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# Determination and Differentiation of *Flos Chrysanthemum* Based on Characteristic Electrochemical Profiles by Capillary Electrophoresis with Electrochemical Detection

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A high-performance capillary electrophoresis with electrochemical detection (CE-ED) method has been developed for the analysis of bioactive ingredients in *Flos Chrysanthemum* in this work. The effects of several factors such as the acidity and concentration of running buffer, the separation voltage, the applied potential, and the injection time were investigated. Under the optimum conditions, the eight analytes could be well separated within 20 min at the separation voltage of 14 kV in a 50 mmol/L Borax running buffer (pH 9.2). A 300 $\mu$m diameter carbon disk electrode has a good response at a potential of +950 mV (vs SCE) for all analytes. Good linear relationship was established over 3 orders of magnitude with detection limits (S/N = 3) that ranged from $1.9 \times 10^{-7}$ to $3.0 \times 10^{-8}$ g/mL. This proposed method has been successfully applied for the determination and differentiation of six kinds of popular *Flos Chrysanthemum* samples based on their characteristic electrochemical profiles, and the results are satisfactory.

**KEYWORDS: Capillary electrophoresis; electrochemical detection; *Flos Chrysanthemum*; characteristic electrochemical profiles**

## INTRODUCTION

There has been more than 3000 years of history of cultivating *Flos Chrysanthemum*. People gradually realized its functions in health care and prolonging life in ancient times. Of all ages, there are many ways of preparing edible *Chrysanthemum*, such as *Chrysanthemum* meals, *Chrysanthemum* wines, and *Chrysanthemum* teas. *Flos Chrysanthemum* is not only well-known as edible flowers but also Chinese traditional herbal medicines, which belongs to the medicinal and edible cognates. Modern research has shown that *Flos Chrysanthemum* extract can be used to reduce oxygen consumption of myocardium, enhance body immunity, resist consenescence, prevent and cure angiosclerosis, and infective disease. Therefore, *Flos Chrysanthemum* is an important constitute of Chinese traditional medicines treating hypertension and flu in clinic, which is consistent with the functions described in ancient works revealing that *Flos Chrysanthemum* mainly possesses the therapeutic effects of scattering cold, clearing heat and toxin, and brightening eyes (*1*). Furthermore, most of the electroactive ingredients in *Flos Chrysanthemum* are important members of phytoestrogens (*2*), which can be an alternative natural source for extracting bioactive ingredients. Hence, it is necessary to develop some simple, economical, and efficient methods for the simultaneous analysis and quantitative measurement of multiple constitutes in *Flos Chrysanthemum* to establish the quality standard.

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Chemical constituent investigations show that phenolic compounds and coumarins, such as acacetin, hesperetin, umbelliferone, chlorogenic acid, kaempferol, apigenin, luteolin, quercetin, etc., are important active constituents in *Flos Chrysanthemum*. The molecular structures of these compounds are shown in **Figure 1**. To our knowledge, GC/MS, HPLC, and CE-UV have been used in the determination of the essential oil (*3*, *4*), sesquiterpenoid alcohols (*5*), phenolic acids (*6*−*8*), or coumarins in *Flos Chrysanthemum* (*9*). The State Pharmacopoeia Commission of China only takes chlorogenic acid as the check compound for evaluating the quality of *Flos Chrysanthemum*. However, the content of flavonoids are relatively high (*10*, *11*), which are also the main active ingredients treating hypertension and coronary heart disease, and showed antimutagenic activities of these compounds against furylfuramide (*12*). To our knowledge, so far only a few reports can be found, including the HPLC approach and polyamide TLC-scanning method being used for the determination of luteolin (*13*), one or two kinds of flavones (*14*) or flavonoids (*15*) in *Flos Chrysanthemum*. HPLC, however, as a prime analytical method used in the analysis of traditional Chinese medicines, often has some shortcomings, including long analysis time, low resolution, and short column lifetime because of easy contamination (*16*−*20*). These above methods mostly rely on photoabsorption detection, and the sensitivity is relative low. Capillary electrophoresis (CE) is increasingly recognized as an important analytical separation technique because of its speed, efficiency, reproducibility, ultra-small sample volume, little consumption

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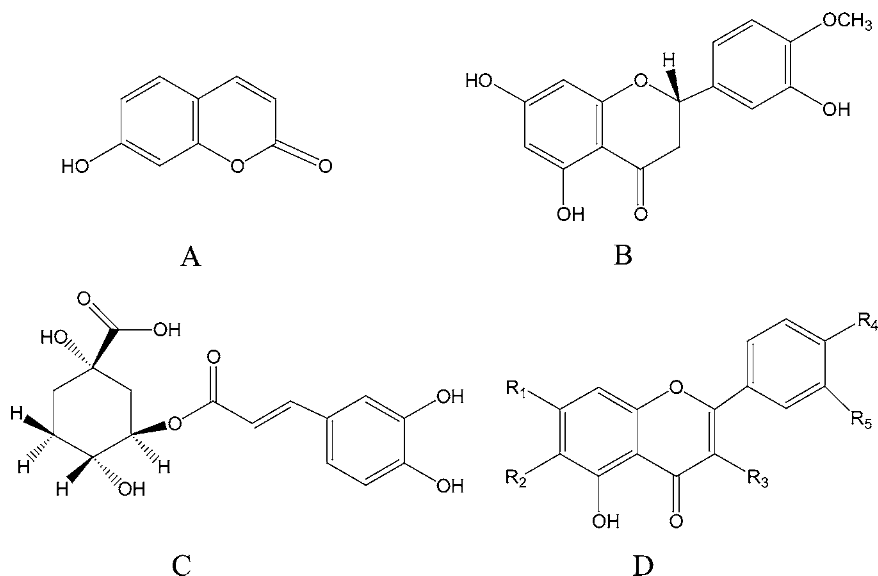


**Figure 1.** Molecular structures of (**A**) umbelliferone, (**B**) hesperetin, (**C**) chlorogenic acid, and (**D**) quercetin ($R_1$, $R_3$, $R_4$, $R_5$ = OH, $R_2$ = H); kaempferol ($R_1$, $R_3$, $R_4$ = OH, $R_2$, $R_5$ = H); luteolin ($R_1$, $R_4$, $R_5$ = OH, $R_2$, $R_3$ = H); apigenin ($R_1$, $R_4$ = OH, $R_2$, $R_3$, $R_5$ = H); acacetin ($R_1$ = OH, $R_2$, $R_3$, $R_5$ = H, $R_4$ = $OCH_3$).

of solvent, and ease of clearing up the contaminants. In addition, with electrochemical detection (ED), CE-ED affords high sensitivity and good selectivity for electroactive species. In comparison with HPLC, CE is often a more efficient separation method without requiring complicated operation and high cost.

In this work, we have developed a sensitive, dependable, and simple method for the simultaneous determination of three categories of electrochemical active ingredients including phenolic acids, coumarins, and flavonoids (**Figure 1**) from *Flos Chrysanthemum* by employing CE-ED, which was successively used for the analysis of six kinds of popular *Flos Chrysanthemum* in Chinese market after a relatively simple extraction procedure. Further on, differentiation of these real-world samples based on their electropherograms or characteristic "electrochemical profiles" can be readily achieved. The objective determination of these compounds as floral markers and the occurrence of species-specific markers for *Flos Chrysanthemum* is also considered.

## MATERIALS AND METHODS

**Apparatus.** The laboratory-built CE-ED system (*21*) was used in this work. A +30 kV high-voltage power supply (Shanghai Institute of Nuclear Research, China) provided a voltage between the ends of the capillary. The inlet end of the capillary was held at a positive potential, and the outlet end was maintained at ground. A 75 cm length of 25 $\mu$m i.d. fused-silica capillary (Polymicro Technologies, Phoenix, AZ) was used for the separation. All samples were injected electrokinetically without preconcentration, applying 14 kV for 10 s.

The design of CE-ED detection was based on the end-column approach in which the working electrode is simply placed at the outlet of the separation capillary and detection is carried out in the same solution reservoir that contains the grounding electrode for CE instrument. A carbon-disk electrode with 300 $\mu$m diameter was employed as the working electrode. Before use, the surface of the carbon-disk electrode was polished with emery sand paper, sonicated in deionized water, and then positioned carefully opposite the capillary outlet with the aid of an Oriel Corp. (Stratford, CT) model 14901 micropositioner. A three-electrode cell system consisting of a carbon-disk working electrode, a platinum auxiliary electrode, and a SCE (saturated calomel electrode) reference electrode was used in combination with a BAS LC-4C amperometric detector (Biochemical System, West Lafayette, IN). The electropherograms were recorded using a chart recorder (Shanghai Dahua Instrument Factory, China). The peak current and concentration of each standard were subjected to regression analysis to obtain the calibration equations and correlation coefficients, which in turn can be used for quantitation of all analytes in real samples.

**Reagents and Solutions.** Acacetin, hesperetin, umbelliferone chlorogenic acid, kaempferol, apigenin, and luteolin were purchased from Sigma (St. Louis, MO), and quercetin was obtained from Shanghai Reagent Factory (Shanghai, China), and all were used as received. *Dendranthema indicum* Des monl. (Anhui, China), *Dendranthema morifolium* cv. Gong-ju (Anhui, China), *Dendranthema morifolium* cv. Chu-ju (Anhui, China), *Dendranthema morifolium* cv. Hang-ju (Zhejiang, China), *Dendranthema morifolium* cv. Yang-ju (Xizang, China), and *Dendranthema morifolium* cv. Huang-ju (Anhui, China) were purchased from Shanghai Crude Drug Co. (Shanghai, China).

Stock solutions of eight analytes ($1.00 \times 10^{-3}$ g/mL, each) were prepared in anhydrous ethanol (A. R. grade) and were diluted to the desired concentration with the running buffer ($H_3BO_3-Na_2B_4O_7$ buffer with a pH value from 8.7 to 9.5). Before use, all solutions were filtered through 0.22 $\mu$m nylon filters. All experiments were performed at room temperature.

**Sample Preparation.** About 2 g of each Flos Chrysanthemum sample (Dendranthema indicum Des monl., Dendranthema morifolium cv. Gong-ju, Dendranthema morifolium cv. Chu-ju, Dendranthema morifolium cv. Hang-ju, Dendranthema morifolium cv. Yang-ju, or Dendranthema morifolium cv. Huang-ju) was ground into powder in a mortar and accurately weighed. Each weighed sample was extracted with 10 mL of anhydrous ethanol (A. R. grade) and water (4:1) for 30 min in an ultrasonic bath. Each of the samples was then centrifuged by a desk centrifuge first, and then filtered through filter paper and a 0.22 $\mu$m syringe filter in turn. After filtration, the solutions were injected directly to the CE-ED system for analysis. Before use, all sample solutions were stored in the dark.

## RESULTS AND DISCUSSION

**Optimum of Analytical Procedure.** Because the phenolic hydroxy groups of the eight analytes can be readily oxidized electrochemically at a relatively moderate potential (*22*), electrochemical detection was used in this work. In amperometric detection, the potential applied to the working electrode directly affects the sensitivity, detection limit, and stability of this method. Therefore, the hydrodynamic voltammetry experiment was investigated to obtain optimum detection. As shown in **Figure 2**, the peak current of analyte increases with the rising of applied potential. However, when applied potential was

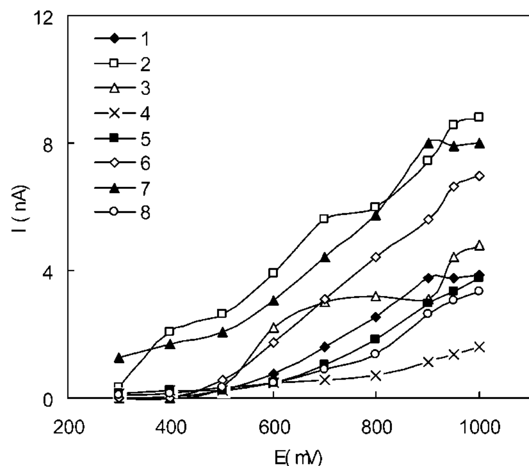


**Figure 2.** Hydrodynamic voltammograms (HDVs) of acacetin (1), hesperetin (2), umbelliferone (3), chlorogenic acid (4), kaempferol (5), apigenin (6), luteolin (7), and quercetin (8) in CE-ED. Fused-silica capillary, 25 $\mu$m i.d. × 75 cm; working electrode, 300 $\mu$m diameter carbon disk electrode; running buffer, 50 mmol/L (pH 9.2); separation voltage, 14 kV; injection time, 10 s (at 14 kV); concentrations of eight analytes, 2.0 × $10^{-5}$ g/mL, each.

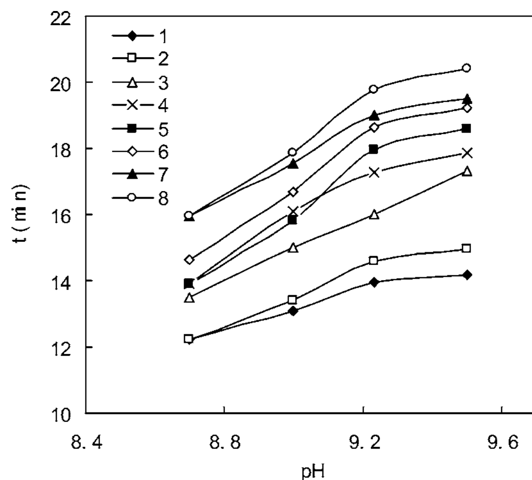


**Figure 3.** Effect of running buffer pH on migration time. Working electrode potential is +950 mV (vs SCE); other conditions are the same as in **Figure 2**.

greater than +950 mV (vs SCE), both the baseline noise and the background current increase, resulting in an unstable baseline, which is a disadvantage for sensitive and stable detection. Therefore, the applied potential of +950 mV (vs SCE) was selected, where the background current is not too high and the S/N ratio is the highest.

The acidity of the running buffer affects the zeta-potential ($\xi$), the electroosmotic flow (EOF), as well as the overall charge of the annlytes, which determine the migration time and the separation of the analytes. The effect of the running buffer pH on the migration time of the analytes was investigated in the pH range of 8.7−9.5, as shown in **Figure 3**. When the pH is lower than 9.0, satisfactory separation of the analytes cannot be achieved. Because the analytes migrate counter-electroosmotic, the migration time increases with increasing pH value, as well as the resolution is improved for all analytes. When the pH is higher than 9.5, apigenin cannot be separated from luteolin. Meanwhile, the peak current is low and the peak shape becomes poor. At pH 9.2, the eight analytes can be well separated within a relatively short time.

Besides the pH value, the running buffer concentration is also an important parameter. The effect of the running buffer concentration on migration time was also studied, and the optimum running buffer concentration is 50 mmol/L (pH 9.2).

For a given capillary length, the separation voltage determines the electric field strength, which affects both the velocity of electrosmotic flow (EOF) and the migration velocity of the analytes, which in turn determines the migration time of the analytes. As expected, a higher separation voltage gives a shorter migration time for all analytes. However, when the separation voltage exceeds 16 kV, baseline noise becomes larger. Therefore, the optimum separation voltage selected is 14 kV, at which good separation can be obtained for all analytes within 20 min.

The injection time determining the amount of sampling affects both peak current and peak shape. The effect of injection time on peak current was studied by varying injection time from 2 to 12 s at 14 kV. It was found that the peak current increases with increasing sampling time. When the injection time is longer than 10 s, peak current nearly levels off and peak broadening becomes more severe. In this experiment, 10 s (14 kV) is selected as the optimum injection time.

Through the experiments above, the optimum conditions for acacetin, hesperetin, umbelliferone chlorogenic acid, kaempferol, apigenin, luteolin, and quercetin have been decided. The applied potential to the working electrode was selected at +950 mV (vs SCE), and the injection time was 10 s (14 kV), and they can be well separated within 20 min at the separation voltage of 14 kV in a 50 mmol/L Borax running buffer (pH 9.2). The typical electropherogram for a standard solution of the eight analytes was shown in **Figure 4A**.

**Method Validations.** To determine the linearity of acacetin, hesperetin, umbelliferone, chlorogenic acid, kaempferol, apigenin, luteolin, and quercetin, a series of standard solutions from 2.0 × $10^{-7}$ g/mL to 2.0 × $10^{-4}$ g/mL were tested. The peak current and concentration of each analyte were subjected to regression analysis to calculate the calibration equations and correlation coefficients. The results of regression analysis on calibration curves were summarized in **Table 1**. The results show that within the concentration range indicated in **Table 1** there was an excellent correlation between peak current and concentration of each analyte.

The limit of detection (LOD) was established with a signal-to-noise ratio of 3. The LOD of eight analytes ranged from 1.9 × $10^{-7}$ to 3.0 × $10^{-8}$ g/mL, and the detailed data are shown in **Table 1**.

The reproducibility of the peak current is estimated by making repetitive injections of a standard mixture solution (2.0 × $10^{-5}$ g/mL for each analyte) under the selected optimum conditions ($n = 7$). The relative standard derivations (RSDs) of the peak current are 1.9%, 0.9%, 2.9%, 3.9%, 2.6%, 1.7%, 1.9%, and 3.2% for acacetin, hesperetin, umbelliferone chlorogenic acid, kaempferol, apigenin, luteolin, and quercetin, respectively ($n = 7$). The reproducibility exhibited in the present study shows that it is feasible to determine the above analytes based on CE-ED.

The stability of standard and sample solutions was determined by monitoring the peak current of standard mixture solutions and sample solutions over a period of 1 week. The results showed that the migration time and peak current of each analyte were almost unchanged (RSD% < 1.9) and that no significant degradation was observed within the given period, indicating the solutions were stable for at least 1 week.

**Recovery.** To evaluate the precision of the method, the recovery experiments under the optimum conditions were also

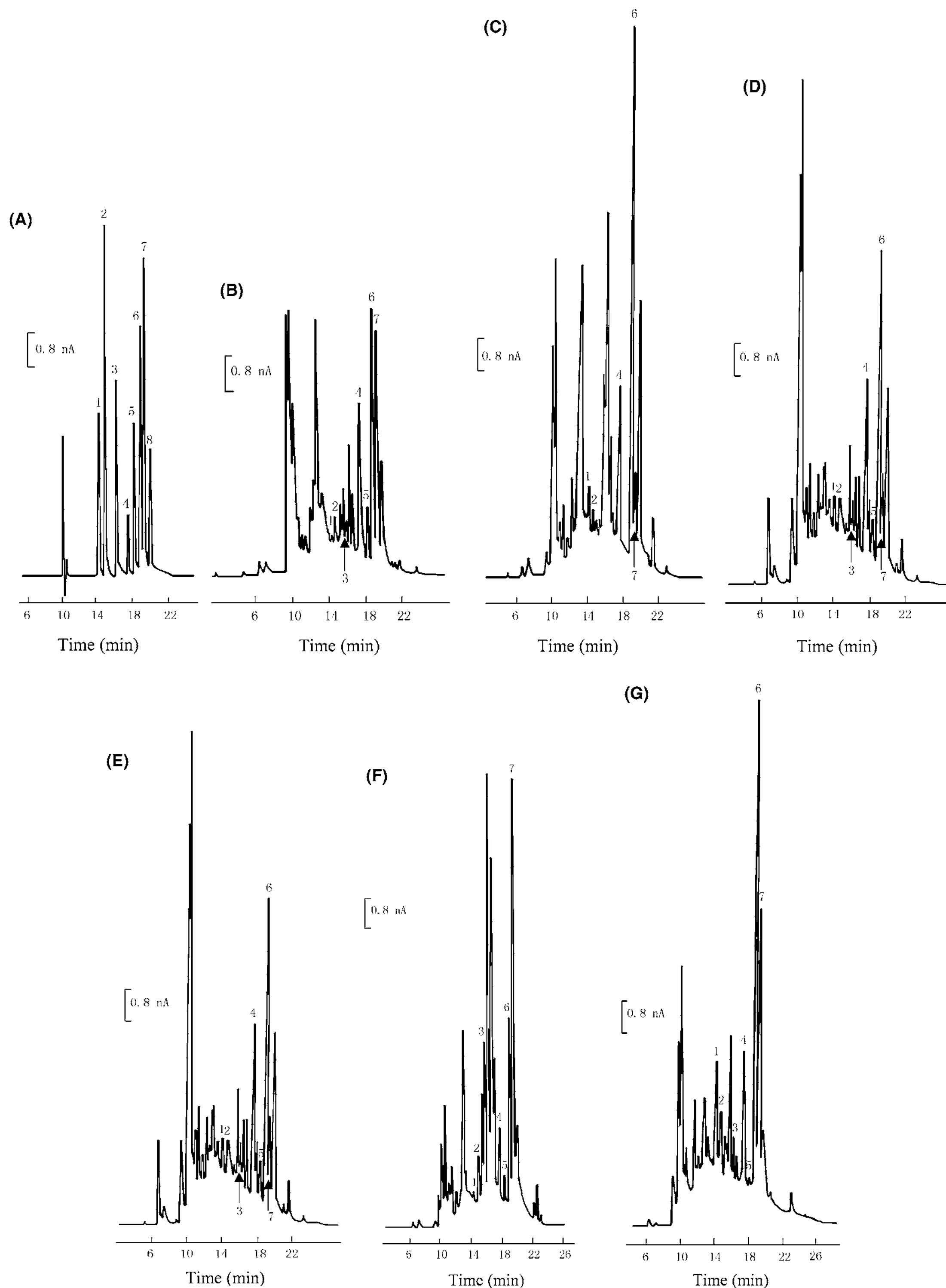


**Figure 4.** The electropherograms of standard solution (**A**), *Dendranthema indicum* Des monl. (**B**), *Dendranthema morifolium* cv. Gong-ju (**C**), *Dendranthema morifolium* cv. Chu-ju (**D**), *Dendranthema morifolium* cv. Hang-ju (**E**), *Dendranthema morifolium* cv. Yang-ju (**F**), and *Dendranthema morifolium* cv. Huang-ju (**G**). Experiment conditions and peak identification are the same as in **Figure 3**.

conducted with *Flos Chrysanthemum* samples. Recovery was determined by the standard addition method, and the results of the recovery experiment of *Dendranthema indicum* Des monl. sample ($n = 3$) are listed in **Table 3**. The results indicate the method is accurate enough for the simultaneous determination of the above analytes.

**Sample Analysis and Discussion.** Under the optimum conditions, the proposed procedure was applied for the determination of active ingredients in six kinds of *Flos Chrysanthemum* samples including *Dendranthema indicum* Des monl., *Dendranthema morifolium* cv. Gong-ju, *Dendranthema morifolium* cv. Chu-ju, *Dendranthema morifolium* cv. Hang-ju, *Dendranthema morifolium* cv. Yang-ju, and *Dendranthema morifolium* cv. Huang-ju, which are main-trend *Flos Chrysanthemum* products in the Chinese market. Typical electropherograms of *Flos Chrysanthemum* samples were shown in **Figure 4B−G**, respectively. By spiking standard analytes and the migration time of analytes as compared to the electropherogram

**Table 1.** Regression Equations and Detection Limits[a]

| compound | regression equation[b] | correlation coefficient | linear range (g/mL) | detection limit ($10^{-8}$ g/mL) |
|---|---|---|---|---|
| acacetin | $y = 1.85 \times 10^5 x + 0.24$ | 0.9992 | $1 \times 10^{-6}$ to $1 \times 10^{-4}$ | 3.7 |
| hesperetin | $y = 4.19 \times 10^5 x + 0.14$ | 0.9995 | $1 \times 10^{-7}$ to $1 \times 10^{-4}$ | 3.0 |
| umbelliferone | $y = 2.44 \times 10^5 x + 0.15$ | 0.9992 | $1 \times 10^{-7}$ to $5 \times 10^{-5}$ | 3.0 |
| chlorogenic acid | $y = 7.18 \times 10^4 x + 0.05$ | 0.9995 | $1 \times 10^{-6}$ to $1 \times 10^{-4}$ | 19 |
| kaempferol | $y = 1.47 \times 10^5 x - 0.06$ | 0.9989 | $1 \times 10^{-6}$ to $1 \times 10^{-4}$ | 11 |
| apigenin | $y = 2.29 \times 10^5 x + 0.26$ | 0.9991 | $1 \times 10^{-7}$ to $1 \times 10^{-4}$ | 3.8 |
| luteolin | $y = 3.47 \times 10^5 x + 0.20$ | 0.9994 | $1 \times 10^{-7}$ to $1 \times 10^{-4}$ | 3.4 |
| quercetin | $y = 1.54 \times 10^5 x - 0.05$ | 0.9994 | $1 \times 10^{-6}$ to $1 \times 10^{-4}$ | 12 |

[a] CE-ED conditions are the same as in **Figure 3**. [b] In the regression equation, the *x* value is the concentration of analytes (g/mL), and the *y* value is the peak current (nA).

**Table 2.** Assay Results for Six Kinds of *Flos Chrysanthemum* Samples ($n = 3$)[a]

| sample | ingredients | found (μg/g) | RSD (%) |
|---|---|---|---|
| *Dendranthema indicum* Des monl. | acacetin | 43.5 | 4.1 |
| | hesperetin | 70.7 | 3.5 |
| | umbelliferone | 70.2 | 4.3 |
| | chlorogenic acid | 2372.5 | 3.1 |
| | kaempferol | 332.6 | 3.5 |
| | apigenin | 973.4 | 2.9 |
| | luteolin | 678.7 | 3.0 |
| | quercetin | NF | |
| *Dendranthema morifolium* cv. Gong-ju | acacetin | 206.5 | 4.3 |
| | hesperetin | 21 | 4.8 |
| | umbelliferone | NF | |
| | chlorogenic acid | 2431.4 | 2.0 |
| | kaempferol | NF | |
| | apigenin | 2236.7 | 2.7 |
| | luteolin | 169.5 | 3.5 |
| | quercetin | NF | |
| *Dendranthema morifolium* cv. Chu-ju | acacetin | 194.9 | 5.0 |
| | hesperetin | 99.7 | 3.7 |
| | umbelliferone | 127.4 | 4.8 |
| | chlorogenic acid | 3098.0 | 3.2 |
| | kaempferol | 244.2 | 3.9 |
| | apigenin | 1338.2 | 2.2 |
| | luteolin | 214.9 | 4.5 |
| | quercetin | NF | |
| *Dendranthema morifolium* cv. Hang-ju | acacetin | 88.4 | 4.2 |
| | hesperetin | NF | |
| | umbelliferone | 1140.5 | 2.1 |
| | chlorogenic acid | 2023.5 | 3.4 |
| | kaempferol | NF | |
| | apigenin | 857.5 | 2.4 |
| | luteolin | 389.6 | 3.9 |
| | quercetin | NF | |
| *Dendranthema morifolium* cv. Yang-ju | acacetin | 44.9 | 4.6 |
| | hesperetin | 151.7 | 4.1 |
| | umbelliferone | 738.1 | 3.0 |
| | chlorogenic acid | 1123.5 | 3.7 |
| | kaempferol | 197.7 | 4.8 |
| | apigenin | 950.8 | 3.0 |
| | luteolin | 1598.4 | 2.3 |
| | quercetin | NF | |
| *Dendranthema morifolium* cv. Huang-ju | acacetin | 681.2 | 4.0 |
| | hesperetin | 141.7 | 4.4 |
| | umbelliferone | 178.6 | 4.1 |
| | chlorogenic acid | 2245.1 | 2.7 |
| | kaempferol | 48.1 | 4.6 |
| | apigenin | 2115.9 | 2.0 |
| | luteolin | 843.4 | 2.3 |
| | quercetin | NF | |

[a] CE-ED conditions are the same as in **Figure 3**.

of the standard mixture solution (**Figure 4A**), the active ingredients acacetin (1), hesperetin (2), umbelliferone (3), chlorogenic acid (4), kaempferol (5), apigenin (6), and luteolin (7) in *Flos Chrysanthemum* samples can be identified and determined, and

**Table 3.** Determination Results of Recovery in This Method with *Dendranthema indicum* Des monl. Sample ($n = 3$)[a]

| ingredient | original amount (g/mL) | added amount (g/mL) | found (g/mL) | recovery (%) | RSD (%) |
|---|---|---|---|---|---|
| acacetin | $0.09 \times 10^{-5}$ | $2.0 \times 10^{-5}$ | $2.11 \times 10^{-5}$ | 101.0 | 3.4 |
| hesperetin | $0.14 \times 10^{-5}$ | $2.0 \times 10^{-5}$ | $2.17 \times 10^{-5}$ | 101.4 | 4.1 |
| umbeliferone | $0.14 \times 10^{-5}$ | $2.0 \times 10^{-5}$ | $2.21 \times 10^{-5}$ | 103.2 | 3.6 |
| chlorogenic acid | $4.71 \times 10^{-5}$ | $2.0 \times 10^{-5}$ | $6.83 \times 10^{-5}$ | 101.8 | 2.3 |
| kaempferol | $0.65 \times 10^{-5}$ | $2.0 \times 10^{-5}$ | $2.58 \times 10^{-5}$ | 97.4 | 3.4 |
| apigenin | $1.97 \times 10^{-5}$ | $2.0 \times 10^{-5}$ | $4.03 \times 10^{-5}$ | 101.5 | 2.4 |
| luteolin | $1.37 \times 10^{-5}$ | $2.0 \times 10^{-5}$ | $3.44 \times 10^{-5}$ | 102.1 | 2.9 |
| quercetin | NF | $2.0 \times 10^{-5}$ | $2.07 \times 10^{-5}$ | 103.5 | 4.5 |

[a] CE-ED conditions are the same as in **Figure 3**.

quercetin (8) cannot be found in all six samples. The assay results are listed in **Table 2**.

As we can see from **Figure 4** and **Table 2**, on one hand, that acacetin, chlorogenic acid, apigenin, and luteolin have higher percentages in all *Flos Chrysanthemum* samples indeed indicated the floral characters, which can be commended as the electrochemical floral markers of *Flos Chrysanthemum*; on the other hand, the significant differences in their electropherograms or "electrochemical profiles" can be found; in other words, the peak structures or peak profiles including peak number, peak height, and peak position are noticeably different. For example, the content of umbelliferone (peak 3) in **Figure 4C** is relatively high, while it has not been determined in **Figure 4E**. Therefore, these electropherograms or characteristic "electrochemical profiles" can be effectively used for the intraspecific differentiation of *Flos Chrysanthemum*. However, as limited by the number of standard samples, all of the constituents are not identified in the present work.

## ABBREVIATIONS USED

ED, electrochemical detection; HDV, hydrodynamic voltammogram.

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