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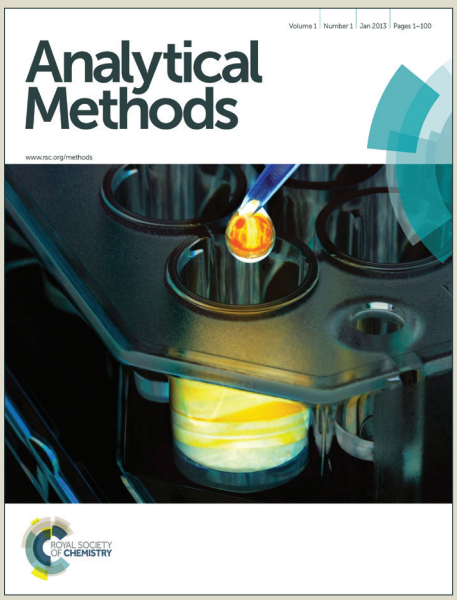

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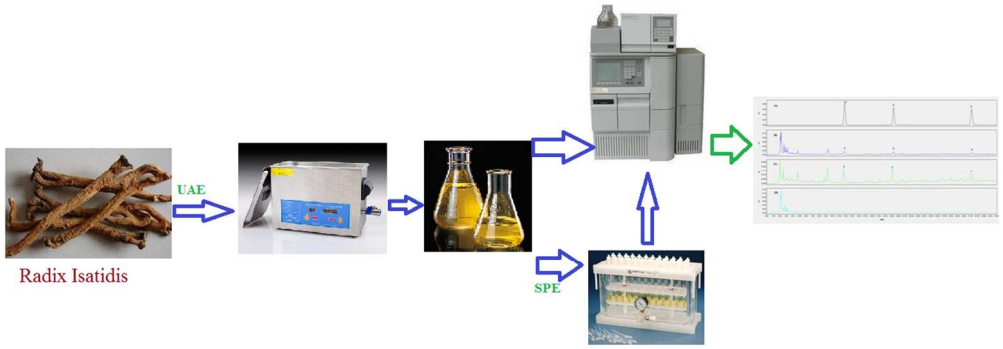

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Ultrasound-assisted extraction coupled with SPE-HPLC-DAD for the determination of three bioactive phenylpropanoids from Radix Isatidis

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Abstract

This study demonstrated a reversed-phase high performance liquid chromatography (RP-HPLC) with photo-diode array detection (DAD) method for simultaneous determination of three phenylpropanoids including syringin, clemastanin B and indigoticoside A in Radix Isatidis. Samples were extracted with the method of ultrasound-assisted extraction (UAE). The optimal UAE conditions were: 80% (v/v) methanol solution, extraction time 45 min, solvent to solid ratio at 20 mL/g, temperature 60 □. Under the optimized conditions, satisfactory extraction yields of the target analytes were obtained. The investigated analytes in extracting solution were concentrated in advance by solid phase extraction (SPE). Optimum conditions for SPE were achieved using 10 mL water as the washing solution and 5 mL water/methanol (50:50, v/v) for elution. Three analytes demonstrated good linearity ($R^2 \geq 0.9995$) in a relatively wide concentration range. The method revealed high

average recovery (range, 96.4%-98.1%) and good precision with inter-day and intra-day variations with less than 0.69%. The validated method was successfully applied to quantify the three phenylpropanoids in eleven batches of Radix Isatidis obtained from different areas of China.

Keywords: Radix Isatidis; ultrasound-assisted extraction; SPE-HPLC; syringin; clemastanin B; indigoticoside A

1. Introduction

Radix Isatidis is the dried root of the plant *Isatis indigotica* Fort[1]. It was first documented as the herbal drug in *The Divine Husbandman's Herbal Foundation Canon,* a famous ancient medical book in the Han Dynasty of China (200 AD). Previously studies indicated that Radix Isatidis has antivirus, anti-inflammatory, antioxidant, anti-bacterial, antitumor, immune regulatory effects, etc.[2-3]. Radix Isatidis was frequently used for the prevention of severe acute respiratory syndrome (SARS) in 2003 and swine flu pandemic in 2009 in China[4-5]. Chemical studies showed that Radix Isatidis contains various phenylpropanoids. 7S,8R,8'R-(-)-lariciresinol-4,4'-bis-O-β-D-glucopyranoside (clemastanin B), (+)-lariciresinol-4-O-β-D-glucopyranoside (indigoticoside A) and syringin are the major phenylpropanoids isolated from Radix Isatidis[6-8]. Our previous screening results showed that clemastanin B and syringin had strong inhibitory effects on influenza virus FM1[9-10]. The latest studies showed that clemastanin B inhibited different subtypes of human and avian influenza viruses at different magnitudes of activity (IC_{50} 0.087-0.72 mg/mL)[11]. Indigoticoside A was seperated from the antiviral active fraction of Radix

Isatidis and had antioxidant effects *in vitro* in our previous study[7,12]. Syringin has been reported to exhibit immunomodulatory, anti-inflammatory and hepatoprotective properties[13-15]. Therefore, quantitative analysis of syringin, clemastanin B and indigoticoside A is very important for ensuring the efficacy and quality of Radix Isatidis.

The UAE is expeditious, inexpensive, efficient and environment protectional[16]. As a conventional extraction technique, it is also a well-established method in the processing of plant material, and in the extraction of analytes from different parts of plants[17]. The UAE had been applied to the extraction of clemastanin B in Radix Isatidis[18-19]. But there was not any literature reported about syringin, clemastanin B and indigoticoside A by UAE simultaneously. So the optimization of the UAE conditions is very important for obtaining the maximum bioactive constituents in a shortest processing time at a low cost.

Solid phase extraction (SPE) is a useful extraction and cleanup method to concentrate and purify these target compounds[20]. It can be regarded as a conventional and valuable sample preparation method because of its simplicity and robustness[21]. The SPE strategy comprises the isolation and pre-concentration of the analyte from a complex matrix by adsorption onto an appropriate sorbent, removal of interfering impurities by washing with a suitable solvent system, and selective recovery of the retained analyte with a suitable solvent[22]. In our previous study, some polar compounds seriously interfered with the determination of phenylpropanoids in Radix Isatidis[19]. There were few reports about simultaneous determination of several

phenylpropanoids in Radix Isatidis by HPLC because of interfering impurities. Therefore, we would like to adopt the SPE technique to remove interfering impurities to ensure the accuracy of phenylpropanoids determination.

The primary purpose of present study was to establish optimum conditions of ultrasound-assisted extraction for syringin, clemastanin B and indigoticoside A (Fig. 1) and to develop a rapid, simple and accurate SPE-HPLC-DAD method for the simultaneous determination of the three phenylpropanoids in Radix Isatidis. The proposed method was applied to quantify these three bioactive compounds in Radix Isatidis from different areas of China successfully. This is the first up-to-date report that the sensitive method was developed for the analysis of the three bioactive compounds in Radix Isatidis, and it provided the novel analytical method comparing with the traditional analysis. This method would contribute to the moderlization of the traditional plant on the determination of its bioactive components for their potential applications in the pharmaceutical industry.

2. Experimental section

2.1. Reagents and materials

Eleven batches of Radix Isatidis were collected from different provinces in China. Its botanical origin was identified by Professor Chen Jian-wei, Nanjing University of Chinese Medicine. The voucher specimens were deposited at the Herbarium in Nanjing University of Chinese Medicine (Nanjing, China).

Methanol for HPLC was purchased from Merck (Darmstadt,Germany). Deionized water was prepared using a Millipore MilliQ-Plus system (Millipore, Bedford, MA).

Syringin, clemastanin B and indigoticoside A were separated and purified in our laboratory. The structures (Fig. 1) were elucidated by their UV, IR, MS, ^{1}H NMR, ^{13}C NMR and 2D NMR data[6-8]. The purity of each compound was more than 98% detected by TLC and HPLC-DAD-ELSD. The commercial C18-SPE cartridge (500 mg/6 mL) was obtained from Agela Technologies (Tianjin, China).

2.2. Apparatus

Sample preparation was performed using UAE in a KQ-500 B ultrasonic device (Kunshan Ultrasound Instrument Company, China) with a frequency of 40 kHz, with ultrasound input power of 500 W, equipped with digital timer and temperature controller. All chromatographic measurements were performed on a Waters 2695 liquid chromatography system (Waters, USA), equipped with a vacuum degasser, a quaternary, low-pressure mixing pump, an autosampler, a thermostated column compartment and a Waters 2998 photodiode array detector. The chromatography column was a Waters Symmetry C18 column (250 × 4.6 mm, 5 μm).

2.3. Chromatographic conditions

The column was operated at 30℃, and UV detection at 280 nm. Gradient elution with (A) water and (B) methanol was 0-10 min 20% B; 10-25min, 20-30% B; 25-35 min, 30% B. The flow rate was set at 1.0 mL/min and injection volume was 10 μL.

2.4. Preparation of standard solutions

The standard stock solutions of syringin, clemastanin B and indigoticoside A were prepared in water with a concentration of 517.0 μg/mL, 724.0 μg/mL, 653.0 μg/mL

respectively. The stock solutions were serially diluted, mixed and used for preparation of standard solutions, which were stored in a refrigerator at 4℃.

2.5.Sample preparation and solid phase extraction

The extraction was performed under the optimized conditions: the dried powder sample (500.0 mg) was placed into a 50 mL conical flask, and then mixed with 10.00 mL water/methanol (20:80, v/v) ultrasonic extraction for 45 minutes. The mixtures were centrifuged at 12,000 rpm for 10 min. The supernatant was concentrated to dry with a vacuum centrifugal concentration meter and then added 4 mL of water to dissolve. The solution was filtered through 0.45 μm membranes. The clean-up procedure was as follows: The C18-SPE cartridge was pre-conditioned with 5mL of methanol and 10 mL of deionized water before use, and then the concentrate was loaded to the pre-conditioned cartridge. The concentrate was passed through the cartridge, washed by 10.00 mL water to remove interferences and eluted with 5 mL water/methanol (50:50, v/v). The eluate was dried by blowing N_2 stream and dissolved in 4.00 mL of water/methanol (50:50, v/v), filtrated by 0.45 μm microfiltration membrane filter prior to injection into the HPLC system.

2.6. *Validation of the developed method*

2.6.1 Calibration curves, limits of detection (LOD) and quantification (LOQ)

Calibration curves were calculated based on the peak areas, obtained from the chromatograms of seven different concentrations with the ranges of 1.148-172.3 μg/mL for syringin, 1.340-241.2 μg/mL for clemastanin B and 1.361-217.7 μg/mL for indigoticoside A by diluting these stocking solutions in series. The limits of detection

(LOD) and quantification (LOQ) for each analyte under present chromatographic conditions were determined at the signal-to-noise ratio (S/N) for each compound of about 3 and 10, respectively. Each concentration level was injected three times.

2.6.2 Precision and accuracy

The intra-day and inter-day precisions were investigated by determining a known concentration mixed standard solution in six replicates during a single day and by duplicating the experiments on three consecutive days. Variations were expressed as the relative standard deviations (RSD).

The recovery was used to evaluate accuracy of the method. Recovery was performed by adding the standard stock solutions of syringin (176 μL), clemastanin B (608 μL) and indigoticoside A (598 μL) to 0.25 g Radix Isatidis powder (collected from Neimenggu, S11). The mixture was extracted and analyzed using the method mentioned above. Three replicates were performed for the test. The quantity of each analyte was subsequently obtained from the corresponding calibration curve. The recovery was calculated as follow:

$$\text{Recovery}(\%) = \frac{(\text{amount found} - \text{original amount})}{\text{amount spiked}} \times 100$$

2.6.3 Stability

The stability was tested with one of the sample solutions mentioned above at room temperature. At 0, 2, 4, 6, 12, 24 and 48 h, the samples were analysed respectively.

2.7. Extraction yield determination

The extraction efficiency were evaluated using the extraction yield as index, which were calculated according to the following equation:

$$\text{Yield}\ (\text{mg/g}) = \frac{\text{weight of analytes extracted}\ (\text{mg})}{\text{weight of dried sample}\ (\text{g})}$$

2.8 Comparison of the presented method with other methods

The presented method was compared with other conventional methods used for determination of the interested analytes. The extraction and determination methods were in accordance with the relevant literatures[18-19]. The contents of the three analytes in different samples were presented in Table 6.

3. Results and Discussion

3.1 Optimization of chromatographic conditions

The chromatographic conditions were optimized to obtain better resolution of adjacent peaks within a shorter analysis time. Three different brands of chromatographic columns were investigated including a Hanbon Hedera C18, a Waters Symmetry C18 and an Agilent Lichrospher C18 column. The Waters Symmetry C18 column offered better resolution. According to the full wavelength scanning (from 200 to 400 nm), 280 nm was selected as the detection wavelength. Two common mobile phase systems, water-methanol and water-acetonitrile were tested, and the water-methanol system resulted in better separation of the investigated compounds. Furthermore, addition of acid in mobile phase was found to have no significant improvement on the separation. In order to obtain the best possible resolution, various linear gradients of methanol-water were investigated at a flow rate of 1.0 mL/min. Finally, the gradient programme described above was chosen as it

allowed the three major peaks to be clearly separated. As shown in Fig.2, the analytes were completely separated with good peak symmetry.

3.2 Optimization of UAE conditions

To achieve the best UAE conditions, different extraction solvents (water, ethanol, methanol and ethyl acetate), various concentrations of extraction solvents (0%, 10%, 30%, 50%, 80%, 100% methanol), six different ultrasonic times (10, 20, 30, 45, 60 ,90 min), six liquid/solid ratios (5, 10, 15, 20, 30, 40 mL/g) as well as increasing temperatures (30, 40, 50, 60, 70 and 80℃) were investigated. When one parameter was optimised it was kept constant while the remaining parameters were optimised. Extraction by methanol was the best choice for the highest total yield of the three phenylpropanoids. Also, it clearly indicated that the highest extraction yields of syringin, clemastanin B and indigoticoside A were obtained using 80% methanol (Fig.3A). Thus, 80% methanol was chosen as the best solvent in the following extraction experiments. Ultrasonic extraction times were trialed ranging from 0 to 45 min, extraction yields of syringin, clemastanin B and indigoticoside A increased gradually. However, the extraction yields had no significant improvements between 45 and 90 min, suggesting that the extraction had reached its maximum efficiency (Fig.3B). Hence, 45 minutes was chosen as the optimum extraction time. Data shown in Fig.3C indicated that 20 mL/g was the optimum ratio of the solvent to solid. In Fig.3D, the extraction yields of the three phenylpropanoids increased while the extraction temperature went up gradually from 30 to 60℃. However, the yields decreased when the temperature was increased beyond these temperatures. The optimum extraction

temperature was 60℃. In conclusion, the ideal UAE condition was 80% methanol using with 20 mL/g of liquid/solid at a temperature of 60℃ for 45 min.

3.3 Optimization of SPE conditions

3.3.1. Determination of maximum loading capacity of solid phase cartridge

Maximum loading capacity of analytes was determined by passing different concentration of standard solution through the C18-SPE cartridge. As it showed in Table 1, the maximum loading capacities for syringin, clemastanin B and indigoticoside A were 160, 640 and 800 μg, respectively.

3.3.2. The flow rate of loading

The flow rate of loading had a significant influence on the recoveries of analytes. The effects of the flow rate of loading (0.5, 1.0, 1.5 and 2.0 mL/min) on the recoveries of syringin, clemastanin B and indigoticoside A were investigated. When the flow rate was 0.5 mL/min, the recovery rates were higher but more time-consuming than other flow rates. However, when the flow rate exceeded 1.0 mL/min, the recoveries of analytes decreased significantly. The experimental results showed that the most appropriate flow rate was 1.0 mL/min.

3.3.3. Influence of the washing solvent and elution solvent

In the SPE, selection of an appropriate washing solvent and elution solvent is the first factor that should be considered because it has a direct effect on desorption efficiency[23]. To obtain a suitable washing solvent and elution solvent, different percentages of methanol in water were investigated. The best washing solvent and

elution solvent for the compounds was examined by using 5 mL of each solvent. Table 2 shows that the three phenylpropanoids were not washed out by water, while some unnecessary compounds were washed out. Therefore, water was selected as a suitable washing solvent. The amount of the three phenylpropanoids increased with the increasing of percentages of methanol when the percentages of methanol ranged from 0% to 50% (v/v). However, the contents had no significant change between 50 and 100% (v/v). The results showed that 5 mL of 50% (v/v) methanol can completely clear the three analytes of adsorption. Therefore, the optimum condition for elution solution was water/methanol (50:50 v/v).

3.3.4. Influence of elution solvent volume

In order to determine a suitable volume of the elution solvent, different volumes (2, 3, 4, 5, 6 and 8 mL) of water/methanol (50:50, v/v) were used for elution of the retained analytes from the cartridge. As shown in Table 3, it can be observed that the extraction amount of the three phenylpropanoids increased with volumes of elution solvent increasing from 2 to 5 mL. When the volume of elution solvent was over 5 mL, the amount of phenylpropanoids remained almost constant. Therefore, the most suitable volume of elution was 5 mL.

3.4.Validation of the developed method

3.4.1 Calibration curves, LOD and LOQ

Linear regression analysis of peak area versus theoretical concentration data was evaluated for each analyst. As shown in Table 4, all the analytes showed good linearity ($R^2 \geq 0.9995$) in a relatively wide concentration range, and their LODs and

LOQs were less than 0.2137 μg/mL and 0.7136 μg/mL respectively.

3.4.2. Precision, accuracy and stability

As shown in Table 5, the intra-day precisions and inter-day precisions RSD values of the three compounds were less than 0.54% and 0.69% respectively. The recovery percentages of the analytes ranged from 96.4% to 98.1%. The Radix Isatidis sample was steady within 48 h (RSD < 3.3%). The results indicated that the developed method was precise and accurate for the quantitative determination of three analytes in Radix Isatidis.

3.5. Comparison of the presented method with other methods

The results of the comparison were shown in Table 6. It showed that better results were achieved by this method than other conventional methods. Generally, UAE extraction of Radix Isatidis contained a lot of co-extracted matters which might interfere with the analysis of syringin, clemastanin B and indigoticoside A (Fig.4B). The sample after being treated with SPE some polar interfering compounds can be removed by deionized water. The result showed that SPE greatly removed the co-extracted matter in Radix Isatidis (Fig.4C, Fig.4D). In summary, the method of sample preparation achieved satisfactory results for the three constituents.

3.6 Application

Identification of the investigated compounds was performed by comparison of their retention times and UV spectra with those obtained injecting standards under the same conditions. Quantification was performed on the basis of linear calibration plots of the peak areas versus the concentration. The application of this approach was

confirmed by the successful analysis of eleven batches of Radix Isatidis. The mean contents of syringin, clemastanin B and indigoticoside A in Radix Isatidis three parallel determinations were summarized in Table 7.

As shown in Table 7, there were some significant differences in the content of the three compounds from different Radix Isatidis samples, which could be due to the factors of species, climate, environment such as altitude and growing conditions. The highest total content of the three compounds was found from Neimengu province (S11, 3.69 mg/g), and the lowest from Anhui provinces (S2, 0.642 mg/g).

4. Conclusions

An efficient UAE method has been developed for the extraction of syringin, clemastanin B and indigoticoside A from Radix Isatidis. Under the optimized UAE conditions, satisfactory extraction efficiencies of the three phenylpropanoids were obtained. A simple and sensitive SPE-HPLC-DAD assay procedure was first developed for the determination of the three bioactive compounds from Radix Isatidis. Our present work proved that the SPE-HPLC-DAD method was a relatively reliable, convenient and high recovery analytical method which was suitable for routine quantitative analysis and quality control of Radix Isatidis. Our research might serve as a reference basis for the further study and better quality control of Radix Isatidis and its related medicine containing the bioactive components.

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Table. 1 The maximum loading of C18-SPE cartridge for adsorption of analytes

Sample loaded (μg)	Recovery (%)		
	syringin	clemastanin B	indigoticoside A
10.0	95.9	100.3	99.6
20.0	98.7	97.8	98.8
40.0	99.3	99.3	98.5
80.0	99.3	99.9	99.3
160	98.4	99.5	98.3
320	62.6	99.9	97.6
640	31.3	99.4	98.3
800	25.3	87.2	97.3
1.00×10^3	20.2	70.3	85.2
1.20×10^3	16.1	58.2	70.1

Table 2. Extracted amounts of the three phenylpropanoids when using different solvents in the washing step.

Washing solvent	Contents (mg/g) ±S.D. (n=3)		
	Syringin	Clemastanin B	Indigoticoside A
Water	-	-	-
10% Methanol	0.186±0.072	0.582±0.075	0.326±0.022
20% Methanol	0.213±0.024	0.659±0.082	0.547±0.040
30% Methanol	0.289±0.092	1.23±0.02	0.921±0.038
50% Methanol	0.359±0.049	1.75±0.06	1.56±0.06
80% Methanol	0.358±0.074	1.75±0.04	1.56±0.07
100% Methanol	0.359±0.023	1.75±0.08	1.56±0.06

"- ": not detected in the washing step.

Table 3. Extracted amounts of the three phenylpropanoids by different volumes of 50% methanol in the elution step.

volumes of elution solvent(mL)	Contents (mg/g) ±S.D. (n=3)		
	Syringin	Clemastanin B	Indigoticoside A
2	0.104±0.062	0.486±0.013	0.284±0.018
3	0.216±0.022	0.982±0.044	0.723±0.011
4	0.323±0.014	1.46±0.08	1.25±0.06
5	0.366±0.032	1.74±0.02	1.56±0.06
6	0.369±0.050	1.74±0.03	1.56±0.04
8	0.368±0.072	1.74±0.05	1.57±0.03

Table 4. Linear regression data, LOD and LOQ of the investigated compounds.

Analyte	Regression equation	R^2	Linear range (μg/mL)	LOD (μg/mL)	LOQ (μg/mL)
Syringin	$Y=1.384\times10^4X-1.734\times10^4$	0.9995	1.148-172.3	0.1682	0.5641
Clemastanin B	$Y=3.938\times10^3X-5.648\times10^3$	0.9999	1.340-241.2	0.2137	0.7136
Indigoticoside A	$Y=5.975\times10^3X-1.328\times10^4$	0.9999	1.361-217.7	0.1778	0.5413

Table 5. Precision, stability and recovery of investigated compounds in Radix Isatidis.

Analyte	Precision (RSD%)		Stability (n= 6, RSD%)	Recovery% ± S.D. （n= 3）
	Intra-day (n= 6)	Inter-day (n= 6)		
Syringin	0.19	0.28	2.5	98.1 ±0.7
Clemastanin B	0.54	0.69	3.3	96.4 ±2.9
Indigoticoside A	0.46	0.68	2.6	96.9 ±2.9

Table 6. Comparison of the presented method with other methods which were used for determination of interested phenylpropanoids.

Method	Extraction solvent	Contents (mg/g) ± S.D.(n=3)			Reference
		Syringin	Clemastanin B	Indigoticoside A	
UAE/HPLC	50% Methanol	0.246±0.022	1.42±0.04	1.28±0.08	[18]
UAE/HPLC	60% Methanol	0.238±0.035	1.48±0.03	1.31±0.06	[19]
UAE/SPE-HPLC	80% Methanol	0.365±0.004	1.76±0.02	1.56±0.03	This research

Table 7. Content of analytes in Radix Isatidis samples under optimum condition.

Samples	District	Concentration of analytes (mg/g) ± S.D. (n=3)		
		Syringin	Clemastanin B	Indigoticoside A
S1	Anhui provinces	BQ	0.763±0.043	0.299±0.042
S2	Anhui provinces	0.0213±0.0091	0.322±0.014	0.298±0.023
S3	Anhui provinces	BQ	0.354±0.012	0.398±0.012
S4	Gansu provinces	0.0237±0.0039	0.813±0.082	0.761±0.105
S5	Gansu provinces	0.0302±0.0073	1.12±0.06	0.426±0.047
S6	Shanxi provinces	BQ	0.423±0.013	0.419±0.082
S7	Heilongjiang provinces	0.0758±0.0123	0.515±0.041	0.507±0.071
S8	Henan provinces	0.247±0.041	1.03±0.08	1.04±0.05
S9	Sichuang provinces	0.0403±0.0137	0.621±0.011	0.348±0.021
S10	Henan provinces	0.0383±0.0052	0.924±0.095	1.04±0.12
S11	Neimengu provinces	0.365±0.074	1.76±0.02	1.56±0.09

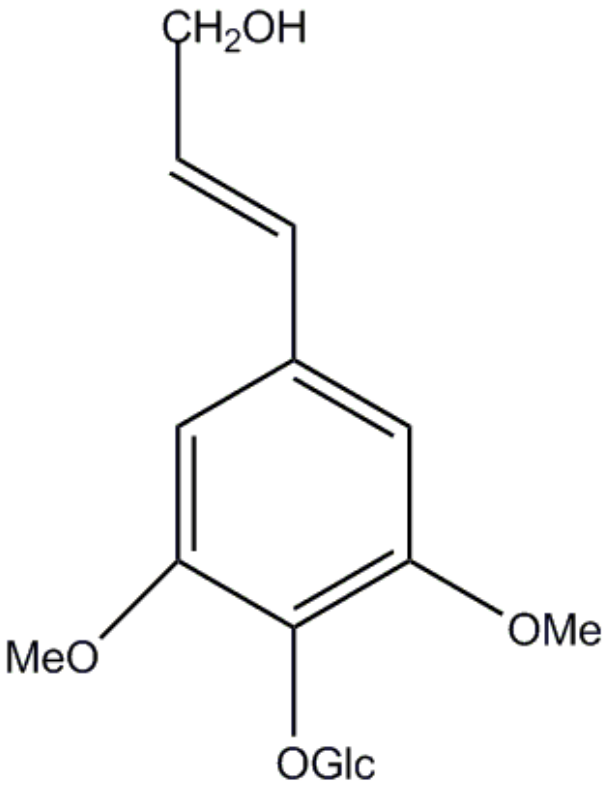
"BQ": below the limit of quantification.

Figures Captions

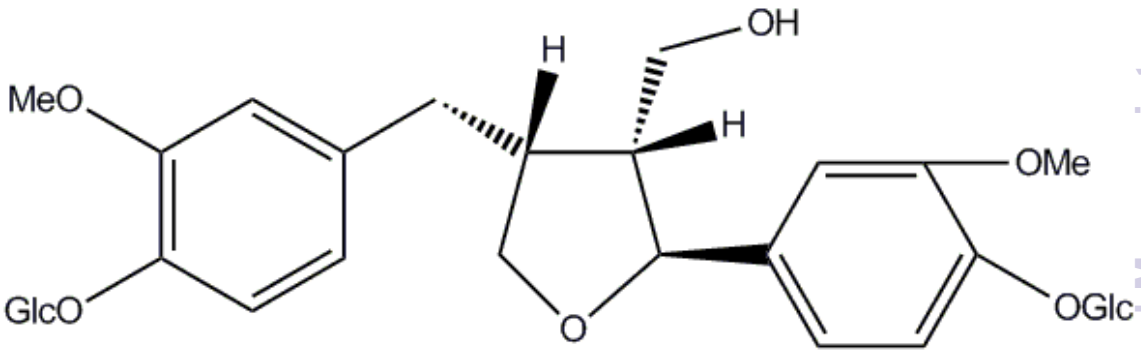
Fig. 1 The chemical structures of the investigated compounds in Radix Isatidis.

Fig. 2 HPLC chromatograms of mixed standards (**A**), UAE extract of Radix Isatidis without (**B**) and with SPE eluted with water/methanol (50:50,v/v) (**C**), washed by deionized water (**D**). **1:**Syringin; **2:**Clemastanin B; **3:**Indigoticoside A.

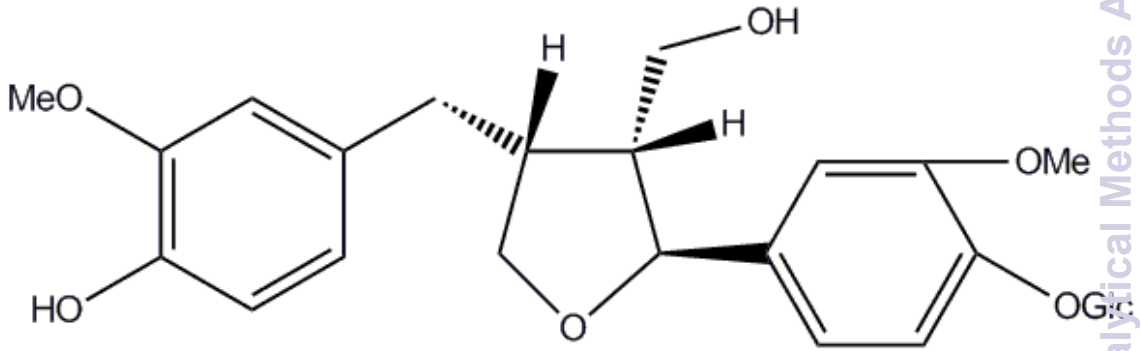
Fig. 3 Effects of four factors on the yield of phenylpropanoids.(**A**) extraction solvent concentration;(**B**) extraction time;(**C**) extraction ratio of liquid/solid; (**D**) extraction temperature.



1. syringin



2. clemastanin B



3. indigoticoside A