# THIN LAYER CHROMATOGRAPHY AND IMAGE ANALYSIS OF SELECTED LIRIODENINE BEARING PLANTS IN THAILAND

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**ABSTRACT:** To determine liriodenine content in selected Thai medicinal plants by TLC image analysis as well as HPLC. Twenty-eight plant materials in Magnoliaceae, Annonaceae and Nelumbonaceae were collected from natural sources in Thailand. Crude extracts were prepared by Soxhlet extraction in 95% ethanol. Liriodenine content was analyzed by TLC image analysis using Scion Image software as well as HPLC-DAD method. Validations of both methods were performed including linearity, accuracy, precision and sensitivity based on International Conference of Harmonization (ICH) guideline. Calibration curves showed good linear correlation coefficients ($R^2$ > 0.995) over the range of concentration 5-200 µg/mL. Both method validation tests showed adequate performances in reliability and sensitivity.TLC image showed a well-defined fluorescent spot of liriodenine at the $R_f$ value of 0.75, while HPLC chromatogram indicating liriodenine peak at 11 min of retention time. Top two highest contents of liriodenine were found in *M. longifolia* and *M. champaca* bark. There was no significant difference between the results of both quantitative methods ($p$>0.05). This study presented that TLC image was advantageous for liriodenine analysis due to its simple, rapid and inexpensive. Both proposed methods could be used as a tool for the quantification of liriodenine in medicinal plants.

**Keywords:** Liriodenine, Medicinal plant, Thin layer chromatography, Image analysis, High performance liquid chromatography

## INTRODUCTION

Liriodenine (Figure 1), an isoquinoline alkaloid has a wide range of biological activities such as a cytotoxic to human cancer cells, anti-platelet, anti-fungal and anti-microbial actions [1-3]. Recently studies revealed that liriodenine had cardioprotective efficacy by reducing the extent of cardiovascular injuries under ischemia-reperfusion conditions [4]. Also, it showed probably a promising drug for treatment of cardiac arrhythmia with heart failure [5]. Hence, the development of a simple and effective method for liriodenine determination in plant samples could be useful for screening to find the new natural sources of this compound.

The present study focuses on developing chromatographic technique for liriodenine quantification. Selected medicinal plants from the families of Magnoliaceae, Annonaceae and Nelumbonaceae were collected for screening of liriodenine content according to their accessibility throughout Thailand. These families were previously reported of liriodenine as chemical constituent.

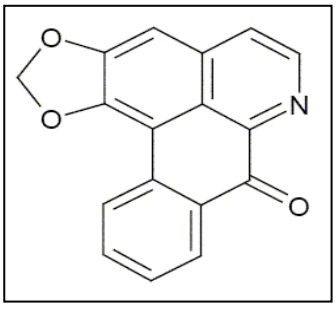


**Figure 1** Liriodenine

However, liriodenine bearing plants endemic to Thailand has not been investigated.

Thin layer chromatography (TLC) and high-performance liquid chromatography (HPLC) are the most commonly used methods for obtaining chemical fingerprints and identification of the crude plant extracts. In addition to many useful application functions, the imaging program, Scion Image, was selected for quantitative analysis in this study due to its simplicity and low cost. The results were then compared to the advance high technology technique as HPLC-DAD [6-8] to confirm its accuracy. Both analytical methods were validated

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**Table 1** Quantitative analysis of liriodenine bearing plants endemic to Thailand

| Family | Scientific name (Thai name) | no. | Part | % Yield | Liriodenine content (% w/w) | |
|---|---|---|---|---|---|---|
| | | | | | HPLC | TLC image analysis |
| Magnoliaceae | *Michelia longifolia* Blume (Champi) | 1 | leaf | 23.42 | 0.0011 | 0.0009 |
| | | 2 | bark | 10.23 | 0.0158 * | 0.0153 * |
| | *Michelia champaca* Linn. (Champa) | 3 | leaf | 30.42 | 0.0019 | 0.0019 |
| | | 4 | bark | 17.71 | 0.0119 * | 0.0116 * |
| | *Magnolia figo* Lour. (Champi kaek) | 5 | leaf | 36.61 | 0.0014 | 0.0014 |
| | *Magnolia sirindhorniae* Noot. & Chalermglin (Champi sirinthon) | 6 | leaf | 30.96 | 0.0013 | 0.0015 |
| | | 7 | bark | 12.80 | 0.0003 | 0.0003 |
| | *Magnolia liliifera* (L.) Baill. Var. *liliifera* (Montha) | 8 | leaf | 25.91 | 0.0008 | 0.0007 |
| | | 9 | bark | 6.48 | 0.0058 | 0.0060 |
| | *Magnolia coco (*Lour.) DC. (Yihup nu) | 10 | leaf | 25.14 | 0.0003 | 0.0003 |
| Annonaceae | *Cananga odorata* (Lam.) Hook.f.&Thomson var. *odoratam (*Kradang nga thai) | 11 | leaf | 43.85 | 0.0006 | 0.0009 |
| | *Cananga odorata* (Lam.) Hook.f.&Thomson var. *fruticosa* (Kradang nga songkhla) | 12 | leaf | 30.85 | 0.0004 | 0.0006 |
| | *Rauwenhoffia siamensis* Scheff. (Nom meaw) | 13 | leaf | 32.94 | - | - |
| | *Anaxagorea javanica* Blume (Champoon) | 14 | leaf | 25.07 | - | - |
| | | 15 | bark | 14.41 | - | - |
| | *Melodorum fruticosum* Lour. (Lamduan) | 16 | leaf | 25.34 | - | - |
| | | 17 | bark | 24.79 | - | - |
| | *Artabotrys hexapetalus* (L.f.) Bhandari (Karawek, Kradang nga cheen) | 18 | leaf | 20.85 | - | - |
| | *Desmos chinensis* Lour. (Saiyood) | 19 | leaf | 26.78 | - | - |
| | *Anomianthus dulcis* J. Sinclair (Nom woa) | 20 | leaf | 13.18 | - | - |
| | *Goniothalamus macanii* Craib (Kao lam) | 21 | leaf | 6.28 | - | - |
| | *Annona squamosa* L. (Noina) | 22 | leaf | 14.42 | - | - |
| | | 23 | bark | 12.08 | 0.0005 | 0.0005 |
| | *Annona reticulata* L. (Noi nong) | 24 | leaf | 14.50 | - | - |
| | | 25 | bark | 11.59 | - | - |
| | *Annona muricata* L. (Thurain thet) | 26 | leaf | 13.97 | - | - |
| | *Mitrephora maingayi* Hook f. & Thomson (Nang daeng, Porkeehad) | 27 | leaf | 20.53 | - | - |
| Nelumbonaceae | *Nelumbo nucifera* Gaertn. (Bua luang) | 28 | leaf | 21.09 | 0.0069 | 0.0074 |

(-) Not found or undetectable for liriodenine

(*) Top 2 medicinal plant secies containing liriodenine

and developed to the optimum conditions for rapid screening and quantification.

## MATERIALS AND METHODS

***Plant materials:***

Twenty-eight plant materials for liriodenine quantitation were collected from several places (Table 1). The samples were authenticated by one of the authors and the national herbarium comparison. The vouchers and numbers of specimens were deposited at College of Public Health Sciences, Chulalongkorn University, Thailand.

***Plant extraction:***

Crude extracts were exhaustively performed with 95 % ethanol in a Soxhlet apparatus, and analyzed for liriodenine content by TLC image analysis using Scion Image software and HPLC. All measurements were done in triplicate.

***TLC image analysis:***

Applied 2 µL of each extract solution in methanol onto TLC silica gel 60 $F_{254}$ plate (0.25mm thickness; Merck, Germany), then firstly developed using 100% methanol to a distance of 6 mm to expand the band length [8]. Ensuring air-drying, the plates were developed with mobile phase using chloroform and methanol (9:1) [9] to a distance of 80 mm, then dried and visualized under UV 365 nm. The image was taken by digital camera (Canon PowerShot A650). TLC images saved as TIFF files were analyzed by Scion Image program for Windows (version Alpha 4.0.3.2, Scion Corporation, Maryland, USA). After importing images, they were resized with scale to fit windows mode and modified grayscale selection with smoothing menu to reduce noises of image. Using the rectangular selection tool and load macros command to create the plot profile, then the areas and gray value of the selection were measured as square pixels [10].

***HPLC analysis:***

Instrumentation was performed with a SHIMADZU gradient system (Kyoto, Japan) equipped with LC-20AD pumps, a CTO-20AC column oven, DGU-20A3 degasser and a SPD-M20A diode array detector (DAD) employed at the wavelength range of 180-800 nm. Separation was carried out with an Inersil ® ODS-3, C-18 column (particle size of the packing 5µm, 4.6 x 250mm) and HPLC guard column (5µm, 4.0x10mm). The mobile phase consisted of A (1% formic acid, adjusted to.pH 4.5 with diethylamine) and B (100% acetonitrile) with the gradient program from 60:40 to 40:60 over 35 minutes [11]. The solvents were filtered through 0.45µm membrane and degassed with ultrasonic bath prior to use. Each methanolic extract solution was filtered through 0.45 µm syringe filter. Aliquot (20 µL) of each sample was injected into the system at flow rate of 1 mL/min with column temperature set at 25ºC.

***Preparation of standard solution:***

Liriodenine (100% by LCMS) was purchased from Specs, Delft, The Netherlands (Lot no. AK-693/ 21087012). The purity of this compound was reconfirmed by NMR. Methanolic standard solution of 5, 25, 50, 100 200 µg/mL was applied on chromatographic conditions to generate calibration curve. The standard curve was analyzed using the linear least-squares regression equation derived from peak area.

***Method validation:***

Linearity, accuracy, precision (repeatability and intermediate precision), limit of detection (LOD) and limit of quantification (LOQ) were evaluated according to ICH guidelines specification [12]. Linearity was estimated by regression line; correlation coefficient, y-intercept and slope. Precision were determined by repeatability (intra-day) and intermediate precision (inter-day) for 3 consecutive days. Three different concentrations of liriodenine within the range of 5-200 µg/mL were analyzed in six determinations each. Data were expressed as relative standard deviation (%RSD) for precision; while % accuracy was calculated from the observed and nominal concentration. LOD and LOQ for TLC image analysis were assessed based on standard deviation (σ) of the y-intercept and the slope (*s*) as 3σ/*s* and 10σ/*s* respectively. For HPLC, they were analyzed by signal to noise. The determination was done in triplicate.

***Data Analysis:***

Quantitative analysis was conducted as mean percentage by dried weight. Calibration curve was analyzed using regression equation. Liriodenine content between two methods: HPLC and TLC image were compared using Student's paired *t*-test.

## RESULTS

***Quantitative determination of liriodenine***

The liriodenine contents measured by TLC image analysis as well as HPLC method were shown in Table 1. Among the 28 samples, there were 14 specimens containing liriodenine. The two highest contents were found in the bark of *M. longifolia* and *M. champaca* with the amount of 0.0153 and 0.0116% w/w respectively from TLC image analysis.

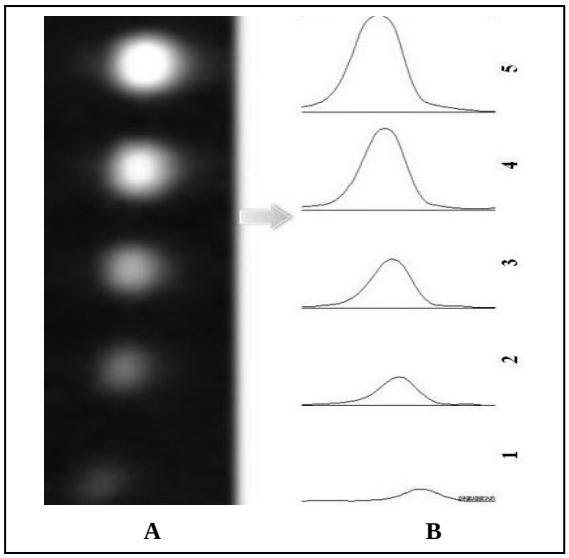


**Figure 2** TLC image of standard liriodenine, 5-200 µg/mL (lane1-5) from Scion Image software
A. Converting to grayscale and smoothing image
B. Chromatogram of plotting area

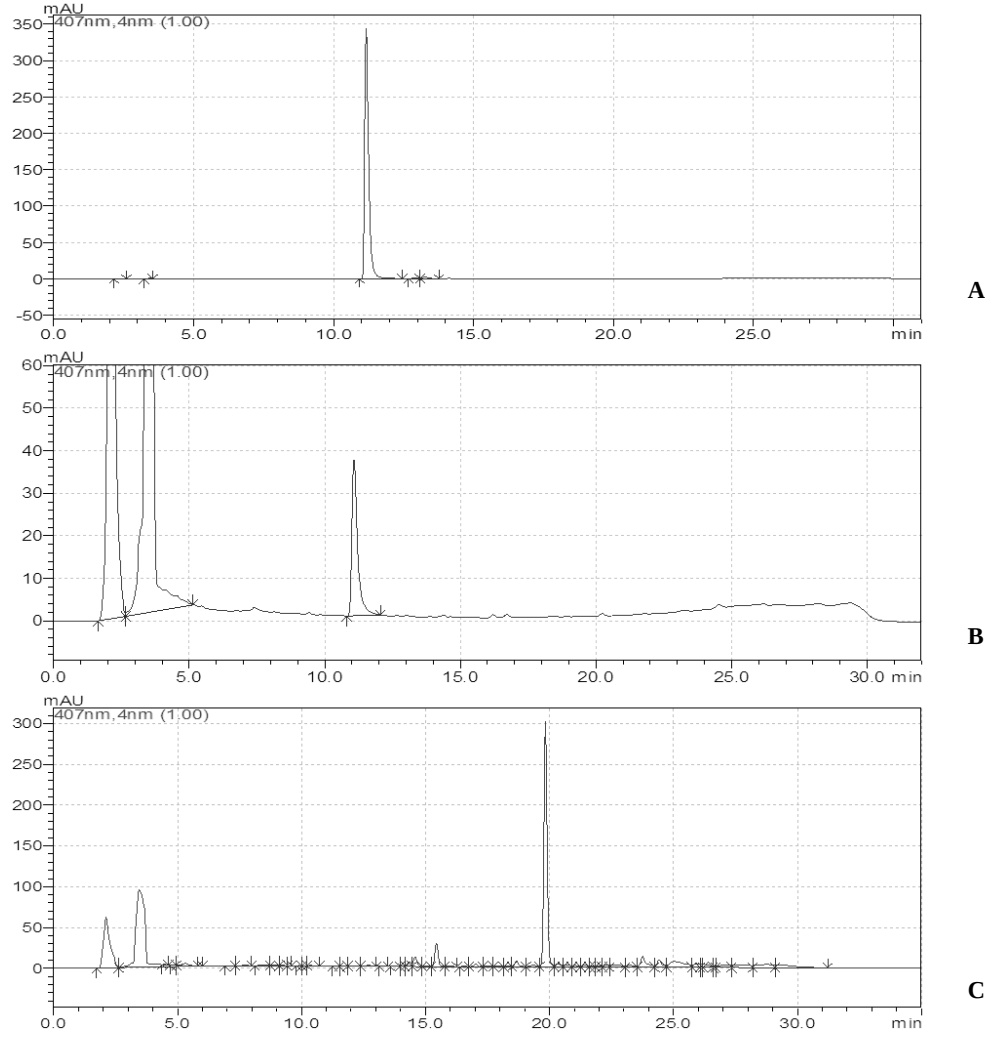


**Figure 3** High performance liquid chromatography (HPLC) chromatograms
A.) Standard liriodenine, 200µg/mL
B.) Crude methanolic extract with liriodenine (*M. longifolia* bark)
C.) Crude methanolic extract without liriodenine (*A. dulcis* leaves)

**Table 2** Repeatability (intra-day precision) and accuracy of liriodenine determined by HPLC and TLC image analysis

| Nominal concentration | Observed concentration (n = 6) | | | |
|---|---|---|---|---|
| **HPLC-DAD (µg/mL)** | **Day** | **Mean ± SD** | **% RSD** | **% Accuracy** |
| 25 | 1 | 25.75 ± 0.17 | 0.68 | 103.02 ± 0.70 |
| | 2 | 25.37 ± 0.15 | 0.59 | 101.46 ± 0.60 |
| | 3 | 25.60 ± 0.08 | 0.29 | 102.38 ± 0.30 |
| 50 | 1 | 49.28 ± 0.13 | 0.26 | 98.57 ± 0.26 |
| | 2 | 50.17 ± 0.07 | 0.14 | 100.34 ± 0.14 |
| | 3 | 50.20 ± 0.06 | 0.13 | 100.40 ± 0.13 |
| 100 | 1 | 101.33 ± 0.07 | 0.07 | 101.33 ± 0.07 |
| | 2 | 101.87 ± 0.05 | 0.05 | 101.87 ± 0.05 |
| | 3 | 101.06 ± 0.17 | 0.17 | 101.06 ± 0.17 |
| **TLC image (µg/spot)** | **Day** | **Mean ± SD** | **% RSD** | **% Accuracy** |
| 25 | 1 | 22.95 ± 0.66 | 2.89 | 91.79 ± 2.66 |
| | 2 | 23.56 ± 0.53 | 2.27 | 94.25 ± 2.14 |
| | 3 | 24.58 ± 0.96 | 3.89 | 98.31 ± 3.82 |
| 50 | 1 | 53.06 ± 0.33 | 0.62 | 106.11 ±0.65 |
| | 2 | 49.69 ± 0.91 | 1.83 | 99.37 ± 1.82 |
| | 3 | 49.00 ± 1.68 | 3.43 | 97.99 ± 3.36 |
| 100 | 1 | 99.89 ± 2.86 | 2.86 | 99.89 ± 2.86 |
| | 2 | 98.43 ± 1.02 | 1.04 | 98.43 ± 1.02 |
| | 3 | 102.19 ± 1.20 | 1.17 | 102.19 ± 1.20 |

**Table 3** Intermediate precision (inter-day precision) and accuracy of liriodenine determined by HPLC and TLC image analysis

| Nominal concentration | Observed concentration (3 days, n=18) | | |
|---|---|---|---|
| **HPLC-DAD (µg/mL)** | **Mean ± SD** | **%RSD** | **% Accuracy** |
| 25 | 25.57 ± 0.20 | 0.82 | 102.29 ± 0.84 |
| 50 | 49.89 ± 0.45 | 0.90 | 99.77 ± 0.89 |
| 100 | 101.42 ± 0.36 | 0.36 | 101.42 ± 0.36 |
| **TLC image (µg/spot)** | **Mean ± SD** | **%RSD** | **% Accuracy** |
| 25 | 23.70 ± 0.98 | 4.14 | 94.78 ± 3.92 |
| 50 | 50.58 ± 2.11 | 4.16 | 101.16 ± 4.21 |
| 100 | 100.17 ± 2.38 | 2.38 | 100.17 ± 2.38 |

**Table 4** Linearity and sensitivity for liriodenine determined using HPLC-DAD and TLC image analysis

| Method | HPLC-DAD | TLC image analysis |
|---|---|---|
| Slope | 17222.1 | 55.57 |
| Y-intercept | -15914.4 | 186.28 |
| $R^2$ | > 0.995 | > 0.995 |
| Limit of Detection (LOD) | 0.994 µg/mL | 3.767 µg/spot |
| Limit of Quantitation (LOQ) | 3.013 µg/mL | 12.557 µg/spot |

***Comparison of methods***

TLC plate developed with chloroform and methanol (9:1) showed a clearly fluorescent spot of liriodenine at 0.75 of *Rf* value under UV 365 nm. The image analysis was shown in Figure 2. The HPLC chromatographic peak of liriodenine was shown in Figure 3 with a retention time of about 11 min. Absorbance detection at both 248 and 407 nm increased accuracy of HPLC analysis of liriodenine in crude extract of plant materials. There was no significant difference between the results of both quantitative methods ($p$>0.05) as analyzed by Student's paired *t*-test.

***Method validation***

Method validation including linearity, accuracy, precission, LOD and LOQ were summarized in Table 2-4. In summary, calibration curve of standard compound showed good linearity relationship for both methods ($R^2$>0.995) over the range 5 – 200 µg/mL. The regression equation from TLC image and HPLC-DAD were $y = -0.1573x^2 + 61.392x +$

151.38 and $y$ = 17222.1$x$ - 15914.4 respectively where $y$ is AUC and $x$ is concentration. Average percentage of accuracy from TLC image analysis and HPLC-DAD revealed 98.57 – 103.02% and 91.79 – 106.11% respectively; high value indicated a satisfactory accuracy. RSDs of all parameters were less than 5 % for repeatability and reproducibility indicating that the proposed methods were precise. LOD and LOQ which calculated based on the standard deviation ($\sigma$) of the y-intercept and the slope ($s$) as $3\sigma/s$ and $10\sigma/s$ respectively were 3.767 and 12.557 µg/spot from TLC image analysis; while LOD and LOQ values analyzed by signal to noise from HPLC-DAD at 407 nm showed 0.994 and 3.013 µg/mL.

## DISCUSSION

The result supported that wavelength at 248 nm was the maximum absorption wavelenght for liriodenine [11]. However, it was found that liriodenine showed good absorbance at 407 nm as well. These dual wavelengths gave more reliable to detect liriodenine in selected plant extracts. Liriodenine bearing plants demonstrated peak of liriodenine at both 248 and 407 nm. The results were in accordance with liriodenine detection by TLC image analysis.

This study revealed liriodenine found in 10 species of families: Magnoliaceae, Annonaceae and Nelumbonaceae which accessible in Thailand. The fluorescent coloring spot which was a dominant characteristic of liriodenine on TLC plate allowed TLC image analysis to be more suitable and convenient for the determination of liriodenine. Moreover, TLC image analysis can be applied for quantitative analysis of this compound because it is simple, rapid and inexpensive.

In addition to method validation, TLC image analysis as well as HPLC developed in this study are sensitive and specific. Moreover, the accuracy and precision of methods are within the acceptable range.

## CONCLUSION

Liriodenine has been found high content in *M. longifolia and M. champaca* bark. There was no significant difference between the results of both quantitative methods ($p$>0.05). This study presented that TLC image was advantageous for liriodenine analysis. Both proposed methods could be used as a tool for the quantification of liriodenine in medicinal plants.

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