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Development of A Fast Method for Fructus Aurantii Identification by Electrochemical Fingerprint

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Electrochemical analysis techniques can be used for the identification of plant samples. This work describes the identification of fructus aurantii and its closely related species by electrochemical fingerprinting. For a better extraction of electrochemically active substances, DMSO-$CHCl_3$-CH_3OH, 2:2:1, v/v was used as a solvent. Electrochemical fingerprints were collected in two different buffer solutions. The collected fingerprint profiles can be used for density plots construction. The plants can be automatically identified by feature extraction of density plot. The oxidation peaks exhibited in the electrochemical fingerprint are most likely the oxidation of narirutin, naringin, hesperidin, and neohesperidin. Therefore, HPLC was used for the validation of the standards and samples. Finally, electrochemical techniques were used to document the electrochemical behavior of these four substances. The results suggest that narirutin, naringin, hesperidin, and neohesperidin may be the most significant substances contributing to the electrochemical fingerprinting of fructus aurantii.

Keywords: Fructus aurantii. Electrochemical fingerprints; Plant identification; Pattern recognition; Feature extraction

1. INTRODUCTION

Fructus aurantii is the cultivated varieties are the dried immature fruits of *Citrus aurantium* L. The main commercial specifications are Chuan fructus aurantii, Xiang fructus aurantii, Su fructus aurantii, Jiang fructus aurantii and Qu fructus aurantia [1–3]. Among them, Qu fructus aurantii is the dried unripe fruit of *Citrus maxima* (Burm) Merr. Modernization and pharmacological studies have

shown that fructus aurantii contains chemical components such as flavonoids, volatile oils and alkaloids [4]. It has pharmacological activities such as antibacterial, hypolipidemic and antidepressant. At present, most of the research on fructus aurantii is focused on cultivation technology, harvesting time, drying and processing technology, concoction method, pharmacological activity [5–7], etc. However, there is a lack of research on the determination of flavonoid glycoside components in fructus aurantii and its relatives. Meanwhile, there are difficulties in how to identify different fructus aurantii.

Electrochemical technology is an analytical technique that uses the oxidation or reduction of electrochemically active substances as a signal [8]. It has been widely used in different fields of detection. In botany-related fields, the use of electrochemical fingerprinting for plant identification and phylogenetic investigation is an analytical technique that has emerged in recent years [9–13]. Since different plants have different electrochemically active substances in their organs, they can present different fingerprint profiles. These profiles can be used for identification purposes once the signal is enhanced. On the other hand, the content of these electrochemically active substances is manipulated by the genes of the plant. Thus, their differences also reflect plant differences at the genetic level. Such differences can be used to understand the phylogenetic status of different plants. In this work, we applied this technique to the identification of fructus aurantii. Our results suggest that electrochemical fingerprinting techniques have the potential to be applied to the identification and quality control of herbal medicines.

2. EXPERIMENTAL

2.1. Plant collection and sample processing

Tangerine, tangerine (seedless), fructus aurantii and *Citrus changshanensis* were collected from four origins: Lishui-laozhu, Lishui-bihu, Quzhou-qiuchuan and Quzhou-tianma, respectively. Table 1 shows the specific information. The prepared form was prepared according to the Chinese Pharmacopoeia. Steps for preparing dry samples of plants: Preheat the oven to 130°C. Spread the sliced plants in a baking tray previously lined with bran, place in the oven and bake for 30 min, remove and sift the bran.

Table 1. Information of plant collected for investigation.

Abbreviation	Location	Name	Longitude (°)	Latitude (°)	Altitude (m)
LL	Lishui-laozhu	Tangerine	119.92	28.46	73
LB	Lishui-bihu	Tangerine (seedless)	119.75	28.33	113
QQ	Quzhou-qiuchuan	Fructus aurantii	118.36	28.82	368
QT	Quzhou-tianma	Citrus changshanensis	118.52	28.89	255

2.2. Sample extraction

The dried plant samples were crushed separately and sieved through No. 3 sieve. About 2.0 g of the sample powder was weighed and placed in a 100 mL round bottom flask, (DMSO–$CHCl_3$–CH_3OH, 2:2:1, v/v) was added and then fixed on a condenser. The sample was refluxed at 70 ℃ for 1.5 h. The sample was filtered and concentrated, and then fixed with 80% methanol into a 100 mL volumetric flask, and then filtered through a 0.45 μm microporous membrane.

2.3. Reagents, instruments and parameters

Neohesperidin, hesperidin, naringenin and brassinidin were purchased from Shanghai Yuanye Biotechnology Co. Acetonitrile was chromatographically pure (Beijing Jingke Ruida Technology Co., Ltd.). Methanol, dimethyl sulfoxide (DMSO), $CHCl_3$, phosphoric acid and other reagents were all analytically pure (Sinopharm Chemical Reagent Co., Ltd.). All other reagents were analytical grade and used without further purification.

A 600-2998 high performance liquid chromatograph (Waters, USA) was used to detect neohesperidin, hesperidin, naringenin and brassinosteroid in the samples. the HPLC was performed on a Waters Xbridge C18 column (4.6 mm×250 mm, 5 μm). The mobile phase was acetonitrile-0.1% phosphoric acid water. The detection wavelength was 283 nm, and the column temperature was 30 ℃. The injection volume was 10 μL.

Shanghai T&H CHI760E electrochemical workstation is used to detect electrochemical fingerprints of samples. A three-electrode system was used to detect the electrochemical fingerprints of plants. Glassy carbon electrode, platinum wire electrode and Ag/AgCl electrode were used as working electrode, counter electrode and reference electrode respectively. Differential pulse voltammetry was used to scan the samples. The electrolytes were 0.1 M phosphatic buffer solution (PBS, pH 7.0) and acetate buffer solution (ABS, pH 4.5). The voltammetric profile (fingerprints) of herbal tissue were recorded using differential pulse voltammetry (DPV) at 0-1.0 V, with a pulse amplitude of 50 mV, a pulse width of 0.05 s and a pulse period of 0.5 s. Except the reproducibility test, the fingerprints of herbal tissue were recorded repeated three times in each condition.

3. RESULTS AND DISCUSSION

Figure 1 shows the electrochemical fingerprints of LL, LB, QQ and QT collected under PBS conditions. It can be seen that all four plants have electrochemically active substances involved in the oxidation reaction under PBS conditions. Both LL and LB exhibited 2 electrochemical oxidation peaks, although there were some differences in the positions. Both QQ and QT exhibit three oxidation peaks. Again, there are differences in the location and current values of the oxidation peaks. Due to the complex composition of substances in herbal medicines, a wide variety of chemical components have different polarities [14–17]. Therefore, the selection of a suitable solvent system plays a crucial role in the feasibility of the method. Folic acids and other large polar chemicals can be effectively extracted with

methanol, while trichloromethane can extract small and medium polar chemicals [18]. Dimethyl sulfoxide has a solubilizing effect and can dissolve organic substances in general, especially for the extraction of heterocycles and their polymers. It can also extract organic substances containing nitrogen, sulfur, oxygen, halogens, etc. It also has some solvency ability for inorganic salts or metal organic compounds [19,20]. Therefore, we chose the mixture (DMSO-$CHCl_3$-CH_3OH , 2:2:1, v/v) to obtain the maximum extraction efficiency.

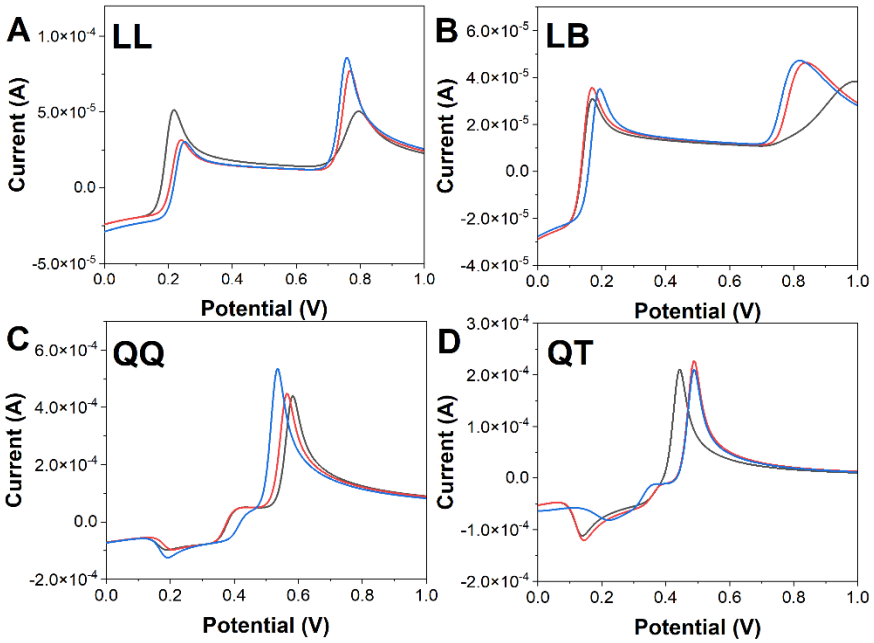

Figure 1. DPV curves of LL, LB, QQ and QT recorded under PBS (0.1 M, pH 7.0) with three individual samples.

To further improve the information abundance of electrochemical fingerprinting, the extracts of the four samples were collected again under ABS conditions. Figure 2 shows the electrochemical fingerprints of LL, LB, QQ and QT collected under ABS conditions. Under ABS conditions, LL and QQ have two distinct oxidation peaks. LB exhibits 2 distinct oxidation peaks and a relatively weak oxidation peak. QT exhibits an oxidation peak at the beginning of the scan, so there is a significant current drop between 0.05 and 0.10 V. Subsequently, it showed a weak oxidation peak and a strong oxidation peak around 0.23 V and 0.38 V, respectively. A large oxidation peak appears again in the QT near 0.75 V. Different potentials are involved in the electrochemical oxidation because of the different pH of the electrolyte. In addition, pH affects the potential of oxidation of the same substance [21,22]. Therefore, electrochemical fingerprinting of samples using different conditions can further improve the abundance of electrochemical fingerprints. Another conclusion can be drawn based on Figure 1 and Figure 2. Although the electrochemical fingerprints of the same plant had some differences, they showed basically the same trend. Therefore, differences between single samples do not affect the identification between different plants. This gives the electrochemical fingerprinting technique the ability to be used for plant identification [23].

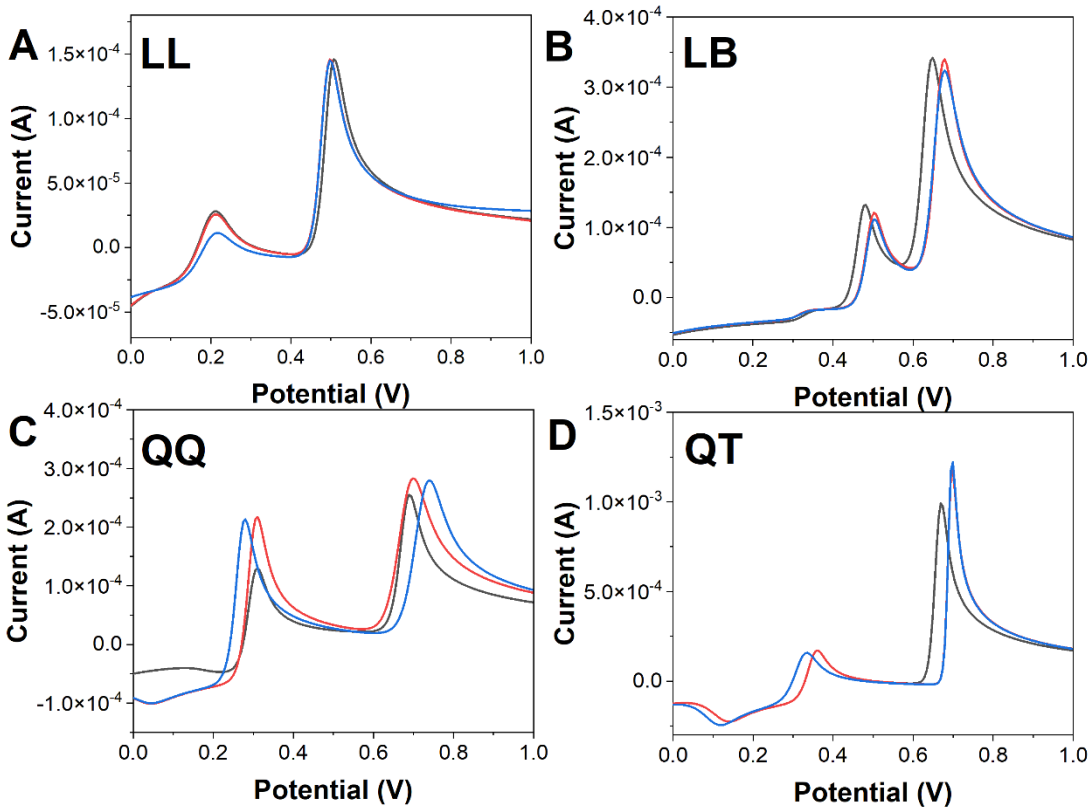

Figure 2. DPV curves of LL, LB, QQ and QT recorded under ABS (0.1 M, pH 4.5) with three individual samples.

To identify the four plant samples, two sets of electrochemical fingerprints were used for the density plot construction. Figure 3 shows the density plots of LL, LB, QQ and QT. It can be seen that the regions with richer amounts of data show highlighting. Subsequently, we tried the feature extraction and fusion methods for plant identification [24]. We extract the features of the image in the following method

$$D = b^2 \cdot n \cdot (v - b + \sigma) \cdot (h - b + \sigma)$$

Where, b: number of edge cell cells in the block region; n: number of histogram channel features; σ: single slide step of the template; υ: number of pixels in the horizontal direction of the cell; h: number of pixels in the vertical direction of the cell [25]. We perform the convolution operation on the density plots to obtain the horizontal and vertical gradient components G_y, G_x, and calculate the current pixel gradient amplitude G.

$$\begin{cases} G_x = \max(G_{rx}, G_{gx}, G_{bx}) \\ G_y = \max(G_{ry}, G_{gy}, G_{by}) \\ G_{x,y} = \sqrt{G_x^2 + G_y^2} \end{cases}$$

Calculate the gradient direction α and Gaussian filtering.

$$\alpha_{x,y} = tan^{-1} \frac{G_x}{G_y}$$

Gaussian noise reduction was applied to the gradient direction to reduce the influence of factors such as cumulative transmission of errors [26]. Statistical intra-cell histogram channel features. The cell pixel feature values are :

$$C_{xy} = 1 - \frac{|\alpha_{x,y} - bin \cdot b - b/2|}{b}$$

The cell histogram channel features are:

$$O_{h,v,b} = \sum_{x=1}^{x} \sum_{y=1}^{y} C_{x,y} \cdot G_{x,y}$$

where h,υ are the coordinates of the block area where the cell is located, b is the number of histogram channel features, X is the number of pixels in the horizontal direction of the cell, and Y is the number of pixels in the vertical direction of the cell.

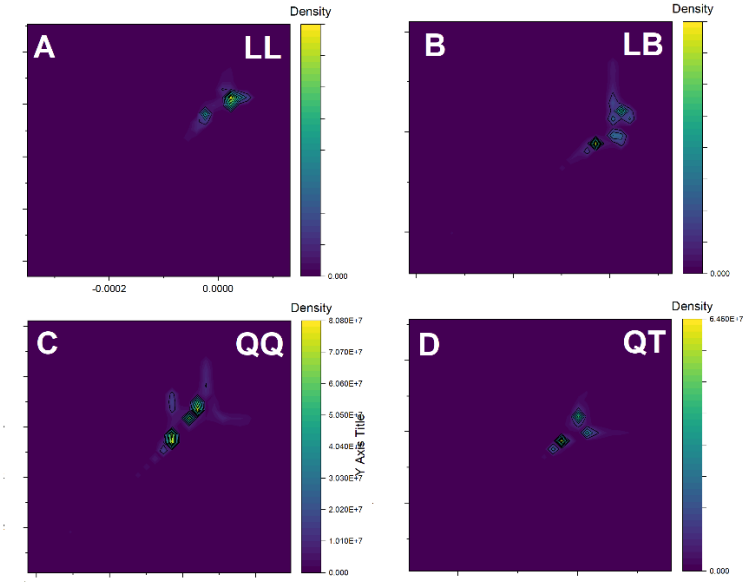

Figure 3. Density patterns of LL, LB, QQ and QT constructed by electrochemical fingerprints recorded under two different conditions.

Table 1 shows the recognition rates of plant samples with different parameters. It can be seen that the automatic recognition rate can reach more than 75%, representing that this recognition technique can achieve automatic recognition of electrochemical fingerprint profiles.

Table 1. Identification rates of plant samples with different parameters.

Image size	b	n	υ	σ	Training time (s)	Test time (s)	Identification rate
64	2	9	8	1	12.21	0.04	64.33
32	2	9	8	1	10.50	0.03	75.01
16	2	6	8	1	9.15	0.03	42.51
32	2	12	8	1	9.24	0.03	69.59

To further verify the composition of the major electrochemical oxidation peaks in the electrochemical fingerprint, we investigated four flavonoid glycoside components in fructus aurantii. Figure 4 shows the HPLC fingerprints of the standards and samples of narirutin, naringin, hesperidin, neohesperidin [27,28]. Table 2 shows the results of the spiked recoveries of QQ. The RSDs of the peak areas of neohesperidin, hesperidin, naringin and narirutin of QQ samples were 0.6841% , 0.0991% , 0.1362% and 0.4988% , respectively. The average contents of neohesperidin, hesperidin, naringin and narirutin in QQ were 249.1474 mg/g, 4.7574 mg/g, 3.1305 mg/g and 1.957 mg/g, respectively, as measured in the repeatability experiment. The RSDs were 0.419%, 0.610% , 0.848% , and 0.311% , respectively. The average spiked recoveries of neohesperidin, hesperidin, naringin and narirutin were 99.63%, 99.26%, 99.03%, and 102.34%, respectively, with RSDs of 1.24%, 1.63%, 2.11%, and 1.85%, respectively.

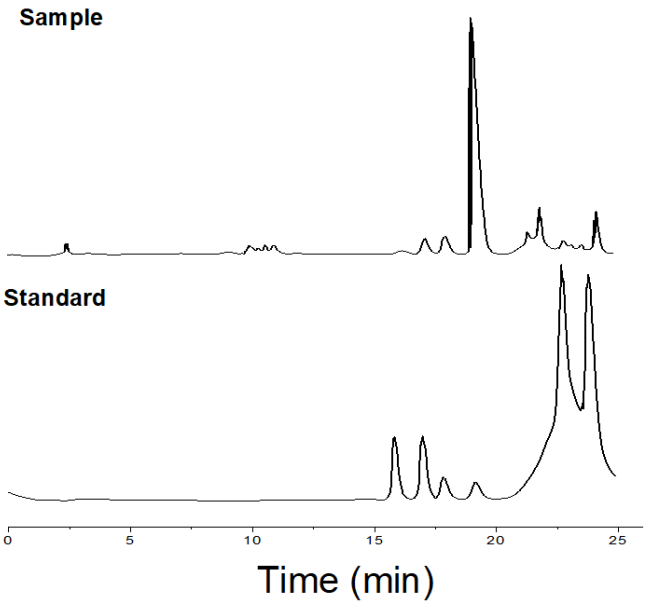

Figure 4. HPLC fingerprints of narirutin, naringin, hesperidin, neohesperidin in mixed standard sample of fructus aurantia.

Table 2. Results of spiked recovery test.

Constituent	Weighing sample (g)	Found (mg)	Added (Added)	Detected (mg)	Recovery (%)	RSD (%)
Narirutin	0.1002	0.1924	0.2000	0.3971	102.34	1.85
Naringin	0.1005	0.3138	0.3000	0.6109	99.03	2.11
Hesperidin	0.1002	0.4775	0.5000	0.9752	99.26	1.24
Neohesperidin	0.0101	2.5173	2.0000	4.5099	99.63	1.63

These four flavonoid glycosides are likely to be the major contributors to the electrochemical oxidation signal in the electrochemical fingerprinting [29]. Therefore, we have collected the electrochemical behavior of these four molecules. Figure 5 shows the electrochemical behavior of narirutin, naringin, hesperidin, and neohesperidin under two conditions (the same conditions as those used for electrochemical fingerprinting acquisition). Narirutin showed an oxidation peak at 0.22 V and 0.76 V in PBS and ABS [30], respectively. Naringin showed an oxidation peak at 0.78 V and 0.23 V for PBS and ABS [31], respectively. Hesperidin showed an oxidation peak at 0.38 V and 0.14 V for PBS and ABS [32], respectively. Neohesperidin showed two oxidation peaks at 0.12 V and 0.24 V under PBS conditions [33]. Under ABS conditions, it showed only one oxidation peak at 0.13 V. The electrochemical oxidation potentials and oxidation peak patterns of these substances were very similar to the electrochemical behaviors in Figures 1 and 2. Therefore, it is likely that the electrochemical behaviors of these four flavonoid glycosides constitute the differences in the electrochemical fingerprints of the different samples.

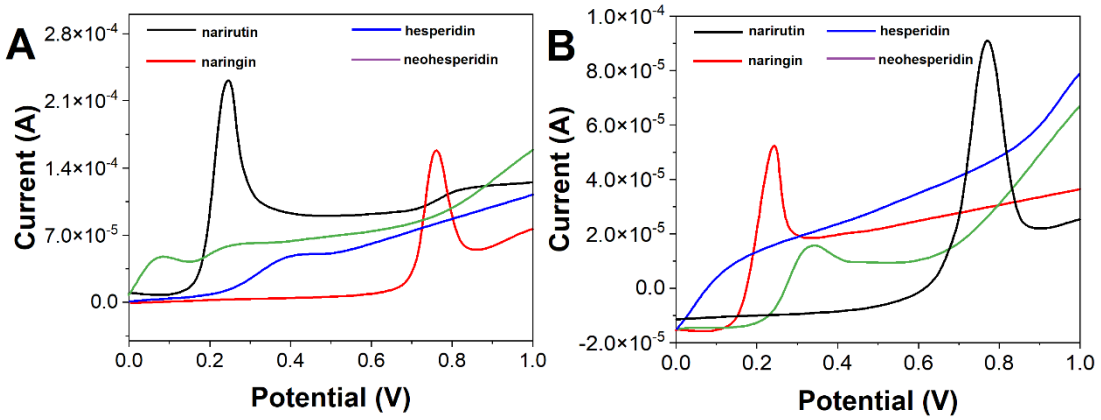

Figure 5. DPV of narirutin, naringin, hesperidin, neohesperidin in (A) PBS (0.1 M, pH 7.0) and (B) ABS (0.1 M, pH 4.5).

4. CONCLUSION

In conclusion, electrochemical fingerprinting was used for the identification of fructus aurantii. PBS and ABS were used as detection environments for fingerprinting. The electrochemical fingerprints showed different behaviors under different buffer solutions. A density plot can be formed for identification purposes using two sets of electrochemical fingerprint profiles. The automatic identification can reach 75% accuracy by feature extraction. The four flavonoid glycosides may contribute significantly to the electrochemical fingerprinting. Therefore, HPLC was used for the separation and detection of the four flavonoid glycosides. In addition, the electrochemical behavior of these four flavonoid glycosides was investigated. The results demonstrated that the electrochemical behaviors of narirutin, naringin, hesperidin, and neohesperidin were very similar to those of fingerprinting.

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