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# Separation and Identification of the Fifteen Constituents in Forsythiae Fructus

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## ABSTRACT

Forsythiae Fructus, a commonly used Chinese herbal medicine derived from the dried seeds of Oleaceae plants, contains rengyoside B **(1)**, matairesinol **(2)**, salidroside **(3)**, quercitrin **(4)**, suspensaside **(5)**, rengyoside C **(6),** matairesinoside **(7),** forsythiaside **(8),** pinoresinol-β-D-glucoside **(9),** cornoside **(10),** acteoside **(11),** arctigenin **(12),** arctiin **(13),** phillyrin **(14)** and pinoresinol **(15)** as its major bioactive constituents. In this study, a high performance liquid chromatography (HPLC) method using a Cosmosil 5C18-AR Π column, and a mixture of phosphate solution and methanol-acetonitrile as the mobile phase with detection of absorbance at 254 or 280 nm was developed to successfully resolve these 15 compounds within 60 min. Peaks were identified by matching the UV spectra and MS information with those of authentic standards or literature data. The method was applicable to LC-MS if the phosphate was replaced with an acetate solution. Calibration curves for 4 of the 15 constituents were obtained around a concentration range of 2 orders of magnitude and showed good linearity. The reproducibility (RSD) of the proposed method was 0.23-1.50% (intraday) and 0.41-2.40% (interday). The detection limits (S/N = 3) for the constituents were determined to be 0.16-0.27 μg/mL.

Key words: Forsythiae Fructus, constituent separation, high performance liquid chromatography, LC-MS

## INTRODUCTION

Forsythiae Fructus, a widely used Chinese herb, have long been used in traditional Chinese medicine for the treatment of gonorrhea, erysipelas[1], pharyngitis, pyrexia, and ulcers[2]. The major bioactive constituents of Forsythiae Fructus are rengyoside B **(1)**, matairesinol **(2)**, salidroside **(3)**, quercitrin **(4)**, suspensaside **(5)**, rengyoside C **(6)**, matairesinoside **(7)**, forsythiaside **(8)**, pinoresinol-β-D-glucoside **(9)**, cornoside **(10)**, acteoside **(11)**, arctigenin **(12)**, arctiin **(13)**, phillyrin **(14)** and pinoresinol **(15)**[3], etc. Forsythiae Fructus has been shown to have antioxidant, antiemetic[4-5], anti-inflammatory, antiviral[6-7], antitumor activities[8], antibacterial, choleretic, antipyretic[9] and free radical scavenging effects[10].

Several methods have been reported for the separation of some of these constituent compounds including HPLC, reversed-phase solid-phase extraction HPLC (RP-SPE-HPLC) or high-speed counter-current chromatography (HSCCC)[11-17]. However, none of the methods is entirely adequate due to poor resolution or long analysis time and, as a result, not all of these compounds can be assayed in a single run. We describe herein, the development of a simple and rapid LC and LC-MS method for the simultaneous separation and identification of these fifteen compounds in Forsythiae Fructus samples.

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## MATERIALS AND METHODS

### I. *Reagents and Materials*

Salidroside, forsythiaside, arctiin and phillyrin were purchased from Wako Pure Chemical Industries, Ltd. (Kyoto, Japan), acetophenone from Aldrich (Milwaukee, WIS, USA), $KH_2PO_4$ from Nacalai Tesque (Kyoto, Japan), phosphoric acid from Acros (New Jersey, USA) and acetic acid, acetonitrile, methanol from Merck (Darmstadt, Germany). Deionized water from a Milli-Q system (Millipore, Bedford, MA, USA) was used to prepare all buffers and sample solutions. Samples of Forsythiae Fructus were provided by the Brion Research Institute of Taiwan and were identified by external appearance and pharmacognostic histological anatomy.

### Π. *Preparation of Forsythiae Fructus Extracts*

A 0.5 g sample of pulverized Forsythiae Fructus was extracted with 70% methanol (8 mL) under reflux for 15 min, followed by centrifugation of the suspension for 5 min at 1500 × *g* (Universal, Hettich Zentrifugen). The extraction was repeated three times. The combined extracts were filtered through a 0.45-μm filter. After the addition of 1 mL of internal standard (10 mL of acetophenone diluted to 100 mL with 70% methanol), the extract was diluted to 25 mL with 70% methanol. This

solution was passed through a 0.45-μm filter once more and an aliquot of the filtrate was injected into the HPLC and LC-MS system.

III. *Apparatus and Conditions*

(I) *LC-UV System*

HPLC was performed on a Shimadzu LC-6AD apparatus equipped with a Shimadzu SCL-6B system controller and a Shimadzu M6A photodiode array detector (254, 280 nm). Satisfactory separation of the marker substances was achieved using a reversed-phase column (Cosmosil 5C18-AR Π, 5 μm, 25 cm × 4.6 mm I.D.) (Nacalai Tesque, Kyoto, Japan) eluted at a flow rate of 0.8 mL/min with a linear solvent gradient of A-B [A = 20 mM $KH_2PO_4$ (pH 4.54); B = $H_2O/CH_3OH/CH_3CN$ (1:3:1, v/v/v)] programmed as follows: 0 min, 95%A: 5%B; 10 min, 70%A: 30%B; 20 min, 60%A: 40%B; 30 min, 50%A: 50%B; 40 min, 40%A: 60%B; 45 min, 20%A: 80%B; 50 min, 100%B; 55 min, 100%B; 60 min, 95%A: 5%B.

**(**Π) *LC-MS System*

The compounds were identified using a Shimadzu LCMS 2010A advantage system. The LC-MS included a Shimadzu LC-M10AD system equipped with a Shimadzu LC-M10AD UV detector, an atmospheric pressure chemical ionization (APCI) and an electrospray ionization (ESI) interface. The chromatographic conditions were similar to that for the LC-UV system, except A = 0.1% acetic acid. After passing through the HPLC UV-detector, the solution was passed through a splitter and a portion of the solution was diverted to the LC-MS ion source at a rate of about 150 μL/min. The APCI source was operated at a nebulizer gas flow of 2.5 L/min, drying gas flow of 0.02 MPa, capillary temperature 250°C and probe temperature 400°C. The ESI source was operated at a nebulizer gas flow of 1.0 L/min, drying gas flow of 1 MPa, probe high voltage of 4.5 kV and capillary temperature of 250°C. The energy level for the collision-induced dissociation (CID) fragmentation was maintained at 50%. Tuning of the MS instrument was operated through a T adapter, the standard solution (1 mg/mL) was injected using syringe pump at a flow rate of 5 μL/min, and the HPLC solvent (mobile phase composition = 50%A, 50%B) was simultaneously injected using the HPLC pump at a rate of 150 μL/min.

IV. *Calibration Curves and Validation*

A stock solution was prepared by dissolving four marker substances (**3**, 13.3 mg; **8**, 10.2 mg; **13**, 7.8 mg; **14**, 11.2 mg) in 25 mL of 70% methanol, and was stored in a refrigerator. The stock solution was then diluted with 70% methanol into a series of standard solutions (dilution factor: 7.8, 12.5, 25, 62.5 and 125). Each of these solutions was analyzed three times by the LC-UV method and standard curves were derived by plotting the peak-area ratios versus concentrations. Samples, spiked with various concentrations of stock solutions, were analyzed. Recovery was determined by comparing the amount of marker substances added with that of the marker substances detected. The limits of detection were based on a signal to noise (S/N) ratio of 3:1 as the minimum. Reproducibility (relative standard derivation, RSD) was calculated from the variation of peak-area ratio or retention time in six replicate injections.

## RESULTS AND DISCUSSION

I. *HPLC Analysis*

All 15 compounds and acetophenone (internal standard) were successfully resolved and identified in a single run by the HPLC and LC-MS methods under the optimal conditions. Separation was achieved by optimizing the pH and the phosphate or acetate concentration of the mobile phase A, the analytical column and the organic modifier. Preliminary experiments were first conducted using a Nucleosil C18 column and a 280 nm detection wavelength with a gradient solvent system [A: MeOH (containing 1% tetrahydrofuran); B: $H_2O$ (containing 10 mM $KH_2PO_4$)][13]. In this case, the active constituents caffeic acid, rutin, forsythoside A, forsythin and forsythigenin were well separated. However, when Forsythiae Fructus crude extracts were applied, the analysis failed owing to overlap with components in addition to the marker substances of the herb. Meanwhile, a chromatograph with low resolution, low theoretical plate number and drifting baseline should not be suitable for the analysis of trace components. Therefore, a new assay system which not only permits the separation of all 15 constituents, thus reducing the difficulties associated with clean-up, but also can be applied to LC-MS analysis was developed. It was necessary to substitute the phosphate in the buffer with acetate.

The choice of column can also affect the resolution, and five reversed-phase columns, Cosmosil 5C18, 5C18-MS, 5C18-MS Π, 5C18-AR and 5C18-AR Π were examined in preliminary experiments. Cosmosil 5C18-AR Π, which gave the highest theoretical plate numbers for most peaks, had the best resolution for the pair of compounds **13**/**14**, (Rs = 1.78). When 5C18-AR Π was used as the analytical column in this experiment, a series of buffer solutions with different phosphate concentrations and pH values were examined. The analyses were performed using 10, 15, 20, 25 and 30 mM $KH_2PO_4$ and the results indicated that a concentration of 20 mM was optimal for the separation of the 15 compounds, especially for the **3**/**4** peak-pair (Rs = 1.25). By adjusting the pH value of mobile phase A with 10% phosphoric acid, a series of eluents with pH 2.65, 3.05, 3.43, 4.00, 4.54 and 4.82 were prepared and examined.

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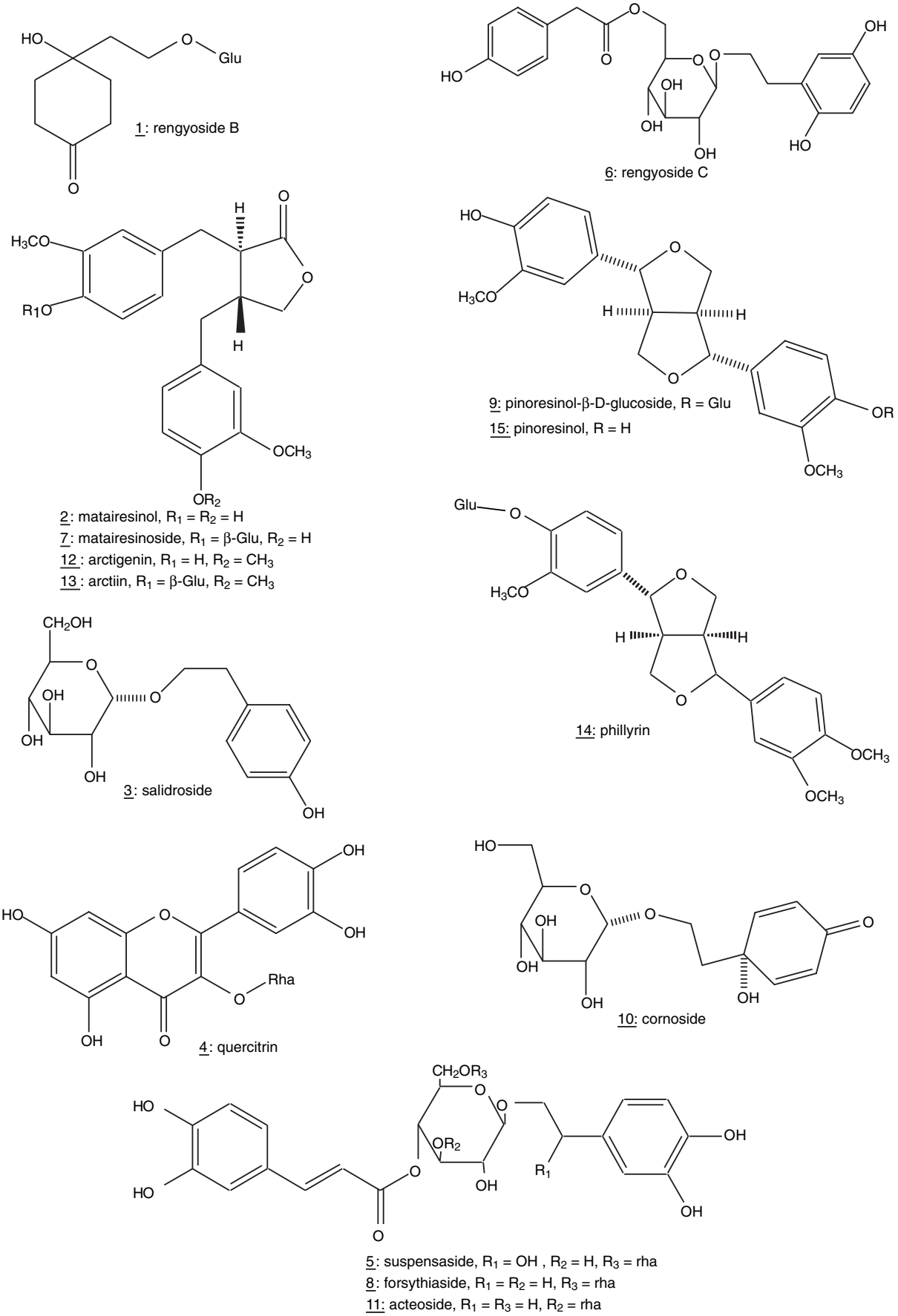


**Figure 1.** Structures of the 15 constituents in Forsythiae Fructus.

The resulting data indicated that all of the compounds could be well separated, except **3**/**4** and **13**/**14**, which were separated completely only at pH 4.54, as shown in Table 1. Therefore, a solution of 20 mM $KH_2PO_4$ adjusted to pH 4.54 with 10% phosphoric acid was used as the mobile phase A.

Mobile phase B was an organic phase composed of water with variable ratios of acetonitrile and methanol. Since the elution power of acetonitrile is stronger than that of methanol, higher acetonitrile concentrations caused the constituents to be eluted earlier; whereas, when higher methanol ratios were used, the lower polarity constituents were not eluted at all within the analysis time. Regarding the resolution of **10**/**11**, different ratios of acetonitrile/methanol/water (0/4/1, 1/3/1, 2/2/1, 3/1/1 and 4/0/1) were examined. The results showed that the resolution of the peak-pair declined with an increase in the ratio of acetonitril. At the ratio of 1/3/1, the resolution of **10**/**11** was optimal, as shown in Figure 2. Based on those three factors – retention time, theoretical plate numbers and resolution, acetonitrile/methanol/water in the ratio of 1/3/1 was used as the mobile phase B. Using mobile phase (A) = 20 mM $KH_2PO_4$ (pH 4.54) and mobile phase (B) = $CH_3CN$/$CH_3OH$/$H_2O$= 1/3/1 (v/v/v) in a gradient elution, typical chromatograms are shown in Figure 3.

## Π. *LC-MS Analysis*

For LC-MS analysis, the phosphate in the mobile phase was replaced by HCOOH, HOAc, etc. in suitable concentrations in water. Preliminary experiments indicated that acetic acid gave the best results. Analyses were performed using 0, 0.025%, 0.05%, 0.075% and 1.0% aqueous acetic acid and the results showed no significant differences in the separation of the 15 compounds in terms of capacity factor. Nonetheless, they did have an effect on the theoretical plate number. With an increase in acetic acid concentration, the peak shape became steeper and the resolution of the two closely spaced peaks **13** and **14** reached their optimal Rs value, 1.25.

The constituents analyzed in this study are mostly phenolic compounds. Since the HPLC separation and qualitative MS analysis must be carried out simultaneously, it becomes necessary to select an ion source suitable for all compounds. Therefore, we compared all the total ionic chromatogram (TIC) data of APCI positive, negative ion modes (APCI ±) and ESI positive, negative ion modes (ESI ±) in order to select a best ion source. It was found that the TIC from ESI yielded a greater abundance of MS peaks than APCI, and the ESI positive mode resulted in a good resolution and a low noise level. Hence, in this study, ESI was chosen as the ion source and cations were monitored. The resulting MS spectra then served as the source of data for qualitative analysis.

## Ш. *Identification of the Constituents*

Fifteen constituents in the Forsythiae Fructus samples were well resolved by the proposed method and their structures were recognized by comparing the

**Table 1.** Effect of different pH values on resolution (Rs)

| pH | 2.65 | 3.05 | 3.43 | 4.00 | 4.54 | 4.82 |
|---|---|---|---|---|---|---|
| Peaks 3/4 | 0.59 | 0.53 | 0.67 | 0.59 | 1.25 | 1.12 |
| Peaks 13/14 | 0.69 | 1.05 | 1.02 | 0.69 | 1.78 | 1.35 |

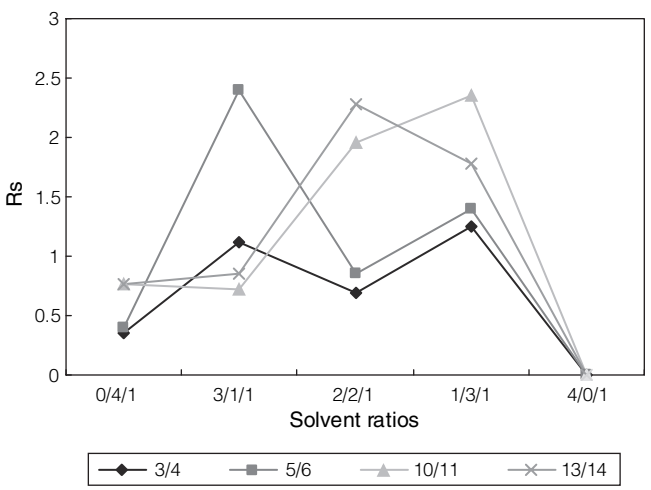


**Figure 2.** Effect of organic solvent ratios on resolution (Rs).

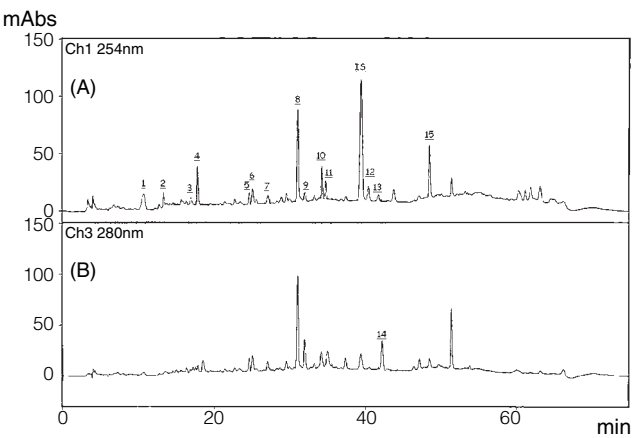


**Figure 3.** HPLC chromatograms of a Forsythiae Fructus extract. (A) 254 nm, (B) 280 nm.

LC-MS data with those reported in the literature[16-23]. The major MS peaks of these compounds are listed in Table 2. Rengyoside B, **1**, gave base peak at m/e = 339 for $[M+H+H_2O]^+$ and had, molecular weight of 320[17]; matairesinol, **2**, $[M+H]^+$ = 359, molecular weight 358[18]; salidroside, **3**, $[M+H]^+$ = 301, molecular weight 300 [compared with authentic standard][16]; quercitrin, **4**, $[M+H]^+$ = 449, $[M+ACN]^+$ = 489, molecular weight 448[19]; suspensaside, **5**, $[M+Na]^+$ = 663, molecular weight 640[20]; rengyoside C, **6**, $[M+Na]^+$ = 473, molecular weight 450[2]; matairesinoside, **7**, $[M+Na]^+$ = 543, molecular weight 520[20]; forsythiaside, **8**, $[M+Na]^+$ = 647, molecular weight 624 [compared with authentic standard][2]; pinoresinol-β-D-glucoside, **9**, $[M+Na]^+$ = 543, molecular weight 520[20]; cornoside, **10**, $[2M+H]^+$ = 633, molecular weight 316[21]; acteoside, **11**, $[M+H_2O]^+$ = 642, molecular weight 624[22]; arctigenin, **12**, $[M+H-H_2O]^+$ = 355, molecular weight 372[20]; arctiin, **13**, $[M+H_2O]^+$ = 552, molecular weight 534 [compared with authentic standard][20]; phillyrin, **14**, $[M+Na]^+$ = 557, molecular weight 534 [compared with authentic standard][20] and pinoresinol, **15**, $[M+H]^+$ = 359, molecular weight 358[23]. UV spectra obtained from the photodiode array detector and elution order postulated according to the molecular polarity were also used to confirm the assignments.

IV. *Calibration Curves and Validation*

Calibration curves were prepared by plotting the peak-area ratios (using acetophenone as an internal standard) against the corresponding concentrations. The regression lines were linear in the concentration range studied and the corresponding coefficients of correlation are shown in Table 3. The detection limits (S/N = 3) for the components were 0.16 - 0.27 μg/mL, as shown in Table 3. The reproducibility (RSD) of the proposed method in terms of peak-area ratio was 0.23-1.50% (intraday, $n$ = 6) and 0.41-2.40% (interday, $n$ = 6). Suitable amounts of marker substances or known drug materials (**3**, 177 μg/mL, **8**, 136 μg/mL, **13**, 104 μg/mL and **14**, 149 μg/mL) were added to a sample containing a known content and the mixture was analyzed by the proposed method. The

**Table 2.** Major peaks of the marker constituents in Forsythiae Fructus

| Compound | $[M+H]^+$ | $[M+Na]^+$ | $[M+H_2O]^+$ | $[M+H+H_2O]^+$ | Other peaks |
|---|---|---|---|---|---|
| 1 | - | - | - | 339 | 159, 163 |
| 2 | 359 | - | - | - | 195, 341 |
| 3 | 301 | - | - | - | - |
| 4 | 449 | - | - | - | 303 |
| 5 | - | 663 | - | - | 163, 325, 477 |
| 6 | - | 473 | - | - | 137, 313 |
| 7 | - | 543 | 538 | - | 195, 341 |
| 8 | - | 647 | - | - | 163, 325, 477 |
| 9 | - | 543 | - | - | 341 |
| 10 | | | $[2M+H]^+$ = 633 | | 155, 163 |
| 11 | - | - | 642 | - | 163, 325, 477, 461, 489 |
| 12 | | | $[M+H-H_2O]^+$ = 355 | $[M+K]^+$ = 411 | 195, 341 |
| 13 | 535 | 557 | 552 | $[M+K]^+$ = 573 | 195, 341 |
| 14 | 535 | 557 | 552 | $[M+K]^+$ = 573 | 341 |
| 15 | 359 | | $[M+ACN]^+$ = 399 | | 341 |

**Table 3.** Calibration curves and detection limits (S/N = 3) of the 4 marker substances

| Compound | y = ax + b (y = peak-area ratio, x = conc.(mg/mL)) | | | $R^2$ | Detection limit | |
|---|---|---|---|---|---|---|
| | Linear range [μg/mL] | Slope a | Intercept b | | μg/mL | ng |
| 3 | 0.53 - 31.92 | 0.7856 | -0.0018 | 0.9991 | 0.266 | 5.32 |
| 8 | 0.41 - 24.48 | 2.0799 | 0.004 | 0.9991 | 0.204 | 4.08 |
| 13 | 0.31 - 18.7 | 57.844 | -0.0711 | 0.9990 | 0.156 | 3.12 |
| 14 | 0.45 - 26.9 | 54.369 | -0.0901 | 0.9991 | 0.224 | 4.48 |

**Table 4.** The recovery and reproducibility of intraday and interday analysis

| Compound | Recovery (%) | Reproducibility (RSD, %) | |
|---|---|---|---|
| | | Intraday (n = 6) | Interday (n = 6) |
| | | Peak-area ratio | |
| 1 | - | 1.26 | 1.09 |
| 2 | - | 1.44 | 1.05 |
| 3 | 102.6 ± 0.005 | 1.08 | 2.08 |
| 4 | - | 1.29 | 2.00 |
| 5 | - | 1.46 | 2.34 |
| 6 | - | 1.21 | 1.28 |
| 7 | - | 1.46 | 2.32 |
| 8 | 102.8 ± 0.002 | 1.50 | 2.40 |
| 9 | - | 0.83 | 0.96 |
| 10 | - | 1.43 | 0.63 |
| 11 | - | 0.79 | 0.64 |
| 12 | - | 1.48 | 1.96 |
| 13 | 99.5 ± 0.008 | 1.42 | 2.23 |
| 14 | 100.2 ± 0.007 | 0.23 | 1.52 |
| 15 | - | 0.81 | 0.41 |

recoveries of the components were 98.6-102.8%. These data are shown in Table 4.

V. *Determination of Four Constituents in Forsythiae Fructus Samples*

By substituting the peak-area ratios of the individual peaks for *y* in the equations listed in Table 3, the contents of the individual components in the sample extracts were determined. The average amounts of the four constituents in extracts of three Forsythiae Fructus samples were (mg/g ± SD): **3**, 1.65 ± 0.03; **8**, 7.18 ± 0.04; **13**, 0.03 ± 0.15; **14**, 0.27 ± 0.03.

## CONCLUSIONS

This work successfully demonstrates that, by optimizing parameters such as phosphate, acetic acid, methanol, acetonitrile concentrations of the eluent system and the column used, high-resolution separations of a complicated mixture can be achieved. Combined LC-UV-ESI-MS can be used not only to recognize the authentic standards but also to identify the peaks interested.

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