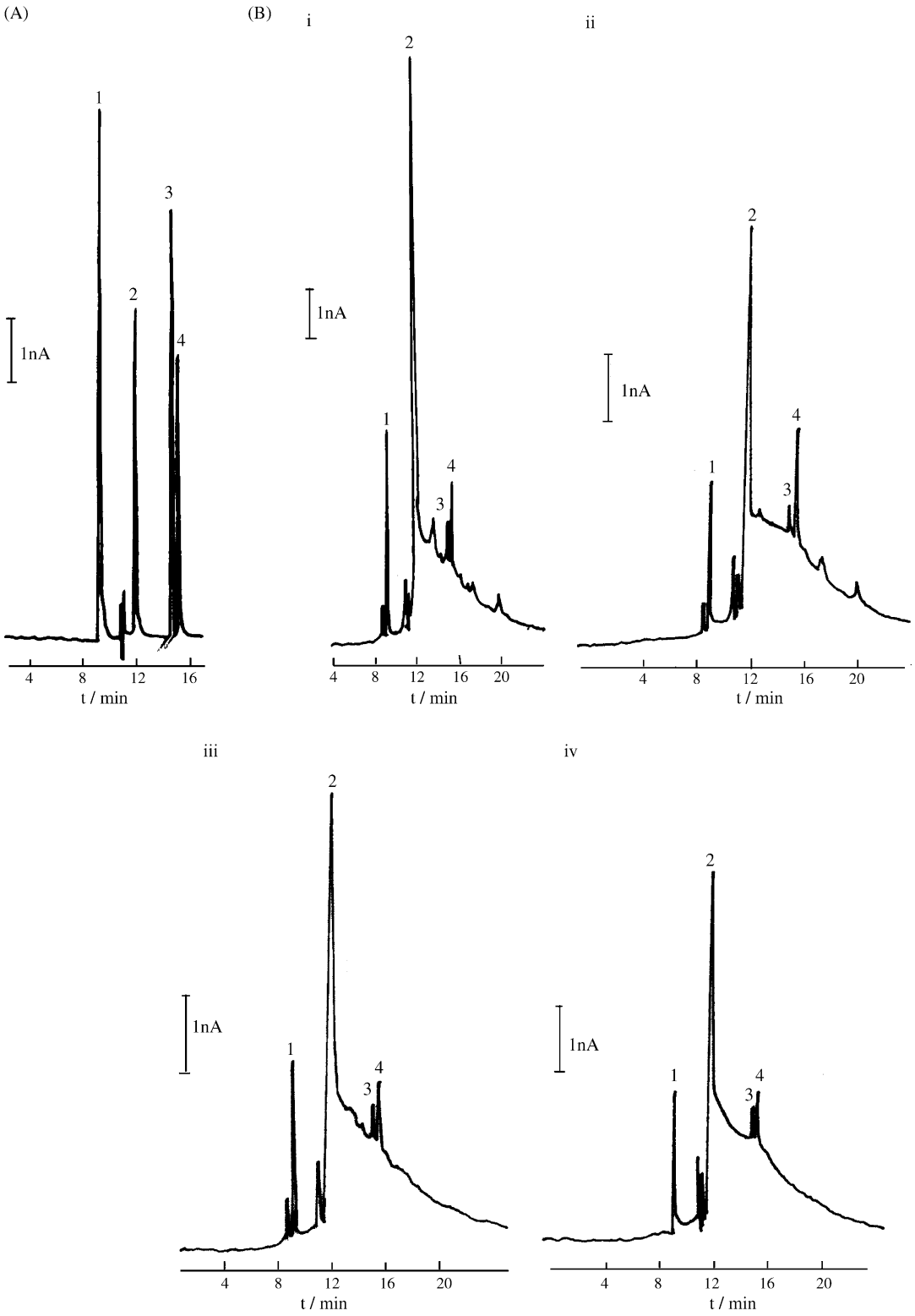

Fig. 3. The electropherogram of a standard mixture solution (1.0×10^{-6} g/mL for hesperidin, 1.0×10^{-5} g/mL for synephrine and naringenin and 2.0×10^{-5} g/mL for naringin) (A) and the typical electropherograms of *Frucus aurantii* (B): (i) Zhangshu of Jiangxi; (ii) Fuzhou of Fujian; (iii) Yibin of Sichuan; (iv) Suzhou of Jiangsu. Dilution for all the samples: 1:200. Peak identification: (1) synephrine; (2) naringin; (3) hesperidin; (4) naringenin. Working potential: +0.85 V (vs. SCE); other conditions as in Fig. 2.

so is naringin and hesperidin. The overall amount of the four flavonoids in *F. aurantii* of Jiangxi province is more than two times as in the other three provinces as shown in Fig. 4, which is in agree with the fact that Jiangxi province is the authentic source of *F. aurantii*. Among different cultivated regions in China, Zhangshu of Jiangxi province produces the best quality of *F. aurantii*, which could be due to its specific geographical location. Zhangshu is 30 m above sea level and receives >1500 mL

Table 1
Injection, intra- and inter-day precision of the analytes in the standard solution[a]

Compound	Precision (%R.S.D.)					
	Peak height			Migration time		
	Injection ($n = 7$)	Intra-day ($n = 10$)	Inter-day ($n = 7$)	Injection ($n = 7$)	Intra-day ($n = 10$)	Inter-day ($n = 7$)
Synephrine	2.6	3.2	3.6	0.9	1.2	1.3
Naringin	2.8	2.5	3.5	0.5	0.8	1.5
Hesperidin	2.0	3.6	3.2	1.0	1.0	1.2
Naringenin	1.8	2.0	4.6	0.8	0.9	1.6

[a] Working potential is +0.85 V (vs. SCE). Other conditions as in Fig. 2.

Table 2
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)
Synephrine	$y = 871428x + 0.1$	$R = 0.9997$	0.2–200	5×10^{-8}
Naringin	$y = 272000x + 0.02$	$R = 0.9997$	1–1000	5×10^{-7}
Hesperidin	$y = 7000000x - 0.12$	$R = 0.9995$	0.05–100	1×10^{-8}
Naringenin	$y = 457142x + 0.16$	$R = 0.9996$	0.5–500	1×10^{-7}

[a] Working potential is +0.85 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.

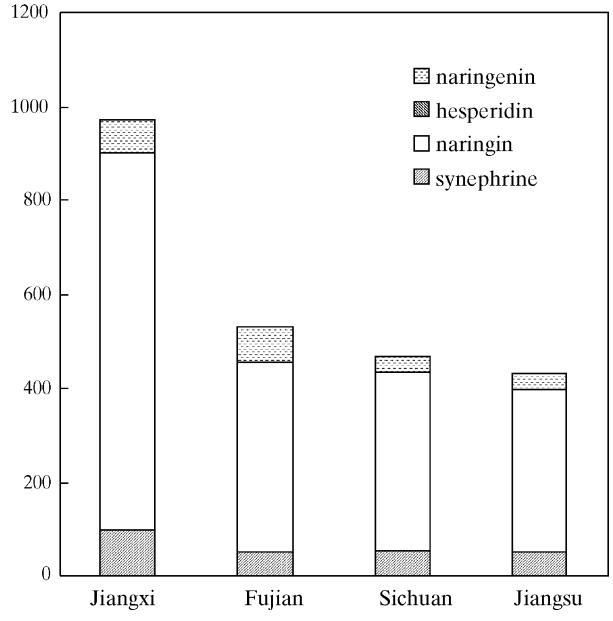

Fig. 4. Cumulative bar chart of the overall amount (10^{-5} g/g) of the four flavonoids in the *Frucus aurantii* of different origins.

of annual rainfall, and the average temperature in a year is ~17.5 °C, the climate is suitable for the growth of medicinal plants. Besides the geographical properties, farmers in Zhangshu have experience of cultivating *F. aurantii* for over 1000 years. Indeed, Zhangshu produces >60% of the total production of *F. aurantii* in China. The proposed method provided a simple, reliable way not only for the determination of the flavonoids markers in *F. aurantii*, but also an excellent method for quality control in medicinal factories and constituent investigation of other plants.

The recovery and reproducibility experiments under the optimum conditions were also conducted to evaluate the precision and accuracy of the method. Recovery was determined by standard addition method, accurate amounts of synephrine, naringin, hesperidin and naringenin were added to the diluted extracts of the samples, and the results obtained from *F. aurantii* (Zhangshu, Jiangxi) are listed in Table 4. The recovery for all the samples varied from 92 to 106%. The above assay results indicate that this method is accurate, sensitive and reproducible, providing a useful quantitative method for the analyses of flavonoids markers in *F. aurantii*.

Table 3
Assay results of the analytes in *Frucus aurantii* of different geographical origin ($n = 3$, 10^{-5} g/g)[a]

Cultivation region	Content[b]			
	Synephrine	Naringin	Hesperidin	Naringenin
Zhangshu, Jiangxi	97.85 ± 1.20	800.0 ± 2.65	2.360 ± 0.06	71.75 ± 0.40
Fuzhou, Fujian	52.02 ± 0.35	402.33 ± 1.36	1.33 ± 0.05	73.09 ± 0.55
Yibin, Sichuan	54.18 ± 0.60	380.58 ± 1.76	1.06 ± 0.11	32.29 ± 0.86
Suzhou, Jiangsu	50.11 ± 0.55	345.63 ± 1.85	1.33 ± 0.0.08	32.29 ± 0.65

[a] Working potential is +0.85 V (vs. SCE). Other conditions as in Fig. 2.
[b] Values are means ± S.D., $n = 7$.

Table 4
Results of the recovery of this method ($n = 3$, 10^{-5} g/g)[a]

Compound	Original amount	Added amount	Found amount	Recovery (%)	R.S.D. (%)
Synephrine	97.85	100.0	189.85	92	3.6
Naringin	800.0	500.0	1270.0	94	2.8
Hesperidin	2.36	5.00	7.66	106	3.5
Naringenin	71.75	55.0	123.45	94	3.0

[a] Working potential is +0.85 V (vs. SCE). Other conditions as in Fig. 2.

4. Conclusions

This work presents the first application of CE-ED for the determination of syneprine, naringin, hesperidin and naringenin in *F. aurantii* of different geographical origin. 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, and the capillary is much easier to wash. The reproducibility of quantitative analysis is satisfactory. ED coupled with CE enable selective and sensitive detection of the electroactive constituents in the crude drug, and simplification of the electropherograms for only electroactive constituents could be detected. Samples do not need derivatization before determination because the analytes could be directly detected on the working electrode. It is concluded that CE-ED is a powerful technique for the chemical markers and fingerprint study of natural products and has become an alternative, competitive and supplementary method for HPLC, because of its special attributes.

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