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Preparation of bioactive amide compounds from black pepper by countercurrent chromatography and preparative HPLC

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ABSTRACT

Amide compounds are the major bioactive substances in black pepper. Black pepper extracts were fractionated by high-speed countercurrent chromatography resulting in three compounds: dehydropipernonaline, piperine and (2E,4E,12Z)-N-isobutyl-octadecatrienamide, and four fractions which were separated by preparative HPLC to yield ten purified compounds: retrofractamide-A, (2E,4E)-N-isobutyl-decadienamide, retrofractamide-C, piperoleine A, dehydropiperoleine A, retrofractamide-B, piperoleine B, pipernonaline, pipercyclobutanamides A and (2E,4E,14Z)-N-isobutyleicosa-2,4,14-trienamide as identified by ESI-MS and NMR analysis. The results of the present study indicated that HSCCC is an effective technique for the preparation of bioactive amide compounds from black pepper.

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1. Introduction

The genus *Piper*, belonging to the Piperaceae, has received considerable attention because pepper fruits have been used in spice and also in folk medicine (Parmar et al., 1997; Stohr et al., 2001). Black pepper, the fruit of *Piper nigrum* L. is widely used as an anodyne and a treatment for stomach disease in China (Tabuneng et al., 1983). Studies on *Piper* species have shown that the major bioactive components are amides (Yang et al., 2002; Ghoshal & Lakshmi, 2002; Woo et al., 2007; Zhang et al., 2007; Hsu and Yen, 2007; McNamara et al., 2005; da Silva et al., 2002; Zhang et al., 2008). It is difficult to obtain purified amides using traditional chromatography since amides can easily suffer from peak tailing and poor efficiency on silica-based columns (Parmar et al., 1997; da-Cunha and Chaves, 2001; da Silva et al., 2002; Reddy et al., 2004). The preparative separation and purification of amides from plant materials by conventional methods usually requires multiple chromatography steps. For example, piperine (**2**), retrofractamide-A (**4**), (2E,4E)-N-isobutyl-decadienamide (**5**), retrofractamide-C (**6**) and retrofractamide-B (**9**) were obtained by column chromatography and preparative TLC on silica gel (Navickiene et al., 2000; Banerji et al., 2002), and dehydropipernonaline (**1**), (2E,4E,12Z)-N-isobutyl-octadecatrienamide (**3**), piperoleine A (**7**), dehydropiperoleine A (**8**), piperoleine B (**10**), pipernonaline (**11**), pipercyclobutanamides A (**12**) and (2E,4E,14Z)-N-isobutyleicosa-2,4,14-trienamide (**13**) were prepared by column chromatography on silica gel and RP-C_{18}, together with preparative HPLC with ODS column (Matsuda et al., 2009).

High-speed countercurrent chromatography (HSCCC) has been used for the separations of valuable natural products due to no adsorptive sample loss and deactivation, tailing of solute peaks and contamination (Liu et al., 2009; Du et al., 2009; Qiao et al., 2012; Ito & Conway, 1996). Thus, it is an effective way to fractionate crude plant extracts for the separation and preparation of purified components. In the present study, we obtained 13 amide compounds by advanced preparative HPLC separations of the amide fractions from HSCCC fractionation of the crude extracts of the fruit of *P. nigrum* L.

2. Materials and methods

2.1. Materials

Ten kilograms of dry black pepper were provided by Hainan Nanhai Farm, Hainan, China. All chemical reagents for HSCCC separation were of analytical grade, and were purchased from Huadong Chemicals (Hangzhou, China).

2.2. Extraction and pre-separation

Ten kilograms of dry black pepper were powdered and then extracted twice with 50 L of 90% aqueous ethanol for 2 h at 50 °C. The extracts were combined and evaporated to syrup. The syrup

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was dispersed into 2 L of 1% aqueous HCl, and then extracted with ethyl acetate (2 L × 2) to remove impurities. The acidic solution was adjusted to pH 10 with aqueous ammonia, and then extracted with chloroform (2 L × 2). The chloroform solution was concentrated at reduced pressure and dried in vacuum to yield 195 g of crude amide extract.

2.3. Selection of solvent system for HSCCC

Selection of solvent system for HSCCC separation can employ TLC, HPLC or other methods which provide comparison of partition coefficients of the components between two phases. Usually TLC method is the most convenient method since the procedure is simple and the result is visual. In the present study, we investigated the partition coefficients of the components between the two phases of a series of solvent systems composed of n-hexane–ethyl acetate–methanol–water with ratios of 2:1:2:2, 2:2:2:2, 2:3:2:2, 2:4:2:2, 2:5:2:2, 2:6:2:2, 2:1:3:2, 2:2:3:2, 2:3:3:2, 2:4:3:2, 2:5:3:2, 2:6:3:2, 2:1:4:2, 2:2:4:2, 2:3:4:2, 2:4:4:2, 2:5:4:2 and 2:6:4:2 (v/v). After a solvent system was prepared, 1 ml of upper phase and 1 ml of lower phase were taken to dissolve about 0.5 mg of the black pepper extracts. The two phases were fully mixed and allowed to settle. The concentration of the components in the upper phase and lower phase was analyzed by TLC. We found that a system composed of n-hexane–ethyl acetate–methanol–water (2:6:3:2) afforded relatively obvious differences of the partition coefficients between the various components (Fig. 1). This system was used for HSCCC separation.

2.4. High-speed countercurrent chromatographic separation

HSCCC separation employed the solvent system composed of n-hexane–ethyl acetate–methanol–water (2:6:3:2, v/v). The upper phase was used as stationary phase while the lower phase was the mobile phase.

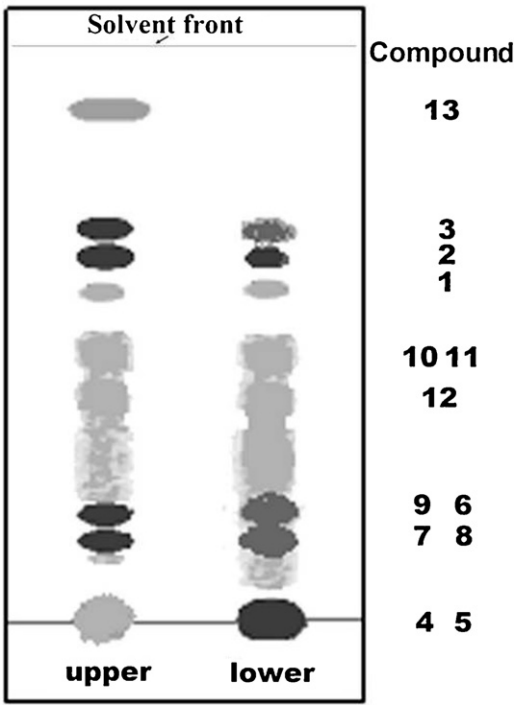

Fig. 1. TLC analysis of the components in upper phase and lower phase of the solvent system composed of n-hexane–ethyl acetate–methanol–water (2:6:3:2, v/v). TLC plate: Silica G 254 Aluminum; developing solvent: ethyl acetate–n-hexane (3:2, v/v); colorizing reagent: 10% ethanolic sulfuric acid.

The high-speed countercurrent chromatograph used in the present study was constructed at the Institute of Food and Biological Engineering, Zhejiang Gongshang University, Hangzhou, China. The apparatus was equipped with a 1200-ml column with 6-layer coils (ca 200 coils) made of a 5.0 mm i.d. polytetrafluoroethene (PTFE) tubing. The beta values of the coils range from 0.44 to 0.88. For the separation a K-1800 Wellchrom preparative HPLC pump (Knauer, Germany), a 100 ml sample loop made of 3 mm i.d. PTFE tubing, and a B-684 collector (Büchi, Switzerland) with 25-ml tube racks were used. The separation procedure began with filling the column with the stationary phase. Then the apparatus was rotated at 1000 rpm and the sample solution (3.5 g crude amide extract in 100 ml mobile phase) was injected into the HSCCC system through the PTFE sample loop by pumping the mobile phase at a flow rate of 5.0 ml/min. The effluent was collected with 25 ml/tube by a fraction collector.

2.5. HPLC analysis and preparation

The fractions from HSCCC separation were analyzed by HPLC. The analytical HPLC system was composed of an Alliance 2695, a 5 μm ODS AQ column (150 mm × 3.9 mm i.d.), a 996 PDA detector and a Millennium HPLC 2010 processing system (Waters, Milford, USA). A gradient elution was performed for the separations with a gradient 40–100% aqueous methanol from 0 to 40 min at a flow rate of 0.8 ml/min. The column temperature was set at 25 °C, and the detection wavelength was 254 nm. The preparative HPLC conditions were decided by analytical HPLC results. The preparative HPLC utilized a 15 μm ODS AQ column (250 mm × 20 mm i.d.), eluted with a flow rate of 10 ml/min for all the mobile phases for the separations of FrA, FrB, FrC and FrG.

2.6. ESI-MS and NMR

All ESI-MS experiments were performed on a Bruker Esquire LC-MS ion trap multiple mass spectrometer (Bremen, Germany) in positive and negative ionization mode analyzing ions up to m/z 2200. ^{1}H-, ^{13}C- and DEPT 90/135-NMR spectra were recorded in $CDCl_3$ on a Bruker Avance 500 (Karlsruhe, Germany) with 500 MHz for ^{1}H-, and 125 MHz for ^{13}C-measurements, respectively.

3. Results and discussion

3.1. HSCCC fraction

The crude amide extracts from black pepper were a complex mixture based on TLC (Fig. 1). With a suitable solvent system a HSCCC separation can yield purified compounds from a complex crude sample. Fig. 2 shows the fractionation effect which afforded six fractions as R_f values from low to high: FrA (tubes 23–28) with one spot, FrB (tubes 39–65) with multiple spots, FrC (tubes 66–95) with three spots, FrD (tubes 96–109) with one spot, FrE (tubes 113–173) with one spot and FrF (tubes 183–215) with one spot. In addition, a fraction FrG was obtained from the extrusion of the stationary phase in the column after the rotation of the column was stopped. HPLC analysis of the fractions showed that FrA, FrB and FrC mainly presented 2, 4 and 3 peaks respectively and FrG showed a major peak with minor peaks, while FrD, FrE and FrF each gave single peak (Fig. 3). Direct concentration and dryness in vacuum of FrD, FrE and FrF resulted compounds **1** (135 mg), **2** (85 mg) and **3** (265 mg), which were subjected to identification of chemical structure by ESI-MS and ^{1}H and ^{13}C NMR. FrA, FrB, FrC and FrG were concentrated and dried in vacuum to yield 510, 1103, 185 and 175 mg samples.

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