Quantitative Thin Layer Chromatographic Analysis of Rosmarinic Acid as a Chemical Marker in *Perilla frutescens* leaves

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บทคัดย่อ

พัฒนาการวิเคราะห์ที่ง่าย รวดเร็ว และเหมาะสม ในการหาปริมาณกรดโรสมารินิกในใบงาขี้ม้อน (*Perilla frutescens* (L.) Britton) โดยใช้วิธีทินเลเยอร์โครมาโทกราฟี-เด็นซิโทเมทรีและวิธีทินเลเยอร์โครมาโทกราฟีโดยวิเคราะห์ภาพถ่าย กรดโรสมารินิกจัดเป็นตัวบ่งชี้ทางเคมีในสารสกัดใบงาขี้ม้อน ซึ่งใช้เสริมสร้างคุณภาพวัตถุดิบสมุนไพรของใบงาขี้ม้อนได้ เก็บใบงาขี้ม้อนจากแหล่งต่าง ๆ 15 แหล่งในประเทศไทย ทำการแยกวิเคราะห์สารด้วย แผ่นทีแอลซีเคลือบซิลิกาเจล 60 จีเอฟ 254 โดยปรับใช้สองวัฏภาคเคลื่อนที่ในการศึกษานี้ คือ โทลูอีน-คลอโรฟอร์ม-อะซีโทน-กรดฟอร์มิก (5:4:1:0.2) เป็นวัฏภาคเคลื่อนที่ลำดับที่หนึ่ง และโทลูอีน-เอทธิล อะซีเตท-กรดฟอร์มิก (5:4:1) เป็นวัฏภาคเคลื่อนที่ลำดับที่สอง วิธีทินเลเยอร์โครมาโทกราฟี-เด็นซิโทเมทรีและวิธีทินเลเยอร์โครมาโทกราฟี โดยวิเคราะห์ภาพถ่ายที่พัฒนาขึ้นสำหรับวิเคราะห์ปริมาณกรดโรสมารินิกในใบงาขี้ม้อนให้ค่าความจำเพาะ ความเที่ยง ความแม่นจากการทำซ้ำในวันเดียวกันและระหว่างวัน ขีดจำกัดของการตรวจพบ ขีดจำกัดของการหาปริมาณ และค่าความคงทนที่ถูกต้อง การวิเคราะห์กรดโรสมารินิกด้วยเทคนิคทินเลเยอร์โครมาโทกราฟีทั้งสองวิธี พบว่ามีปริมาณเท่ากับ 2.504 ± 1.631 และ 2.485 ± 1.606 กรัมต่อ 100 กรัม โดยน้ำหนักแห้งตามลำดับ วิธีทินเลเยอร์โครมาโทกราฟีโดยวิเคราะห์ภาพถ่ายอาจเป็นทางเลือกหนึ่งในการหาปริมาณของกรดโรสมารินิกในใบ *Perilla frutescens*

คำสำคัญ: ใบงาขี้ม้อน, กรดโรสมารินิก, ทินเลเยอร์โครมาโทกราฟี-เด็นซิโทเมทรี, ทินเลเยอร์โครมาโทกราฟี โดยวิเคราะห์ภาพถ่าย

Abstract

The simple, less time consuming and selective methods for determination of rosmarinic acid content in *Perilla frutescens* (L.) Britton leaf were developed using TLC-densitometry and TLC-image analysis. Additionally, rosmarinic acid as a chemical marker in *Perilla frutescens* leaf extracts could be used to strengthen the quality of the starting herbal raw materials of *Perilla frutescens* leaf. *Perilla frutescens* leaves were collected from 15 different locations in Thailand. The analytical separation was performed on the silica gel60 GF_{254} TLC plate. Two mobile phases were optimized for this study. The first one was toluene-chloroform-acetone-formic acid (5:4:1:0.2) and the second

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one was toluene-ethyl acetate-formic acid (5:4:1). Both quantitative TLC methods to determine rosmarinic acid content in the leaf provided valid specificity, accuracy, repeatability, intermediate precision, limit of detection, limit of quantitation and robustness. Quantitative TLC of rosmarinic acid revealed 2.504 ± 1.631 and 2.485 ± 1.606 g per 100 g of dried leaves by densitometry and image analysis respectively. TLC-image analysis could be one of alternative methods of rosmarinic acid quantification in *Perilla frutescens* leaf.

Keywords: *Perilla frutescens leaf,* Rosmarinic acid, TLC- densitometry, TLC-image analysisIntroduction

Introduction

Perilla frutescens (L.) Britton, belonging to Lamiaceae family is the aromatic plant native to India and China. Presently, the plant can be found globally as it is one of the important economic crops but highly cultivate in China, India, Korea and Japan (Singh et al., 2017). This plant is popular for its uses as culinary herb and herbal medicine in many Asian countries. The leaves are used as garnishes in Japanese cuisine as well as pickles and vegetable wrap in Korean food. In medicinal aspect, the leaves are eaten as fresh vegetable with fish and crab as an antidote against allergy. Additionally, it has been used to treat various symptoms and diseases such as cough, asthma, cold, vomiting and abdominal pain in traditional Chinese medicine for centuries (Yu et al., 2017). In India, the leaves are used to treat asthma, cold and some intestinal disorders which are similar to the use of *P. frutescens* leaves in Thai folk medicine (Saklani, Kothiyal, & Gautam, 2011).

Numerous reports revealed that the edible leaf has been proved to be the natural source of antioxidant due to its flavonoid and phenolic acid constituents (Asif, 2012; Izumi et al., 2012). The main phenolic constituent in *P. frutescens* leaves has been proved to be rosmarinic acid (Figure 1). This phenolic compound is derived from the combination of caffeic acid and 3, 4-dihydroxyphenyl-lactic acid (Osakabe et al., 2002). Moreover, the compound has shown various remarkable pharmacological and biological activities such as antioxidant, anti-inflammatory, cytoprotective, anti-diabetic, anti-allergic and neuroprotective activities (Oh, Park, Ahn, Park, & Kim, 2011; Runtuwene et al., 2016; Senol et al., 2017; Zdařilová, Svobodová, Šimánek, & Ulrichová, 2009).

Currently, raw material products of *P. frutescens* leaf can be commonly found in herbal markets. Unlike modern medicines, the active phytoconstituent in the starting herbal raw materials may vary due to many variables including multiple sources, best harvesting time, collection sites and processing methods. Thus, active phytoconstituent determination of *P. frutescens* leaf in raw material is needed to provide for the consumers and strengthen the quality of medicinal plant as per World Health Organization (WHO) strategy (World Health Oraganization, 2017). In this study, the validated analytical methods to quantitate rosmarinic acid content in *P. frutescens* leaf from 15 different regions in Thailand was developed using TLC-densitometry and TLC-image analysis.

Objectives

To investigate the rosmarinic acid contents in *P. frutescens* leaves in Thailand using TLC-densitometry and TLC-image analysis.

Materials and Methods

Chemical and reagents

Rosmarinic acid was purchased from Sigma-Aldrich (Missouri, USA). All chemicals and reagents used were of analytical grade.

Plant material collection

The leaf samples of *P. frutescens* were collected from 15 locations throughout Thailand. The plant materials were authenticated by Associate Professor Dr. Nijsiri Ruangrungsi. The voucher specimens were deposited at College of Public Health Sciences, Chulalongkorn University, Thailand. The samples were cleaned prior to dry in hot air oven at 40°C. The dried samples were pulverized by grinding machine and kept in the airtight container.

Preparation of ethanolic extract

Each powder sample (5 g) was exhaustively extracted in 95% ethanol by a Soxhlet apparatus. The obtained ethanolic extract was filtered through Whatman filter paper No.4 and allowed to evaporate at 50°C. The percent yield of each ethanolic extract was recorded and the ethanolic extract was re-dissolved in 1 ml of 95% ethanol.

Preparation of standard solution

Rosmarinic acid standard solution was prepared by dissolving the compound in 95% ethanol. The standard solution was diluted to give concentrations of 0.25, 0.4, 0.8, 1 and 2 mg/ml.

TLC-densitometric method

Four microliters of each standard and sample solution was applied as bands of 4 mm in length onto the silica gel60 GF_{254} TLC plate (20 cm x 20 cm, E. Merck, Darmstadt, Germany) by CAMAG Linomat 5 (Muttenz, Switzerland). Initially, the plate was developed in the mixture of toluene-chloroform-acetone-formic acid (5:4:1:0.2, v/v) and removed when the mobile phase migrated to the eluent front. Then the plate was allowed to dryness at room temperature. After that, the plate was developed again in the mixture of toluene-ethyl acetate-formic acid (5:4:1, v/v) as the second mobile phase and allowed to dryness at room temperature. Finally, the developed TLC plate was subjected for quantitative determination of rosmarinic acid by the CAMAG TLC Scanner 4 (Muttenz, Switzerland) at the wavelength of 330 nm. The peak area obtained from chromatographic developments were used to quantify the rosmarinic acid content using the winCATS version 1.4.9 software for the integration. Each standard and sample were done in three replicates.

TLC-image analysis method

The images of developed TLC plates were captured using Canon PowerShot A560 IS digital camera of 21.1 million pixels resolution, 24x combined zoom with Optical Image Stabilizer System (Canon, USA) in a Spectrolines model CC-80 UV-Fluorescence analysis cabinet with 365/ 254 nm UV lamp (New York, USA). In this study, the camera was set into the shutter speed priority (TV) mode and configured the shutter speed at 15 seconds with the optimal ISO speed for the light level at 800. Then the digital images were transferred to the computer in the TIFF (.tif) format. ImageJ, a Java-based image processing as a free analytical software for scientific research (National Institutes of Health, USA) was used to analyze the intensity of each bands on the TLC plate. Briefly, the captured image was subjected to the program to generated chromatograms, then the peak area of each selected chromatogram was measured yielding the value which proportional to the intensity of the spotted band containing certain amount of compound applied on the TLC plate. This process was done in the same TLC plate using with TLC-densitometric method.

Method validation

The method validation of both analytical procedures using TLC-densitometer and ImageJ software were investigated by following the International Conference on Harmonisation guideline (The International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use, 2005).

Calibration range

The calibration curve of standard rosmarinic acid was constructed at the concentrations of 0.25, 0.4, 0.8, 1 and 2 mg/ml using the peak area *versus* standard concentrations.

Limit of detection (LOD) and the limit of quantitation (LOQ)

The LOD and LOQ of *both analytical methods are based on* the residual standard deviation of a regression line (σ) and the slope (S) from the calibration curve. The results of LOD and LOQ were calculated using these following formulas: 3.3 σ/S and 10 σ/S respectively.

Accuracy

The accuracy was carried out as the percentage recovery of the sample spiked with known amount of standard rosmarinic acid at concentrations of 0.1, 0.6 and 1.2 mg/ml.

Precision

The precision was evaluated by 9 determinations (3 concentrations/ 3 replicates each) in the same day (repeatability) and three different days (intermediate precision). The obtained results were expressed as the percentage of relative standard deviation (RSD).

Specificity

Specificity was evaluated according to the similarity of the absorbance spectra at the peak apex among all samples and standard rosmarinic acid as well as the similarity of the absorbance spectra at up-slope, apex, and down-slope of the peak. The maximum absorbance of rosmarinic acid is at 330 nm.

Robustness

The robustness was performed using variation in the ratio of the second solvent system and the results were expressed as %RSD.

Results and Discussion

In this present study, rosmarinic acid in *P. frutescens* leaf extract was quantified using TLC-densitometry and TLC-image analysis. Two mobile phases were optimized in order to separate the compound in the leaf extract and the first mobile phase, toluene-chloroform-acetone-formic acid (5:4:1:0.2, v/v) was used to decrease the spotted tailing of rosmarinic acid on the TLC plate. The investigations indicated that both analytical methods were shown to be valid (Figure 2-4). The accuracy was found to be around 85 - 100 % of the recovery in the leaf extract. The repeatability and intermediate precision obtained from both methods showed %RSD less than 5.50. The lowest concentration of rosmarinic acid in samples that could be determined with suitable precision and accuracy were found in TLC-densitometric method (LOD = 0.18 µg/spot, LOQ = 0.55 µg/spot) and TLC-image analysis method (LOD = 0.20 µg/spot, LOQ = 0.60 µg/spot). Both methods were found to be robust with no alteration of peak area after varied the ratio of the solvent system (Table1). Rosmarinic acid determination from TLC-densitometric and TLC-image analysis methods were found to be 2.504 ± 1.631 and 2.485 ± 1.606 % w/w of dried leaves, respectively (Table2). The contents of rosmarinic acid from both methods were not significant different ($p>0.05$ by paired t-test). Thus, the two methods could be used to quantify rosmarinic acid content in *P. frutescens* leaf. Rosmarinic acid contents in fresh leaves of *P. frutescens* obtained from five prefectures in Japan were reported as 0.47 -1.44 % w/w. The fresh leaves were extracted by refluxing twice for 60 min at 80°C in 50 mL of water–acetone–hydrochloric acid (20:80:1 v/v/v) and analyzed by liquid chromatography–mass spectrometry (Natsume, Muto, Fukuda, Tokunaga, & Osakabe, 2006). Rosmarinic acid contents in *P. frutescens* leaves might vary due to the extraction methods, seasoning periods and growing areas

Table 1 Method validity of TLC-densitometry and TLC-image analysis

P. frutescens leaves Parameter	TLC-densitometry	TLC-image analysis
Accuracy (%recovery)	98.75 – 101.73	92.10 – 102.25
Repeatability (%RSD)	1.38 ± 0.68	0.70 ± 0.60
Intermediate precision (%RSD)	4.32 ± 2.95	5.15 ± 2.95
Robustness (%RSD)	1.10 ± 0.51	1.43 ± 0.87
Limit of detection (LOD)	0.183	0.199
Limit of quantitation (LOQ)	0.553	0.602

Table 2 Rosmarinic acid content (g/100g) in *P. frutescens* leaf was from 15 sources throughout Thailand (each source was done in triplicate)

Source	Rosmarinic acid (g/100g *P. frutescens* leaf)	
	TLC-densitometric method	TLC-image analysis method
1	1.932	2.016
2	3.322	3.302
3	4.044	4.133
4	4.943	4.778
5	3.528	3.579
6	3.291	3.165
7	0.189	0.186
8	0.267	0.276
9	0.299	0.301
10	2.823	2.776
11	2.963	3.006
12	3.565	3.551
13	4.600	4.463
14	1.094	1.043
15	0.707	0.706
Mean ± SD	2.504 ± 1.631	2.485 ± 1.606

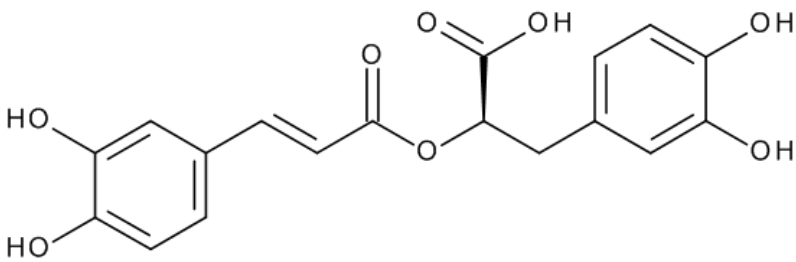
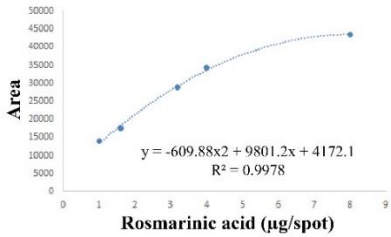
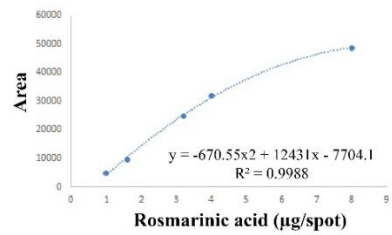


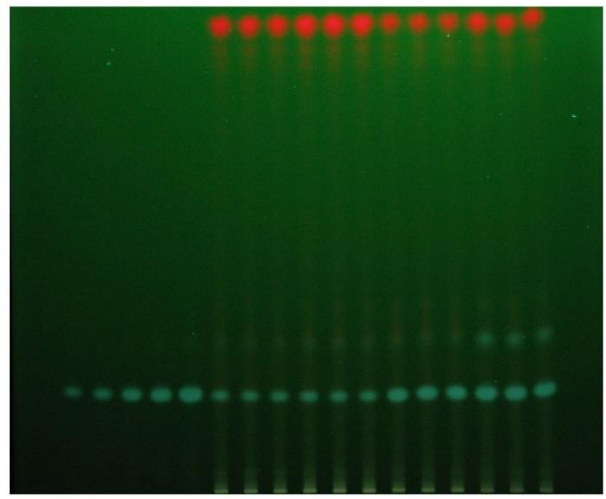
Figure 1 Molecular structure of rosmarinic acid



(a) Standard curve of rosmarinic acid in *P. frutescens* leaf by TLC-densitometry



(b) Standard curve of rosmarinic acid in *P. frutescens* leaf by TLC-image analysis



(c) TLC chromatogram of rosmarinic acid in *P. frutescens* leaves (Tract 1-5) and sample (Tract 6-15) at 365 nm

Figure 2 Standard curves and TLC chromatograms of rosmarinic acid in *P. frutescens* leaf

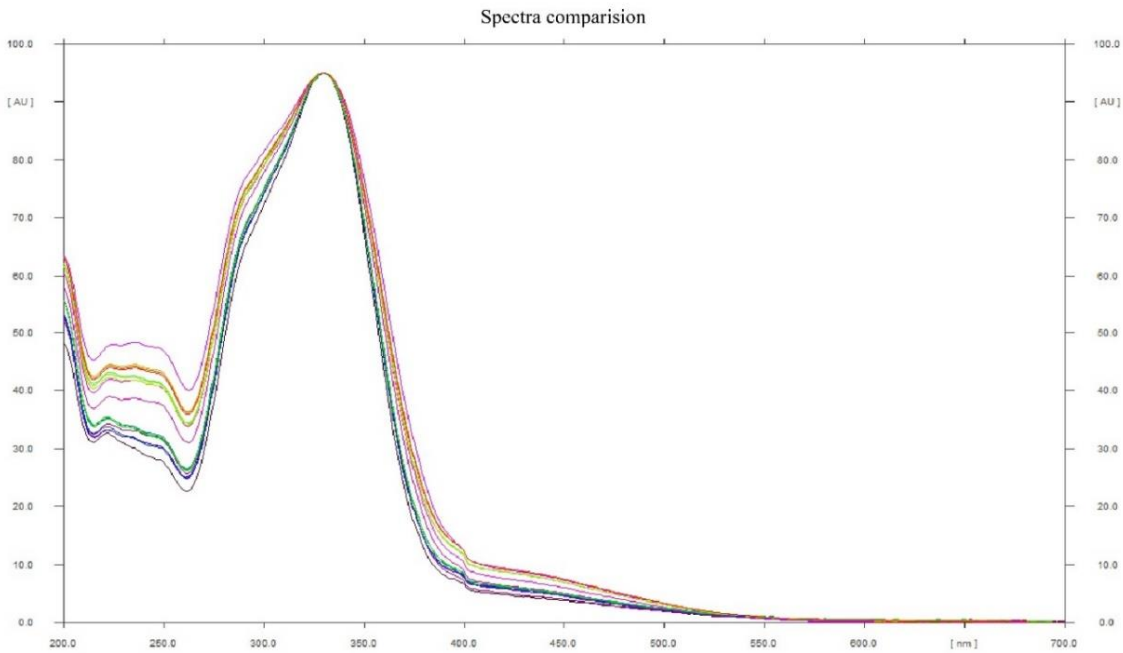


Figure 3 Peak identity determination by comparison of the absorbance spectra of rosmarinic acid among samples and standard rosmarinic acid

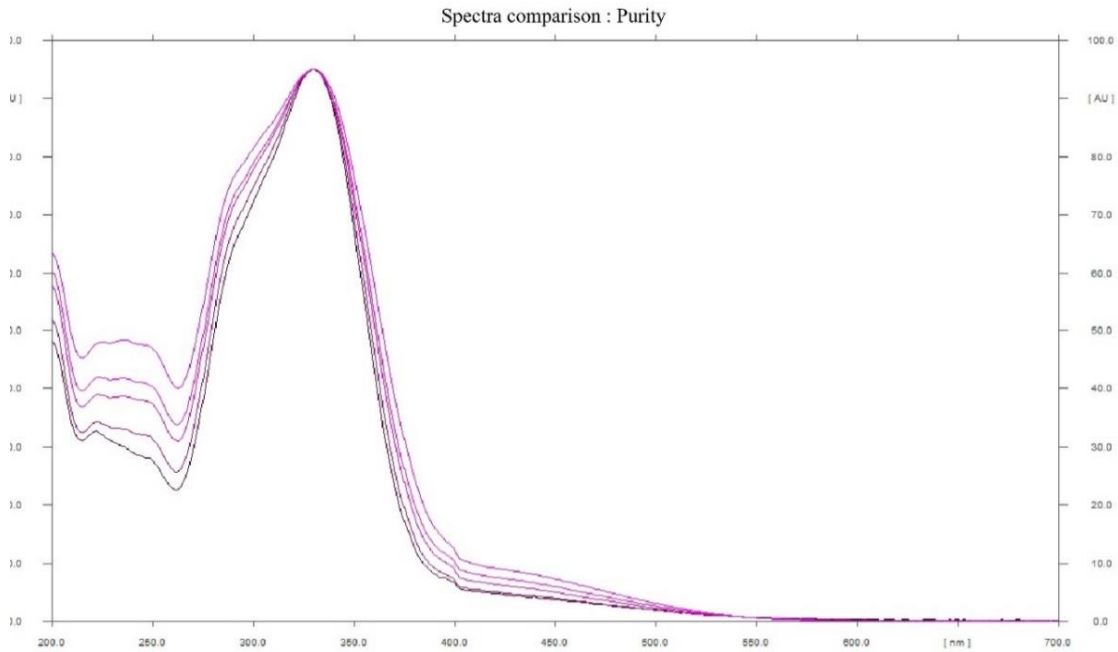


Figure 4 Peak purity determination using up-slope, apex and down-slope of the peak

Conclusion

TLC-densitometry and TLC-image analysis were firstly developed for quantitative determination of rosmarinic acid in ethanolic extracts of *P. frutescens* leaves. Both method validities were demonstrated. TLC-image analysis using ImageJ, a free analytical software could be used as an alternative method for rosmarinic acid quantification in *P. frutescens* leaf as its simplicity, less time consuming and inexpensive instruments. Rosmarinic acid contents in *P. frutescens* dried leaves in Thailand were found to be 2.504 ± 1.631 and 2.485 ± 1.606 % w/w by developed TLC-densitometry and TLC-image analysis respectively.

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