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Extraction and electrochemical fingerprinting of total flavonoids from *Hovenia spp.*

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The effective components of medicinal and edible plant are an important raw material for biological pharmacy and health food, thus flavonoids have become a research focus in biomedical field. *Hovenia spp.* is rich in flavonoids with a number of pharmacological effects. In this work, ultrasonic assisted and ethanol reflux method was first to be adopted to extract the total flavonoids of *Hovenia spp*. The effects of solvent concentration, liquid-to-solid ratio, ultrasonic/ethanol reflux temperature and extraction time in the extraction process were investigated. Afterwards, the electrochemical fingerprinting of total flavonoids were recorded. Three factors extracted from PCA analysis can reach an interpretation rate of 98%. The identification of each species can be achieved by locating the hot area of the 2D density pattern, which is much easier compared with using electrochemical fingerprinting.

Keywords: Electrochemical fingerprint; Total flavonoids; *Hovenia*; Extraction; PCA analysis

1. INTRODUCTION

Hovenia has three species and two varieties includes *H. acerba*, *H. dulcis*, *H. acerba* var. kiukiangensis, *H. trchocarpa* var. trcfocarpa and *H. trchocarpa* vat. robusta. According to literature, *H. acerba* mainly contains alkaloids, flavonoids, saponins, organic acids and glucans. Takai et al. [1] isolated peptide alkaloids: frangulanine and des-N-methylfrangulanine from the root bark of *H. acerba*, among which des-N-methylfrangulanine is ion selective for the induction of mitochondrial lysis tumors. The structures of flavonoids obtained from *H. acerba* are mainly flavonols, flavanols and dihydroflavonols, such as quercetin II, dihydrokaempferol I, (+)-3, 3′, 5, 5′, 7-pen-tahydroflavanone III, [(+)-dihy-dromyricetin IV, emodin, kaempferol, vanillicacid, apigenin, myricetin and 4′, 5, 7-trihydroxy-3′, 5′-methoxy-flavanone [2–8]. The genetic resources of these plants have potential value of application in interspecific distant hybrid breeding within the genus *Hovenia*, thus the taxonomic and

phylogenetic study is not limited to a theoretical issue, but also a basis for application [9–11]. It is of great significance for an in-depth study of the phylogeny of the species of the genus *Hovenia* as well as for the introduction of its cultivation and utilization [12,13].

Flavonoids are widely distributed in the plant kingdom, mainly found in the families Leguminosae, Asteraceae, Aromaticaceae, Ginkgo, Labiatae, and Umbelliferae. They are an important class of polyphenolic natural products of plants through photosynthesis. According to the degree of oxidation of the central three-carbon chain, the B-ring connection position and the possibility of forming a ring with the three-carbon chain, the main flavonoids can be divided into flavonoids, isoflavonoids, chalcones, anthocyanins and flavanones [14–17]. Most flavonoids are crystalline solids, while a few of them are amorphous powders with high melting points and rotational properties. Glycosides, monoglycosides, and disaccharides exist in different states in flavonoids, and their solubility varies. Generally speaking, molecules that are more planar, such as flavonoids and flavonols, are difficult to be soluble in water due to the tight arrangement among molecules and the gravitational force among molecules [18,19]. Dihydroflavones and dihydroflavonols are non-planar molecules, thus they have less intermolecular gravitational force and are more soluble than flavonols. In contrast, the solubility of free glycosides in water is very low, which is almost insoluble, however, they are more soluble in organic solvents such as ethanol and methanol [20,21].

The methods of extracting flavonoids often include maceration method, decoction method, percolation method, solvent reflux extraction method. In recent years, with the development of various modern extraction and separation techniques, ultrasonic-assisted extraction, microwave-assisted extraction, supercritical fluid extraction, semi-biomimetic extraction and enzymatic extraction have also been applied to the extraction process of various flavonoids to improve the extraction efficiency.

Electrochemical fingerprinting is a technique for collecting electrochemically active substances in plant tissues. Since the electrochemical signal is proportional to the type and content of the substance, it can reflect the difference of electrochemically active substance profile in plants. This technique has been applied in recent years to the study of plant phylogeny and has become an alternative technique to provide evidence [22–33]. In this work, ultrasonic assisted and ethanol reflux method was first to be adopted to extract the total flavonoids of *Hovenia spp*. The effects of solvent concentration, liquid-to-solid ratio, ultrasonic/ethanol reflux temperature and extraction time in the extraction process were investigated. Afterwards, the electrochemical fingerprinting of total flavonoids was recorded. The results of the obtained cluster analysis and the proposed claims were studied comparatively.

2. EXPERIMENTAL

2.1 Materials

Rutin was purchased from Shanghai Jinsui Biotechnology Co. Anhydrous ethanol was purchased from Tianjin Tianli Chemical & Reagent Co. Sodium nitrite and sodium hydroxide were purchased from Tianjin Kaitong Chemical Reagent Co. Aluminum nitrate was purchased from Shanghai Shanpu Chemical Co. *H. acerba*, *H. dulcis*, *H. acerba* var. kiukiangensis, *H. trchocarpa* var. trcfocarpa and *H.*

trchocarpa vat. robusta were purchased from Bozhou Guoyuan Chinese herbal medicines and tablets Co.

2.2 Instruments

The UV spectrophotometer is a 2450UV produced by Shimadzu, Japan. All the electrochemical fingerprint recordings were conducted with a CHI600 electrochemical workstation. A commercial glassy carbon electrode (GCE), an Ag/AgCl electrode and a Pt electrode were adopted as the working electrode, reference electrode and counter electrode, respectively. The automatic balance centrifuge is TDZ5 produced by Changsha Pingfan Instrument Co and the rotary evaporator is RE-52B produced by Shanghai Yarong Biochemical Instrument Co.

2.3 Ultrasonic-assisted extraction of total flavonoid

The pre-treated dried plant sample was accurately weighed 10.0 g and placed in a standard mill-mouth three-neck flask. According to the experimental conditions, the sample was placed in an ultrasonic cleaner and extracted with a certain concentration of ethanol solution at a certain extraction time, temperature and solid-liquid ratio. The extraction solution was filtered under reduced pressure, cooled, and fixed to 500 mL with the same concentration of ethanol used in the extraction. 1 mL of the extraction solution was accurately measured from 10 mL solution.

2.4 Ethanol reflux extraction of total flavonoid

10.0 g of the pretreated and dried plant was accurately weighed and an amount of ethanol was added. After reflux extraction under set conditions, the filtrate was filtered, cooled, and fixed with the same concentration of ethanol solution used in the extraction. 1 mL of the extract was accurately measured from a 10 mL volume.

2.5 Determination of total flavonoids

The determination of the total flavonoid content of *Hovenia spp*. was performed using the UV spectrophotometric method with rutin being the control. 10.0 mg of rutin standard control was weighed at 120℃, dissolved with 80% (v/v) ethanol, and poured into a 50 mL volumetric flask to make a control solution of 0.2 mg/mL of total flavonoids. A certain amount of rutin standard solution was selected in a 10 mL volumetric flask with 1 mL of 5% sodium nitrite solution added, and the solution was well shaken. After standing for 6 min, 1 mL 10% aluminum nitrate solution was added and well shaken. After standing for 6 min, 4 mL 4% NaOH solution was added, then the volume was fixed to 10 mL with 30% (v/v) ethanol solution and well shaken. After standing for 15 min, it was centrifuged at 4000r/min for 6 min, and used as the standard solution after color development.

2.6 Electrochemical fingerprinting

Electrochemical fingerprinting was performed at two conditions include 0.1 M of PBS (phosphate buffer solution, pH=7.0) and 0.1 M of ABS (acetic acid buffer solution, pH=4.5). A glassy carbon electrode was adopted as a working electrode for recording the electrochemical fingerprint. A Pt wire and an Ag/AgCl (3M KCl) were adopted as counter electrode and reference electrode, respectively. A differential pulse voltammetry (DPV) was applied as scan method in recording the electrochemical fingerprint. The scan range is between -0.4 V to 1.8 V (pulse amplitude: 50 mV; pulse width: 0.05 s; pulse period: 0.5 s). A normalization process was conducted for all recorded electrochemical fingerprints, where the ratios between the current and the maximum peak current were obtained at different potentials. The taxonomic analysis was performed with hierarchical clustering method [34]. 2D density pattern and principal component analysis (PCA) were carried out for all electrochemical fingerprints with the Origin. The electrochemical data recorded under PBS was used as x axis, while the data recorded under ABS was used as y axis for 2D pattern generation.

3. RESULTS AND DISCUSSION

The parameters of ultrasonic-assisted extraction of total flavonoids were optimized first. As shown in Figure 1A, the increase of total flavonoid extraction is more obvious with the increase of ethanol concentration. When the concentration of ethanol reaches 60%~80%, the increase of total flavonoid extraction rate is flat and the maximum value appeared. When the concentration of ethanol increases to 90%, the extraction rate of total flavonoids decreases. According to the principle of similar solubility, the maximum solubility can be achieved with similar polarity [35,36]. When the concentration of ethanol is 60% to 80%, the polarity of the solution is close to the polarity of total flavonoids from *Hovenia spp*., which is closest to 70%, and the total flavonoid extraction rate reaches its peak. Therefore, when the ethanol concentration continues to increase [37], the total flavonoid extraction rate tends to decrease, thus the concentration of ethanol was chosen to be around 70%.

From Figure 1B, it can be seen that the extraction rate of total flavonoids increases significantly as the liquid-solid ratio increases from 10:1 to 15:1, which indicates that the increase of solvent amount affects the diffusion process of total flavonoids from the inside to the outside of the raw material [38–40]. After the liquid-solid ratio increases to 15:1, the increase in the liquid-solid ratio does not have a significant effect on the extraction rate of total flavonoids from *Hovenia spp*. In order to reduce the difficulty of the subsequent process and lower the cost, the experiment was conducted directly with a liquid-solid ratio of 15:1.

Figure 1C shows that the extraction rate of total flavonoids increases with the temperature. When the temperature exceeds 50℃, the total flavonoids can be partially degraded by the high temperature [41]. Therefore, considering the cycle time and energy consumption of the production process, the extraction temperature of about 50℃ was used as the selection range for the orthogonal experiment.

Figure 1D presents that the extraction rate of total flavonoids increases more from the stage of 25 min to 45 min of sonication. With the extension of ultrasonic action time, the extraction rate of total flavonoids after more than 45 min decreases instead. In the initial stage of ultrasonic intensification, ultrasonic cavitation accelerates the dissolution of active ingredients on the surface of the material and damages the cell wall to some extent [42,43]. As a result, the leaching rate of total flavonoids increases accordingly. However, after 45 min of sonication, the concentrations inside and outside the cells reach equilibrium with the extension of time. The oxidation time of total flavonoids in air increases, the dissolved impurities also increases correspondingly, and the final result is the relative decrease of flavonoid yield [44,45]. Therefore, this experiment chose the sonication time of about 45 min as the selection range.

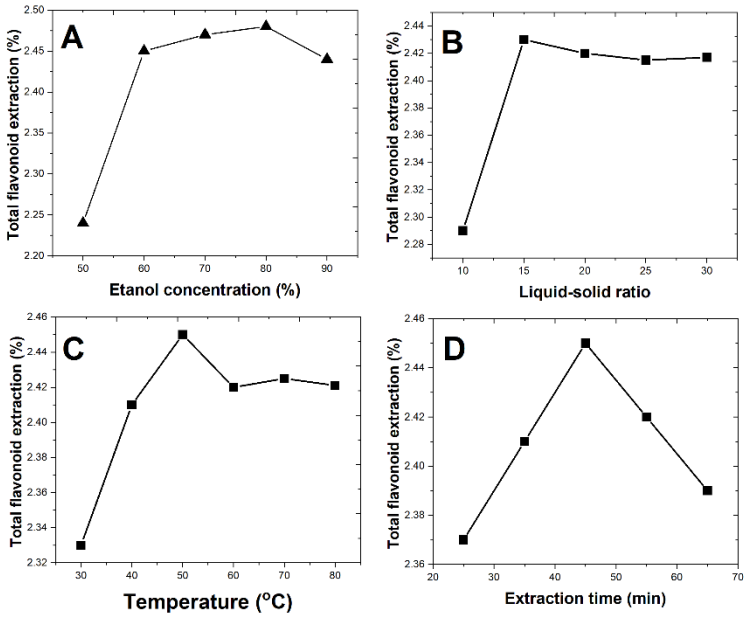

Figure 1. Effect of (A) ethanol concentration, (B) liquid-solid ratio, (C) temperature, (D) time on ultrasonic-assisted extraction of total flavonoid.

The parameters of ethanol reflux extraction of total flavonoid were optimized as well. As shown in Figure 2A, the extraction rate of total flavonoids increases with the extraction time. When the temperature reaches 80 min, it tends to be more stable and the variation is not significant [46]. According to the economic principle, the extraction time of 80 min was chosen as the appropriate time for the experiment.

It can be noted from Figure 2B that the extraction rate of total flavonoids increases with the ethanol volume fraction. The maximum value is reached as the volume fraction of ethanol is 70%, after which the extraction rate decreases, the possible reason for which is that total flavonoids exist mainly in the form of flavonoid sapogenins and glycosides [47–49]. The glycosidic components are easily soluble in ethanol and the glycosides are easily soluble in water [50]. At 70% ethanol volume fraction, the best

ratio of water to ethanol can be achieved. Therefore, the concentration of ethanol was chosen to be about 70% for the experiment.

Figure 2C shows that the extraction rate of total flavonoids increases with the temperature. The extraction rate reaches the maximum as the extraction temperature is 70°C, after which the extraction rate starts to decrease with the increase of temperature. The reason for this phenomenon may be that the soluble proteins in the plant are denatured by solubilization when the temperature increases, which increases the viscosity of the extraction solvent [51], hindering the solubilization of flavonoids from the cells, which in turn decreases the extraction rate of flavonoids. Therefore, the experimental extraction temperature of about 70°C was chosen as the appropriate temperature.

It can be seen from Figure 2D that the total flavonoid extraction rate shows an increasing trend with the increase of ethanol dosage. However, the increase is not significant as the liquid-solid ratio reaches 30:1, and it tends to be stable when the material-liquid ratio is 30:1. Therefore, the experimental selection of the material-liquid ratio of 30:1 is appropriate.

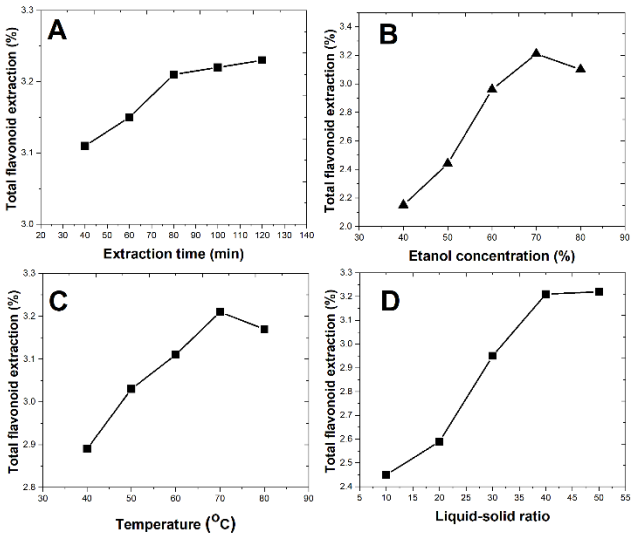

Figure 2. Effect of (A) extraction time, (B) ethanol concentration, (C) temperature, (D) liquid-solid ratio on ethanol reflux extraction of total flavonoid.

Since the total flavonoids extracted with ethanol reflux extraction method is higher than that extracted with the ultrasonic-assisted extraction method, this technique was adopted for the electrochemical fingerprinting study of the extraction of total flavonoids in this study. The electrochemical fingerprints of total flavonoid of *H. acerba*, *H. dulcis*, *H. acerba* var. kiukiangensis, *H. trchocarpa* var. trcfocarpa and *H. trchocarpa* vat. robusta were recorded under PBS first (Figure 3). A series of oxidation peaks can be seen between –0.3 and 1.8 V for either species, representing the involvement of substances in the electrochemical oxidation reaction in the flavonoids [52]. According to previously published papers, these flavonoids are likely to be xanthophylls, quercetin, chenopodophylls, daidzein and catechins [53–56]. The electrochemical fingerprints vary considerably

among the different species, indicating the diversity of flavonoids in different species of *Hovenia spp*. However, some of these species show a similar profile, which suggests that highly similar substances in the flavonoids can be electrochemically oxidized in PBS at pH 7.0. The reason for this phenomenon is that these total flavonoid extracts all come from one genus. The electrochemically active substances possessed by different species are also similar due to their close affinity to each other [57,58]. However, this does not exactly mean that these species have the same flavonoid components, thus there are some molecules that are not electrochemically active. In addition, there are some molecules that are electrochemically active only under acidic or basic conditions. Therefore, in this work, the electrochemical fingerprints were further recorded under different pH values. As shown in Figure 4, the fingerprints of these species of total flavonoids present a very significant difference under ABS condition, the reason for which is that there are electrochemically active substances that can only participate in electrochemical oxidation under acidic conditions. Adjusting the acquisition conditions of electrochemical fingerprinting allows the fingerprints to show more comprehensive information [59].

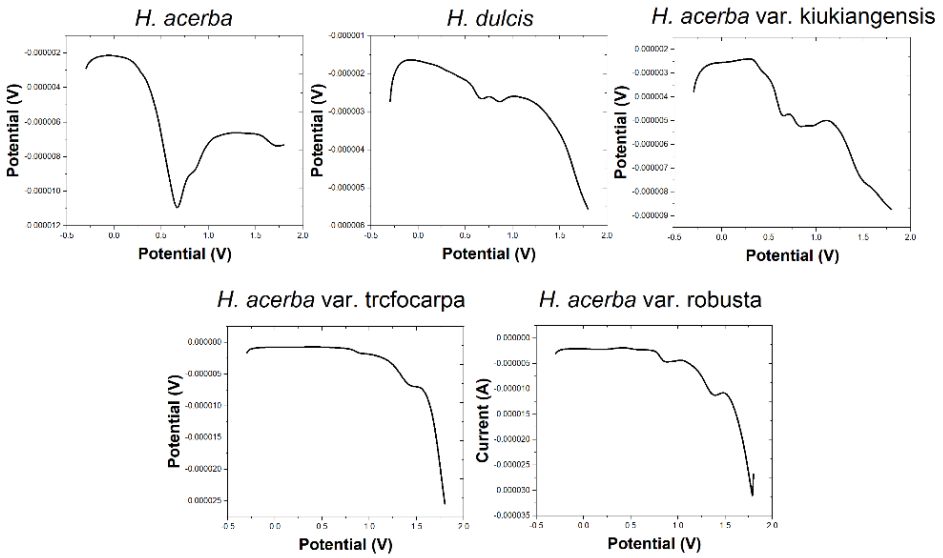

Figure 3. Electrochemical fingerprint of total flavonoid of *H. acerba, H. dulcis, H. acerba var. kiukiangensis, H. trchocarpa var. trcfocarpa* and *H. trchocarpa vat. robusta* recorded under PBS.

In this study, PCA was tried to be adopted to statistically analyze all species. The statistical analysis can explain the similarity of electrochemically active substances among different species. As shown in Figure 5, three eigenvalues of principal components can well represent the difference of all data sets. Three factors extracted from PCA can reach an interpretation rate of 98%. These results indicate that the electrochemical fingerprints among different species are not easily distinguished in a particularly obvious way.

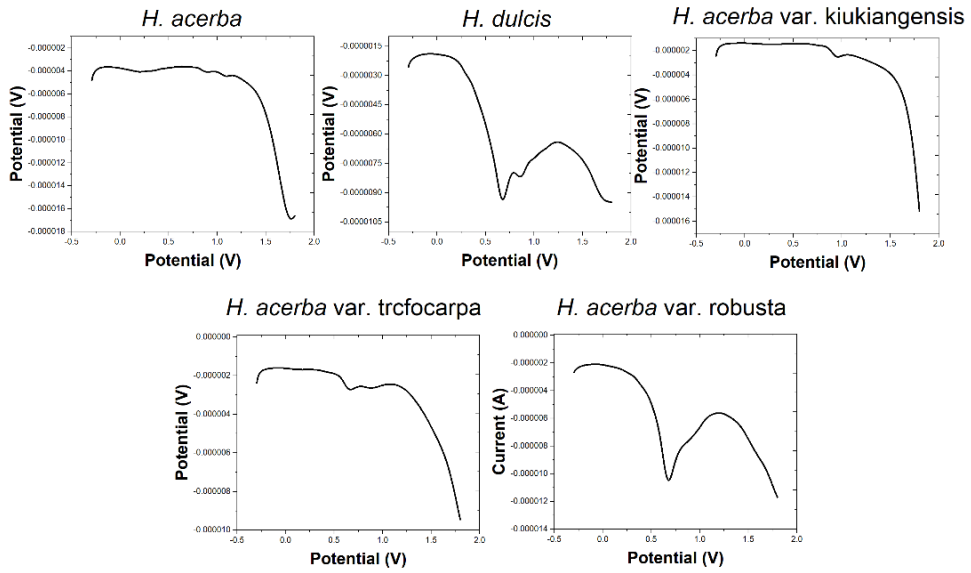

Figure 4. Electrochemical fingerprint of total flavonoid of *H. acerba*, *H. dulcis*, *H. acerba var. kiukiangensis*, *H. trchocarpa var. trcfocarpa* and *H. trchocarpa var. robusta* recorded under ABS.

The distance between *H. acerba* and *H. dulcis* can be seen from the results of PCA, which represents a greater variability of their electrochemical fingerprint data. This result is consistent with the curves presented in Figures 3 and 4. On the contrary, the proximity of *H. trchocarpa* var. *trcfocarpa* and *H. trchocarpa* var. *robusta* means that their electrochemical fingerprints have less variability in distance. The reason for the above is that these two samples are variants of the same species, which is also consistent with Figures 3 and 4.

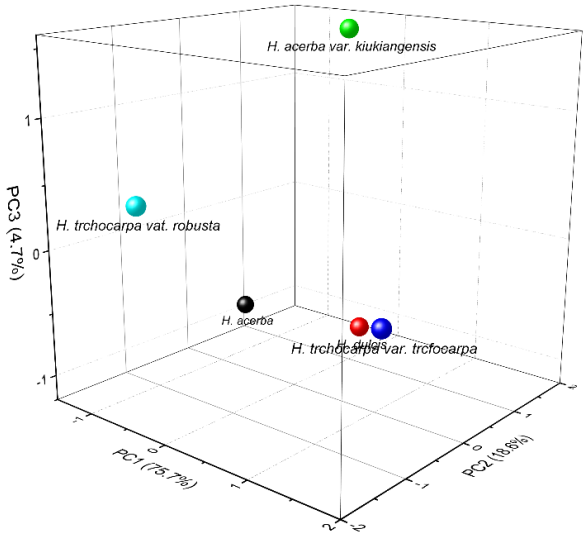

Figure 5. PCA analysis of *H. acerba*, *H. dulcis*, *H. acerba var. kiukiangensis*, *H. trchocarpa var. trcfocarpa* and *H. trchocarpa var. robusta*.

Figure 6 shows the 2D density patterns of *H. acerba, H. dulcis, H. acerba var. kiukiangensis, H. trchocarpa var. trcfocarpa* and *H. trchocarpa vat. robusta*. It can be seen from the figure that the identification of each species can be achieved by locating the hot area of the pattern, which is much easier compared with using electrochemical fingerprinting. Moreover, it is found that this 2D density pattern allows the confidence of electrochemical fingerprinting to be complicated by dimensionality increase, which is highly effective in dealing with cases where there is little variability among data sets. For example, as shown in Figures 3 and 4, the fingerprint patterns of *H. trchocarpa* var. *trcfocarpa* and *H. trchocarpa* var. *robusta* are very similar, however, they can be clearly distinguished in 2D density pattern.

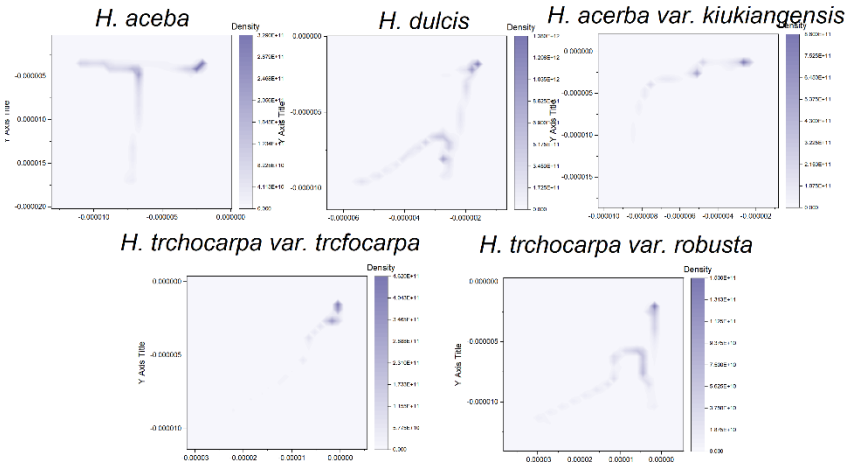

Figure 6. 2D density patterns of *H. acerba, H. dulcis, H. acerba var. kiukiangensis, H. trchocarpa var. trcfocarpa* and *H. trchocarpa vat. robusta*.

4. CONCLUSION

In conclusion, the total flavonoids were extracted from *H. acerba, H. dulcis, H. acerba var. kiukiangensis, H. trchocarpa var. trcfocarpa* and *H. trchocarpa vat. robusta* with the methods of ultrasonic-assisted extraction and ethanol reflux extraction in this work. The electrochemical fingerprints of the 6 species of *Hovenia* were recorded with total flavonoids under PBS and ABS. These electrochemical fingerprints can be used for identification investigation. Three factors extracted from PCA analysis can reach an interpretation rate of 98%. The identification of each species can be achieved by locating the hot area of the 2 D density pattern, which is much easier compared with using electrochemical fingerprinting.

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