

## Development of the chromatographic fingerprint analysis of dioscorealides and dioscoreanone from *Dioscorea membranacea* Pierre

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### Abstract

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High-performance liquid chromatography-photodiode array detection (HPLC-PDA) was developed for chromatographic fingerprint analysis of the ethanolic extract from *Dioscorea membranacea* Pierre (known as Hua-Khow-Yen in Thai medicinal plant). Chromatographic fingerprint, together with the contents of the dioscorealide A, dioscorealide B and dioscoreanone, which are three markers of the main constituents were first established and applied for quality control. The samples were separated with reversed phase HPLC by linear gradient elution using water and acetonitrile as mobile phase at a flow rate of 1.0 ml/min and detector wavelength at 245 nm for dioscoreanone and at 270 nm for dioscorealide A and dioscorealide B. This HPLC fingerprint analysis has proven its usefulness for identification and assessment for quality evaluation of *D. membranacea* extract.

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**Key words :** chromatographic fingerprint, *Dioscorea membranacea*, dioscorealide A, dioscorealide B, dioscoreanone

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## บทคัดย่อ

อนุศักดิ์ ทิริคดิธรรม<sup>1</sup> จุติมา ชูโหม<sup>1</sup> และ อรุณพร อิฐรัตน์<sup>2</sup>

การศึกษาลายพิมพ์ทางโครมาโทกราฟีของ dioscorealides และ dioscoreanone จากสารสกัดชั้นเอทานอลของหัวข้าวเย็น

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พัฒนาการศึกษาลายพิมพ์ทางโครมาโทกราฟีของสารสกัดชั้นเอทานอลจากหัวข้าวเย็น (*Dioscorea membranacea* Pierre) โดยใช้เทคนิคโครมาโทกราฟีของเหลวสมรรถนะสูงแบบรีเวอร์สเฟส ที่มีตัวตรวจวัดสารชนิดโฟโตไดโอดอาร์เรย์ โดยอาศัยลายพิมพ์ทางโครมาโทกราฟีและปริมาณของสารเทียบของค์ประกอบหลัก ได้แก่ dioscorealide A dioscorealide B และ dioscoreanone สำหรับการควบคุมคุณภาพของสารสกัดจากหัวข้าวเย็น แยกสารประกอบโดยใช้การระเหยตัวอย่างแบบเปลี่ยนแปลงสัดส่วนของโมบายเฟส โมบายเฟสประกอบด้วย อะซิโตนไนไตรล์ และน้ำ อัตราการไหลของโมบายเฟส 1.0 มล./นาที ตรวจวัดการดูดกลืนแสงของสาร dioscoreanone ที่ความยาวคลื่น 245 นาโนเมตร ตรวจวัดการดูดกลืนแสงของสาร dioscorealide A และ dioscorealide B ที่ความยาวคลื่น 270 นาโนเมตร การวิเคราะห์โดยใช้ลายพิมพ์ทางโครมาโทกราฟีได้พิสูจน์ว่ามีประโยชน์สำหรับการพิสูจน์เอกลักษณ์ และนำมาใช้ควบคุมคุณภาพของสารสกัดจากหัวข้าวเย็น

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Thai traditional medicines have been widely used to prevent and cure human disease for a long time. Because of its low toxicity and effective therapeutically performance, Thai herbals have attracted considerable attention in many fields. Due to the significant expansion of the use of this alternative medicine, it is very critical to develop a high standard of quality control in assessing the active components in raw plant materials to guarantee their identity, consistency and authenticity (WHO, 2000). Recently, chromatographic fingerprint technique, as a more meaningful quality control method of herbal samples, has been attracting more and more people's attention because the fingerprint technique emphasizes on the integral characterization of compositions of samples with a quantitative degree of reliability and focus on identifying and assessing the stability of the plants. Chromatographic fingerprint is a kind of method to show chemical information of medicines with chromatograms, spectrograms and other graphs by analytical techniques (Gan, and Ye, 2006). Up to now, varieties of chromatographic techniques involving fingerprint include TLC, gas

chromatography, high-performance liquid chromatography (HPLC), CE, etc (Pietta *et al.*, 1991; Hasler *et al.*, 1992; Aichholz, and Lorbeer, 2000; Vierling *et al.*, 2003). Because of its advantages and popularization, HPLC fingerprint analysis has been regarded as the first choice.

Thai medicinal plants locally known as "Hua-Khao-Yen" have mostly been used in Thai traditional medicines as common ingredients in several preparations, including those used in the treatments of lymphopathy, dermatopathy, venereal diseases, leprosy, bacterial infections and cancers (Itharat *et al.*, 2003; Itharat *et al.*, 2004). Especially the ethanol extract from *Dioscorea membranacea* Pierre rhizome; one of the *Dioscorea* of the *Dioscoreaceae*, was potently cytotoxic against various cancer cell lines (Tewtrakul *et al.*, 2006). Regarding compounds isolated from Hua-Khao-Yen, two novel derivatives of naphthofuranoxepins including dioscorealide A and dioscorealide B and a new 1,4-phenanthraquinone, dioscoreanone, have been recently identified as the key active components of the extract of *D. membranacea*. The content of each component varies significantly

due to difference in geographic origin, climate condition, environment and other factors (Itharat *et al.*, 2003). Therefore, the compendia quality control method of the extract from Hua-Khao-Yen is HPLC fingerprinting of these dioscorealides and dioscoreanone.

However, fingerprint analysis shows only the result of similarity calculated based on the relative value; retention time, with the selected marker compound as reference standard, and does not display the absolute quantity. Obviously, quantitative determination of some marker components is necessary. In this study, we aimed at developing the simple, reliable and reproducible method to establish characteristic HPLC-UV fingerprints of *D. membranacea* extract together with the determination of the marker substance dioscorealide A, dioscorealide B and dioscoreanone. Both the chromatographic fingerprint and contents of the markers were suitably applied for quality control and guarantee clinical efficacy of Hua-Khao-Yen crude drug.

## Experimental

### 1. Materials and reagents

Ethanol and methanol were both analytical grade, and acetonitrile was chromatographic grade (Labskan, Bangkok, Thailand). Water was purified by a Milli-Q academic water purification system (Milford, MA, US).

The rhizomes of *D. membranacea* Pierre were collected from Amphor Pa-tue, Chumporn Province, Thailand. Dioscorealide A, dioscorealide B and dioscoreanone were isolated from *D. membranacea* in our laboratories. The chemical structures of the standards were shown in Figure 1.

### 2. Instrumentation and chromatographic condition

The HPLC apparatus was a Agilent 1100 Binary HPLC Pump system (Agilent, America) equipped with a photodiode array detector (PDA) (Agilent G1365B). Data acquisition and processing were performed by ChemStation software (Agilent G2175BA). Chromatographic separation

was carried out at room temperature using a  $\mu$ Bondapak<sup>TM</sup> C18 analytical column (300 mm  $\times$  3.9 mm I.D., 10  $\mu$ m).

The mobile phase consisted of water (A) - acetonitrile (B) with gradient elution as follows: 70-55% A at 0-10 min, 55% A for 5 min, 55-30% A at 15-17 min, 30% A for 5 min, 30-70% A at 22-25 min, 70% A for 10 min. The flow rate was 1.0 ml/min. The sample volume injected was 20  $\mu$ l. UV spectra were recorded over the range of 200-400 nm, and the quantification wavelength of these chromatograms was set at 245 nm for dioscoreanone and 270 nm for dioscorealide A and dioscorealide B.

### 3. Preparation of standards

Stock solutions of each compound; 2.0 mg/ml, were prepared by dissolving weighed quantities of standard compounds in acetonitrile and diluting to the desired concentration. By using the stock solutions, a series of mixed working standard solutions were prepared with the concentrations of 0.10-15.0  $\mu$ g/ml. All the solutions were stored under refrigeration.

### 4. Preparation of plant extract

Plant materials were dried at 50°C, powdered and extracted by methods corresponding to those practiced by Thai traditional doctors. The ground dried rhizomes of plant was exhaustively percolated with 95% ethanol, and then concentrated to dryness under reduced pressure. The sample was prepared by accurately weighing 20.0 mg of plant extract into a 10 ml volumetric flask. Approximately 6 ml of 100% acetonitrile was added, and the solution was sonicated for 15 min. The solution was allowed to cool to room temperature before being filled up to the final volume of 10.0 ml. After centrifugation for about 10 min, 5 ml of the supernatant was diluted to 100 ml volumetric flask by acetonitrile and filtered through a 0.45- $\mu$ m filter membrane before analysis. Twenty microliters of the sample solution was directly injected into the HPLC column and separated under above chromatographic conditions.

## Result and discussion

### 1. Optimization of HPLC systems

Selection of detection wavelength was one of the key factors contributing to a reliable and reproducible HPLC fingerprint of dioscorealide A, dioscorealide B and dioscoreanone. Photodiode array detector (DAD) was applied to select the optimized wavelength of constituents in the fingerprint. In a full-scan experiment, chromatogram at 270 nm shows more components information and better separation than at other wavelength for analyzing of dioscorealide A and dioscorealide B, and that for determining dioscoreanone was 245 nm. Because in constant speed elution, some of these components in the sample have a long retention time, we have chosen linear gradient elution instead. Satisfactory results were obtained within 35 min for the HPLC separation. In this method, mobile phase modifier such as acetic acid and formic acid were not suitable for determination of the active constituents. Especially, formic acid could decrease HPLC response of target peaks.

### 2. Determination of dioscorealide A, dioscorealide B, and dioscoreanone

#### 2.1 Standardization of fingerprint

For traditional quality control system, dioscorealide A, dioscorealide B and dioscoreanone are used as the marker substances to evaluate the quality of extract of *D. membranacea*. The contents of these compounds were determined at the above-mentioned optimum conditions. The quantitative method was assessed by reproduc-

ibility, linearity and detection limit. The reproducibility was estimated by making repetitive injection of a standard mixture solution (5.0 µg/ml for each) under the optimum conditions (n = 6). R.S.D. values of the retention time and the peak area were 0.27 and 0.98, 1.01 and 0.28, and 1.21 and 0.61% for dioscorealide A, dioscorealide B and dioscoreanone, respectively. To determine the linearity equations and linear scope for the analytes, a series of mixed standard solutions range from 0.10-15.0 µg/ml were tested. The detection limit was also evaluated on the basis of a signal-to-noise ratio of 3. The results are summarized in Table 1. All results indicated that the conditions for the fingerprint analysis were satisfactory.

#### 2.2 HPLC sample analysis and recovery

The contents of the dioscorealide A, dioscorealide B and dioscoreanone from the extracts of *D. membranacea* were determined at the above-mentioned optimum conditions. The content of dioscorealide A, dioscorealide B and dioscoreanone in the herbal extracts are 0.83% w/w (0.03% R.S.D., n = 3), 0.95% (1.34% R.S.D., n = 3), and 4.44% (1.64% R.S.D., n = 3) respectively. During sample analysis, the UV absorbance of the targeted peaks was compared with those of standards for confirmation. Recoveries were also determined to evaluate the precision and accuracy of the method. By standard addition of mixture standard solution to extract sample, recoveries was determined and the average values were 92.2, 102.5 and 92.6% for dioscorealide A, dioscorealide B and dioscoreanone, respectively (n = 3), with R.S.D. values less than 2%. The assay results

**Table 1. The regression equations and detection limits for dioscorealide A, dioscorealide B and dioscoreanone**

Compound	Regression equation	Correlation coefficient	Linear range (µg/ml)	Detection limit (µg/ml)
Dioscorealide A	$y = 76.7x + 5.29$	0.9995	0.22-5.60	0.08
Dioscorealide B	$y = 61.7x - 1.49$	0.9994	0.10-5.20	0.05
Dioscoreanone	$y = 8.22x - 5.29$	0.9993	1.50-15.0	0.40

The y value is the peak area of analytes; the x value is the concentration of the analytes (µg/ml).

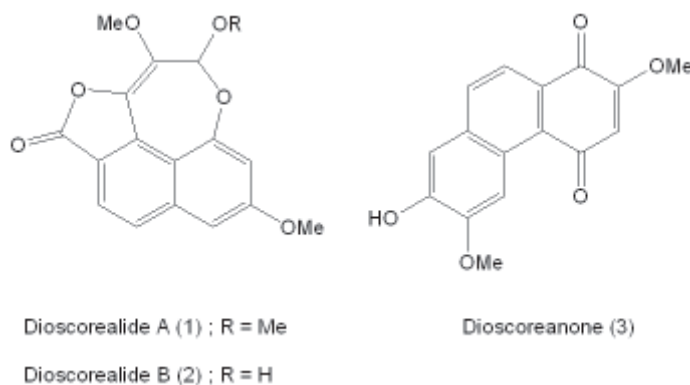


Figure 1. Chemical structures of the identified compounds in the HPLC fingerprints: (1) dioscorealide A; (2) dioscorealide B; (3) dioscoreanone

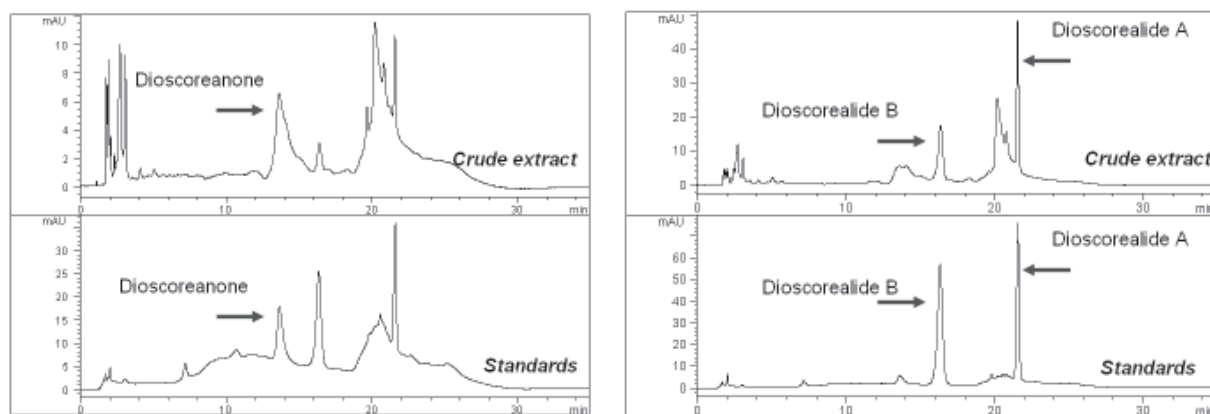


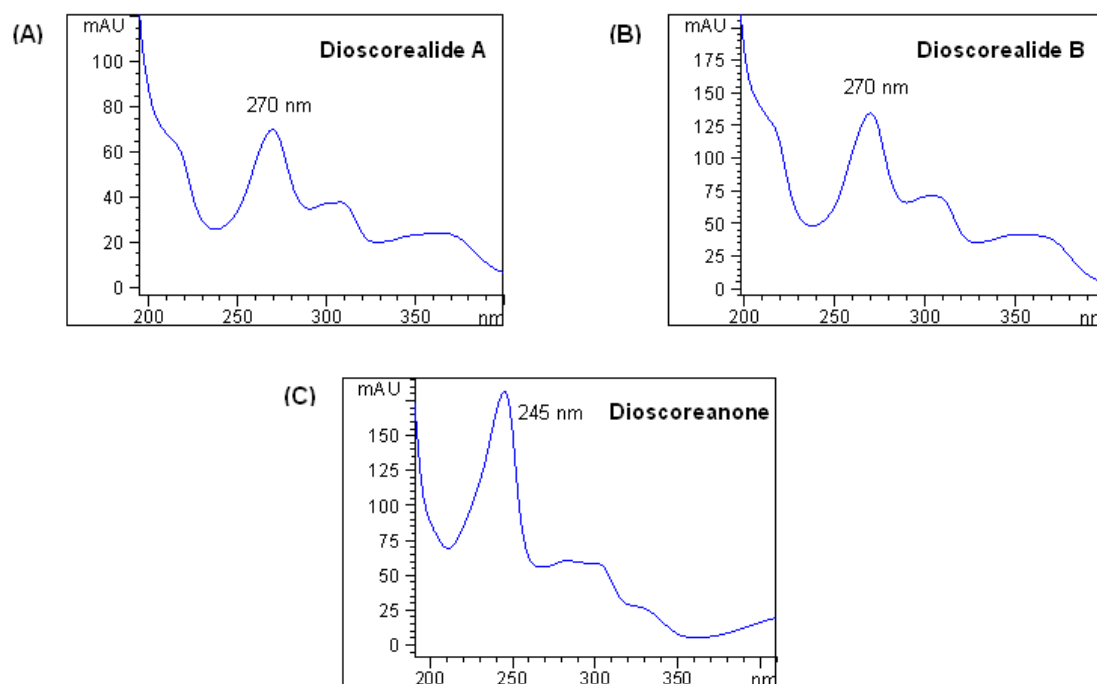
Figure 2. Representative HPLC fingerprint of standards and the extract of *D. membranacea* with UV detection at (A) 245 nm for dioscoreanone; and (B) 270 nm for dioscorealide A and dioscorealide B.

indicate that this method is accurate, sensitive and reproducible, and it is a useful method for quantitative analysis of dioscorealide A, dioscorealide B and dioscoreanone in the extract of *D. membranacea*.

### 3. Development of fingerprint of dioscorealides and dioscoreanone

In our study, it can be generalized that the major constituents in *D. membranacea* extracts are dioscorealide A, dioscorealide B and dioscoreanone. Therefore, HPLC fingerprint analysis was performed based on the relative retention time.

The retention time was 13.6, 16.3 and 21.5 min for dioscoreanone, dioscorealide B and dioscorealide A, respectively. A representative HPLC fingerprint of standards and herbal sample are shown in Figure 2. It can be seen that 245 nm is the maximum for the UV spectrum of dioscoreanone, whereas 270 nm is the  $\lambda_{\max}$  of dioscorealide A and dioscorealide B. The HPLC detection of each active constituent based on the chromatographic retention time was confirmed by using diagnostic UV absorption spectral patterns obtained from the photodiode array detection as shown in Figure 3.



**Figure 3.** Typical UV spectra of the representative compounds in the ethanol extract of *D. membranacea* obtained on the PDA detector. (A) Dioscorealide A; (B) Dioscorealide B; and (C) Dioscoreanone.

### Conclusion

A HPLC method was successfully developed for fingerprint analysis of *D. membranacea*. The HPLC fingerprint of dioscorealide A, dioscorealide B and dioscoreanone represent the characteristic markers of these herb's constituents for the first time. The fingerprint obtained by using our established method provides a good repeatability in separation pattern which demonstrated that the fingerprint presented in this paper is a rapid, reliable and effective method suitable for quality evaluation and quantitative determination of *D. membranacea*.

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