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Determination of Anthraquinones and Stilbenes in Giant Knotweed Rhizome by HPLC with UV Detection

INTRODUCTION

Giant knotweed rhizome, the dried rhizome and root of *Polygonum cuspidatum* Sieb. et Zucc. is a common medicinal plant in China (Figure 1). Chinese Pharmacopeia Edition 2005 (Ch. P 2005) regulates its use as an herbal medicine.¹ It is used with the belief that it cures angiocardopathy, skin inflammations, liver diseases, reduces fever, and relieves arthritis pain etc.



Figure 1. Giant Knotweed Rhizome.

There are many compounds that are considered active components in giant knotweed rhizome, including anthraquinones (for example, anthraglycoside A, anthraglycoside B, emodin, physcion, rhein, and chrysophanol) and stilbenes (for example, resveratrol and

polydatin). Their chemical structures are shown in Figure 2, and their UV absorption spectrums are shown in Figure 3. Ch. P 2005 regulates giant knotweed rhizome with two different HPLC methods for the determination of emodin and polydatin, respectively.² The quality control for giant knotweed rhizome, therefore, is inconvenient (that is, two methods) and inadequate, as the methods only determine two of the putative active components. Besides determining emodin and polydatin, some reported

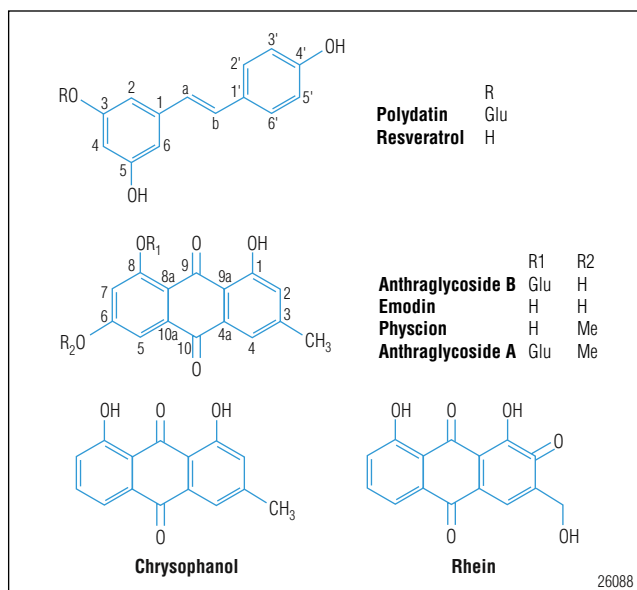


Figure 2. Structures of some compounds in giant knotweed rhizome.

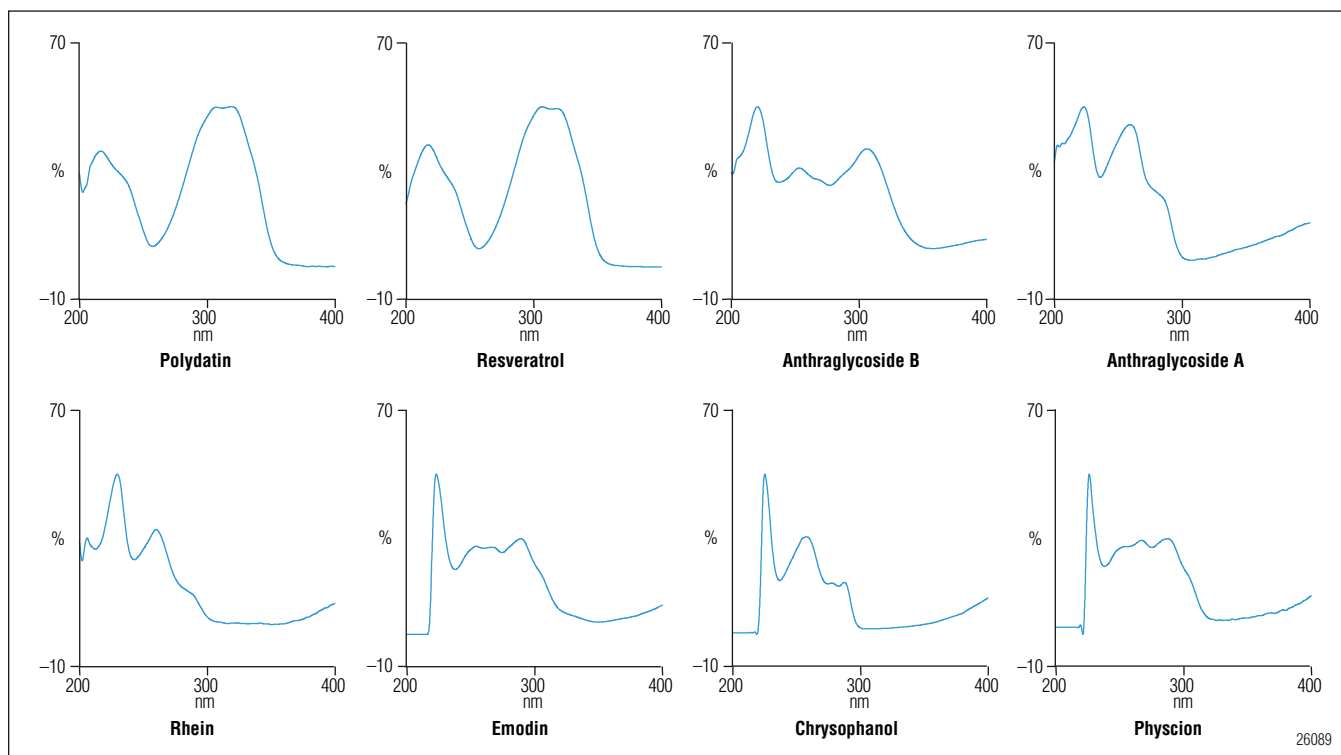


Figure 3. UV absorption spectra (obtained by auto background subtraction using Chromeleon software).

evaluation methods for the quality control of giant knotweed rhizome use the HPLC method to determine anthraglycoside A, anthraglycoside B, resveratrol, emodin, and physcion.^{3,4} However, these methods are still inadequate for evaluating the quality of giant knotweed rhizome as one method determines the first three compounds listed previously and the second method determines the last four compounds.

In this application note (AN), we report an efficient HPLC method that in a single injection determines the eight main active components of giant knotweed rhizome: anthraglycoside A, anthraglycoside B, emodin, physcion, rhein, chrysophanol, resveratrol, and polydatin. Samples were extracted using ultrasonic extraction and the ASE[®] 200 Accelerated Solvent Extractor respectively, with satisfactory results obtained for both extraction methods.

EQUIPMENT

Dionex UltiMate[®] 3000 HPLC system consisting of:
 HPG 3400A pump
 WPS 3000TSL autosampler
 TCC-3200 thermostatted column compartment
 VWD-3400 UV-vis detector

Dionex Summit[®] 680 HPLC system consisting of:
 HPG 680 pump with SOR 100 Air Solvent Rack
 ASI-100[™] Automated Sample Injector
 TCC-100 thermostatted column compartment
 PDA 100 UV-vis detector
 Chromeleon[®] 6.80 SP5 chromatography data system
 Kudos[®] SK3200LH ultrasonic generator, Kudos
 Ultrasonic Instrumental Co., Shanghai, China
 ASE 200 Accelerated Solvent Extractor

REGENTS AND STANDARDS

Water (Milli-Q[®] Gradient A10)
 Methanol (CH₃OH) (HPLC grade, Fisher Scientific)
 Acetonitrile (CH₃CN) (HPLC grade, Fisher Scientific)
 Acetic acid (HAc) (analytical grade, SCRC, China)
 Ammonium acetate (NH₄Ac) (analytical grade, SCRC, China)
 Anthraglycoside A, anthraglycoside B, and polydatin, ≥98% (Kaimi Chem. Co., Shanghai, China)
 Resveratrol, emodin, rhein, chrysophanol, and physcion, ≥98%, (Research Center of Standardization of CTM, Shanghai, China)

CONDITIONS

ASE Conditions

Solvent:	Methanol
Temperature:	100 °C
Pressure:	1500 psi
Static Time:	5 min
Static Cycles:	2
Flush:	20%
Purge:	60 s

Chromatographic Conditions

Guard Column:	Acclaim® 120 C18 column, 5 µm, 4.3 × 10 mm, P/N 059446 with guard column holder, P/N 59526
Analytical Column:	Acclaim 120 C18 column, 5 µm, 4.6 × 250 mm, P/N 002762
Column Temp.:	30 °C
Mobile Phase:	(A) CH ₃ CN (B) 20 mM NH ₄ Ac
Gradient:	See Table 1
Flow Rate:	1.0 mL/min
Inj. Volume:	5 µL
UV Detection:	Absorbance at 254 nm

Table 1. Gradient Elution Program

Time (min)	25 mM NH ₄ Ac (%)	CH ₃ CN (%)	Curve
0.0	75	25	
10.0	60	40	5
15.0	0	100	5
20.0	0	100	5
20.1	75	25	5
23.0	75	25	5

PREPARATION OF STANDARDS AND SAMPLES

Stock Standard Solution

Accurately weigh 100 mg of resveratrol and polydatin, 40 mg of anthraglycoside B, physcion and chrysophanol, and 20 mg of emodin, rhein, and anthraglycoside A, respectively; add them to individual 10 mL volumetric flasks and dilute to the mark with methanol. The concentration is 1000 µg/mL for resveratrol and polydatin, 400 µg/mL for anthraglycoside B, physcion, and chrysophanol, and 200 µg/mL for emodin, rhein, and anthraglycoside A.

Sample Preparation

Five giant knotweed rhizome samples from different regions in China were purchased for this study. Sample details are shown in Table 2.

Table 2. Five Purchased Giant Knotweed Rhizome Samples

Sample	Source
1	Zhejiang
2	Hebei
3	Hubei
4	Beijing Tongrentang 1
5	Beijing Tongrentang 2

Accurately weigh 0.25 g of each giant knotweed rhizome sample, dried and powdered, through a 250 µm sifter.

Ultrasonic method: Add the weighed sample to a 25 mL volumetric flask, and then add methanol to the mark. After 30 min of ultrasonic extraction of the uncovered flask, supplement the methanol to the mark to replace liquid lost by evaporation.

ASE method: Add the weighed sample to a 5 mL stainless steel extraction cell equipped with two cellulose filters on the bottom. Sample should nearly fill the cell. Extract with ASE using the conditions above and transfer each extract into a 25 mL volumetric flask. Bring to volume with methanol.

RESULTS AND DISCUSSION

Effects of Buffer pH Value

Giant knotweed rhizome is the dried rhizome and root of *Polygonum cuspidatum*. Like many Chinese medicines, it is a complex mixture of compounds. Although extraction is performed before analysis, the sample extract still contains many polar and nonpolar compounds that can interfere with determination of the target analytes. Given the complexity of the sample, an acetonitrile-ammonium acetate buffer prepared at different pH values is a good HPLC mobile phase choice to separate the target analytes from the other sample components.

To determine the optimum mobile phase pH value we evaluated the separation of the sample 4 extract at different pH values (Figure 4). Retention of both peaks I

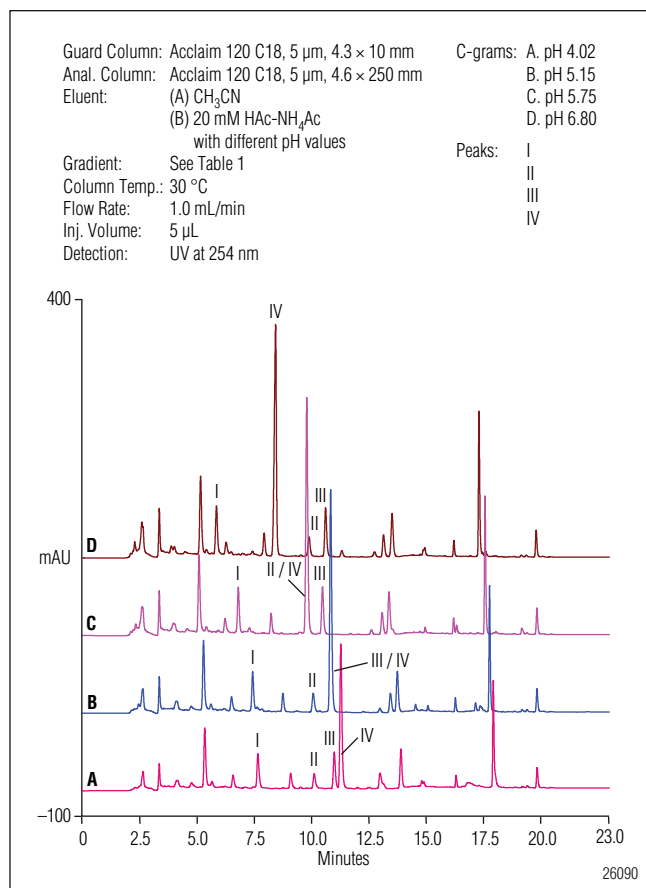


Figure 4. Chromatograms of sample 4 at different pH values of acetate buffer. The sample was extracted using the ultrasonic method.

and IV decrease with increasing mobile phase pH. Peak IV coelutes with peak III at pH 5.2, and with peak II at pH 5.8. The best separation of the components in the giant knotweed rhizome sample extract is at pH 6.8.

After investigating the effect of mobile phase pH value on the sample extract separation, we investigated the effect of mobile phase pH on an eight-component standard. As shown in Figure 5, retention of rhein (peak 2) and anthraglycoside B (peak 3) decrease with increasing mobile phase pH. Anthraglycoside B coelutes with resveratrol (peak 4) at pH 5.8. Notably, an impurity (unknown peak) can interfere with the determination of anthraglycoside B between pH 4.0–5.2. The impurity peak is well-separated from other components when $\text{pH} \geq 5.5$. Similar to the study with the sample extract, this study suggests that 20 mM NH₄Ac (pH 6.8) was the optimum mobile phase composition. At pH 6.8, the main

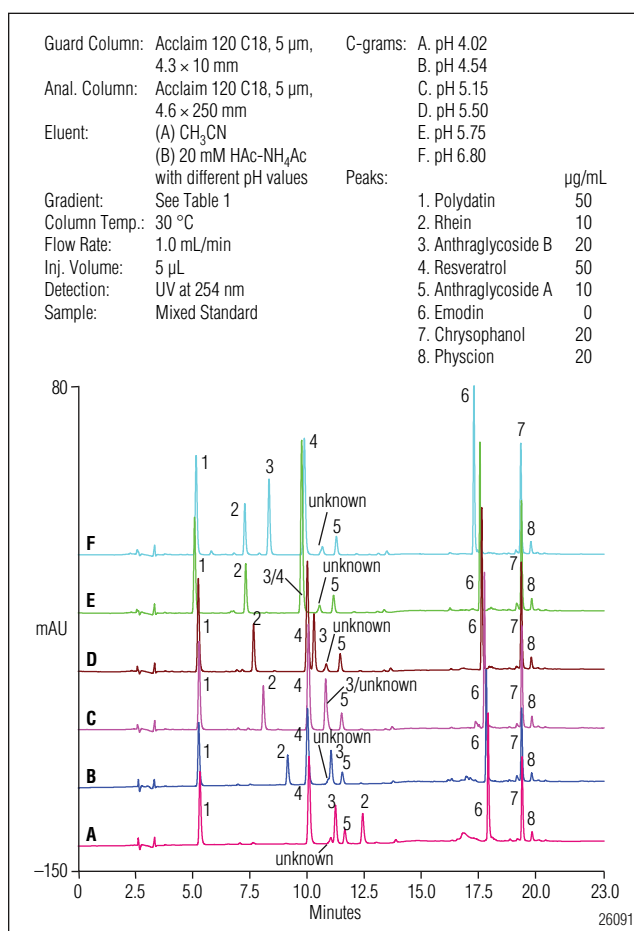


Figure 5. Chromatograms of a mixed standard at different pH values of acetate buffer: Compounds polydatin, rhein, anthraglycoside B, resveratrol, anthraglycoside A, emodin, chrysophanol, and physcion maintain the same peak numbers in Figures 6, 7, and 8. The numbering is not related to their order of elution.

components in the giant knotweed rhizome extract and the eight target analytes in the mixed standard are well-resolved, and the impurity found in the mixed standard does not interfere with the analysis. While ammonium acetate has little buffering capacity at pH 6.8, our studies found no reproducibility issues with the analysis of these samples under the conditions presented in this application note.

Reproducibility

Method reproducibility was estimated by making nine consecutive replicate injections of the mixed standard. Figure 6 is the overlay of the nine chromatograms and Table 3 summarizes the retention time and peak area precision data. The figure and table suggest that this method has good intraday reproducibility.

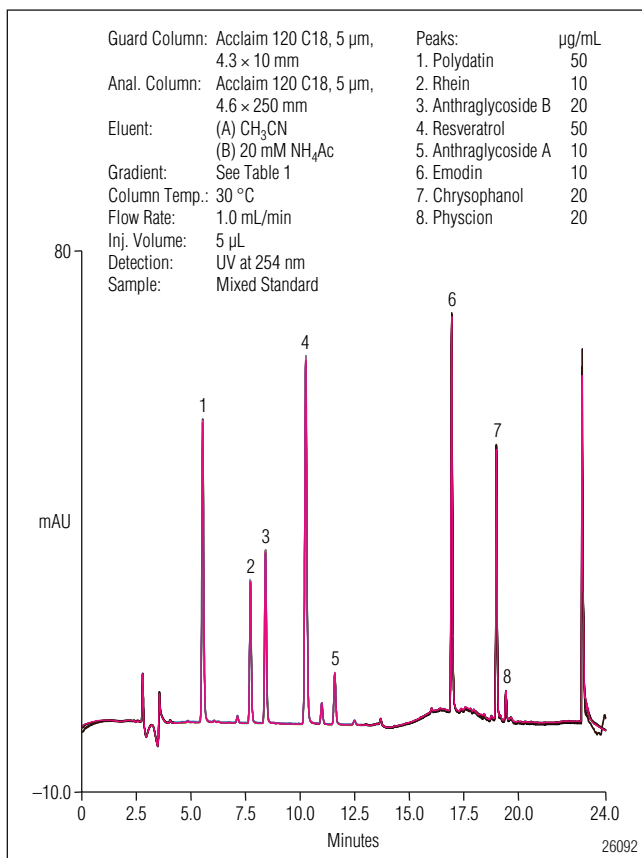


Figure 6. Overlay of chromatograms of nine consecutive injections of a mixed standard.

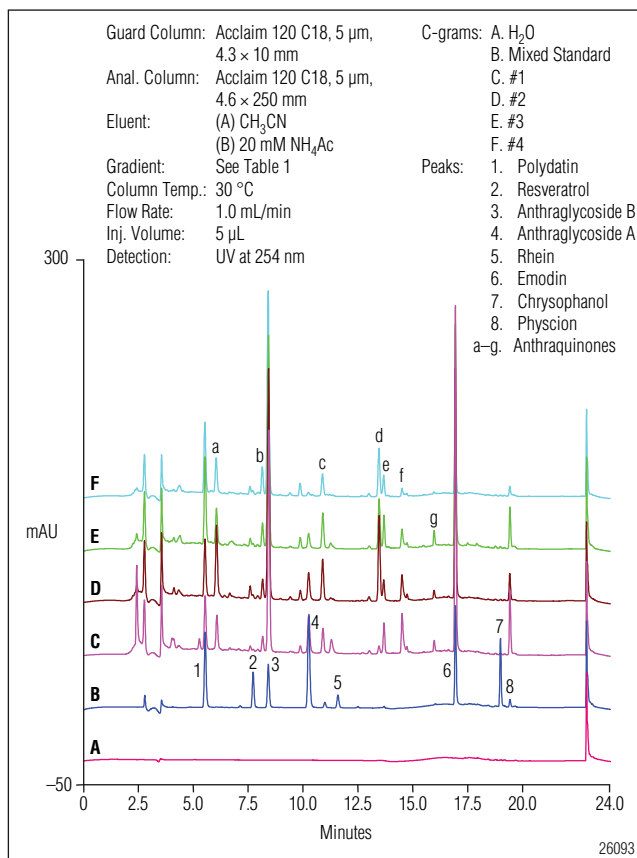


Figure 7. Overlay of chromatograms of water (chromatogram A), the mixed standard (chromatogram B), and samples 1-4 (chromatograms C-F, respectively).

Table 3. Retention Time and Peak Area Reproducibility

Analyte	Retention Time RSD	Peak Area RSD
Polydatin	0.075	0.385
Resveratrol	0.036	0.753
Anthraglycoside B	0.052	0.661
Anthraglycoside A	0.000	0.633
Rhein	0.036	0.953
Emodin	0.022	0.987
Chrysophanol	0.015	0.526
Physcion	0.019	0.727

Discrimination of the Components in Giant Knotweed Rhizome Samples

The putative active components in giant knotweed rhizome are stilbenes and anthraquinones. Stilbenes have three UV absorption maxima: 217, 307, and 318 nm. Anthraquinones have one UV absorption peak (~230 nm)

and three UV absorption zones (240–260 nm, and 305–389 nm contributed by the benzene structure; and 262–295 nm contributed by the quinone structure). The absorption peak wavelength and intensity of UV absorption zones are related to the structure, including the number and position of the substituents. Figure 7 shows the chromatograms of the samples and the mixed standards. Six components, polydatin (peak 1), rhein (peak 2), anthraglycoside B (peak 3), resveratrol (peak 4), emodin (peak 6), and physcion (peak 8) were identified in each sample by comparing retention times and UV absorption spectra. Anthraglycoside A (peak 5) and chrysophanol (peak 7) were not found. Another seven peaks (peaks a–g) were found in each sample (peak c is the unknown peak shown in Figure 5). They each have one UV absorption peak (~230 nm) and three UV absorption zones between 240 and 295 nm with different absorption peak wavelengths and intensities, similar to the UV absorption spectra of anthraglycoside A, anthraglycoside B, and rhein (Figure 3) suggesting that they are anthraquinones.

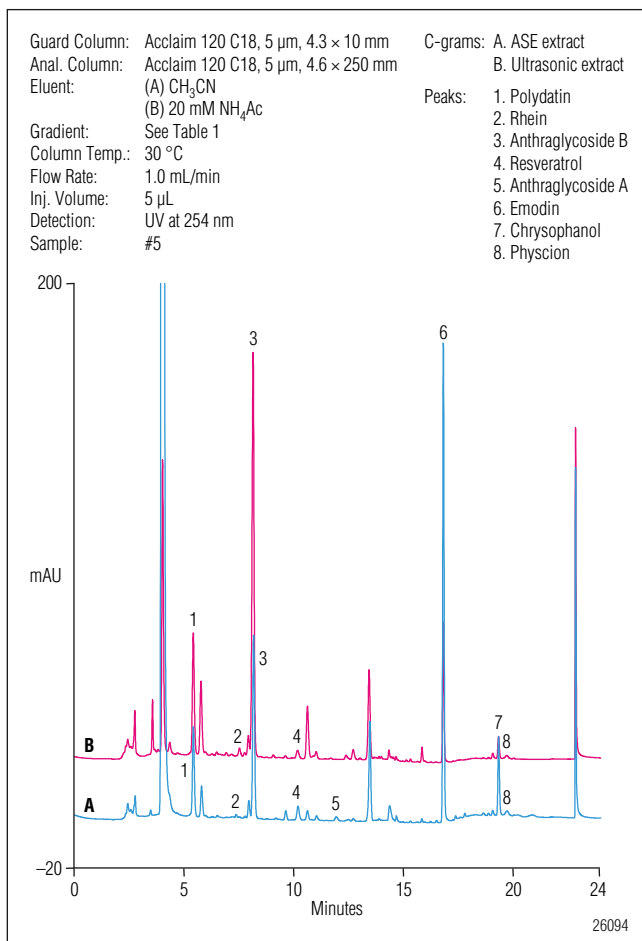


Figure 8. Chromatograms of sample 5 extracted using A) ASE and B) the ultrasonic method.

Using ASE for Sample Preparation and the UltiMate 3000 HPLC System for Chromatography

The giant knotweed rhizome sample 5 was extracted using ASE under different conditions (that is, temperatures and static cycles). The final conditions are as described in this AN. Figure 8 shows the chromatograms of the same sample extracted using ASE and the ultrasonic method. The separations were performed on the UltiMate 3000 HPLC system. Table 4 shows a comparison of the extraction methods using the same extraction solvent (methanol). The biggest difference is anthraglycoside A (peak 5), present in the

Table 4. Comparison of Extraction Efficiency Using ASE and Ultrasonic Extraction			
Peak No.	Analyte	Peak Area (mAU*min)	
		Peak Area Obtained by ASE	Peak Area Obtained by Ultrasonic
1	Polydatin	3.45	5.16
2	Rhein	0.07	0.12
3	Anthraglycoside B	7.11	16.6
4	Resveratrol	0.71	0.45
5	Anthraglycoside A	0.21	0.00
6	Emodin	13.4	3.88
7	Chrysophanol	3.27	1.05
8	Physcion	0.88	0.21

ASE extract, but not found in the ultrasonic extract. Peak 5 in the chromatogram of the ASE extract matched the UV spectrum of anthraglycoside A. This ASE method shows higher recovery for the later eluting analytes and lower recovery of the early eluting analytes compared to the ultrasonic method, but it is possible that all high results (ASE and ultrasonic extraction) are a result of coextractables. Without a reference standard, it is difficult to compare the quality of the extraction methods for this application, but these results suggest that ASE, which is automated to save time and improve reproducibility, can be used for this application.

CONCLUSION

This AN describes an HPLC method that baseline resolves eight active components in giant knotweed rhizome using 20 mM NH₄Ac (pH 6.8) as part of the mobile phase. This method can be used for the quality control of giant knotweed rhizome, a common medicinal plant in China, and is superior to existing methods that measure only a few of the purported active components of giant knotweed rhizome. Sample extractions can be automated with ASE to save time and improve reproducibility.

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