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Simultaneous determination of flavonoid and alkaloid compounds in Citrus herbs by high-performance liquid chromatography–photodiode array detection–electrospray mass spectrometry

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Abstract

The major active biological constituents in Citrus herbs are flavonoids, especially hesperidin, naringin and alkaloids, mainly synephrine, with beneficial medical effects on human health. They are used as the markers to control the quality of Citrus herbs. In this paper, a new ion pairing chromatographic method was developed to exclude the most polar solute (synephrine) from the viod volume and to maintain selectivity between the two other solutes (hesperidin and naringin). Perfluorinated carboxylic acids, which are appropriate for MS detection due to their volatility, were used as ion-pairing agents. The problems of the synephrine separation, such as band tailing and low retention, were solved successfully by using perfluorinated carboxylic acids. The effect of heptafluorobutyric acid (HFBA) was the best in the three investigated perfluorinated carboxylic acids. For the flavanone glycosides, the influence of the perfluorinated acids on retention time was rather weak. The two different kinds of the analytes were separated satisfactorily in one run using an isocratic eluent and the total analysis time takes less than 10 min. The abundance of pseudomolecular ions was recorded using selected ion monitoring (SIM) mode of *m/z* 135.1, 273.1 and 303.1 for synephrine, naringin and hesperidin, respectively. The contents of hesperidin, naringin and synephrine in several Citrus herbs were simultaneously determined by the proposed method.

Keywords: HPLC-DAD-ESI/MS; Perfluorinated carboxylic acids; Hesperidin; Naringin; Synephrine; Citrus herbs

1. Introduction

The Citrus herbs (*Cirtus aurantium* L.), as traditional Chinese herbs, have been used for a long time. Recent studies show that citrus herbs have beneficial effects, such as used as antioxidant [1], useful for treatment of obesity [2,3] and have anti-carcinogenic activity [4,5]. A number of citrus species have been recorded in the Chinese Pharmacopoeia as appropriate for medical use, such as *Pericarpium citri reticulatae virde (qingpi), Pericarpium citri reticulatae (chenpi), Fructus aurantii (zhiqiao), Fructus aurantii immaturus (zhishi)*.

The major active biological constituents in these herbs are flavonoids, especially hesperidin, naringin and alkaloids, mainly synephrine [6]. Hesperidin and naringin are effective at inhibiting the in vitro proliferation of human breast cancer cell [7], possess antioxidant [8] and blood lipid-lowering activities [9]. Synephrine is a highly water-soluble sympathomimetic amine which can increase cardiac output and blood pressure [10]. Usually, the two different kinds of compounds are used as the markers to control the quality of the Citrus herbs. However, no method for the simultaneous analysis of the above-mentioned two kinds of bioactive constituents in Citrus herbs has yet been reported.

Recently, there have been a number of methods published on the separation and determination of hesperidin and naringin, either alone or in combination with other flavonoid glycosides in citrus juices, biological fluids or pharmaceutical formulations, including HPLC [11–15], HPLC–UV [16–18], LC–MS [19–22], HPLC–ECD [23,24], μHPLC–ECD [25], CE–ECD [26,27] and CE–UV [28]. However, these methods are not validated for simultaneous determination of synephrine; most of them are time-consuming in sample pretreatment and were used to determine the contents of flavonoids in the extract of only one or two

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species of Citrus herbs. He et al. [29] developed HPLC–ESI-MS method to analyze the phytochemical constituents of an extract of sour orange; however, under their HPLC conditions, synephrine could not be retained on the stationary phase and was eluted almost immediately.

For the determination of synephrine, several methods have been reported. These methods included HPLC–EC [30,31] or HPLC–UV [32,33]. However, most of the mobile phases were not suitable for the LC–ESI-MS analysis owing to the presence of high-concentration sodium salts including sodium dodecyl sulfate (SDS) [34]. In our lab, recently, an aqueous solution of ionic liquid was used as mobile phases for the successful separation of adrenergic amines by liquid chromatography [35]. But the ionic liquid was not suitable for the LC–ESI-MS analysis either. In this work, perfluorinated ion pair agents, which are appropriate for MS detection due to their volatility, were investigated to replace SDS and ionic liquid. In the literature, heptafluorobutyric acid (HFBA) had been used as ion-pairing reagent to improve the separation and retention of herbicides and amino acids [36], but there are no reports on the flavonoids using perfluorinated ion pairing agents.

For the extraction those compounds from plants, the methods of refluxing [29] and sonication [27] were usually adopted. But these methods are time-consuming and need large amount of organic solvents. Clearly, a simple and rapid extraction method for the simultaneous extraction would be very useful. Microwave-assisted extraction (MAE), which is a powerful sample pretreatment technique [37], has been proved to be more efficient to extract the effective constituents from the Chinese herbal medicine compared to refluxing and sonication methods [38]. Herewith, MAE was tried to extract the effective constituents from Citrus herbs.

In this paper, a simple and reliable HPLC-DAD–ESI-MS method was developed to separate simultaneously the flavonoids and alkaloids compounds in Citrus herbs using perfluorinated carboxylic acids as ion-pairing agents. This developed method was successfully applied to quantification of these components contained in several commercially available herbs.

2. Experimental

2.1. Reagents and materials

The standards of hesperidin, naringin and synephrine were purchased form National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China); their chemical structures are shown in Fig. 1. Analytic grade formic acid, trifluoroactic acid (TFA), pentafluoropropionic acid (PFPA) and heptafluorobutyric acid (HFBA) were obtained from Sigma (St. Louis, MO, USA). The traditional Chinese herbs of *Pericarpium citri reticulatae virde (qingpi), Pericarpium citri reticulatae (chenpi), Fructus aurantii (zhiqiao), Fructus aurantii immaturus (zhishi)* were purchased from local pharmaceutical stores (Changsha, China). HPLC-grade methanol and acetonitrile were obtained from Tedia (Fairfield, Ohio, USA). Water was deionized in a Milli-Q water purification system (Millipore Bedford, MA, USA).

2.2. Instrumentation

A Waters-600 HPLC system (Milford, MA, USA) equipped with DAD detection in the range of 210–400 nm was interfaced to Micromass ZQ 4000 electrospray mass spectrometer (Manchester, U.K.). Masslynx V 4.0 software (Micromass) was used for data acquisition and processing. A column of symmetry C_{18} (3.9 mm × 150 mm, 5 μm; Milford, MA, USA) was utilized. An isocratic eluent of 80% A (12 mmol HFBA and 0.05% formic aqueous solution) and 20% B (acetonitrile) at a flow rate of 0.8 ml/min was used and 20% eluent was allowed to flow into the mass spectrometer by solvent splitting. The injection volume was 10 μl. The ESI-MS spectra were acquired in positive-ion

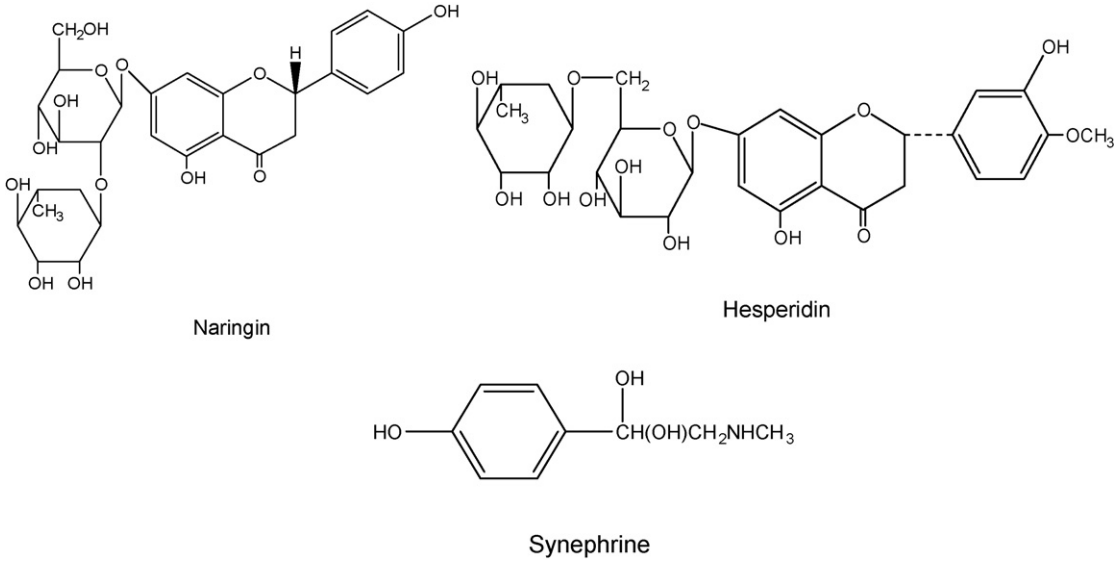

Fig. 1. Chemical structures of hesperidin, naringin and synephrine.

mode by scanning over the *m*/*z* range of 100–800 u. The capillary voltage was set at 4.0 kV and cone voltage was 60 V. Temperatures of the electrospray source and desolvation were 95 and 280 °C, respectively. Nitrogen was used as both nebulizing gas and desolvation gas at flow rates of 50 and 350 l/h, respectively. Quantifier ions used for synephrine, naringin and hesperidin were *m*/*z* 135.1, 273.1 and 303.1, respectively. Selected ion recording (SIR) was used to monitor the abundance of the quantifier ions.

2.3. Microwave-assisted extraction

A household microwave oven with a maximal power of 700 W (Tianjing, PR China) was modified in our laboratory with the addition of a water condenser. The whole system was open and run at atmospheric pressure. 0.1 g well-grounded sample was placed into the cartridge vessel; then appropriate solvent was added. With water running through the condensation pipe, the sample was treated under microwave irradiation in an intermittent way, i.e. irradiation–cooling–irradiation. The irradiation time was kept for 10 s, and 2 min was taken to cool the sample solution between two irradiations. The total optimized extraction time did not include the cooling cycle's time. Prior to LC–MS analysis, the resulted solution was filtered through a 0.45 μm microporous membrane.

2.4. Standard solutions

A mix standard solution containing synephrine (100 μg/ml), naringin (1000 μg/ml) and hesperidin (500 μg/ml) was prepared in methanol. By diluting the standard solutions, a series of standard solutions between 0.01 and 100, 500 μg/ml was obtained, the solutions were stored in refrigerator before using.

3. Results and discussion

3.1. Optimization of the HPLC conditions

The flavonoids and alkaloids were separated individually, based on their different chromatographic behaviours. Firstly, the separation of the flavonoids (hesperidin and naringin) was studied. Acetonitrile aqueous solution was selected as the mobile phase. It was found that for the separation of hesperidin and naringin, the 20% acetonitrile aqueous solution was the best eluent. At higher ratio of the acetonitrile, the peaks of hesperidin and naringin were overlapped. At lower of the ratios, the peaks were broadened and the retention times became longer. Then, under the condition of the 20% acetonitrile aqueous solution as the eluent, the influence of perfluorinated acids on the flavonoids separation was investigated. With the increase of concentrations of perfluorinated acids, the retention times of hesperidin and naringin were affected slightly.

For the separation of synephrine, 5% aqueous acetonitrile containing 0.1% H_3PO_4, 5% aqueous acetonitrile containing 0.1% formic acid and acetonitrile–acetate buffer were tested. In all cases, short retention time (<1.617 min) and peak tailing were observed. Tris and triethylamine were also tested as the ion-pairing agents, little effect on the retention behaviour was observed. However, when 5% acetonitrile with perfluorinated carboxylic acids aqueous solution were used, symmetrical peak shape and strong retention behaviour were obtained. For optimizing the separation conditions of synephrine, the concentrations of perfluorinated carboxylic acids in the mobile phase were investigated. Under the condition of using 5% acetonitrile aqueous solution as the eluent, the relationships between the concentrations of perfluorinated carboxylic acids and the retention time of the synephrine were studied. It was found that the retention of synephrine enhanced significantly with the increase of the concentration of HFBA, the band tailing of the peak was also suppressed. When the concentration of HFBA was over 12 mM, the retention time increased slowly. The influences of PFPA and TFA on synephrine were weaker than HFBA. So HFBA was selected, at the concentration of 12 mM. In order to obtain the suitable retention time of the synephrine, using 12 mM HFBA aqueous solution as the eluent, the influence of acetonitrile concentration was investigated. The result showed that the higher the acetonitrile concentration, the shorter the retention time. When 20% acetonitrile was used, an appropriate retention time of 2.57 min for the synephrine was obtained. Considering the separation condition of the hesperidin and naringin, the isocratic eluent of 80% A (12 mmol HFBA aqueous solution) and 20% B (acetonitrile) was adopted for the separation of the three analytes.

Fig. 2 shows typical HPLC profiles of the alkaloid and flavone glycosides in one run. The peaks are completely separated and the shapes are symmetrical. By using the photodiode array detector (DAD), the max absorption wavelength (λ_{max}) of target peaks was obtained. The optimal wavelengths for simultaneous analysis of synephrine, naringin and hesperidin were set at 273.5 nm and 283 nm, respectively. The chromatograms at 273.5 nm and 283 nm for the analytes are shown in Fig. 2.

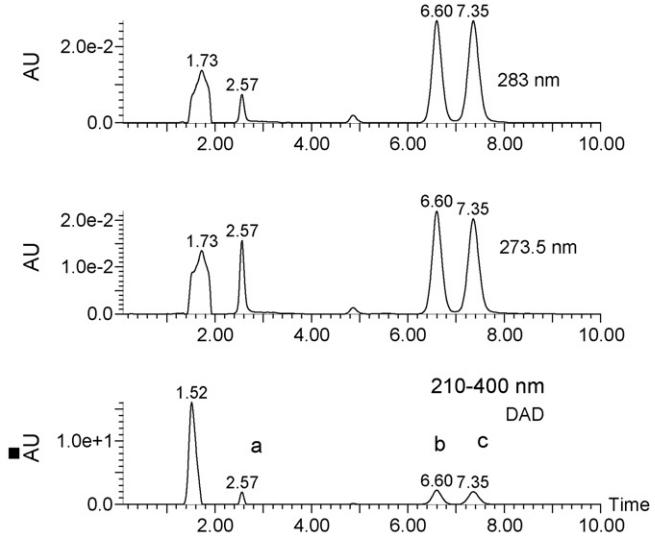

Fig. 2. The typical HPLC profiles of the alkaloid (a, synephrine) and flavone glycosides (b, hesperidin and c, naringin) in one run. The chromatograms for synephrine were recorded at 273.5 nm and at 283 nm for hesperidin and naringin.

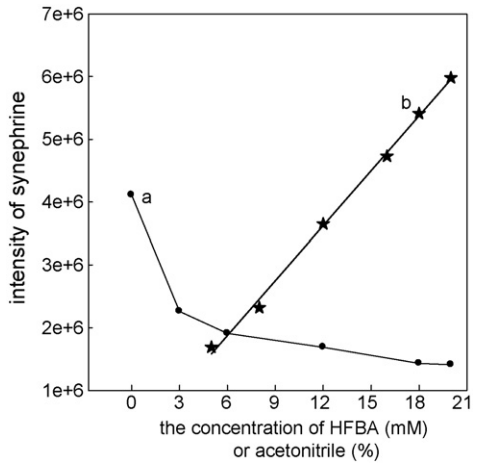

Fig. 3. The influences of the HFBA concentration (curve a) and the content of acetonitrile (curve b) on intensity of synephrine.

3.2. Optimization of MS condition

In order to obtain the best ESI mass spectra, the influences of the capillary voltage, cone voltage, desolvation gas and desolvation temperature were investigated by flow injection analysis (FIA) mode. Significant variation in the intensity of the analytes was not observed when capillary voltage, desolvation gas were varied from 2500 to 4000 V, and from 150 to 350 l/h, respectively. And the capillary voltage was set at 4000 V; desolvation temperature was set at 280 °C. In-source collision-induced dissociation (CID) was used to generate useful fragment ions by varying the cone voltage from 20 to 100 V. The maximum signal intensity of the useful fragment ions was gained at cone voltage of 60 V, while maintaining sufficient $[M+Na]^+$ response. At cone voltage above 60 V, more insignificant small fragment ions were produced and the signal intensity was decreased. In this work, in order to obtain the useful fragments and high intensity, an optimum cone voltage setting of 60 V was chosen for analysis.

3.2.1. Influence of perfluorinated acids on the intensity of the analytes

With the HFBA as additive, the suppression of ionization of analytes was observed. Take synephrine, for example; Fig. 3a shows the influence of the HFBA concentration on intensity of synephrine using 5% acetonitrile as mobile phase. The intensity of synephrine decreased quickly, then slowly. Under the condition of 12 mM HFBA as eluent, the intensity was improved with the increase of acetonitrile content, as shown in Fig. 3b. Comparing Fig. 3a and b, it can be seen that the effect of acetonitrile is stronger than the suppression effect of HBFA. In order to improve the signal intensity further, formic acid was tested as additive in the mobile phase [39]. In this work, 0.05% formic acid was used. Compared to the signal intensity with no formic acid added, the intensities of the analytes were improved slightly.

3.3. HPLC–MS identification of hesperidin, naringin and synephrine

Fig. 4 shows the positive-ion ESI-MS spectra for the compounds at cone voltages 60 V. The signals at *m/z* 135.1 and

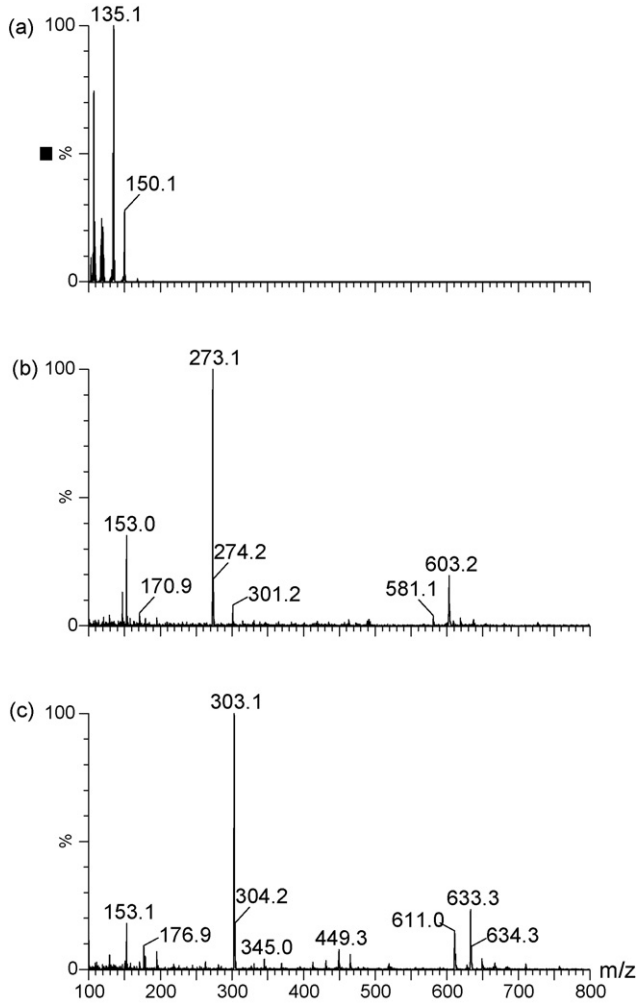

Fig. 4. The positive-ion ESI-MS spectra for the compounds at cone voltage 60 V; (a) synephrine, (b) naringin and (c) hesperidin.

150.1 in synephrine mass spectrum (Fig. 4a) indicate fragments $[M-NH_3\text{-}CH_3]^+$ and $[M-OH]^+$, respectively. Naringin (Fig. 4b) shows both weak molecular ion $[M+H]^+$ at 581.3 and adduct ion $[M+Na]^+$ at *m/z* 603.2, and an intense aglycone ion $[A+H]^+$ at *m/z* 273.1, resulting from the elimination of two sugars. Hesperidin (Fig. 4c) shows an intense aglycone ion $[A+H]^+$ at *m/z* 303.1, weak molecular ion $[M+H]^+$ at *m/z* 611.0 and adduct ion $[M+Na]^+$ at *m/z* 633.3. In our experiments, selected ion recording mode, which was more sensitive than full scan, was used to monitor the analytes and the abundant ions at *m/z* 135.1, 273.1 and 303.1 were selected as quantifier ions for synephrine, naringin and hesperidin, respectively. The SIR chromatograms are shown in Fig. 5 under the above analysis condition. Therefore, based on the standard compounds retention times, UV (λ_{max}) spectra and HPLC–MS-SIR chromatograms, this proposed method can be used as a simple method for identification of these compounds in complex samples.

3.4. Optimization of microwave-assisted extraction

During microwave-assisted extraction from the traditional Chinese herbs, there are many variables affecting the extrac-

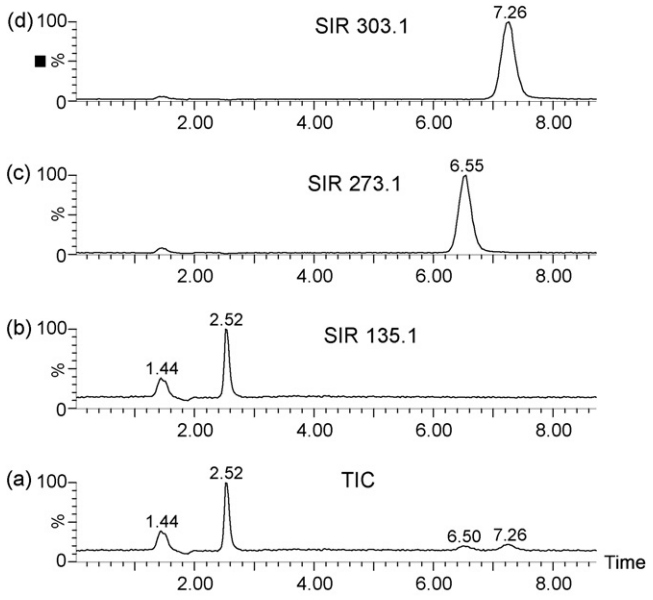

Fig. 5. (a) HPLC–TIC-MS chromatograms of the three analytes; (b) HPLC–MS-SIR chromatogram for synephrine at *m/z* 135.1; (c) HPLC–MS-SIR chromatogram for naringin at *m/z* 273.1 and (d) HPLC–MS-SIR chromatogram for hesperidin at *m/z* 303.1.

tion efficiency. However, studying all the parameters is time-consuming and unnecessary. In this work, four major factors, i.e. microwave power, irradiation time, solvent volume and extraction solvent, were tested to extract the Citrus herbs.

Taking *Pericarpium citri reticulatae*, for example, the peak areas of compound's quantifier ions were used to calculate the extraction efficiencies. The peak areas of hesperidin, naringin and synephrine under different microwave powers are shown in Fig. 6a. Results indicated that 50% microwave power was the optimal irradiation powder. Under the 90% microwave power, the three components might be decomposed and 50% microwave power is beneficial for the extraction.

Fig. 6b gives the relationships between extraction efficiencies and methanol concentration in water. For these analytes, 80% methanol aqueous solution is a suitable extraction solvent. The influence of the solvent volume on extraction efficiencies is shown in Fig. 6c. Firstly, the extraction efficiency is increased with the increase of the solvent volume. Then a weaker increase of extraction efficiency is obtained by further increasing of the solvent volume. When the solvent volume is above 20 ml, the extraction efficiency is increased very slowly. In this work, 20 ml solvent volume was chosen as the optimized volume.

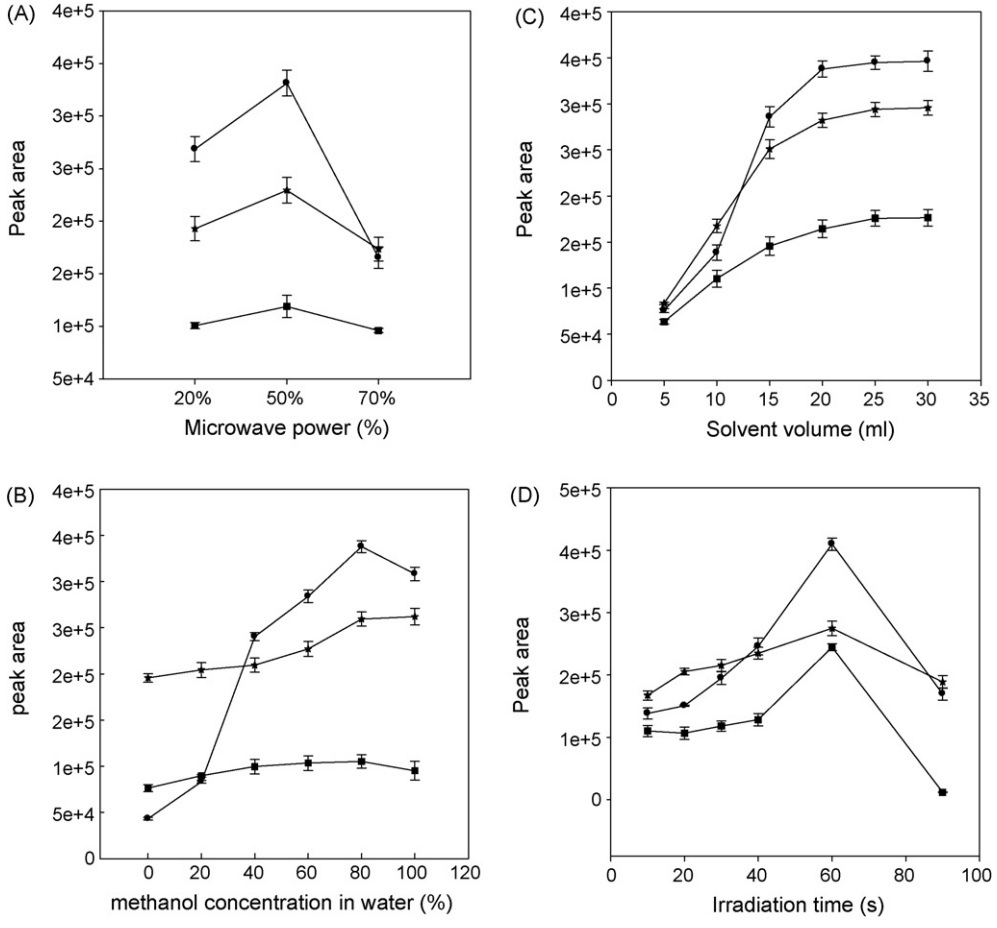

Fig. 6. Effects of microwave power (a), methanol concentration in water (b), solvent volume (c) and irradiation time (d) on the peak areas of hesperidin (●), naringin (■) and synephrine (⋆).

Fig. 6d shows the influence of the irradiation time on extraction efficiencies under the conditions of 50% microwave power and 20 ml 80% methanol aqueous solution. When the irradiation time was increased from 10 to 90 s, the extraction efficiencies of the analytes increased at first, and then decreased. Longer extraction time (>60 s) might cause degradation or conversion of the analytes. So extraction time was set at 60 s.

Based on the above experiments, the optimum microwave-assisted extraction conditions for the extraction of hesperidin, naringin and synephrine from Citrus herbs are as follows: 50%

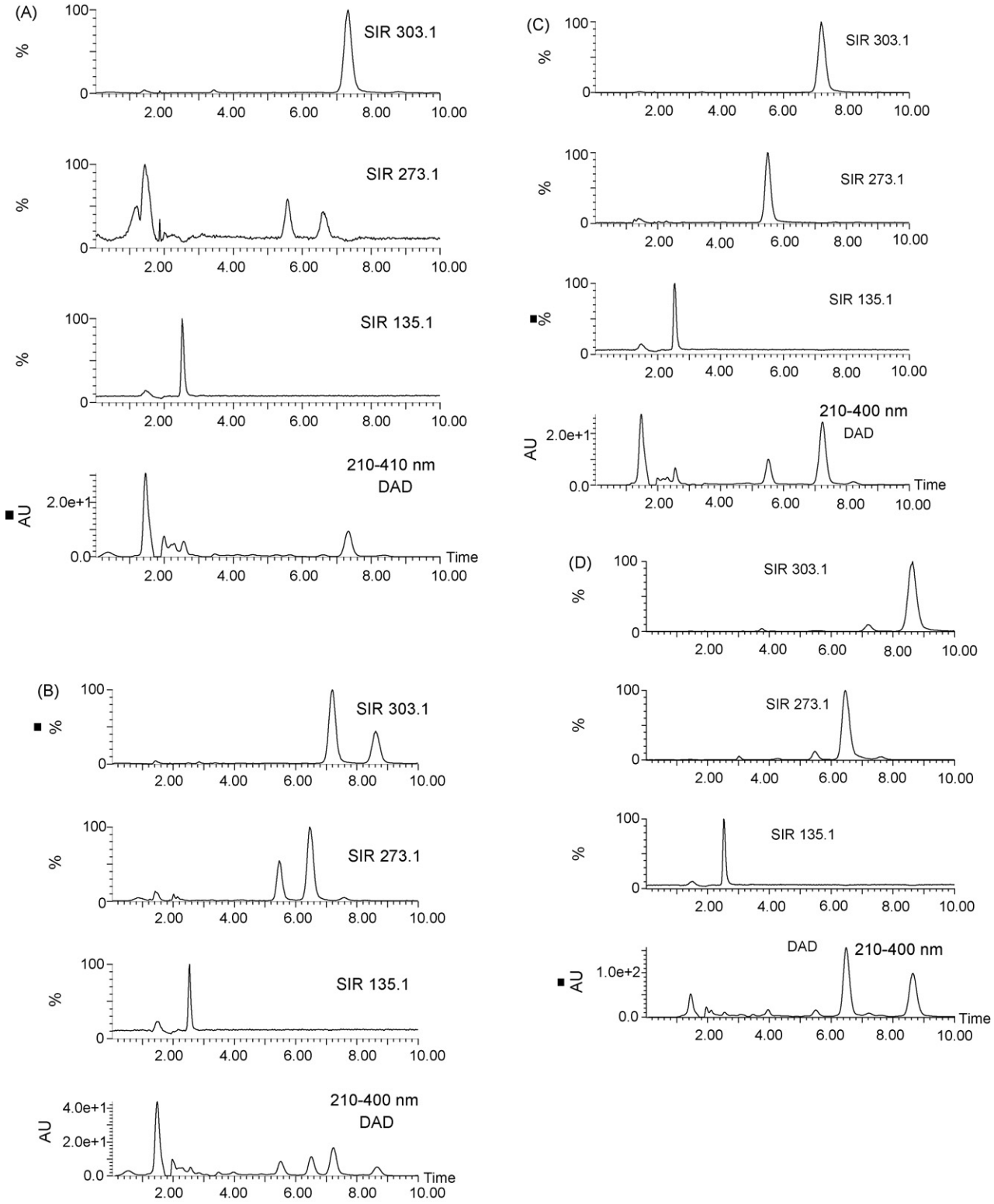

Fig. 7. HPLC-DAD chromatograms and HPLC–MS-SIR chromatograms of the Citrus herbs extracts. (a) *Pericarpium citri reticulatae virde*, (b) *Pericarpium citri reticulatae*, (c) *Fructus aurantii immaturus* and (d) *Fructus aurantii*.

Table 1
Linear range, correlation coefficients (r) and limits of detection (LOD) of the compounds

Compounds	Linear range (μg/ml)	r	Recovery (%)	LOD (μg/ml)
Synephrine	1–100	0.9995	101.7	0.2
Naringin	1–200	0.9997	102.5	0.03
Hesperidin	1–200	0.9993	102.3	0.02

microwave power, 80% (v/v) methanol aqueous solution as extraction solvent, 20 ml solvent for 0.1 g sample and 60 s irradiation times.

3.5. Method validation

3.5.1. Linearity and limit of detection

Under the above optimum analysis conditions, the UV linearity was studied over the concentration range of 1–100 μg/ml for synephrine, and 1–200 μg/ml for naringin and hesperidin. Good linear relationships between the corresponding peak areas and the concentrations were obtained. The limit of detection (LOD) was measured by injecting serial diluted standard solutions, taking the signal-to-noise ratio of 3 as criteria. The linear ranges, correlation coefficients (r) and limits of detection are summarized in Table 1.

3.5.2. Reproducibility

The reproducibility of the method was evaluated by five consecutive injections of the standard solutions. The relative standard deviations (RSDs) of the intra-daily were 1.5% for hesperidin, 1.6% for naringin and 1.2% for synephrine. And the RSD of inter-daily (three consecutive days) were less than 3.5% for all the compounds.

3.5.3. Recovery

The recovery of the method was studied by calculating the mean recoveries of the analytes using the standard addition method. The reference standards were added at three different concentration levels (approximately equivalent to 0.5, 1.0 and 1.2 times of the concentration of the matrix) with three parallels at each level. The solutions were prepared in accordance with the sample preparation procedure. The recoveries of synephrine, hesperidin and naringin were tested with *Pericarpium citri reticulatae virde* as the matrix. The obtained mean recoveries are shown in Table 1. The results showed the recovery of the proposed method was satisfactory.

3.6. Sample analysis

Four kinds of Citrus herbs were extracted under the optimum focused microwave-assisted extraction conditions and the extracts were analyzed by the proposed HPLC-DAD-ESI method. Fig. 7 shows the HPLC–UV and HPLC–ESI–SIR chromatograms of active compounds in the four extracts. The contents were calculated with external standard method. The mean contents of synephrine, naringin and hesperidin in samples from three parallel determinations are summarized in Table 2. The contents of hesperidin, naringin and synephrine are quite different in Citrus herbs. Synephrine was detected in all tested citrus herb samples. Hesperidin was the major flavonoid glycoside found in *Pericarpium citri reticulatae virde, Pericarpium citri reticulatae and Fructus aurantii immaturus* extracts. In *Fructus aurantii* sample, naringin was the main flavonoid glycoside. Hesperidin cannot be detected by UV detector, but in SIR chromatogram, it was found at a very low level. It is shown that the practicability of this method was satisfactory. And the developed HPLC–UV–ESI method can be used as quality control method for the Citrus herbs.

Table 2
Contents (mg/g) of synephrine, naringin and hesperidin in Citrus herbs (mean ± S.D.; $n = 3$)

Sample	Synephrine	Naringin	Hesperidin
Pericarpium citri reticulatae virde	3.66 ± 1.34	0.53 ± 0.32	12.89 ± 0.86
Pericarpium citri reticulatae	1.65 ± 1.06	8.9 ± 1.57	15.06 ± 1.01
Fructus aurantii	4.63 ± 1.36	78.9 ± 0.13	ND
Fructus aurantii immaturus	3.34 ± 1.22	ND	22.73 ± 0.96

ND, not detected.

4. Conclusions

In this paper, an HPLC-DAD-ESI-MS method for simultaneous analyses of alkaloid and flavanone glucoside compounds in extracts of Citrus herbs has been developed. Sample pretreatment using microwave-assisted extraction is simple and rapid (irradiated for 60 s) compared to sonication extraction. Volatile perfluorinated acid HFBA was used as a mobile phase additive for improving the retention behaviour and peak shape of synephrine. Using an isocratic eluent of 80% A (12 mM HFBA and 0.05% formic acid aqueous solution) and 20% B (acetonitrile), the three analytes were separated in a one run and the total analysis time only needs 10 min. The proposed HPLC-DAD-ESI-MS technique provides more qualitative and quantitative information comparing with conventional HPLC. The contents of synephrine, naringin and hesperidin in Citrus herbs were accurately determined by this method, which provides a simple and effective whole quality control about the extract of Citrus herbs for pharmaceutical practice. Furthermore, this method will also be useful in further developing analytical method for other related herbal medicines.

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