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Short Communication

Optimization of microwave-assisted extraction of cajaninstilbene acid and pinostrobin from pigeonpea leaves followed by RP-HPLC-DAD determination

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ABSTRACT

In the present study, a microwave-assisted extraction (MAE) method followed by reversed-phase high-performance liquid chromatography-photodiode array detector (RP-HPLC-DAD) was developed for the determination of cajaninstilbene acid (CSA) and pinostrobin (PI) in pigeonpea leaves. Compared to conventional extraction methods, MAE showed higher efficiency and better recovery yields. The effect of microwave irradiation on cell destruction of plant material was observed by scanning electron microscopy (SEM). The optimum MAE conditions were as follows: sample diameter $\leq$0.5 mm, extraction solution 80% ethanol, liquid to solid ratio 30:1 (mL g^{-1}), temperature 65 °C, and 2 extraction cycles with each cycle 1 min. The relative standard deviations (RSDs) for intra-day repeatability and inter-day reproducibility were <5% and <6%, respectively. The recoveries for CSA and PI were 96 ± 3% and 95 ± 4%. The developed MAE method, followed by RP-HPLC-DAD technique, was successfully applied in the rapid extraction and accurate determination of CSA and PI in pigeonpea leaves.

1. Introduction

Pigeonpea [*Cajanus cajan* (L.) Millsp.] is a valuable perennial or annual woody food crop, and is widely distributed in various parts of tropical and subtropical areas of the world. Besides being a popular food in many developing countries, pigeonpea has been widely used as folk medicine. For example, it is used for treating diabetes, expelling bladder stones, applying to sores and jaundice in India and Java (Grover et al., 2002; Milliken, 1997), coping with genital and other skin irritations, especially in females in Argentina (Morton, 1976), helping hepatitis and measles in Africa (Abbiw, 1990), and stabilizing the menstrual period and dysentery in South America (Duke and Vasquez, 1994). In China, pigeonpea leaves have been used for the treatment of the ischemic necrosis of femoral head, aphtha, bedsore, and tumors, etc. (Fu et al., 2006; Morton, 1976).

Phytochemical investigations indicated that pigeonpea contains some important active components, including flavonoids and stilbenes (Ingham, 1976; Cooksey et al., 1980). Cajaninstilbene acid (formulated as 3-hydroxy-4-prenyl-5-methoxystilbene-2-carboxylic acid, CSA) and pinostrobin (formulated as 5-hydroxy-7-methoxyflavanone, PI) belong to stilbenes and flavanones, respectively. Their structures are shown in Fig. 1. Different studies have revealed the potential effects of CSA and PI on human health. Sun et al. (1995) demonstrated that the preparation of CSA had notable anti-inflammatory property better than salicylic acid. Stilbene extracts that are rich in CSA from pigeonpea have been found to have hypocholesterolemic and hypoglycemic effects for treatment for postmenopausal osteoporosis and they inhibit β-amyloid synthesis (Luo et al., 2008; Inman and Hoppe, 2002, 2003; Zheng et al., 2007; Ramakrishma et al., 2003). PI has showed many bioactivities such as inhibiting the human placental aromatase, DNA topoisomerase I, decreasing the proliferation of MCF-7 cells, inducing mammalian phase 2 detoxication enzymes, antioxidant enzymes, and anti-*Helicobacter pylori* activities (Fahey and Stephenson, 2002; Le Bail et al., 2000; Bhamarapravati et al., 2006). Moreover, in our previous studies (Wu et al., 2009; Kong et al., 2009), CSA and PI were found in higher contents in pigeonpea leaves. Therefore, more selective extraction and determination of

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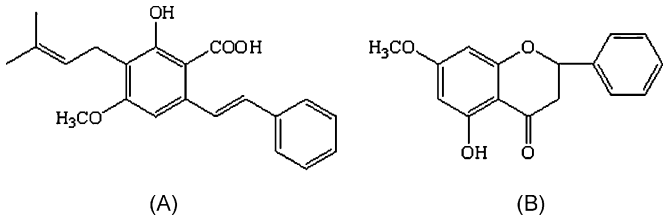

Fig. 1. Structures of CSA (A) and PI (B).

CSA and PI in a shorter processing time are needed for their more efficient consumption.

CSA and PI are relative non-polar compounds, and are difficult to dissolve in water. Hence, higher concentrations of organic solvent and higher temperature are needed to guarantee a better extraction performance. There is a lack of information on the extraction of CSA and PI, because CSA was only found in pigeonpea by now, and for PI, more interest was focused on the activity study. The conventional extraction methods for pigeonpea (maceration, homogenization, or Soxhlet extraction) were mostly reported with ethanol and methanol as solvents (Cooksey et al., 1980; Ohwaki et al., 1993; Green et al., 2003; Duker-Eshun et al., 2004; Jonglertjunya et al., 2009). However, these procedures have mainly been used for phytochemical investigations; they are normally time-consuming and laborious, involving lengthy operation techniques and the thermal decomposition of target compounds; also, the extraction yields are lower.

In our previous study, supercritical fluid extraction (SFE) has been applied in the extraction of CSA and PI from pigeonpea leaves (Kong et al., 2009). However, a long extraction period of 2 h was inevitable in that process. Recently, microwave-assisted extraction (MAE) has been used as an alternative to conventional methods in the extraction of natural products (Fulzele and Satdive, 2005; Rostagno et al., 2007; Chen et al., 2008), environmental sample (Serrano and Gallego, 2006; Jamali et al., 2009), medicine and food safety analyte (Desrosiers et al., 2009; Fernández et al., 2009; Khajeh, 2009). MAE is an extraction technique that combines microwave and traditional solvent extraction (Khajeh, 2009). When compared to conventional methods, it has showed many advantages such as reducing the consumption of extraction time and solvent, improving extraction rate (Eskilsson and Björklund, 2000). At some special conditions, MAE could cause selective migration of target compounds from the material to the extraction solvent at a more rapid rate (Dai et al., 2001; Zhou and Liu, 2006). Even compared to SFE, these advantages were also obvious in this study.

Considering the interest in stilbenes and flavanones as well as previous works by other authors and ourselves, the main objective of this work was to develop a fast and efficient MAE method for the accurate determination of CSA and PI in pigeonpea leaves. For this purpose, the operational parameters of the MAE procedures affecting the extraction yields of CSA and PI, including MAE power, sample diameter, temperature, irradiation time, ratio of liquid to solid and number of extraction cycles were optimized. Moreover, a RP-HPLC-DAD method was developed and validated for the determination of these two targeted compounds. MAE followed by RP-HPLC-DAD method for the determination of CSA and PI is simple, rapid and selective.

2. Materials and methods

2.1. Plant materials

Pigeonpea leaves were collected from Hainan province, China, and authenticated by Prof. Shao-Quan Nie from the Key Laboratory of Forest Plant Ecology, Ministry of Education, Northeast Forestry University, PR China. Voucher specimens were deposited in the herbarium of this Key Laboratory. The leaves were dried in the airy shade for 15 days and stored in dark for 30 days. Then, the dried leaves were pulverized, and parts of them were sieved to different sizes of 2–5, 1–2, 0.5–1 and ≤0.5 mm, respectively. The pulverized and sieved leaves with different sizes were kept in a dry place at room temperature.

2.2. Chemicals and reagents

Reference compounds of CSA and PI were separated and purified in the same Key Laboratory, the structures were confirmed by comparing their UV, MS, ^{1}H NMR and ^{13}C NMR data with the data from literature (Ohwaki et al., 1993; Cooksey et al., 1980; Green et al., 2003; Pompimon et al., 2009), and the purities were higher than 96%.

Ethanol (EtOH) and formic acid were analytical grade from Beijing Chemical Reagents Co. (Beijing, China). Methanol of HPLC grade was purchased from J&K Chemical Ltd. (USA). Deionized water was purified by a Milli-Q Water Purification system (Millipore, MA, USA). All solutions prepared for HPLC were filtered through 0.45 μm nylon membranes (Millipore, USA) prior to use.

2.3. Extraction procedures

MAE was performed on a MARS-II (1000 W, 2450 MHz) microwave accelerated reaction system from SINEO Microwave Chemistry Technology (China), equipped with a TFT multicolor liquid crystal screen, a power sensor (the power range 0–1000 W), an infrared temperature sensor, a temperature controller, a special three necks round-bottomed flask, and electromagnetic stirrer. Extraction of CSA and PI was performed by adding 1.0 g of pulverized leaves into 100 mL of ethanol/water mixture solvent in a flask. The flasks were placed symmetrically in the microwave field. The extractions were carried out under different conditions, according to the design. First, the output power of MAE instrument was optimized with pulverized and un-sieved leaves. And then, experiments were conducted to investigate the effects of sample diameter, extraction temperature, irradiation time, ratio of liquid to solid and number of extraction cycles on MAE efficiency for CSA and PI.

Maceration (MAC) was carried out with 1.0 g un-sieved pigeonpea leaves in 100 mL of extraction solvents in a 250 mL flask at 25 °C for 12 h.

Ultrasound-assisted extraction (UAE), a KQ-250DB ultrasonic bath (Kunshan Ultrasonic Instrument Co. Ltd., Jiangsu, China) with six transducers at the bottom was used as an ultrasound source. Extraction of CSA and PI was executed with 1.0 g un-sieved pigeonpea leaves and 100 mL of extraction solvent in a 250 mL flask placed in the ultrasonic bath set at 250 W in frequency of 40 kHz. Extraction was conducted for 60 min at the temperature of 65 °C.

Refluxing extraction (RE), 2.0 g un-sieved pigeonpea leaves was placed into a 500 mL round-bottomed flask with 200 mL of ethanol–water solutions. It was carried out at 65 °C for 60 min.

For all the four methods, the extracts were properly concentrated under vacuum to 100 mL, then, filtered by 0.45 μm membrane filtrations and injected to HPLC analysis. The extraction yields of CSA and PI were defined as follows: Extraction yield (w/w) = mass of CSA (or PI) in extraction solution/mass of material (pigeonpea leaves).

Meanwhile, the morphological changes of leaves before and after extractions by different methods were observed by SEM as described by Zhou and Liu (2006) with some modifications. Briefly, after removing the solvent, the samples were subjected to a

thermal treatment at 40 °C under vacuum for 6 h, fixed on a specimen holder with aluminum tape and marked, then sputtered with gold in a sputter-coater. All samples were examined with a Quanta-200 SEM (FEI Company, USA) under high vacuum and at an accelerating voltage of 15.0 kV.

2.4. Chromatographic separation

Based on previous studies (Wu et al., 2009; Kong et al., 2009), a more simple and rapid RP-HPLC-DAD analysis was developed. The Waters liquid chromatography system (Waters Corporation, USA) equipped with a Model Delta 600 pump, a 2996 photodiode array detector (DAD), Millennium32 system software and a Symmetry Shield^{TM} RP_{18} reversed-phase packing column (150 mm × 3.9 mm i.d., Waters Corporation, made in Ireland) was employed to analyze CSA and PI. The mobile phase of MeOH:water:formic acid 78:21.9:0.1 (v/v/v) with isocratic elution was used for the chromatographic separation. The mobile phase was filtered through a 0.45 μm membrane filter and then deaerated ultrasonically prior to use. The retention times (Rt) of CSA and PI were 4.52 and 14.63 min respectively with flow rate of 1 mL min^{-1} and the column temperature at 35 °C. The injection volume was 10 μL. All samples were filtered through 0.45 μm membrane filters before injection.

Chromatographic peaks of CSA and PI were identified by comparing their UV spectra and retention times with those of corresponding reference compounds. Quantification of CSA and PI was carried out using external standard method at 259 and 288 nm, respectively. Standard solutions of CSA and PI at seven levels in the concentration ranges of 1.5–300 and 1.0–100 μg mL^{-1}, respectively, were injected in triplicate. A linear response with a correlation coefficient of 0.999 was obtained. The calibration curves for CSA and PI were $Y = 41{,}216X - 257{,}570$ ($R^2 = 0.999$), $Y = 79{,}292{,}396X - 93{,}241$ ($R^2 = 0.999$), respectively, where Y is the peak area of the analyte, and X is the concentration of the analyte (μg mL^{-1}). Quantification (LOQs) and detection (LODs) limits were studied by gradually diluting the individual standard solution with methanol. LOQs for CSA and PI were 1.50 and 1.0 μg mL^{-1} calculated for S/N ratio of 10, and LODs were 0.50 and 0.28 μg mL^{-1} for S/N ratio of 3. The chromatogram and UV spectra of standards are presented in Fig. 2.

2.5. Statistical analysis

Data was expressed as mean ± standard deviation (SD) of three replicates. Statistical comparisons were performed using the statistical software (SAS Institute, UAS) with analysis of variance (ANOVA). Means for significant differences were compared by using Duncan's test.

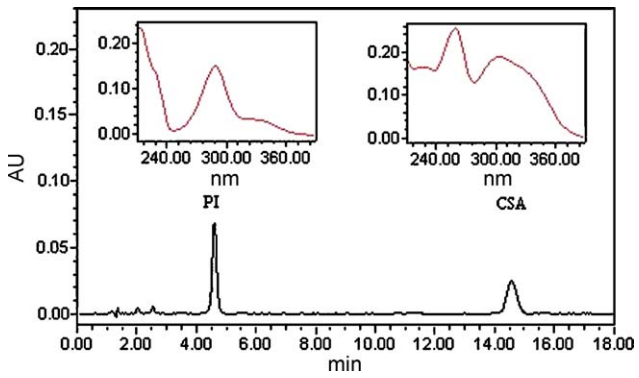

Fig. 2. HPLC chromatogram of CSA and PI, and their UV absorption spectra.

3. Results and discussion

3.1. Comparison of MAE and other methods

3.1.1. Extraction efficiency

Generally, different extraction methods give different results. Sample preparation with an appropriate extraction method should satisfactorily recover the desired components from the matrix. MAC at 25 °C for 12 h, UAE at 65 °C for 60 min, RE at 65 °C for 60 min and MAE at 65 °C for 2 min were conducted to exhaustively extract CSA and PI from pigeonpea leaves. The details of extraction conditions were described in Section 2.3, and for MAE 2.0 g of unsieved pigeonpea leaves was extracted with 100 mL extraction solution for one cycle, the extraction time was 2 min, and the power was 300 W.

The results are shown in Fig. 3, which demonstrates that the extraction yields of CSA and PI were influenced by different extraction methods and EtOH concentration. The extraction yields of CSA and PI by MAC were the lowest among the four extraction methods. For the extraction yields of PI, there is no significant difference among UAE, RE and MAE with all solvents. The extraction yields of CSA by MAE and RE with 80% EtOH or RE with 90% EtOH were the highest as compared with other conditions. However, the highest extraction yields of CSA and PI could be achieved in 2 min using MAE. Because microwave radiation could penetrate through extraction medium, increase the contacts between solvents and materials, and enhance the solubility of substances in the medium. The results also suggested that the influence of EtOH concentration on CSA yields using MAE was significant. Therefore, MAE with 80% EtOH was selected as a simple and efficient method for the extraction of CSA and PI from pigeonpea leaves, and consequently used for the optimization of the extraction process in the following tests.

3.1.2. Morphological changes observed by SEM

In order to study the morphological changes during the different extraction methods, the samples were examined by SEM. Fig. 4A–D showed the micrographs of the untreated sample, UAE sample for 60 min, RE sample for 60 min and MAE sample for 2 min, respectively. It is well known that ultrasounds disrupted vegetal cells via cavitation bubble collapse produced by the passage of an ultrasonic wave (Vinatoru, 2001; Romdhane and Gourdon, 2002; Toma et al., 2001). However, UAE did not seriously

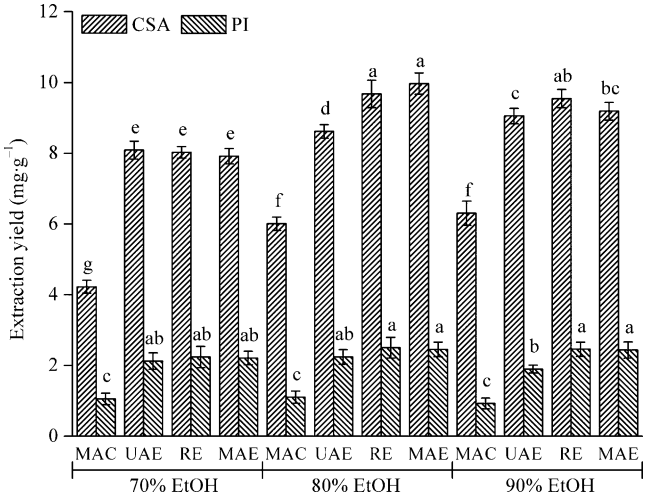

Fig. 3. Effects of different extraction methods and ethanol concentrations on the extraction yields of CSA and PI. The different letters represent a significant difference at $P < 0.05$ level for CSA and PI.

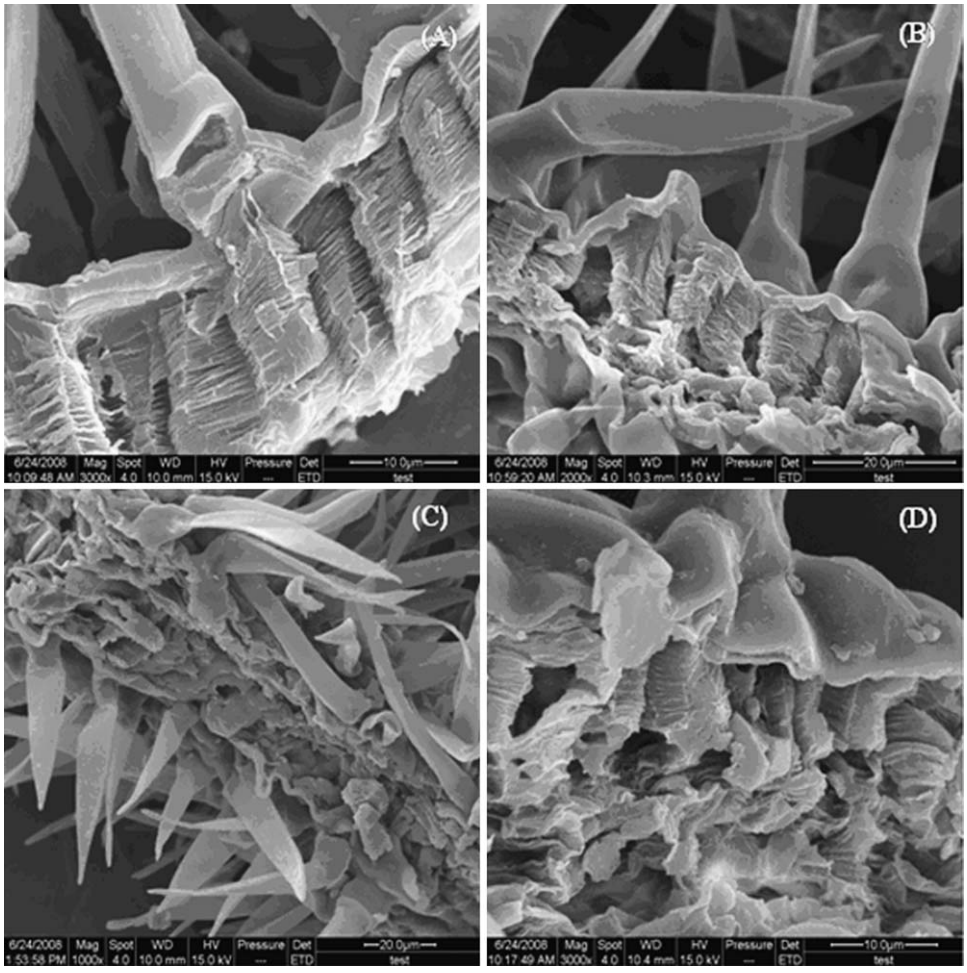

Fig. 4. Scanning electron micrographs of pigeonpea leaves. (A) Untreated, (B) UAE for 60 min, (C) RE for 60 min and (D) MAE for 2 min.

affect the sample in the present study. As shown in Fig. 4B, except for few ruptures on the surface, the observed changes for UAE sample were not considerably different from those of the untreated samples. It could be attributed to the densely tomentose on the pigeonpea leaf epidermis which may decrease the transfer of ultrasonic energy to the inner tissue. The morphological changes observed for RE and MAE were great in comparison to those of UAE sample. There was a shrink phenomenon of the sample particle in RE sample (Fig. 4C), which may due to long time heating results in the denaturation of some cellular compositions, such as protein, chlorophyll, etc. However, the sample structure was seriously destroyed after MAE in a shorter time (Fig. 4D). This observation indicated that microwaves (i.e., the irradiation and the temperature rise in sudden) disrupted the structure of vegetal cell (Ferhat et al., 2006; Zhou and Liu, 2006), then helped the rapid release of substances inside of plant cell to solvents. The results were in accordance with the high extraction yields by MAE.

3.2. Optimization of MAE conditions

3.2.1. Effects of MAE power, sample diameter, extraction temperature and time

Generally, four factors including MAE power, sample diameter, extraction temperature and time have interrelated effects on the extraction yields. Energy produced by microwave is pulsed, so instantaneous high temperature caused by huge energy tends to disintegrate the target compounds totally or partly (Li et al., 2003). A smaller sample diameter leads to increased surface area of sample, a higher extraction temperature is beneficial for extraction due to the increased solubility, and a suitable extraction time is necessary for a method. To avoid the breakages of CSA and PI during the MAE process, the power of 200, 300, 400, 500 and 700 W were optimized firstly with un-sieved pigeonpea leaves with 80% EtOH as solution. The sample diameter of 2–5, 1–2, 0.5–1 and $\leq$0.5 mm were compared, extraction temperature and time were also investigated.

The results are shown in Fig. 5. As shown in Fig. 5A, the power of 300 W was proper for the extraction of CSA and PI. The extraction yields of CSA decreased a little at power higher than 300 W, but the effect on extraction yields of PI was not obvious. Meanwhile, when the power was higher than 300 W, the extraction solution boiled acutely, even overflowed the cooling pipe. Therefore, considering the consumption of energy and safety, the power of 300 W was chosen for all of the following experiments.

The effect of sample diameter on the extraction yields of CSA and PI is presented in Fig. 5B. MAE was conducted at 65 °C, sample 1.0 g, solvent 80% ethanol of 30 mL, and 5 extraction cycles. The extraction yields of CSA and PI depended on the sample diameter (Fig. 5B). For the diameter of 2–5, 1–2 and 0.5–1 mm, the similar extraction yields of about 8.0 ± 0.3 mg g^{-1} for CSA and 2.0 ± 0.4 mg g^{-1} for PI were obtained. When the sample diameter was smaller than 0.5 mm, the extraction yields were 19.1 ± 0.5 mg g^{-1} (CSA) and 3.7 ± 0.5 mg g^{-1} (PI), which were 2-fold and 1-fold higher than those at the diameters of larger than 0.5 mm. Sample diameter reduction by milling not only increases the contact area between solvent and samples but also ruptures cell walls (del Vallea

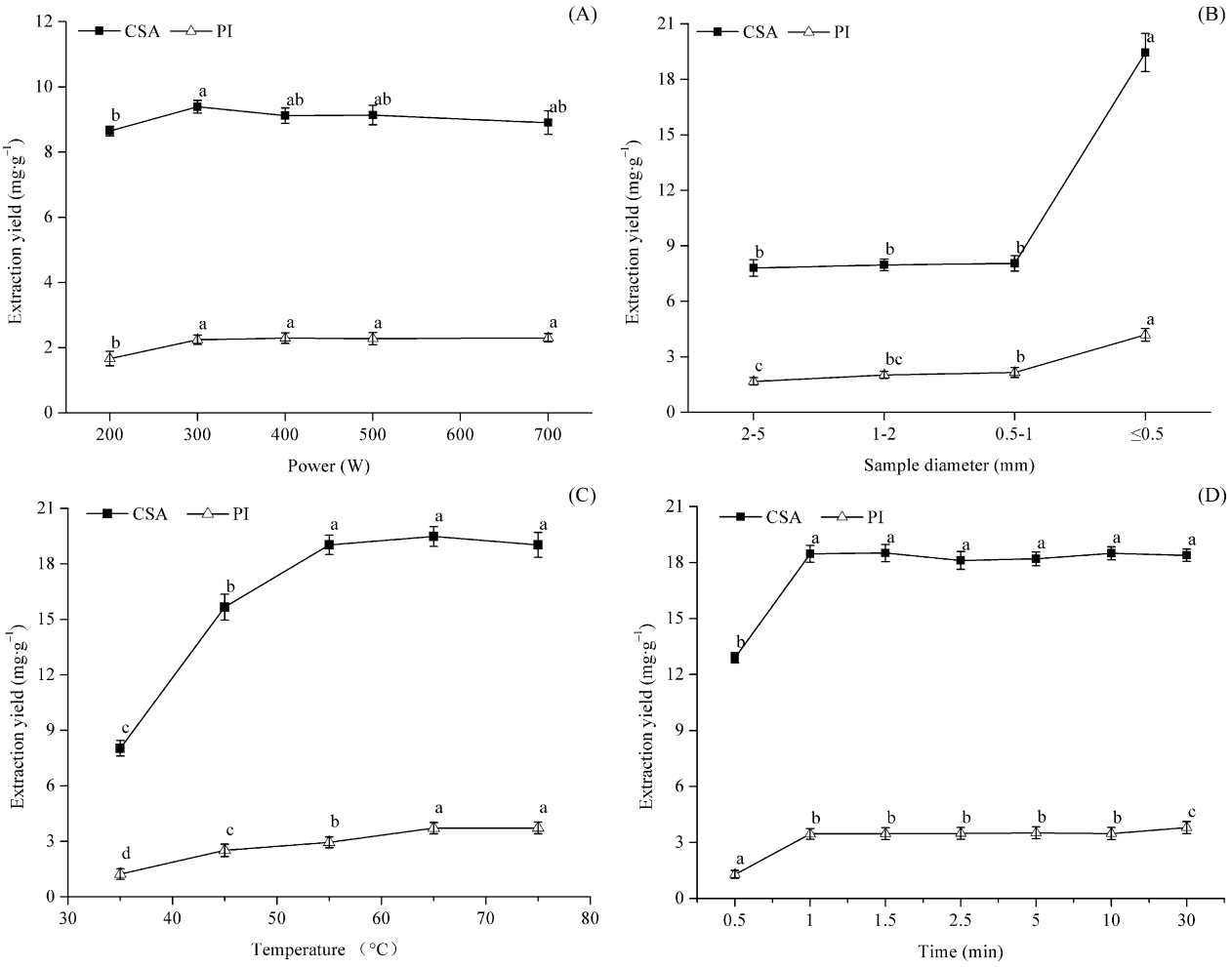

Fig. 5. Effects of different MAE extraction conditions on the extraction yields of CSA and PI. (A) MAE power; (B) sample diameter; (C) temperature; (D) time. The different letters represent a significant difference at $P < 0.05$ level for CSA and PI.

and Uquiche, 2002). In the sample with smaller diameter, there are more cells directly exposed to extraction solvent as well as shorter distance for transfer of target compounds through cell walls to solvent, thus the extraction yields of CSA and PI were higher than those in large sample diameter. Therefore, in consideration of the feasibility, a sample diameter of ≤0.5 mm was chosen for the subsequent tests.

The effects of extraction temperatures (35, 45, 55, 65 and 75 °C) on the extraction of CSA and PI are shown in Fig. 5C. It clearly demonstrated that the extraction yields of CSA significantly increased when the temperature was raised from 35 to 55 °C. There is no significant influence with a further increased temperature (from 55 to 75 °C), the extraction yields of CSA were 19.0 ± 0.5, 19.5 ± 0.5 and 19.0 ± 0.7 mg g^{-1}, respectively. For PI, the yields increased with increasing temperatures, and reached constant after 65 °C. Extraction yields at 65 and 75 °C were 3.7 ± 0.3 mg g^{-1}. Higher temperature caused the decrease of intermolecular interactions within the solvent, gave rise to higher molecular motion, and increased the solubility. The increasing temperature may also improve the release of targeted compounds from the cytoplasm of plant materials, and as a result, the availability of CSA and PI increased. At higher temperature, the solvent viscosity decreased and the diffusivity increased, the extraction efficiency increased. Moreover, in the open-end microwave vessel used in this study, the temperature of the solvent could only quickly reach the boiling point temperature. Thus, the temperature of 75 °C was the upper limit of the solvent used. Considering the consumption of energy, 65 °C was used in the following extraction tests.

To achieve a relatively complete extraction of CSA and PI by MAE, the extraction time was increased from 0.5 to 30 min to study the effect of extraction time under the above optimized conditions (1.0 g sieved pigeonpea leaves with sample diameter ≤0.5 mm, 30 mL of 80% ethanol at 65 °C). Fig. 5D showed the effects of different extraction time. It was found that CSA in the sample was absolutely extracted within 1 min, the extraction yield was up to 18.5 ± 0.5 mg g^{-1}. When the extraction time was increased to 30 min, the extraction yield was 18.5 ± 0.3 mg g^{-1}, which was not significant higher than that at 1 min. In addition, the extraction yield of PI 3.5 ± 0.3 mg g^{-1} at 1 min nearly reached the highest value of 3.8 ± 0.3 mg g^{-1} at 10 min. The results indicated the advantage of MAE in effective extraction of CSA and PI with much shorter time. Thus, 1 min was selected as the appropriate irradiation time in MAE tests.

3.2.2. Effects of liquid to solid ratio and number of extraction cycles

The effect of liquid to solid ratio on the extraction yields in MAE is more obvious than those in other conventional extraction methods (Mandal et al., 2008). To evaluate the effect of liquid to solid ratio on the extraction yields of CSA and PI, a series of extractions were performed at 65 °C with the sample diameter ≤0.5 mm and the 80% ethanol at different liquid to solid ratios (10:1, 15:1, 20:1, 30:1, mL/g). The extraction yields of CSA and PI at

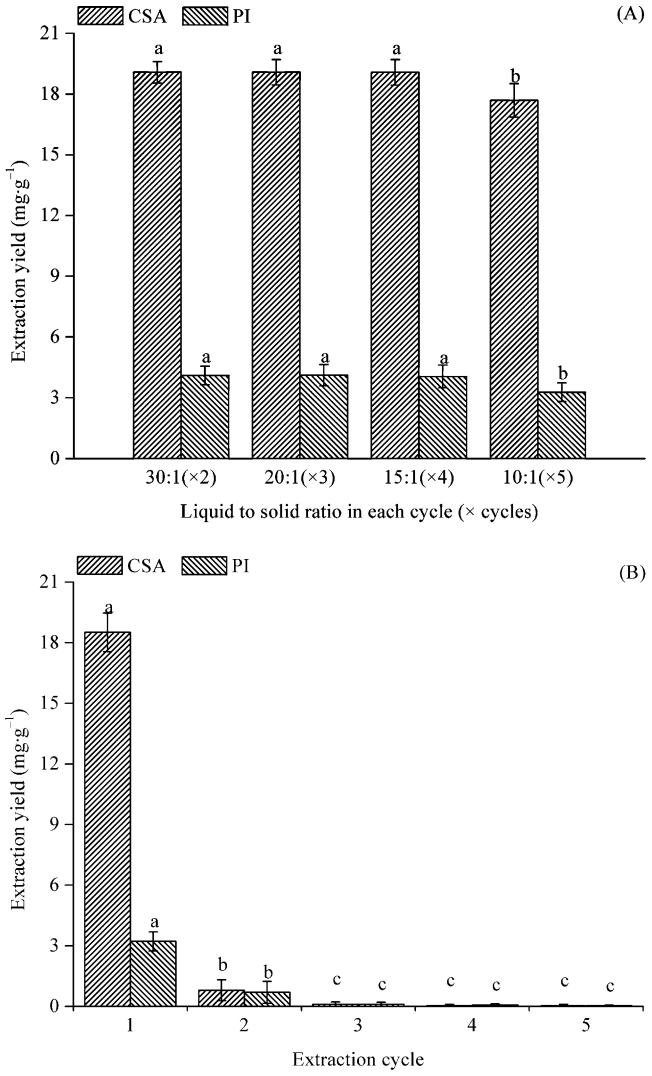

Fig. 6. Effects of liquid to solid ratio and extraction cycles on the extraction yields of CSA and PI. (A) Liquid to solid ratio of 10:1, 15:1, 20:1, and 30:1 for 5, 4, 3 and 2 cycles, (B) different extraction cycles (1–5 cycle) with liquid to solid ratio of 30:1.

1 min and 2–5 extraction cycles under different liquid to solid ratios were compared. The results in Fig. 6A demonstrated that a ratio of 10:1 with 5 extraction cycles gave the lowest extraction yields of 17.7 ± 0.5 mg g^{-1} (CSA) and 3.0 ± 0.4 mg g^{-1} (PI), while the ratio of 15:1 with 4 cycles, 20:1 with 3 cycles and 30:1 with 2 cycles gave almost equal extraction yields of CSA around 19.0 mg g^{-1} and PI from 3.4 to 3.8 mg g^{-1}. Furthermore, Fig. 6B presented the extraction yields of CSA and PI with the ratio of 30:1 in 5 successive extraction cycles, respectively. The sum of CSA yields after 2 cycles was 99% of the total 5 cycles and PI was 94%. Therefore, in order to save time and labor, the ratio of 30:1 with 2 extraction cycles were selected.

By investigating the influences of six parameters on the extraction yields of CSA and PI, it indicated that sample diameter and extraction temperature are the most important parameters, because more than doubles of the amount were extracted when compared to other variables. Moreover, the shorter extraction time of 1 min make it interest for a more selective extraction of CSA and PI by MAE.

3.3. Method validation

To evaluate the repeatability of the developed MAE followed by RP-HPLC-DAD method, the extraction and analysis of CSA and PI in pigeonpea leaves were conducted for five times within one day under the optimum extraction conditions (sample diameter ≤0.5 mm, 80% ethanol, 65 °C, liquid to solid ratio 30:1 with 2 extraction cycles, each cycle 1 min). The obtained relative standard deviations (RSDs) for CSA and PI were <5%. The reproducibility was evaluated by calculating the extraction yields obtained from five independent extractions carried out on five consecutive days, the RSDs were <6%. The extraction yields of CSA and PI were 18.8 ± 0.6 mg g^{-1} and 3.5 ± 0.5 mg g^{-1}, respectively (n = 14).

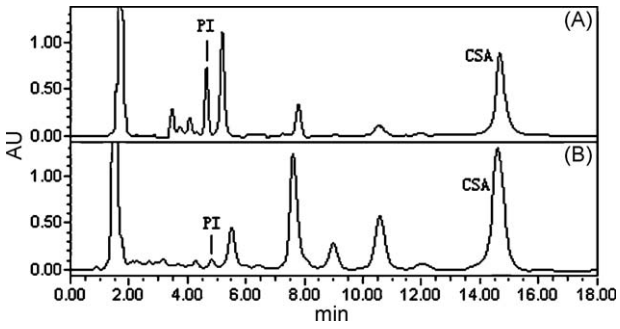

Fig. 7. Chromatograms of pigeonpea leaves extracts at (A) 288 nm and (B) 259 nm.

To test the recovery, reference compounds of CSA 20.0 mg and PI 4.0 mg were respectively added into pigeonpea leaves sample, mixed, then extracted and determined. The recoveries of CSA and PI were 96 ± 3% and 95 ± 4%, indicating the method is adequate for quantitative extractions.

Fig. 7 showed the RP-HPLC-DAD chromatograms of pigeonpea leaves extracts by MAE. Only several constituent peaks were found, which indicated that the extraction of CSA and PI by MAE was more selective. In the extracts, more polar compounds in pigeonpea leaves such as glycosides, tannins, etc., were less extracted in very short duration, hence, it was helpful for the subsequently accurate determination.

4. Conclusions

In the present study, a MAE method followed by RP-HPLC-DAD was presented for the determination of CSA and PI in pigeonpea leaves. The maximum extraction yields of CSA and PI using MAE were 18.8 ± 0.6 mg $g^{-1}$and 3.5 ± 0.5 mg g^{-1} (n = 14). The optimum MAE conditions were: sample particle diameter ≤0.5 mm, extraction solvent 80% ethanol solution, ratio of liquid/solid 30:1, temperature 65 °C, and two extraction cycles, each cycle 1 min. The enhanced extraction was partly related to the cells rupture of the plant material according to SEM observation. The MAE method gave the highest yields with the shortest extraction time when compared with the other conventional methods. The developed MAE method, followed by RP-HPLC-DAD method, showed acceptable repeatability and reproducibility for CSA and PI, which were <5% and <6%, respectively. Moreover, it was satisfactorily applied for the rapid extraction and determination of CSA and PI in pigeonpea leaves.

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