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Development of Gradient Centrifugal Partition Chromatography Method and Its Application for the Isolation of 3,5-Dimethoxyphenanthrene-2,7-diol and Batatasin-I from *Dioscorea opposita*

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Abstract − Gradient centrifugal partition chromatography (GCPC) method was developed and applied to isolate 3,5-dimethoxyphenanthrene-2,7-diol (DMP) and batatasin-I (BA-I) from the dichloromethane soluble extract of *Dioscorea opposita*. In this method, the lower phase of *n*-hexane-methanol-water system (HMW, 10 : 9 : 1, v/v) was used as a mobile phase A (MpA) and water was used as a mobile phase B (MpB). This gradient CPC method is comparable to that of reversed-phase HPLC method in that the stationary upper-phase of HMW (10 : 9 : 1 v/v) works as if it were reversed-phase silica gel due to its hydrophobic property, while the lower phase (MpA) and water (MpB) functioned as hydrophilic mobile phases. The initial condition of the mobile phase was 20% MpA/ 80% MpB and maintained for 150 min to obtain DMP (1.2 mg), and then MpA was increased up to 50% to elute BA-I (1.7 mg). The purities of DMP and BA-I were 94.1% and 98.3% with the recovery yields of 83% and 86%, respectively. Similar results were obtained by linear-gradient CPC. The CPC peak fractions were identified by comparing their retention time to those of authentic samples of DMP and BA-I and their spectroscopic data (^{1}H NMR and ^{13}C NMR) to those of literature values.
Keywords − Gradient centrifugal partition chromatography (GCPC), *Dioscorea opposita*, 3,5-dimethoxy-phenanthrene-2,7-diol, batatasin-I, *n*-hexane-methanol-water system (HMW, 10 : 9 : 1, v/v)

Introduction

Countercurrent separation (CS) technique including high speed countercurrent chromatography (HSCCC) and centrifugal partition chromatography (CPC) is a liquid-liquid chromatography in which the separation mechanism is based on the distribution of compounds over two immiscible liquid phases (Murayama *et al*., 1982; Marchal *et al*., 2003). The advantages of CS are that there is no irreversible adsorption or chemical reaction and therefore the recovery yields of target compounds are superior to those of solid support based conventional column chromatography. For aforementioned reasons, CS technique has been applied to isolate and purify diverse natural products and synthetic compounds (Cortez *et al*., 1999; Himbert *et al*., 2004; Kim and Kim, 2007; Yoon *et al*., 2008).

So far, most of CS methods have been performed by isocratic mobile phase condition. In order to achieve desirable countercurrent separation, selection of optimal two-phase solvent system must be carried out to find suitable partition coefficient value of target compound, which is the most important step but requires the great parts of time and labor. Moreover, the static mobile phase is occasionally not effective to isolate target compounds that show big differences in partition coefficients value to each other. Thus, several countercurrent separations have been accomplished by gradient elution of mobile phase to isolate compounds with considerably difference in polarity (Chu *et al*., 2005; Du *et al*., 2004; Leitao *et al*., 2005; Liu *et al*., 2004a; Liu *et al*., 2004b; Oliveira *et al*., 2005).

Plants belong to the genus *Dioscorea* have long been used as edible tuber crops in many tropical and subtropical areas and as a traditional herbal medicine in oriental countries including China, Japan and Korea (Bae *et al*., 1999., Coursey *et al*., 1967). In the previous study, we developed the validation method utilizing HPLC-

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DAD to determine 3,5-dimethoxypheanthrene-2,7-diol (DMP) and batatasin-I (BA-I) as non-polar marker compounds of *Dioscorea* species cultivated in Korea (Yoon *et al*., 2007). In the course of qualitative and quantitative analyses of DMP and BA-I, there was great need for developing simple and efficient isolation method to obtain DMP and BA-I.

In the present study, step- and linear-gradient CPC method were developed and applied to isolate and purify DMP and BA-I from dichloromethane soluble extract of *D. opposita* using *n*-hexane-methanol-water system (HMW, 10 : 9 : 1, v/v) and water. With this gradient method, it was possible to isolate DMP and BA-I without assessing their partition coefficient values which must be considered in conventional CS process.

Experimental

Apparatus – The CPC instrument used in this study is an LLB-M high-performance centrifugal partition chromatography (Sanki Eng., Kyoto, Japan) with a four-way switching valve operating in either descending or ascending mode, and the total cell volume is 230 ml. The CPC system was equipped with binary Gilson 321 pump (Middleton, WI, USA), a Gilson FC 203B fraction collector (Middleton, WI, USA), a Gilson 151 UV/VIS detector (Middleton, WI, USA) and a Rheodyne valve (Cotati, CA, USA) with a 5 ml sample loop for manual injection. Analytical HPLC system was consisted of two Waters 520 pumps (Milford, MA, USA), a Waters 996 photodiode array detector (Milford, MA, USA), a Waters 717 plus autosampler (Milford, MA, USA), and Empower software was employed to carry out data acquisition. ^{1}H NMR and ^{13}C NMR data were obtained on a Bruker AVANCE 400 spectrometer (Bruker, Karlsruhe, Germany).

Chemicals and Plant Materials – All organic solvents for CPC separation were analytical grade and purchased from Daejung-Chemical and Metals Co., Ltd. (Kyunggi-Do, Korea). Deionized water was prepared by Millipore Rios^{TM} 100 water purification system (Millipore, Bedford, MA, USA). Acetonitrile and water for HPLC analyses were chromatography grade and obtained from Fisher Scientific Korea Ltd. (Seoul, Korea).

The fresh rhizomes of *Dioscorea opposita* Thunb. were provided by Tong Yang Moolsan Co., Ltd (Nonsan, Korea) and identified by Prof. Gwang Jin Chang, the Korea National Agriculture College.

Preparation of Sample Solution – The rhizomes of *D. opposita* (1000 g) were sliced, and extracted with methanol [(2 L, 3 hr) × 3] at room temperature to obtain a methanol extract (72 g). The methanol extract was dissolved in deionized water and then partitioned with dichloromethane (2 L) to give dichloromethane soluble extract (1.9 g). Sample solution for CPC experiment was prepared by dissolving the dichloromethane soluble extract with upper (4 mL) phase of *n*-hexane-methanol-water system (HMW, 10 : 9 : 1, v/v).

Preparation of CPC Solvent System – A two-phase solvent system composed of HMW (10 : 9 : 1, v/v) and water was used to isolate and purify DMP and BA-I in the present study. HMW (10 : 9 : 1, v/v) was thoroughly equilibrated in a separatory funnel at room temperature, and then upper phase and lower phase were separated. The upper- and lower phases from HMW (10 : 9 : 1, v/v) and water were degassed by ultrasonication for 30 min. The upper phase was used as a stationary phase, the lower phase was used as a mobile phase A (MpA), and water was used as a mobile phase B (MpB) for a gradient elution.

Centrifugal Partition Chromatography Procedure – In step-gradient CPC, the column was fully filled with stationary phase in a descending mode at a flow rate of 5 mL/min. Then, the CPC apparatus was rotated at 700 rpm while 20% MpA was eluted by binary pump at a flow rate of 2 mL/min. Sample solution was loaded on CPC apparatus after the mobile phase mixture was emerged in the effluent and hydrostatic equilibration was established in the column. After 150 min, mobile phase was changed to 50% MpA and kept for 350 min. The linear-gradient CPC process was identical to that of step-gradient CPC except the mobile phase condition as shown in Fig. 4A. The detection of CPC peak fractions was performed by combining the tail outlet of the CPC apparatus with UV detector (254 nm) and effluent was collected by a fraction collector in 10 min per each test tube. The relationship between the retention of stationary phase and the alteration of mobile phase (0 - 100% MpA) was investigated by equal procedure described above except that sample loading procedure was skipped and run time for each CPC experiment was 300 min. After CPC experiment, all solvents in the column were collected by pushing them out with N_2 gas to measure the retention of stationary phase.

HPLC Analysis – The methanol extract of *D. opposita*, dichloromethane soluble extract and CPC peak fractions were analyzed by HPLC-PDA with Gemini 5 μ C18 110A column (150 × 4.6 mm ID, 5 μm, Phenomenex, USA). Linear-gradient mobile phase composed of water and acetonitrile (0 - 45 min, 20 - 80% acetonitrile) was applied. Flow rate was 1 mL/min and all samples were

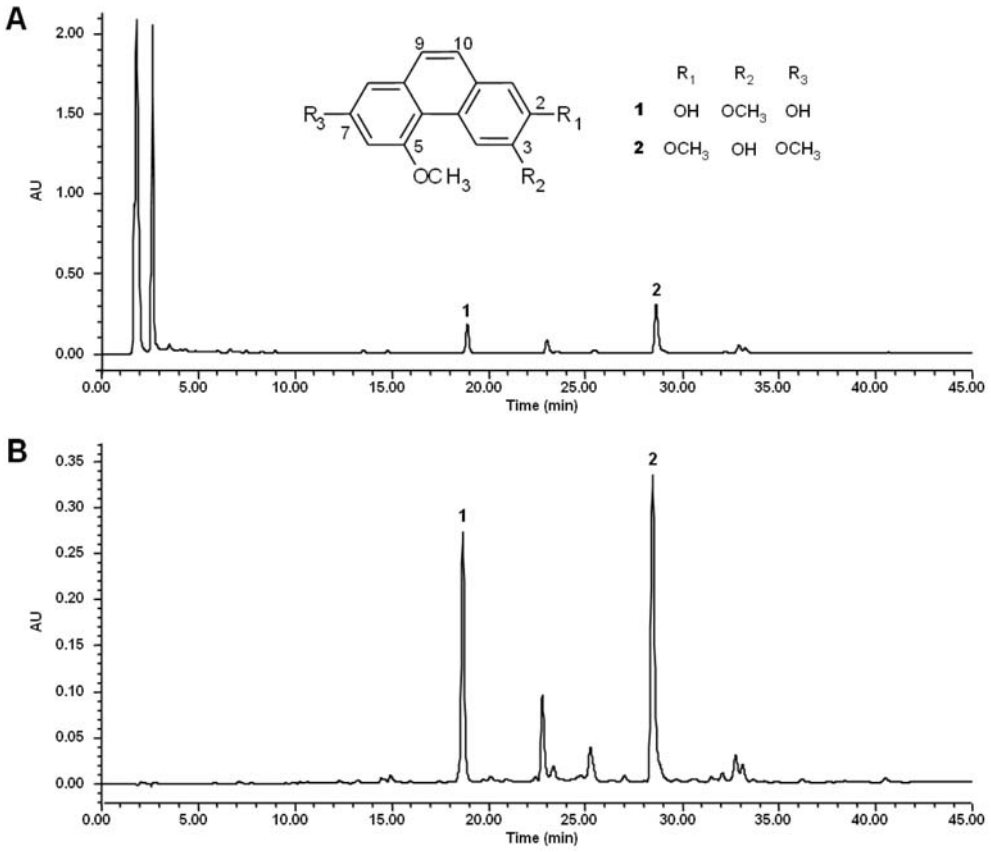

Fig. 1. HPLC chromatograms of methanol extract of *D. opposita* (A) and dichloromethane soluble extract of *D. opposita* (B). HPLC condition - Column: Gemini 5 μ C18 110A column (150 × 4.6 mm ID, 5 μm, Phenomenex, USA); Mobile phase: 0-45 min, 20-80% acetonitrile; Flow rate: 1 mL/min; UV wavelength: 254 nm. Peak 1: DMP; Peak 2: BA-I.

injected with the volume of 20 μL.

Identification of CPC Peak Fractions – CPC peak fractions were identified by comparing their retention time with authentic samples of DMP and BA-I isolated in previous study, and their ^{1}H and ^{13}C NMR spectroscopic data with literature values.

Results and Discussion

HPLC Analysis – *Dioscorea* species are known to be abundant in polar constituents such as purine derivatives, starches, steroidal saponins, and polysaccharide polymers (Yoon *et al*., 2008; Akahori *et al*., 1965; Fu *et al*., 2005, 2006; Jayakody *et al*., 2007; Zhang *et al*., 2004; Zhao *et al*., 2005), while the content level of non-polar constituents such as DMP and BA-I are relatively low (Yoon *et al*., 2007). As shown in Fig. 1A, the methanol extract of *D. opposita* has polar constituents as majority, but target compounds, DMP (t_R = 18.9 min) and BA-I (t_R = 28.7 min), were found as minor compounds. In order to eliminate polar substances and concentrate DMP and BA-I for effective CPC separation, the methanol extract was partitioned with dichloromethane (Fig. 1B), and this process enabled us to isolate DMP and BA-I with satisfactory recovery yields and higher purities.

Development of Gradient CPC – In the present study, linear- and step-gradient CPC method was developed using *n*-hexane-methanol-water system. Although *n*-hexane and methanol are known to be immiscible organic solvents, they were mixed each other to a certain extent. Fig. 2 shows the volume ratio (upper phase/lower phase) of the two-phase system composed of *n*-hexane-methanol-water (H : M : W = 10 : X : Y, v/v, X = 10-Y). The HMW (10 : 10 : 0, v/v) showed the volume ratio of 0.25, but it was increased dramatically with the addition of water and remained unchanged from 10 : 8 : 2 (v/v) to 10 : 0 : 10 (v/v). In HMW (10 : 9 : 1, v/v), the two-phase was well established within short settling time (< 10 sec) and the volume ratio was 0.82 (upper phase : lower phase = 45 : 55). Based on the results, the upper phase of HMW (10 : 9 : 1, v/v) was used as a stationary phase (*n*-hexane-rich phase) and lower phase was used as mobile phase A

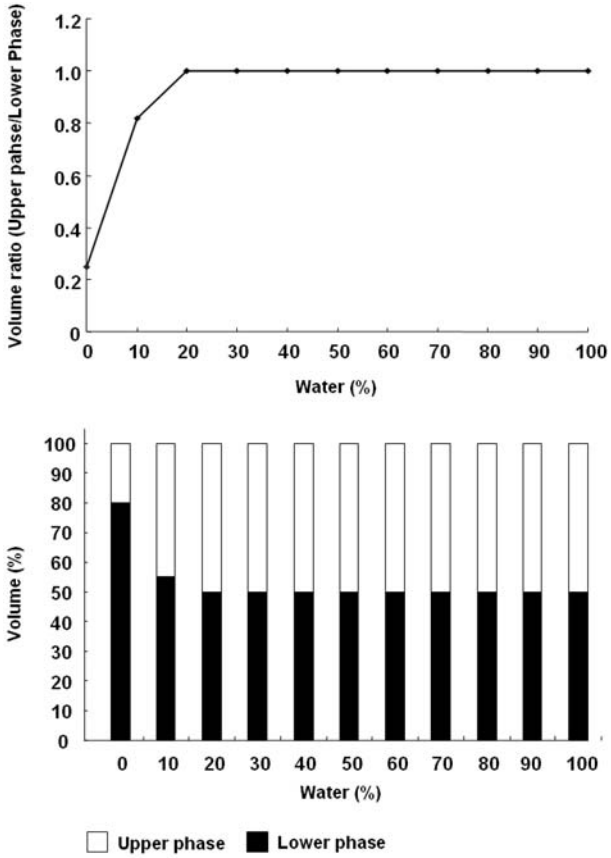

Fig. 2. The change of the volume ratio in two-phase solvent system composed of *n*-hexane-methanol-water (H : M : W = 10 : X : Y, X = 10-Y, v/v).

(MpA, methanol-rich phase) to give similar polarity comparable to that of methanol. In addition, water was prepared additionally to use as a mobile phase B (MpB).

The retention of stationary phase is important because it affects the column resolution and is a criterion of column stability in CS (Foucalut *et al*., 2005). Thus, the relationship between the retention of stationary phase and the alteration of mobile phase was investigated. The retention of stationary phase remained satisfactory level at 68.4 ± 2.1% (0 - 100% of MpA), and the variation was less than 9.7% indicating that the solvents composed of the upper- and lower-phase of HMW (10 : 9 : 1, v/v) and water are very stable system for gradient CPC (Fig. 3).

The CPC solvent system composed of the upper- and lower phase of HMW (10 : 9 : 1, v/v) and water are comparable to those of reversed-phase column chromatography method in that the stationary upper-phase of HMW (10 : 9 : 1 v/v) works as if it was reversed-phase silica gel due to its hydrophobic property, and the lower phase (MpA) acts as an organic mobile phase, finally the water (MpB) functions as an aqueous mobile phase.

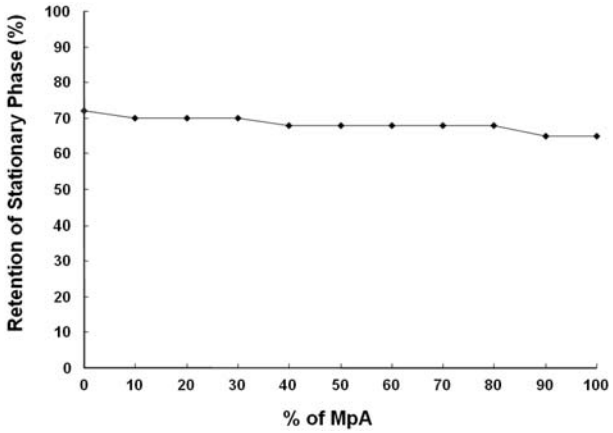

Fig. 3. The relationship between the retention of stationary phase and the alteration of mobile phase A.

CPC Separation – The dichloromethane soluble extract of *D. opposita* was separated by step- and linear gradient CPC method using HMW (10 : 9 : 1, v/v) and water (Fig. 4). The initial baselines of two CPC chromatograms were somewhat unstable, but they were stabilized with the increase of MpA ratio and it was enough to collect CPC peak fractions. In step-gradient mobile phase condition (Fig. 4A), the initial ratio of mobile phase was 20% MpA and maintained in 150 min to obtain DMP and elute relative polar constituents of dichloromethane soluble extract of *D. opposita*, and then MpA was increased up to 50% to get BA-I. As a result, DMP (peak fraction 1, 45 - 110 min, 1.2 mg) and BA-I (peak fraction 2, 290 - 430 min, 1.7 mg) were successfully isolated, and the purities of DMP and BA-I were 94.1% and 98.3%, respectively (Fig. 5). At a glance, the absolute amounts of isolated compounds seemed to be very small because the content levels of DMP and BA-I are very low in dichloromethane soluble extract of *D. opposita*, but the recovery yields of DMP and BA-I were 83% and 86%, respectively, determined by quantitative HPLC analysis. Similar results were obtained in linear-gradient CPC (Fig. 4B) and these results reveal that the upper- and lower phase of HMW (10 : 9 : 1, v/v) and water are very effective to isolate and purify DMP and BA-I from dichloromethane soluble extract of *D. opposita* with high purities and recovery yields. Furthermore, this solvent system can be utilized to fractionate or isolate diverse natural products by altering the polarity of mobile phase like reversed-phase column chromatography.

So far, the isolation of phenanthrenes from *Dioscorea* species were mainly performed by solid support based column chromatography method which requires tedious multiple steps. In addition, the recovery yields of target

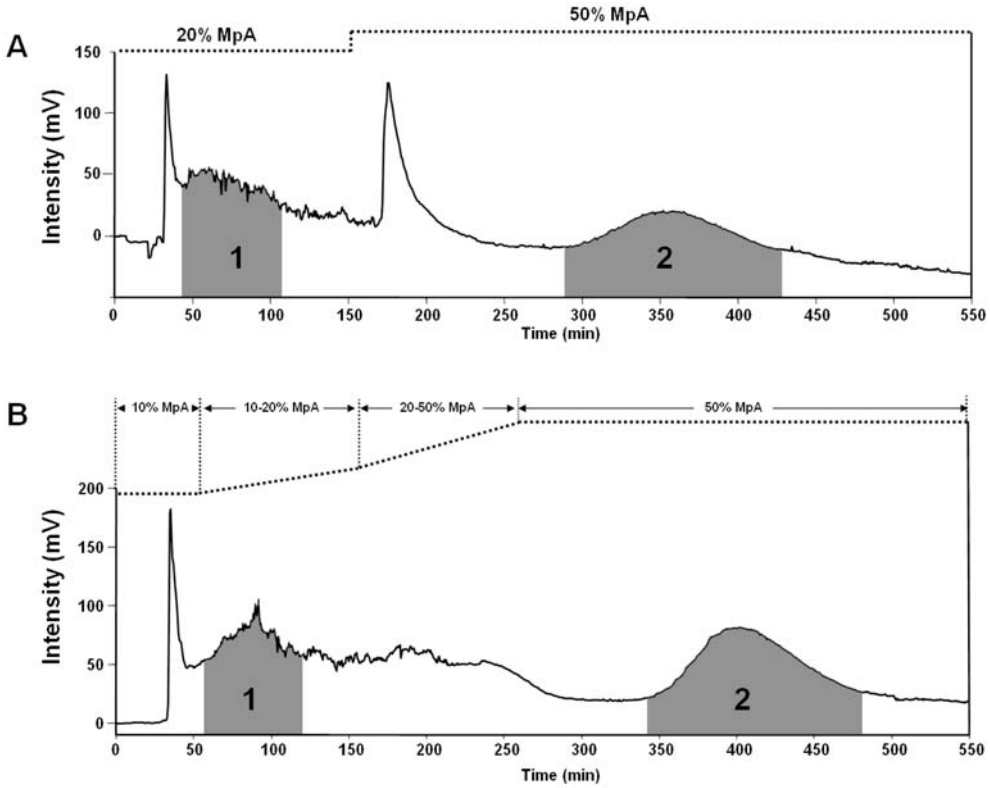

Fig. 4. CPC separation of dichloromethane soluble extract of *D. opposita* by step-gradient elution (A) and linear-gradient elution (B).

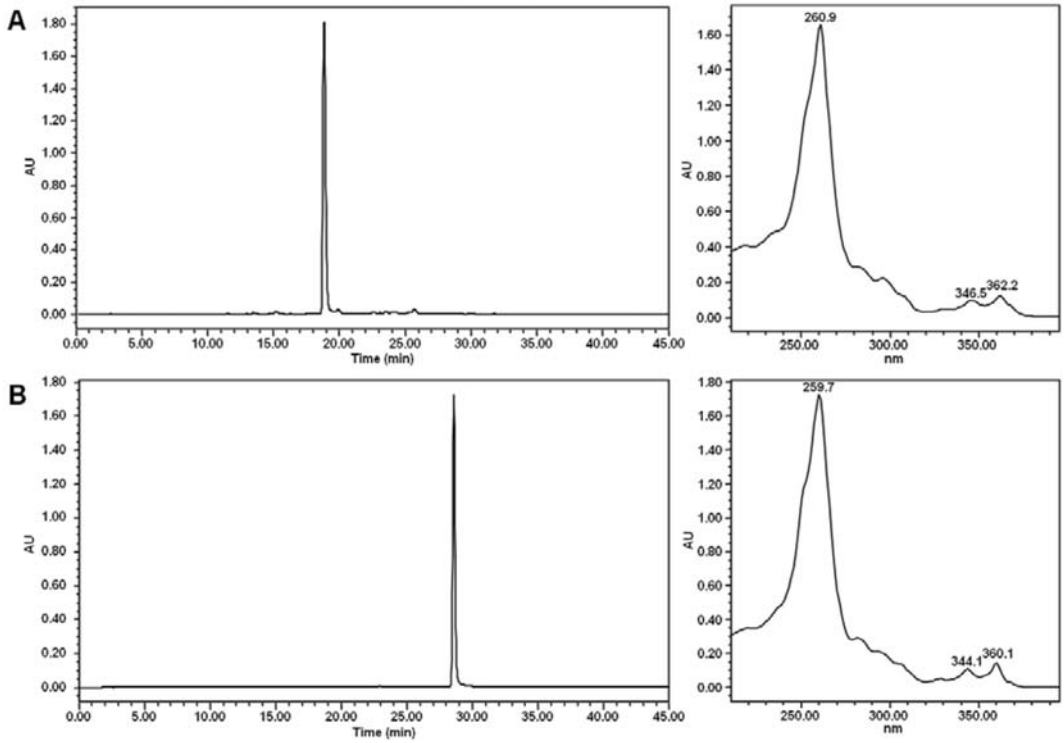

Fig. 5. The HPLC analysis of CPC peak fractions. HPLC condition - Column: Gemini 5 μ C18 110A column (150 × 4.6 mm ID, 5 μm, Phenomenex, USA); Mobile phase: 0-45 min, 20-80% acetonitrile; Flow rate: 1 mL/min; UV wavelength: 254 nm. Peak 1: DMP; Peak 2: BA-I.

materials were low because the irreversible absorption onto solid support matrix such as silica gel. The employment of CPC enabled us to separate pure phenanthrens with high recovery yields via one-step process. The result of the present study demonstrates that countercurrent separation is efficient separation technique.

Structure elucidation of CPC peak fractions – The CPC peak fractions were identified by comparing their retention time with authentic samples of DMP and BA-I obtained in the previous study and their spectroscopic data (^{1}H and ^{13}C NMR). The peak fraction 1 and 2 were good agreement with those of literature values (Coxon *et al*., 1982; Leong *et al*., 1997; Takasugi *et al*., 1987).

Summary – Gradient CPC has been developed using *n*-hexane-methanol-water system. The upper phase of *n*-hexane-methanol-water system (HMW, 10 : 9 : 1, v/v) was used as a stationary phase, and the lower phase and water were used as a mobile phase A and a mobile phase B, respectively. This solvent system worked like a reversed-phase column chromatography and was successfully employed to isolate and purify DMP (1.2 mg) and BA-I (1.7 mg) with high purities (> 94%) from the 400 mg of dichloromethane soluble extract of *D. opposita*. The recovery yields of DMP and BA-I were 83% and 86%, respectively. The results of the present study clearly demonstrate that CPC with gradient elution can provide reference compounds from *D. opposita*, and the two-phase solvent system composed of HMW (10 : 9 : 1, v/v) and water can be utilized as an alternative method for the fractionation or isolation of diverse natural products instead of solid supported column chromatography.

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