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Pressurized liquid extraction of berberine and aristolochic acids in medicinal plants

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Abstract

Berberine and aristolochic acids I and II present naturally in medicinal plants were extracted using a laboratory-made pressurized liquid extraction (PLE) system in the dynamic mode. As the target analytes were present naturally in the medicinal plants, spiking was not done and comparison with ultrasonic extraction and Soxhlet extraction was performed to assess the method accuracy. The effect of temperature, volume of solvent required and particle size were investigated. Method precision (RSD, $n=5$) between 1.98 and 3.4% was achieved for the extraction of berberine and aristolochic acids I and II in medicinal plants and lower than 8% for lower levels of aristolochic acid II in medicinal plants.

Keywords: Pressurized liquid extraction; Berberine; Aristolochic acids

1. Introduction

Berberine (Fig. 1) is a well known alkaloid found in medicinal plants such as *Coptidis rhizoma* (huanglien) which exhibits antibacterial activity [1,2]. Analysis of berberine in medicinal plants using ultrasonic extraction followed by high-performance liquid chromatography (HPLC) was reported [3,4]. Aristolochic acids (I & II) (Fig. 1) are commonly found in medicinal plants such as *Radix aristolochiae* [5]. It was reported to cause acute hepatitis and end stage renal failure [6–8]. Quantitative analysis of aristolochic acids I & II in medicinal plants using ultrasonic extraction with preliminary extraction by hexane followed by methanol with final analysis by HPLC has been reported [9]. Another method extracts aristolochic acids by immersing the medicinal plant in a 10% formic acid solution at room temperature for 18 h [10]. An alternative method includes using a 5% ammonia solution to extract aristolochic acids in medicinal plants overnight [11]. In all cases, the method of extraction can be rather long and tedious.

Pressurized liquid extraction (PLE) is a relatively new extraction method that packs solid samples into an extraction cell and uses an organic solvent at high pressures and temperature above the boiling point. The main reasons for enhanced performance when using PLE are: (1) the higher solubility of analytes in solvent at higher temperature, (2) higher diffusion rate as a result of higher temperature and (3) disruption of the strong solute–matrix interaction caused by van der Waals forces, hydrogen bonding and dipole attractions between solute molecules and active sites on the matrix [12]. The other advantage of PLE is that parameters other than the temperature

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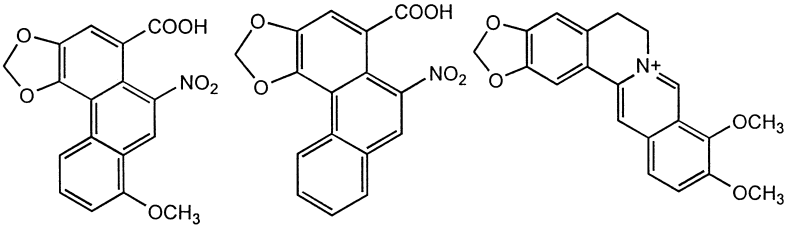

aristolochic acid I aristolochic acid II berberine

Fig. 1. Chemical structures of aristolochic I & II and berberine.

can be varied, the polarity of the extraction solvent can be chosen from a wide range and adapted to the respective matrix. Applications of PLE to the extraction of contaminants in environmental [13–16] and food matrices [17], components in medicinal plants [18] as well as additives in polymers [19–21] were well reported. However, most of the reports mentioned above used commercially available equipment based on static extraction. Vandenburg and co-workers used a PLE system in the dynamic mode for the extraction of additives in polymers [19,20].

The aim of this work is to develop a laboratory-made PLE system in the dynamic mode for the analysis of berberine in *Coptidis rhizoma* and aristolochic acids I and II in *Radix aristolochiae*. The effects of temperature, time of collection/amount of solvent required and particle size were studied. The present extraction system was compared with ultrasonic and Soxhlet extraction to study the efficiency as well as any possible degradation effect at high temperature.

2. Experimental

2.1. Chemicals

Aristolochic acids I (69%) and II (19%) and berberine were purchased from Sigma (St. Louis, MO, USA). Methanol of analytical-reagent grade and HPLC grade were purchased from Hayman (Witham, UK). Acetonitrile of HPLC grade was obtained from Ajax (Auburn, Australia). Ammonium acetate and acetic acid were analytical-reagent grade purchased from Hayashi (Osaka, Japan) and E. Merck (Darmstadt, Germany). Sand purified by acid (about 40 to 100 mesh) was purchased from BDH (Poole, UK).

2.2. Pressurized liquid extraction system

Fig. 2 is a schematic of the PLE system used in this work. The stainless steel tubings used were 1/16 in. O.D.×0.18 mm I.D. (1 in.=2.54 cm). The back pressure was generated based on the sample materials and sand packed in the extraction cell and the tubing used, as no restrictor or back pressure regulator was used in the present system. The extraction cells were of stainless steel with 150×4.6 mm I.D. (2.50 ml) and 100×10 mm I.D. (7.85 ml). The extraction cell was heated in a HP5890, gas chromatograph oven (Hewlett-Packard, USA). The pump used was a ternary gradient HP1050 HPLC pump (Hewlett-Packard, Waldbronn, Germany). The pump flow was set at 1.5 ml/min and the oven temperature was set at 80, 100/105, 120 and 140°C, respectively. The pressure in the system indicated by the HPLC pump was between 9 to 11 bar. The extraction cell was prefilled with methanol to check for possible leakage before setting the temperature of the oven to the required value. Extraction with methanol was carried out for a period of 15 to 20 min and the volume of solvent collected was about 25 to 30 ml. In between runs, the system was washed with methanol.

2.3. HPLC conditions

For all experiments, a Shimadzu LC 10 series (Shimadzu, Kyoto, Japan) equipped with a binary gradient pump, autosampler, column oven and diode array detector was used. For berberine, the mobile

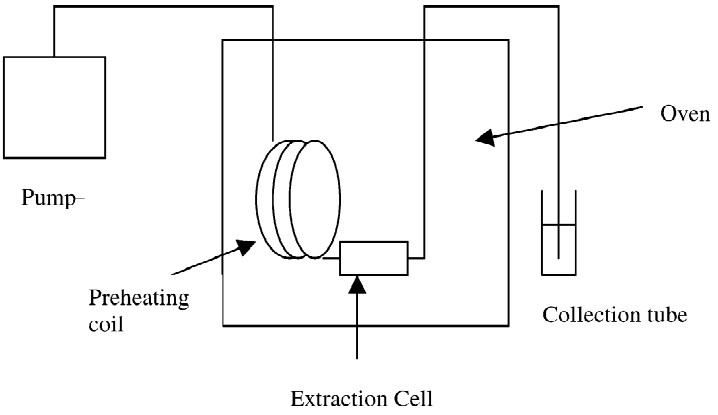

Fig. 2. Schematic illustration of a pressurized liquid extraction system.

phase used was 50 m*M* of ammonium acetate–2% acetic acid–acetonitrile (70:30) with detection at 270 nm [3,4]. For aristolochic acid, gradient elution with mobile phase consisting of (A) 1% acetic acid in water and (B) methanol was used. The initial condition was set at 40% of B and gradient up to 100% B in 15 min before returning to initial conditions. Detection was at 254 nm and 310 nm for berberine and aristolochic acids, respectively. Oven temperature was set at 40°C and flow-rate was set at 1.0 ml/min for the determination of berberine and aristolochic acids. For all experiments, 20 μl of standards and sample extract were injected. The column used for both separations was a Hypersil Elite (Hypersil, Runcorn, UK) C_{18} (250×4.6 mm I.D., 5 μm).

2.4. Preparation of reference standards

For berberine, a series of standards in the range of 0 to 300 mg/l were prepared in methanol. For aristolochic acids I & II, standards in methanol of concentration of 0 to 100 mg/l and 0 to 30 mg/l were prepared, respectively. Quantitation was done using external standard calibration.

2.5. Preparation of medicinal plant extracts

The medicinal plant sample was ground using an IKA MF10 microfine grinder (Staufen, Germany) with a sieve insert of hole size 0.5 mm. In the experiment on the effect of particle size, sieve inserts with hole sizes of 0.25, 0.5 and 1.0 mm were used to obtain particles of different sizes for medicinal plants containing aristolochic acids.

For extraction using PLE, 0.1 g of *Coptidis rhizoma* and 0.5 to 1.0 g of *Radix aristolochiae* samples were weighed directly into the extraction cell and all voids were filled with sand before extraction was carried out. The final conditions selected are: extraction temperature of 120°C and a particle size of less than 0.5 mm. For berberine in *Coptidis rhizoma*, 30 ml of the methanolic extracts was collected in a 50-ml volumetric flask and made up to the mark with methanol. As for aristolochic acids in medicinal plants, 25 ml of the methanol extract was collected in a 25-ml volumetric flask.

For ultrasonic extraction, 0.1 to 1.0 g of sample was weighed into a test tube and extraction was carried out with 20 ml of methanol for 30 min.

For Soxhlet extraction, 0.1 g of *Coptidis rhizoma* and 1.0 to 2.0 g of *Radix aristolochiae* samples were weighed into the thimble. The target analyte (berberine and aristocholic acids) and co-extract gave a yellow color with the extraction solvent. The yellowish color turned lighter and lighter through the course of the extraction. Hence, after extraction with 100 ml of methanol for 7 to 8 h, the extraction solvent was essentially colorless. The excess solvent was evaporated under a gentle stream of nitrogen with gentle heating. The extracts were finally transferred to a 50- or 100-ml volumetric flask.

All extracts were filtered through a 0.45-μm membrane filter (Target) before injecting into the HPLC system.

For the first set of *Coptidis rhizoma* sample prepared for PLE, the herb was ground using the mortar.

3. Results and discussion

3.1. System precision, linearity and limit of detection of HPLC

HPLC system precisions for berberine at 20.8 mg/l and aristolochic acids I and II at 16.2 and 4.46 mg/l were found to be less than 2.0% (RSD, $n=6$). Linearity was achieved from 0 to 300 mg/l for berberine ($0.99 \leq R$) and 0 to 100 mg/l and 0 to 30 mg/l ($0.99 \leq R$) for aristolochic acids I and II, respectively. The limit of detection (LOD) estimated by three times the standard error of the intercept [22–24] for aristolochic acids I and II are 0.32 mg/l and 0.10 mg/l, respectively. The limit of quantitations (LOQs) estimated by 10 times the standard error of the intercept for aristolochic acids I and II were found to be 1.3 mg/l and 0.24 mg/l, respectively. The method's LOQ based on 1 g of sample extracted were found to be 30 mg/kg and 6 mg/kg. For berberine, the LOD and LOQ were estimated to be 0.98 and 4.68 mg/l, respectively. The method's LOD and LOQ based on 1 g of sample extracted were found to be 49 and 234 mg/kg, respectively.

3.2. Optimization of extraction procedures

In the present work, PLE was done in the dynamic mode as compared to static extraction using commercially available accelerated solvent extraction (ASE) equipment. Methanol was selected as the solvent for the extraction of berberine and aristolochic acids from medicinal plants as it was found to have sufficient solubility and is used in the Chinese Pharmacopoeia and other reports [3–5,9]. Just as in commercially available ASE equipment, the amount of solvent required was an important parameter to evaluate. Two different medicinal plants namely *Coptidis rhizoma* and *Radix aristolochiae* were used in this study and the target analytes are essentially non-volatile and rather polar. The amount of berberine and aristolochic acid I present in the plant extract was determined in each 10-ml fraction collected separately as shown in Fig. 3. From the data obtained, it was found that a significant fraction of the target analyte was extracted with 30 to 40 ml of solvent. Based on the data obtained, the amount of solvent to be collected for the extraction was set at 30 ml for berberine and 25 ml for aristolochic acids in medicinal plant.

The effect of temperature on the proposed mode of extraction was investigated and the results are presented in Table 1. It was proposed in several reports [12,21] that the temperature applied for PLE should be higher than the boiling point of the solvent used. Hence, temperatures lower than 80°C were not used. It was found that the extraction efficiency increased with temperature up to 120°C for berberine in *Coptidis rhizoma* and aristolochic acids I & II in *Radix aristolochiae*. However, the amount of target analyte found in the plant extract did not differ significantly when extraction was performed at 100 or 120°C.

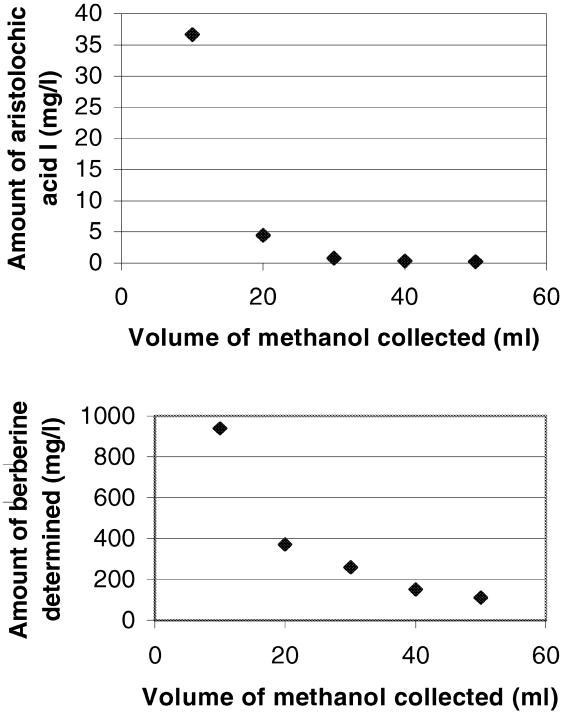

Fig. 3. Amount of (A) aristolochic acid I found in *Radix aristolochiae* and (B) berberine found in *Coptidis rhizoma* over volume of methanol collected over time by PLE.

Table 1
Effect of temperature on PLE for berberine in *Coptidis rhizoma* and aristolochic acids I & II in *Radix aristolochiae*

Temperature for PLE (°C)	Berberine (mg/kg)	Aristolochic acid I (mg/kg)	Aristolochic acid II (mg/kg)
80	53 000	670	50
100	72 000[a]	750	56
120	71 000	759	60

[a] Extraction temperature at 105°C.

A setback for the proposed system was that extraction at 140°C gave poor efficiency. The amount of aristolochic acid I extracted at 140°C was found to be half of that extracted at 120°C. The backpressure was insufficient to keep the solvent in the liquid phase at 140°C. The backpressure generated by the tubings and packing materials in the proposed system was found to be sufficient to ensure high extraction efficiency below 120°C. It was also reported [12,21,25] that the pressure applied would not affect the extraction efficiency significantly and sufficient pressure need to be applied to keep solvent at the liquid state.

Table 2
Effect of particle size on the extraction efficiency of aristolochic acids in *Radix aristolochiae* by PLE at 120°C

Particle size (mm)	Aristolochic acid I (mg/kg, $n=2$)	Aristolochic acid II (mg/kg, $n=2$)
1.0	350	19
0.5	420	28
0.25	450	37

From the results obtained in Table 2, it was found that sample with finer particles gave higher extraction efficiency. Similarly, for berberine in *Coptidis rhizoma* (Table 3), samples prepared with the microfine grinder with sieve insert of 0.25 mm hole size gave results with higher precision and extraction efficiency compared to the sample prepared using a mortar. Despite of the higher temperature and pressure used in PLE, the particle size of the sample played an important part in the extraction efficiency.

3.3. Method precision and accuracy

To further assess the extraction efficiency and investigate the possibility of thermal degradation, the

Table 3
Precision of PLE compared to ultrasonic extraction for the determination of aristolochic acids I and II in medicinal plants at 120°C

	PLE (RSD, %) (mg/kg)	Ultrasonic extraction (RSD, %) (mg/kg)
Aristolochic acid I		
Radix aristolochiae (qingmuxiang),	715±20 (3.1, $n=5$)	475±12 (2.6, $n=5$)
Radix aristolochiae fangchi,	431±15 (3.4, $n=6$)	270±7 (2.4, $n=6$)
herb	646 ($n=2$)	508 ($n=2$)
Aristolochic acid II		
Radix aristolochiae (qingmuxiang),	762±18 (2.3, $n=5$)	543±22 (4.0, $n=5$)
Radix aristolochiae fangchi,	65±5 (8.1, $n=5$)	41+3 (7.5, $n=5$)
herb	91 ($n=2$)	68 ($n=2$)
Berberine		
Coptidis rhizoma 1[a]	71 000±25 000 (3.8, $n=5$)	
Coptidis rhizoma 2	84 000±1600 (2.0, $n=5$)	67 000±1000 (1.5, $n=4$)

[a] *Coptidis rhizoma* 1 was prepared using mortar and pestle.

amount of target analyte extracted from different medicinal plants using PLE was compared with ultrasonic extraction and Soxhlet extraction, the results are tabulated in Table 4. Spiking of target analytes in medicinal plant was not done as it does not mimic any analyte–matrix interaction in the real situation. Ultrasonic extraction was first performed on the medicinal plant to evaluate the sample homogeneity. Method precision (relative standard deviation, $n=5$) between 1.98 to 3.4% was achieved for the extraction of berberine, aristolochic acids I and II using PLE. Higher RSD values between 7.5 and 8.1% was observed for aristolochic acid II in *Radix aristolochiae fangchi* using PLE and ultrasonic extraction as the level of aristolochic acid II present was close to the instrument's LOQ.

From the data obtained in Table 4, the volume of extract collected by PLE showed that the extraction efficiency was comparable to that of Soxhlet extraction. Only in one of the medicinal plant, *Radix aristolochiae* (qingmuxiang), higher extraction efficiency was obtained by PLE. The comparison with Soxhlet extraction with the experiments on effect of temperature in Table 1 showed that extraction by PLE at 120°C did not result in significant degradation of the target analyte.

Higher extraction efficiency for PLE versus ultrasonic extraction or Soxhlet extraction for aristolochic acids and berberine in medicinal plants was observed. The results showed the advantage of PLE over ultrasonic extraction where strong bonds which may be present between the analytes and matrix in samples with complex matrix is difficult to be destroyed.

The results obtained was similar to earlier reports where PLE in static mode was used in the extraction of semivolatile organic compounds from pine needles and mosses [13] and the extraction of polychlorinated biphenyls in solid environmental samples [16]. In both reports, the extraction efficiency of PLE in the range of 110 to 200% versus that by ultrasonic extraction or certified values were observed for samples with more complex matrix. Similarly, a higher extraction yield was observed for the application of PLE using static extraction of target analytes in medicinal plants such as St. John's wort herb, Horse chestnut seed and Turmeric rhizome compared to methods according to Pharmacopoeia monographs [16].

3.4. Specificity of method

A slight drawback of using methanol as the only extraction solvent was that a defatting procedure was not applied. Non polar components in the plant matrix can be extracted at the same time which can result in serious matrix interference in the separation method used. A gradient elution procedure was used in the study of aristolochic acids I and II in medicinal plants so that any hydrophobic compounds extracted can be eluted in a single run. For berberine, aristolochic acids I and II, the UV spectra were rather unique and can be used for identification purposes. By comparing the UV spectra in the chromatograms (Fig. 4) obtained for the target analytes in plant extracts and the standard, significant matrix interference was not observed for berberine and aristolochic acids in the medicinal plants analyzed.

4. Conclusions

The proposed method of extraction was found to be simple, rapid and have lower solvent consumption

Table 4
Comparison of the proposed method of extraction by PLE at 120°C with Soxhlet extraction[a]

	Extraction by PLE ($n=2$)	Extraction by Soxhlet ($n=2$)
Berberine in *Coptidis rhizoma*[b]	7.1% (w/w)	6.7% (w/w)
Aristolochic acid I in *Radix aristolochiae* (qingmuxiang)	739 mg/kg	638 mg/kg
Aristolochic acid II in *Radix aristolochiae* (qingmuxiang)	341 mg/kg	298 mg/kg
Aristolochic acid I in *Radix aristolochiae fangchi*	564 mg/kg	603 mg/kg
Aristolochic acid II in *Radix aristolochiae fangchi*	61 mg/kg	66 mg/kg

[a] Extraction of *Radix aristolochiae* was performed on a different batch of medicinal plant compared to Table 3.
[b] *Coptidis rhizoma* was prepared using mortar and pestle.

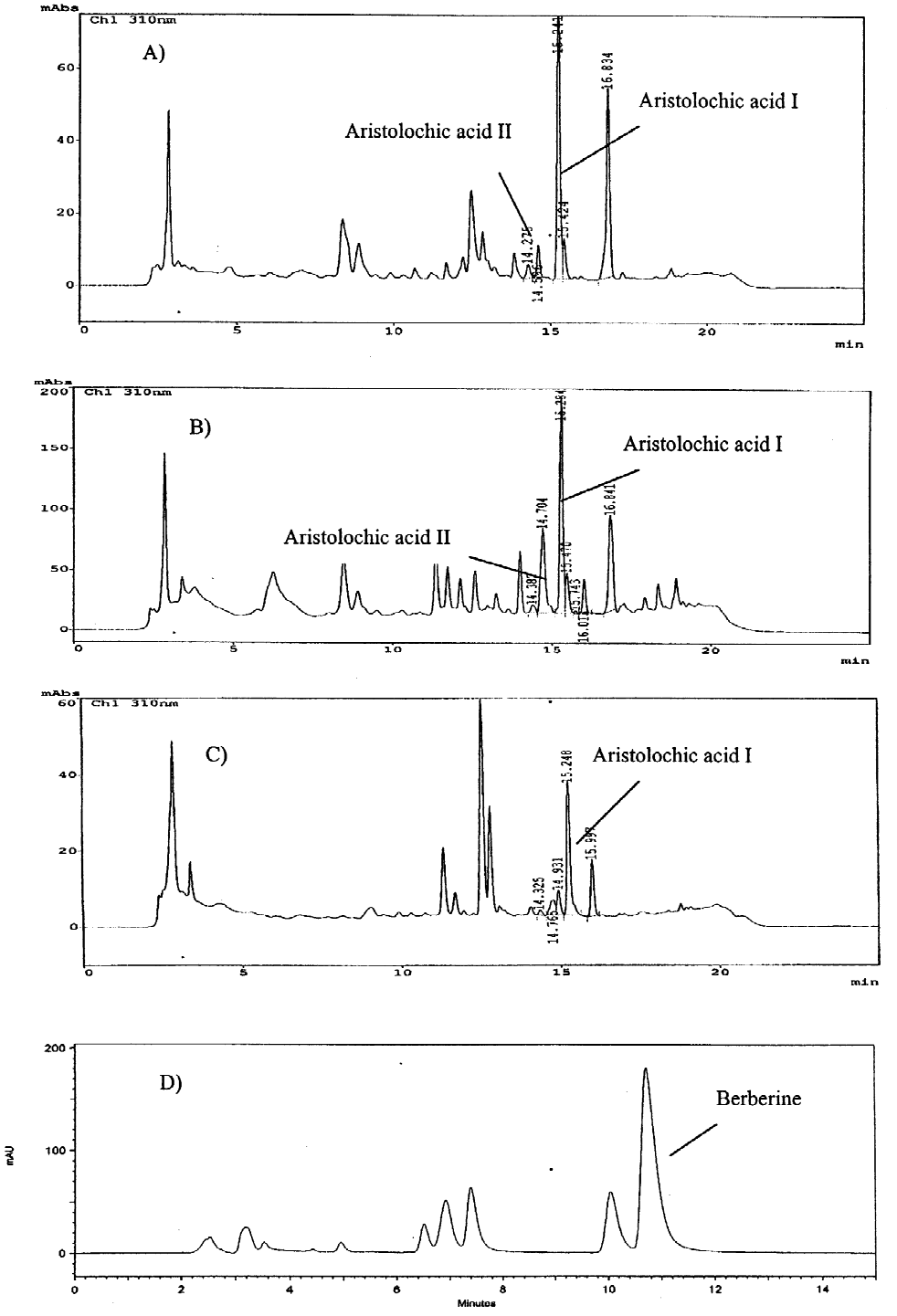

Fig. 4. Chromatograms obtained for aristolochic acids from (A) *Radix aristolochiae*, (B) *Radix aristolochiae fangchi*, (C) unknown herb and (D) berberine in *Coptidis rhizoma*.

compared to classical methods of extraction. A slight drawback of using PLE in the dynamic mode was that the solvent consumption was higher compared to ASE in static mode. Higher extraction efficiency compared to ultrasonic extraction and good method precision was achieved for the analysis of berberine and aristolochic acids I and II in medicinal plants. However, the performance of the method can be affected by parameters such as temperature, solvent, particle size of sample prepared and volume of solvent required just the same way as PLE in static mode. The other advantages of using the proposed PLE system in dynamic mode was that fresh solvent was introduced all the time which can result in higher mass transfer. The proposed system can be assembled together using available components with lower cost compared to ASE by Dionex and does not require the fitting of nitrogen gas.

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