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One Step Purification of Piperine Directly from *Piper nigrum* L. by High Performance Centrifugal Partition Chromatography

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Abstract: In this study, a preparative high performance centrifugal partition chromatography (HPCPC) method for isolation and purification of the bioactive component piperine directly from the ethanol extract of *Piper nigrum* L. was successfully established by using *n*-hexane-ethyl acetate-methanol-water as the two-phase solvent system. The upper phase of *n*-hexane-ethyl acetate-methanol-water (6:5:6:5, v/v) was used as the stationary phase of CPC. Under the optimum conditions, 40 mg of piperine at 98.5% purity, as determined by HPLC, was yielded from 300 mg of the crude extract in a single CPC separation. The peak fraction of CPC was identified by ^{1}H NMR and ^{13}C NMR.

Keywords: High performance centrifugal partition chromatography, piperine, *Piper nigrum* L., preparative chromatography

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INTRODUCTION

Piper nigrum L. (Piperaceae), known as Hujiao in Chinese, is used worldwide as a household spice such as in food additives and condiments. In addition, it is used as an important ingredient for various medicinal purposes in traditional medicine in many Asian countries (1). The pharmacological studies have demonstrated that the fruits of *P. nigrum* have been used in the treatment of cholera and dyspepsia, as well as a variety of gastric ailments and arthritic disorders (2). Previous research on the chemical constituents of *P. nigrum* has resulted in the isolation of several classes of compounds such as terpenes, steroids, lignans, flavones, and alkaloids/alkamides (3,4). Piperine, a nitrogenous pungent substance, is a major alkaloid presents in the fruits of *P. nigrum*. Thus, numerous studies have focused on investigating the bio-activities of piperine. Recent pharmacological studies have shown that piperine possesses anti-inflammatory, antioxidant, anticonvulsant, anti-ulcer, anti-depressant, analgesic, and cytoprotective effects (5–8). The chemical structure of piperine is shown in Fig. 1.

CPC is a continuous liquid–liquid partition chromatographic method generally referred to as countercurrent chromatography (CCC) (9), even though there is no counter-current between the phases. One is mobile and the other one is kept stationary inside of the column by a constant centrifugal field. The column itself is a rotor built as a disk stack in which partition cells are engraved. The CPC technique has the ability to separate fragile compounds because there is no irreversible adsorption or chemical reaction of the injected material with a solid support (10). Moreover, there is generally no problem due to saturation of the solid-state phase, allowing CPC to be efficient in preparative separations (11). A high selectivity is obtained by a careful choice of the biphasic solvent system, thus allowing the separation of compounds with very similar structures (12). Due to the unique features, CPC has been widely used in the separation and purification of various natural and synthetic products (13–16). Following the general research trend devoted to the development of separation science, we report here a CPC method for preparative isolation and purification of

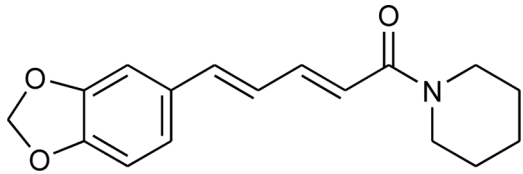

Figure 1. The chemical structure of piperine.

piperine from the crude extract of *P. nigrum*. Although preparation of piperine from *P. nigrum* has been studied (17,18), multiple steps are always needed in the established protocols. Therefore, the purpose of this study is to develop an effective CPC approach for one step preparative purification of piperine directly from the crude extract of *P. nigrum*.

EXPERIMENTAL

Apparatus

The isolation and purification of piperine from *P. nigrum* was performed by a HPCPC instrument (Sanki Engineering, Kyoto, Japan). The total cell volume is 240 mL (total cell No. 2136). A four-way switching valve incorporated in the CPC apparatus allows operation in either the descending or the ascending mode. The revolution speed of the apparatus can be regulated with a speed controller in the range of 0 and 2000 rpm. In addition, it was equipped with an ÄKTA prime system (Amersham Pharmacia Biotechnique Group, Sweden) to pump the two-phase solvent system and perform the UV absorbance measurement, a Rheodyne valve (Rheodyne, USA) with a 2-ml sample loop, and a BSZ-100 fraction collector for solute collection.

The high-performance liquid chromatography (HPLC) used was an Agilent 1100 system including a G1311A QuatPump, a G1322 Degasser, a G1314A variable wave detector, a model 7725i injection valve with a 20 μl loop, a PT100 column oven and Agilent ChemStation for LC. ^{1}H NMR and ^{13}C NMR. NMR experiments were carried out using a Bruker Advance 400 NMR spectrometer (Bruker, Germany).

Reagents and Materials

All organic solvents used for CPC were of analytical grade and purchased from Huadong Chemicals, Hangzhou, China. Reverse osmosis Milli-Q water (18MΩ) (Millipore, Bedford, MA, USA) was used for all solutions and dilutions. Methanol used for HPLC analysis was of chromatographic grade and purchased from Merck, Darmstadt, Germany. The dried roots of *P. nigrum* were purchased from a local drug store.

Preparation of the Crude Extracts of *P. nigrum*

The roots of *P. nigrum* were dried to constant mass at 55°C in a vaccum oven and then pulverized. About 1 kg of pulverized sample was extracted

with 4 L of 95% ethanol for 2 hours under reflux. The extraction procedure was repeated three times. The extracts were combined together and concentrated to dryness, yielding 70 g of the crude ethanol extract, which was stored in a refrigerator (4°C) for the subsequent HPCPC separation.

Preparation of the Two-Phase Solvent Systems and Sample Solution

The two-phase solvent system used was composed of *n*-hexane-ethyl acetate-methanol-water at various volume ratios. The solvent mixture was thoroughly equilibrated in a separatory funnel at room temperature and the two phases were separated shortly before use. The sample solution was prepared by dissolving the crude sample in a solvent mixture consisting of equal volumes of both upper and lower phases at suitable concentration according to the preparative scale of CPC separation.

CPC Procedure

The CPC column was first filled with the organic stationary phase and then rotated at 1200 rpm while mobile phase was pumped into the column in a descending mode at the flow-rate used for the separation (2 ml/min). When the mobile phase emerged from the CPC, equilibrium was reached. The sample (300 mg) dissolved in a 1:1 (v/v) mixture of each phase was injected through the Rheodyne injection valve. UV detection was performed at 254 nm from the outlet of the column, and the fraction was collected in a 10-ml tube in a BSZ-100 fraction collector. Reproducible results were obtained by three repeated CPC experiments for purification of piperine from *P. nigrum*.

HPLC Analysis and Identification of CPC Peak Fractions

HPLC analyses of the crude sample and CPC peak fractions were performed with a Zorbax Extend C18 column (150×4.6 mm i.d., 5 μm). The mobile phase was methanol (solvent A) and water (solvent B) at the gradient: A from 70% to 90% and B from 30% to 10% for 30 min. The flow rate was 0.8 ml min^{-1}, and the effluent was monitored at 254 nm. Identification of the CPC peak fraction was performed by ^{1}H NMR and ^{13}C NMR. NMR experiments were carried out using a Bruker Advance 400 NMR spectrometer with chloroform ($CDCl_3$) as solvent and TMS as internal standard.

RESULTS AND DISCUSSIONS

Optimization of Two-Phase Solvent System

In the present work, the HPLC method for analysis of the crude sample and peak fractions of CPC was established at first. In order to select an appropriate elution system for HPLC analysis of the crude ethanol extract, different kinds of solvent systems were employed. The results indicated that when methanol and water were used in the gradient mode (methanol: 0–30 min, 70–90%) and the flow rate was 0.8 ml min^{-1}, the target compound could be separated with other components. The HPLC chromatogram of crude extract of *P. nigrum* is given in Fig. 2a. The content of piperine was 15.9%, as determined by the HPLC peak area.

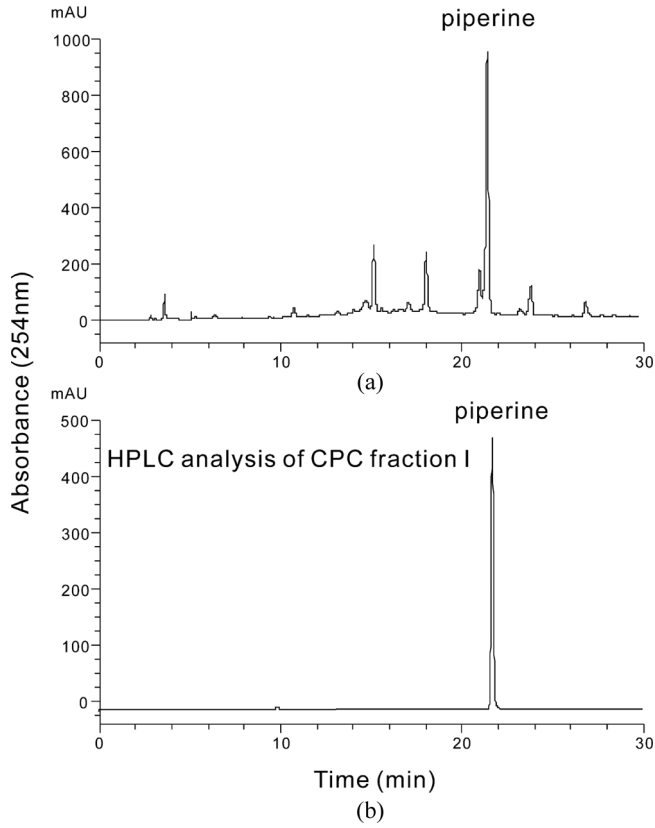

Figure 2. HPLC chromatograms of the crude extract of *P. nigrum* (a) and CPC fraction I (b).

CPC is extremely useful for separation and purification of natural products. The selection of the two-phase solvent system is the most important, and is also the most difficult step because any change of the mobile phase composition is likely to change the stationary phase composition or volume; it is estimated that about 90% of the entire work in CPC is invested in solvent system selection (14). In order to select a suitable system, previous articles on CPC should be carefully consulted, and some rules need to be considered. For example, the target compound should be soluble and stable in the solvent system; the settling time of the solvent system should be short (<30 s); the partition coefficient (K) of the target compounds between two phases should be appropriate; and the retention of the stationary phase should be satisfactory. In addition, the higher the retention of the stationary phase, usually the better the peak resolution. Low partition of the target compounds in the stationary phase results in poor resolution, while high partition tends to give better resolution but broader peak and more dilute peak fraction due to a longer elution time.

In the present work, considering the relative hydrophobic properties of the target compound, a two-phase solvent system composed of *n*-hexane-ethyl acetate-methanol-water at various volume ratios (2:3:2:3, 5.6:5:6, 1:1:1:1, 6:5:6:5, 3:2:3:2, v/v) was selected for the preliminary evaluation in terms of K values and peak resolution. It was necessary to point out that the selected solvent family was recognized as a good first try in CPC separation and exhibited overwhelming applications than other solvent system families (15,19). According to the literature (14,15), the measurement of the K values was performed as follows: a small amount (0.5 mg) of crude sample was added into a test tube to which about 2 ml of each phase of the pre-equilibrated two-phase solvent system were added. After shaken vigorously for 10 min, the mixture was separated by centrifugation for 3 min. Then, an aliquot of each phase (100 μl) was delivered into a test tube separately, each was diluted with an equal volume (1 ml) of methanol and analyzed by HPLC. The K value was expressed as the peak area of target compound in the upper phase divided

Table 1. The K (partition coefficient) values of piperine in several solvent systems

Solvent systems (v/v)	*K-value*
n-hexane-ethyl acetate-methanol-water (2:3:2:3)	2.58
n-hexane-ethyl acetate-methanol-water (5:6:5:6)	1.96
n-hexane-ethyl acetate-methanol-water (1:1:1:1)	1.59
n-hexane-ethyl acetate-methanol-water (6:5:6:5)	1.03
n-hexane-ethyl acetate-methanol-water (3:2:3:2)	0.62

by that in the lower phase. Table 1 shows the K values of piperine in different two-phase solvent systems. The results indicated that when the two-phase solvent system at a volume ratio of 2:3:2:3 was used, the K value of piperine was too large for CPC separation (longer separation time). When it changed from 5:6:5:6 or 1:1:1:1, the partition coefficient of the target compound was greatly reduced. However, it still needed several hours to elute piperine out of the CPC column. When the two-phase solvent system at a volume ratio of 6:5:6:5 was used, the separation of the target compound was achieved with satisfactory peak resolution, and the retention of the stationary phase was good (65%). When it further changed to 3:2:3:2, although the separation time was even shorter, the peak resolution was relatively pool. Therefore, the two-phase solvent system composed of *n*-hexane-ethyl acetate-methanol-water (6:5:6:5, v/v) was selected for the separation of piperine from the crude sample of *P. nigrum* in the present study.

Separation of Piperine by CPC

Although the revolution speed of the apparatus could be regulated with a speed controller in a range between 0 and 2000 rpm, the speed of 1200 rpm was used invariably in the present study. The influence of the flow rate of the mobile phase was also investigated. The results indicated that reducing the flow rate could improve both the peak resolution and the reservation of the stationary phase to some degree, but the chromatogram peaks were broadened at the same time. At last, a flow rate of 2.0 ml min^{-1} was employed in the experiment.

Figure 3 shows the preparative CPC separation of 300 mg of the crude sample using the solvent system composed of *n*-hexane-ethyl acetate-methanol-water (6:5:6:5, v/v). In order to save solvents and time, other eluting substances after the target compound were removed by pumping out the stationary phase instead of eluting them with the mobile phase because the stationary phase was used only once. Each CPC peak fraction was analyzed by HPLC and the chromatogram is shown in Fig. 2b. As a result, 40 mg of piperine with the purity of 98.5% was yielded in a single-step separation, which clearly indicated that the two-phase system was very efficient for CPC separation of piperine from *P. nigrum*.

Structural Identification of Purified Piperine

The structural identification of CPC fraction I in Fig. 3 was carried out by ^{1}H NMR and ^{13}C NMR as follows: ^{1}H NMR (400 MHz,

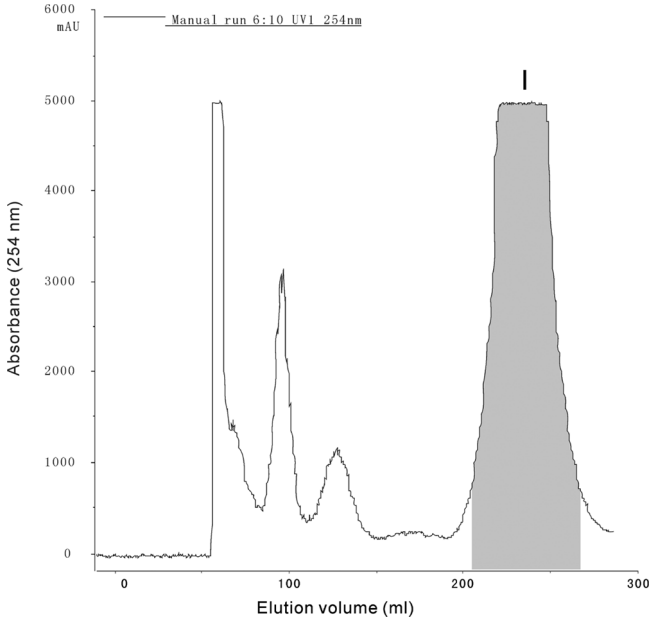

Figure 3. Preparatve HPCPC separation of piperine directly from the ethanol extract of *P. nigrum*. CPC separation conditions: solvent system, *n*-hexane-ethyl acetate-methanol-water (6:5:6:5, v/v); mobile phase, lower phase; rotary speed, 1200 rpm; elution mode, desending, flow rate, 2.0 ml min^{-1}; detection, 254 nm; sample size, 300 mg of crude sample dissolved in 1 ml upper phase and 1 ml lower phase; retention of the stationary phase, 65%.

$CDCl_3$): $\delta = 1.60$ (4H, m), 1.66 (2H, m), 3.58 (4H, br, m), 5.97 (2H, s), 6.43 (1H, d, J = 14.60 Hz), 6.74 (1H, m), 6.75 (1H, m), 6.78 (1H, d, J = 8.05 Hz), 6.89 (1H, dd, J = 1.35, 8.05 Hz), 6.98 (1H, d, J = 1.30 Hz), 7.31 (1H, m). ^{13}C NMR (100 MHz, $CDCl_3$): $\delta = 165.7$ (C-1), 120.2 (C-2), 142.8 (C-3), 125.8 (C-4), 138.5 (C-5), 131.3 (C-1′), 105.9 (C-2′), 148.3 (C-3′), 148.4 (C-4′), 108.7 (C-5′), 122.7 (C-6′), 101.5 ($-OCH_2O$), 43.2 (C-1″), 25.7 (C-2″), 24.8 (C-3″), 26.3 (C-4″), 46.9 (C-5″). According to the literature (4,5), CPC peak I was identified as piperine.

CONCLUSIONS

In conclusion, an effective HPCPC method was developed for the preparative isolation and purification of piperine from the ethanol extracts of *P. nigrum*. 40 mg of piperine at 98.5% purity was obtained directly from a 300 mg amount of the crude extract in a one-step CPC separation.

These results clearly demonstrated that the present CPC method is powerful for the separation of piperine from *P. nigrum.*

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