Rapid Separation and Determination of 12 Kinds Phenolic Acids and Isoflavone in Iris Pseudoacorus Based on Ultra-performance Convergence Chromatography

ZHU Qing-Qing1,2, CHEN Nian-Lai∗1, CHU Run1, MIAO Qiang-Qiang3

1College of Resources and Environment, Gansu Agriculture University, Lanzhou 730070, China
2College of Chemical Engineering, Northwest Minzu University, Lanzhou 730000, China
3Research Institute for National Defense Engineering of Academy of Military Science PLA China, Luoyang 471023, China

∗Corresponding author’s e-mail: chennl@gsau.edu.cn

Abstract. A method was developed for the rapid separation and determination of 12 kinds phenolic acids and isoflavones in Iris pseudoacorus based on Ultra performance convergence chromatography. The compounds were separated on a Waters Acquity UPC2 HSS(100mm×3.0mm, 1.7µm) column at 30℃ by using CO2 and acetonitrile as the mobile phase at a flow rate of 0.4mL/min, and then analyzed by a UV detector at wavelength of 270nm, the whole analysis progress was only 36 min. The results showed that the limits of detection (LOD) and the limits of quantitation (LOQ) of 12 compounds were between 0.413 and 0.458µg/mL, and 0.19 and 0.183µg/mL, respectively. The spiked recoveries were between 95. 81% and 98. 73%, and the relative standard deviations (RSD) were between 3. 20 and 4. 77(n=6). This method is simple, fast, accurate and reproducible, and the result is reliable. The method is applicable for the determination of 12 phenolic acids and isoflavones in Iris pseudoacorus.

1. Introduction

Ultra performance convergence chromatography(UPC2) technology is a separation technology introduced by Waters in March 2012. This technology combines the advantages of gas chromatography (GC) and LC in separation and analysis. At the same time, it makes up for the shortcomings of supercritical fluid chromatography (SFC) and liquid chromatography (LC) while improving the analytical speed and column [1-6] of high performance liquid chromatography.

At present, the main determination methods of phytophenolic acids are high performance liquid chromatography (HPLC)[7-10], gas chromatography (GC)[11], gas chromatography/mass spectrometry (SGC/M)[12], capillary electrophoresis (CE)[13] and so on. The detection methods of plant isoflavones are mainly near-infrared spectroscopy[14], high performance liquid chromatography [15-18], liquid chromatography tandem mass spectrometry[19], quantitative nuclear magnetic resonance spectroscopy[20]. For liquid chromatography-mass spectrometry, because of its high price, it has not been widely used in China. Chromatography and high performance liquid chromatography are not specific, and there are many interfering substances and inaccurate quantitation. The analysis time is long and cannot be used for simultaneous determination of various substances [21]. Although the
analytical results of high performance liquid chromatography are accurate, the sample processing operation is complicated, the analysis time is long and the cost is high.

The Iris is born on the hillside grass, the forest edge grassland and the wetland along the riverside ditch. It can be used for the purification of pollutants in constructed wetlands, and has high horticultural value and medicinal value[22-23]. Iris is a dried rhizome of Iridaceae plant. Iris is dried by Belamcanda chinensis in Sichuan and other places. Iris is is included in the 2005 edition of Chinese Pharmacopoeia in the name of Sichuan Belamcanda chinensis[24]. The latest study found that the isoflavones contained in the iris leaves are similar to the main chemical components in the rhizome of the iris, so the iris and its isoflavones are worthy of attention [25]. Phenolic acid is an organic acid with phenolic groups in its structure, which is the second largest secondary metabolite in plants after flavonoids. In recent years, the problem of continuous cropping obstacles caused by phenolic acids has become a research hotspot [9].

Beside visible light, the solar radiation which strikes the Earth’s atmosphere also contains ultraviolet (UV) and infrared irradiation. Based on the biological effects it induces, UV is divided into UV-C (100-280 nm), UV-B (280-320 nm) and UV-A (320-400 nm). UV-C, the most dangerous, is completely absorbed by the ozone layer in the atmosphere. As a consequence, UV-B is the shortest wavelength component of the sunlight which reaches the surface of the Earth. As an integral part of solar radiation, UV always accompanies visible light.

The UV-B range is absorbed by many constituents of the cell with harmful consequences. UV-B is cytotoxic, damaging the cell at many levels, including nucleic acids, lipids, photosynthetic pigments and proteins [26]. Higher levels of UV-B cause the production of reactive oxygen species (ROS) and activate general stress signaling pathways [27].

Data compiled from the last two decades suggest that nearly 50% of crop plants are affected by elevated levels of solar UV-B. Studies on a number of cultivated and native plant species have shown that ambient and enhanced levels of UV-B have detrimental effects on plant growth, development and morphology, photosynthesis, and biomass production [28-32].

In this study, Ultra performance convergence chromatography(UPC2) was used to establish a simple and rapid detection method for 12 phenolic acids and isoflavones, which provided a reference for the rapid analysis of phenolic acids and isoflavones in related plants and the promotion and application of UPC2 technology.

2. Materials and methods

2.1 UV-B treatments

This experiment consisted of 3 treatments: control under natural UV-B radiation during the entire experiment (CK:168 μw·cm⁻²) and two levels of enhanced UV-B radiation, the low ( UV-B1: 210 μw·cm⁻² ) and the high ( UV-B2:252μw·cm⁻² ) along with ambient were selected for detailed study. The dose selection of enhanced UV-B radiation was based on screening experiment as described in earlier finding( CHU Run et al., 2017 ). Enhanced UV-B radiation was obtained by UV-B lamps(Qin Brand, 40W power, 313nm wavelength peak, China ). Samples were exposed to enhanced UV-B radiation daily during 9:00 to 17:00. Each sample in open field was receiving ambient level (168 μw·cm⁻²) of UV-B along with enhanced UV-B radiation i.e. UV-B1 and UV-B2. In order to remove all incident UV-C (< 280nm), radiation was filtered through 0.13 nm cellulose acetate (China). With the help of power meter (Beijing Normal University Photoelectric Instrument Factory) UV-B irradiance under the lamp at the surface of fronds was measured. All the parameters were analyzed after 21d of treatment.

2.2. Instruments, raw materials and reagents

Ultra performance convergence chromatography (Waters, USA), equipped with Waters EmpowerTM 3 data processing system; 3K30 refrigerated centrifuge (SIGMA, USA); MS3 vortex (IKA, Germany); pipette gun (Thermo Electron,USA, 100-1000 mL, 1. 0-5. 0 mL); T25 homogenizer (IKA, Germany).
gallic acid, vanillic acid, syringic acid, ferulic acid, tectoridin, resveratrol, rutin, iridin, genistein, tectorigenin, irigenin, iris florentin (Nanjing Dilger Medical Technology Co., Ltd., with purity above 98%), CO2 (purity 99.997%), Lanzhou Huineng Company; methanol, acetonitrile, isopropanol, hexane (chromatographic purity, Germany) Merck KGaA Company; the rest of the reagents are analytical pure.

2.3. Conditions of Ultra performance convergence chromatography
Chromatographic column: (100mm × 3.0mm, 1.7 μm); mobile phase: A is CO2, B is acetonitrile; flow rate is 0.4 mL/min; injection volume: 1 μL; column temperature is 30 °C; detection wavelength is 270 nm; dynamic back pressure (ABPR): 1600 Psi. Gradient elution: Table 1

| time (min) | flow rate (mL/min) | ratio of CO2 (%) | ratio of acetonitrile (%) |
|-----------|--------------------|------------------|--------------------------|
| Initial   | 0.4                | 95               | 5                        |
| 4         | 0.4                | 90               | 10                       |
| 15        | 0.4                | 80               | 20                       |
| 24        | 0.4                | 70               | 30                       |
| 32        | 0.4                | 60               | 40                       |
| 34        | 0.4                | 95               | 5                        |
| 36        | 0.4                | 95               | 5                        |

2.4 Sample treatment
Accurately weigh 0.3000g of crushed appendix sample in 50 mL polyethylene tube, add 10 mL methanol solution, homogenize for 3 minutes, centrifuge for 10 minutes at 4°C, 10 000 r/min, take all supernatant, and blow nitrogen to nearly dry, the residue was made up to 1 mL with 70% methanol water, vortexed for 1 min, filter with 0.22 μm microporous membrane, and then analyze by ultra-high performance phase chromatography with external standard method.

2.5 Preparation of Standard Solution
Standard Reserve Solution: Take standard reserve solution: Take gallic acid, vanillic acid, syringic acid, ferulic acid, iris, resveratrol, rutin, iridin glycoside, genistein, iris aglycone, iris aglycone and secondary iris in a proper amount, dissolve in acetonitrile solution and fix the volume to 100 mL, and make them into gallic acid, vanillic acid, syringic acid, ferulic acid, iris, resveratrol, rutin, rutin, rutin, etc. The standard reserve solutions of iris, genistein, iris aglycone, iris aglycone and iris flavin 500 mg/L were refrigerated at 4 °C until use.

3. Results and discussion

3.1 Contents of Methodological Investigation

**stability experiment**

The samples of iris were treated according to the above conditions. According to the above chromatographic conditions, samples were analyzed at 0, 4, 12, 24 and 48 hours. RSD scores of peak area of 12 main compounds (gallic acid, vanillic acid, syringic acid, ferulic acid, tectoridin, resveratrol, rutin, iridin, genistein, tectorigenin, irigenin, iris florentin) in iris were obtained 0.31%, 0.54%, 0.72%, 0.28%, 0.85%, 0.64%, 0.38%, 0.90%, 0.45%, 0.33%, 0.61% and 0.93% respectively. The results showed that the sample solution was stable within 48 hours.

**Specific experiment**

Taking the sample solution No. 1, the mixed standard solution, and the negative test solution were analyzed according to the above chromatographic conditions. The negative samples were compared with mixed standard solution chromatogram and sample chromatogram in 12 compounds (gallic acid,
vanillic acid, syringic acid, ferulic acid, tectoridin, resveratrol, rutin, iridin, genistein, tectorigenin, irigenin, irisflorentin). There was no interference in the peak position of iris flavin, which indicated that the method was specific.

![Fig. 1](image)

Fig. 1 Ultra performance convergence chromatography (UPC²) of 12 kinds of isoflavones and phenolic acids

A: Mixture of standards; B: Sample of number, Peak number: 1: gallic acid; 2: vanillic acid; 3: syringic acid; 4: ferulic acid; 5: tectoridin; 6: resveratrol; 7: rutin; 8: iridin; 9: genistein; 10: tectorigenin; 11: irigenin; 12: irisflorentin

**Linear range and limit of quantitation**

The standard reserve liquids of 12 compounds were precisely measured at 0.2, 1.00, 5.00, 10.00 and 20.00 mL. They were placed in 100 mL Brown volumetric flask and dissolved in acetonitrile until calibration. A series of solutions with mass concentration ranging from 1 to 100 μg/mL were obtained. According to the above chromatographic conditions, each concentration was injected 1.0 µg/L in turn, the concentration (µg/mL) was taken as abscissa, and the peak area was taken as ordinate. Linear regression was carried out. The results are shown in Table 2. It can be seen from Table 2 that the 12 compounds have a good linear relationship within a certain range.

| Analyte       | Linear equation       | Linear range (µg/mL) | correlation coefficient (r) | LOD(µg/mL) | LOQ(µg/mL) |
|---------------|-----------------------|----------------------|-----------------------------|------------|------------|
| Gallic acid   | Y=18950 X -13962      | 1~100                | 0.9992                      | 0.18       | 0.63       |
| vanillic acid | Y=19837 X +13643      | 1~100                | 0.9995                      | 0.097      | 0.34       |
| syringic acid | Y=22690X+21829        | 1~100                | 0.9991                      | 0.082      | 0.29       |
| ferulic acid  | Y=30881 X -53808      | 1~100                | 0.9994                      | 0.12       | 0.42       |
| tectoridin    | Y=48731 X -85782      | 1~100                | 0.9994                      | 0.078      | 0.27       |
| resveratrol   | Y=32405 X -71408      | 1~100                | 0.9991                      | 0.14       | 0.49       |
| rutin         | Y=24700 X -22713      | 1~100                | 0.9991                      | 0.11       | 0.38       |
| iridin        | Y=58568 X -103092     | 1~100                | 0.9994                      | 0.067      | 0.23       |
| genistein     | Y=68430 X -11890      | 1~100                | 0.9994                      | 0.064      | 0.22       |
| tectorigenin  | Y=82036 X -155221     | 1~100                | 0.9990                      | 0.051      | 0.19       |
| irigenin      | Y=72798 X -136063     | 1~100                | 0.9990                      | 0.054      | 0.19       |
| irisflorentin | Y=61401X -80918       | 1~100                | 0.9994                      | 0.062      | 0.22       |

**Precision test**

The precision was investigated separately: according to the above chromatographic conditions, 12 compounds in iris (gallic acid, vanillic acid, syringic acid, ferulic acid, tectoridin, resveratrol, rutin,
iridin, genistein, tectorigenin, irigenin, irisflorentin) were injected repeatedly for 6 times, and the RSD of the precision was between 0.52% and 0.81%. The density of RSD ranges from 0.36% to 1.19%. It can be seen that the method has good daytime and intraday precision for detecting 12 compounds in the appendix, and can meet the detection requirements of 12 compounds in the appendix.

**Repeatability test**

Six samples of the same iris were accurately weighed and processed according to the above conditions. According to the above chromatographic conditions, the contents of 12 compounds and RSD were calculated. RSD of gallic acid, vanillic acid, syringic acid, ferulic acid, tectoridin, resveratrol, rutin, iridin, genistein, tectorigenin, irigenin and irisflorentin were 1.51%, 2.36%, 2.78%, 2.15%, 2.44%, 3.09%, 3.11%, 3.40%, 3.43%, 3.57% and 35% respectively. The results show that the method has good repeatability.

**Standardized recovery test**

Six iris samples of the same batch (No. 3C) were accurately weighed and added with standard solutions of high (25 μg/mL), medium (5 μg/mL) and low (1 μg/mL) at different concentrations. The samples were treated according to the above conditions. Sample analysis was carried out under the above chromatographic conditions. The recovery rate of standard addition showed that gallic acid, vanillic acid, syringic acid, ferulic acid, iridin, resveratrol, rutin and iris. The average recoveries of tail glycoside, genistein, iris aglycone, iris aglycone and iris flavin were 95.81%-98.73%, respectively. The RSD ranged from 3.20-4.77 for the recovery of 12 compounds in iris. The results are shown in Table 3. The results show that the method is accurate and reliable.

**Table 3 Recovery of 12 Compounds in Iris wilsonii samples**

| Analyte      | Initial amount (mg/kg) | Added amount (mg/kg) | Total amount (mg/kg) | Recovery (%) | Average recovery (%) | RSD (%) |
|--------------|------------------------|----------------------|----------------------|--------------|----------------------|--------|
| Gallic acid  | Absent                 | 1                    | 1.02                 | 101.85       | 98.07                | 3.71   |
|              |                        | 5                    | 4.89                 | 97.78        | 96.83                | 3.56   |
|              |                        | 25                   | 23.65                | 94.59        | 92.15                | 4.05   |
|              |                        | 1                    | 24.43                | 101.90       | 92.15                | 4.05   |
| vanillic acid| 23.41                  | 5                    | 28.35                | 98.89        | 98.46                | 3.73   |
|              |                        | 25                   | 47.06                | 94.59        | 94.59                | 3.73   |
|              |                        | 1                    | 1.80                 | 100.12       | 97.28                | 3.69   |
| syringic acid| 0.80                   | 5                    | 5.66                 | 97.12        | 96.69                | 3.56   |
|              |                        | 25                   | 24.11                | 93.25        | 92.15                | 4.05   |
|              |                        | 1                    | 188.20               | 99.87        | 97.28                | 3.69   |
| ferulic acid | 187.20                 | 5                    | 192.05               | 97.00        | 96.34                | 4.05   |
|              |                        | 25                   | 210.24               | 92.15        | 92.15                | 4.05   |
|              |                        | 1                    | 3.77                 | 101.25       | 92.15                | 4.05   |
| tectoridin   | 2.76                   | 5                    | 7.68                 | 98.48        | 98.23                | 3.20   |
|              |                        | 25                   | 26.50                | 94.97        | 94.97                | 3.20   |
|              |                        | 1                    | 10.67                | 100.89       | 100.89               | 3.20   |
| resveratrol  | 9.66                   | 5                    | 14.62                | 99.15        | 97.73                | 4.16   |
|              |                        | 25                   | 32.95                | 93.14        | 93.14                | 4.16   |
|              |                        | 1                    | 4.91                 | 101.45       | 97.33                | 4.77   |
| rutin        | 3.90                   | 5                    | 8.83                 | 98.56        | 97.84                | 4.10   |
|              |                        | 25                   | 27.28                | 93.52        | 93.52                | 4.10   |
|              |                        | 1                    | 2.87                 | 101.75       | 97.33                | 4.77   |
| iridin       | 1.85                   | 5                    | 6.74                 | 97.75        | 97.33                | 4.77   |
|              |                        | 25                   | 24.98                | 92.50        | 92.50                | 4.77   |
|              |                        | 1                    | 2.88                 | 100.56       | 97.28                | 3.69   |
| genistein    | 1.87                   | 5                    | 6.76                 | 97.85        | 97.28                | 3.69   |
|              |                        | 25                   | 25.23                | 93.44        | 93.44                | 3.69   |
|              |                        | 1                    | 3.32                 | 99.85        | 99.85                | 3.69   |
| tectorigenin | 2.32                   | 5                    | 7.09                 | 95.46        | 95.81                | 4.05   |
|              |                        | 25                   | 25.35                | 92.12        | 92.12                | 4.05   |
3.2 Determination
Eighteen batches of iris samples were collected and accurately weighed at 0.3000g. The samples were processed according to the above conditions. The contents of gallic acid, vanillic acid, syringic acid, ferulic acid, tectoridin, resveratrol, rutin, iridin, genistein, tectorigenin, irigenin and irisflorentin were analyzed three times.

4. Conclusion
Twelve compounds in iris were determined by ultra-high performance liquid chromatography for the first time. Through methodological investigation, the method has high sensitivity, good reproducibility, good stability and short detection time. Compared with high performance liquid chromatography (HPLC), except for the 5 minutes used by the equilibrium instrument, the first analysis is only 36 minutes, and the analysis speed is nearly four times faster than that of high performance liquid chromatography. It can be used for the high-throughput determination of compounds in iris, providing reference and technical branch for the quality control of iris. Ultra-high performance liquid chromatography (UPC²) provides a new direction for the chromatographic analysis of compounds in iris. This method was used to determine the contents of main isoflavones and phenolic acids in 18 batches of Iris iris from different sources. The results showed that the content of isoflavones and phenolic acids varied greatly, which may be due to the different intensity of UV-B radiation.

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