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Matrix Solid-Phase Dispersion Extraction Followed by HPLC-DAD Method for the Determination of Major Constituents in *Piper chaba* Root

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ABSTRACT

Piplartine and piperine were extracted from *Piper chaba* by matrix solid-phase dispersion (MSPD) and determined by high-performance liquid chromatography (HPLC) with diode array detection (DAD). The experimental conditions for the MSPD were optimized. Silica gel was selected as dispersion adsorbent and methanol as elution solvent. The calibration curve showed good relationship (r>0.9998) in the concentration range of 5-100 μgmL^{-1} for piplartine and piperine. The recoveries were between 90.6% and 100.9%. The proposed method consumed less sample, time and solvent compared with conventional methods, including ultrasonic and soxhlet extraction.

1. Introduction

Piper chaba hunter (Piperaceae), a glabrous shrub, mainly grows in the subtropical areas of Asia, such as India, Malaysia and Bangladesh [1]. This plant has been traditionally used for many medicinal purposes in Ayurveda. The dried roots and fruits of this plant have been used to treat asthma, bronchitis, fever, pain in abdomen and as a stimulant in haemorrhoidal afflictions [2, 3].

Piplartine is an alkaloid/amide present in *Piper* species. Biological activities described for this amide includes antifungal, antiplatelet, cytotoxic and antitumor activities [4–10].

Piperine is a major plant alkaloid/amide present in black and long peppers (*Piper nigrum* and *Piper longum*), which is one of the most common spices consumed by a large number of people worldwide. It is shown to possess diverse pharmacological activities [11]. Concerning anticancer related properties, piperine has been reported to inhibit lung metastasis [12], and to possess antitumor activities in different animal models [13].

High performance liquid chromatography (HPLC) and high performance thin layer chromatography (HPTLC) methods have been used earlier to isolate, identify and quantify constituents of the genus *Piper* [14-19].

As one of the most promising techniques, matrix solid-phase dispersion (MSPD) has recently been introduced for sample preparation of complex matrices. This technique was first developed in the year 1989 [20]. This combined disruption, extraction and clean up in one step and effectively extracted solid, semisolid and highly viscous samples [21]. MSPD has unique properties as a sample preparation technique compared to classical methods, such as low cost, limited consumption of organic solvents, use of mild extraction conditions, and possibility to integrate extraction and purification in the same step [22]. Recently, MSPD has been successfully applied to the extraction of a wide range of drugs, pesticides, micro contaminants and other compounds from a wide variety of complex samples [23]. However, only a few reports using MSPD technique to extract constituents in medicinal plants were published till date [24–26].

In this study, MSPD as extraction method followed by HPLC separation and diode array detection was first applied for the extraction and determination of to piplartine and piperine in the roots of *P. chaba*. The effect of several extraction parameters, including dispersing sorbent, elution solvent, volume of the elution solvent and the ratio of dispersing sorbent to sample, were examined and optimized. The results obtained in samples with optimized MSPD procedure were evaluated and compared with the classical ultrasonic and soxhlet extraction methods.

2. Experimental Methods

2.1 Plant Material, Chemicals and Standards

Three kinds of *P. chaba* (named as sample PC-R-1, PC-R-2, PC-R-3) cultivated in different areas were bought from local drug store and authenticated by Dr. K. Madhava Chetty, Department of Botany, Sri Venkateswara University, Thirupati, India. They were shade dried, fine powdered and used for analysis. HPLC grade acetonitrile (ACN), acetone, ethyl acetate, chloroform, methanol (MeOH) and light petrolium used for the HPLC analysis were obtained from Merck India. Ultra-pure water for chromatographic use was obtained from a MilliQ system (Millipore Corp., Bedford, MA, USA). Silica gel (230-400 mesh), C18 (230-400 mesh), C8 (230-400 mesh) and diatomaceous earth material were obtained from Sigma Aldrich Pvt Ltd (India). Neutral alumina was obtained from Rankem laboratories, Mumbai. All the samples were filtered through 0.45 µ membrane filter before injecting into HPLC. Two bioactive compounds, piplartine and piperine were isolated from hexane extract in our laboratory as described earlier [27]. Their structures were identified by comparison of their spectral data (UV, IR, MS, ^1H NMR, ^{13}C NMR and 2D NMR) with those published in references. The purity of each compound was determined to be more than 98% by normalization of the peak area detected by HPLC. Chemical structure of the analytes was shown in Fig. 1.

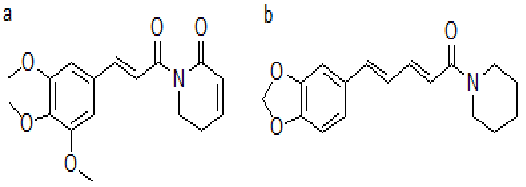

Fig. 1 The chemical structures of (a) piplartine and (b) piperine

2.2 Preparation of Standard Solution

The two standards were accurately weighed and then dissolved with methanol to prepare stock solution. The piplartine and piperine standard stock solutions were stored in 4 °C. Working solutions were prepared by diluting the stock solution with the mobile phase.

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2.3 Extraction of Piplartine and Piperine

2.3.1 MSPD Extraction

An aliquot of 0.5 g of the previously milled sample and 1.5 g of silica gel were placed in an agitate mortar and blended together using an agitate pestle to obtain a complete disruption and dispersion of the sample on the silica gel sorbent. Once completely dispersed, the homogenized sample was loaded into a cartridge that was pre packed with adsorbent cotton at the bottom. A second layer of adsorbent cotton was added on the top of the mixture by gentle compression with syringe plunger. And then column was eluted with 15 mL of methanol by gravity flow. The eluate containing purpose analysts was collected in volumetric flask and raised to mark with methanol. The solution was filter through the 0.45 µm PTFE filter membrane and used as a sample solution.

2.3.2 Ultrasonic Extraction

0.5 g of sample was put into a 50 mL flask, into which 45 mL of methanol was added. The flask was immersed in the water bath of an ultrasonic cleaner (Power sonic 400 series, Hwashin technology, Korea), and sonicate for 30 min. Then the extract was diluted to 50 mL with methanol. The resulting extract was filtered sequentially with filter paper and 0.45 µm PTFE membrane filter, the resulting solution constituted the sample solution.

2.3.3 Soxhlet Extraction

0.5 g of sample and 90 mL of methanol were put into a soxhlet distilling flask. The mixture was heated for 15 hrs at room temp. The extract was transferred into a 100 mL volumetric flask and diluted to the mark with methanol after filter through a 0.45 µm PTFE filter membrane and used as the sample solution for the HPLC analysis.

2.4 HPLC and Chromatographic Conditions

Chromatographic separation was achieved by reverse-phase chromatography using the isocratic elution. Chromatography was performed on Phenomenex Luna C8 column (5 µ, 250 x 4.6). The mobile phase consisted of acetonitrile and water (0.1% formic acid) with 60:40% v/v. The flow rate was set at 1 mL/min and peaks were measured at 340 nm. Chromatographic separation was performed using HPLC equipped with a degasser (G1397A), binary pump (G1312A), autosampler (G1329A), autosampler thermostat (G1329B) and diode array detector (G1315B) of Agilent Technologies 1100 series (Germany). The sample injection volume was 20 µL and the column temperature was maintained at ambient conditions. The data were acquired and processed using Chemstation. The representative chromatograms are shown in Fig. 2.

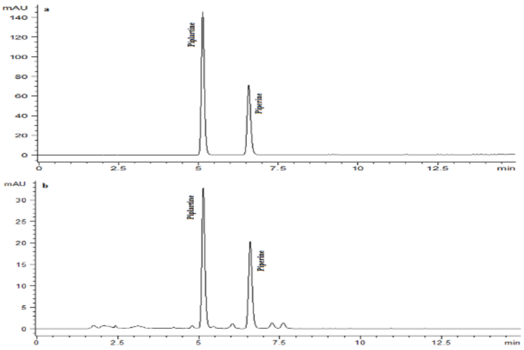

Fig. 2 Representative HPLC chromatograms of (a) standard mixture solutions (concentration of both Piplartine and piperine was 100 µgmL^{-1}), (b) *Piper chaba* roots sample extracted using MSPD

2.5 Stability

Storage stability of standard solutions and sample extracts were measured. Stock standard solutions were checked and found to be stable for at least 1 month at 4 °C. Concentration difference found in working standard solutions were less than 0.2% after 7 days of preparation. Concentration difference for sample extracts maintained at room temperature for 24h were less than 1%.

3. Results and Discussion

3.1 Optimization of Chromatographic Conditions

Chromatographic condition, especially the composition of mobile phase, plays a critical role in achieving good chromatographic behavior and appropriate ionization. Different mobile phases (methanol-water, acetonitrile-water with or without formic acid or ammonium acetate) were investigated using Phenomenex Luna C8 column (5 µ, 250 x 4.6 mm) column to optimize the analytical performance. It was observed that acetonitrile was found to be better in terms of resolution and peak shapes as compared with methanol. Using acetonitrile-water (0.1% formic acid) good peak shape, considerable response and baseline separation were achieved.

3.2 Optimization of MSPD

In the MSPD procedure, the dispersing sorbent is one of the most important parameter, which not only used as an adsorption separation material but also a blending solid support to disrupt and disperse the sample. In this step to find the most effective dispersing sorbent including silica gel, diatomaceous earth, neutral alumina, and C_{18} were examined in order to find the most suitable dispersion adsorbent. The experimental results shown in Fig. 3. Indicate similar results were obtained when the five kinds of dispersion adsorbents were used. Silica gel was selected as dispersion adsorbent.

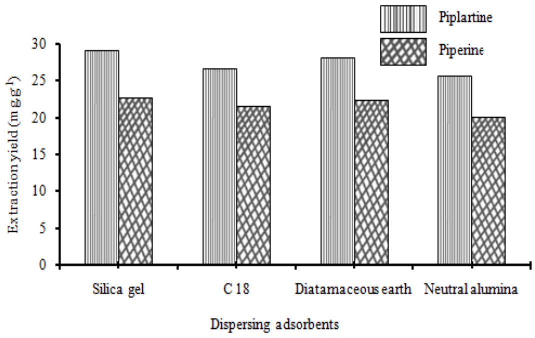

Fig. 3 The effect of the dispersing adsorbents on extraction yields of piplartine and piperine

The ratio of dispersing sorbent to sample can also affect extraction yield and must be considered as major variable during the method development. The four different ratios of silica gel to sample mass: 1:1, 2:1, 3:1 and 4:1 were tested. The results are shown in Fig. 4 shows that the mass ratio has no significant effect on the extraction yields. But when the mass ratio is 3:1, the extraction yields of piplartine and piperine are all slightly higher than other mass ratios. Thus this ratio was selected in this study.

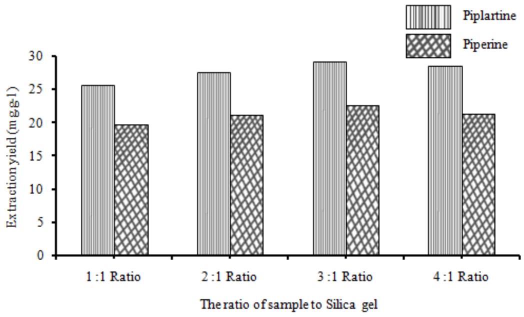

Fig. 4 The effect of the ratio of adsorbent to sample on the extraction yields

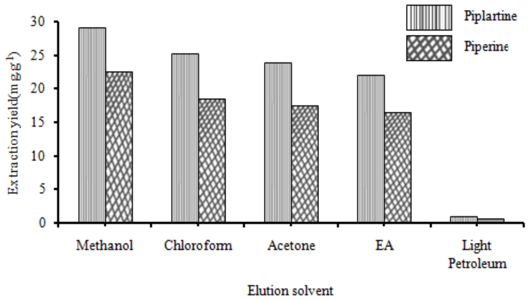

Fig. 5 The effect of elution solvents on the extraction yields

The effect of elution solvent was studied in order to obtain the highest extraction yields for the analysts. Methanol, chloroform, acetone, ethyl acetate and light petroleum were tested because they were commonly used in MSPD. These solvents present dissimilar polarities. The stronger the polarity of the solvent, the stronger the capability to elute the analysts.

Methanol was selected as elution solvent for the further work, because it shows better result in Fig. 5 than any other solvent. Additionally, to elute the target compounds completely with the minimum volume of elution solvent, the effect of elution solvent volume on extraction yields of target compounds was also studied. The results were shown in Fig. 6 indicate that the extraction yields of piplartine and piperine increased with the increase in methanol volume from 5-10 mL no significant increase in extraction yield is observed when the volume of elution solvent is from 15-20 mL . So 15 mL of methanol was chosen as the elution solvent in further experiments.

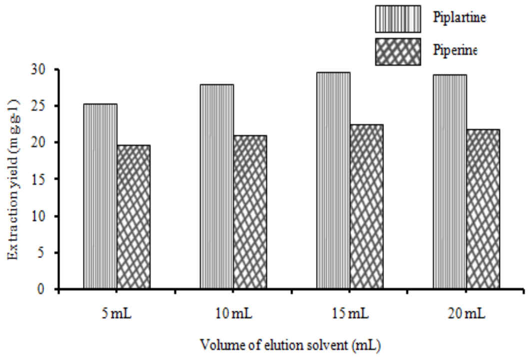

Fig. 6 The effect of volume of elution solvent on extraction yields

3.3 Method Validation

To evaluate the proposed MSPD-HPLC-DAD method, linearity, limit of detection (LOD) and limit of quantification (LOQ), precision and recovery were tested.

3.3.1 Linearity

The linearity of the method was determined by analyzing a series of standard solutions at concentration from 5-100 µg/mL for both analysts. The calibration curves were obtained by plotting peak area against the concentration of the analysts. The typical equation of the calibration curves for piplartine and piperine obtained were y=5.972x+1.45 and y=7.541x-7.62 with the correlation co efficient of 0.9998 and 0.9991, respectively. Where y is the peak area, x is the concentration in µgmL^{-1}.

3.3.2 LOD and LOQ

The standard stock solutions were further diluted with methanol to provide a series of solutions with appropriate concentrations. The LOD and LOQ for piplartine and piperine were estimated at signal to noise ratio of 3:1 and 10:1, respectively. The LODs for piplartine and piperine were 1.21 and 1.24 µgmL^{-1}. The LOQs for piplartine and piperine were 3.70 and 4.09 µgmL^{-1}, respectively. These indicated that sensitivity of the method is adequate for a reliable quantification of the samples.

3.3.3 Precision

The precision of the proposed method was assessed by study of repeatability and intermediate precision. Repeatability (intra-day) of the assay method was evaluated by six replicates of MSPD extraction samples solution in one day and the relative standard deviation of six values was calculated to determine intra-day precision. Intermediate precision (inter-day) at the same sample solution was determined on three successive days. The percentage RSD values for the precision study were between 1.29% and 3.1% for the intra-day and between 2.25% and 5.58 % for inter-day precision of piplartine and piperine, respectively. The results of the precision study indicated that the method is reliable and reproducible.

Table 1 Recovery studies of piplartine and piperine from samples with known concentration

Compound (Intraday; n=3)	Amount added (µgmL^{-1})	Amount found (µgmL^{-1})	%RSD	%Recovery
Piplartine	5	4.78	2.32	95.60
	20	19.62	2.78	98.10
	100	90.66	2.54	90.66
Piperine	5	4.61	2.74	92.32
	20	19.01	2.49	95.05
	100	100.95	1.75	100.95

3.3.4 Recovery

To evaluate the accuracy and applicability of the proposed method, the spiked samples at three fortification levels were analyzed, and each test was performed in triplicate. The *Piper chaba* samples were fortified with the standard stock solutions and followed by an air-drying for 24 h at ambient temperature. This procedure must be careful to avoid the loss of target compounds. Unspiked "blank" samples were previously analyzed to determine the presence of piplartine and piperine. As shown in Table 1, the recoveries were in a range of 90.66-100.95% for piplartine and piperine with RSDs ranged from 1.7 to 2.7%. Excellent recoveries demonstrated that the MSPD is suitable to extract the piplartine and piperine from *Piper chaba.*

3.4 Comparison of MSPD, Ultrasonic and Soxhlet Extraction

In order to evaluate the performance of optimized MSPD, The comparison among MSPD, ultrasonic and soxhlet extraction was made. The results are shown in Table 2. The extraction yields of piplartine and piperine for the MSPD procedure were higher than ultrasonic and Soxhlet extraction. Moreover the MSPD procedure require shorter time, lower solvent volume in the determination of pipalrtine and piperine in the roots of *P. chaba* than ultrasonic and soxhlet extraction procedure. MSPD procedure did not require heating during the extraction avoided the possible loss and degradation of the piplartine and piperine. Considering the extraction yields and consumption of sample, time and solvent, MSPD extraction should be comparatively better method.

Table 2 Comparison of MSPD, ultrasonic and Soxhlet extraction

	MSPD	Ultrasonic	Soxhlet
Extraction yield of Piplartine (mean ± SD, mg/g)	29.282 ± 0.242	26.857 ± 0.110	28.578 ± 0.304
Extraction yield of Piperine (mean ± SD, mg/g)	22.605 ± 0.057	19.795 ± 0.239	20.666 ± 0.173
Sample (g)	0.5	0.5	0.5
Solvent (mL)	15	50	100
Time (h)	0.5	1	15

3.5 Application in Sample Analysis

The proposed method that combines MSPD combined with HPLC-DAD applied to analyze three samples cultivated in different areas. The analytical results are summarized in Table 3. In all three samples, the contents of piplartine and piperine are in the range of 19.33-29.29 mgg^{-1} and 15.55-22.60 mgg^{-1} respectively. The differences in piplartine and piperine concentrations in these samples are due to the difference in cultivated area, growth conditions and picking period.

Table 3 The analytical results of samples

Samples	Piplartine		Piperine	
	Extraction yield (mg/g)	RSD (%, n=3)	Extraction yield (mg/g)	RSD (%, n=3)
PC-R-1	29.28	0.82	22.60	0.25
PC-R-2	25.06	0.63	19.65	0.69
PC-R-3	19.33	2.48	15.55	1.98

4. Conclusion

In this study MSPD extraction followed by HPLC-DAD can be applied as a simple, rapid and cost-effective alternative to determine piplartine and piperine in the *P. chaba*. The method uses the silica gel dispersant based on the MSPD column and methanol as elution solvent, compared with ultrasonic and soxhlet extraction techniques, the MSPD extraction requires smaller amount of samples (0.5 g), lower consumption of organic solvents (15 mL) and shorter extraction times (0.5 hrs). The proposed method has been successfully validated in terms of linearity, precision and recovery. The developed method can also be applied to the extraction of piplartine and piperine from other plant materials and pharmaceutical formulations.

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