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Quantitative Determination of Usnic Acid Content in *Usnea siamensis* by TLC-Densitometry and TLC Image Analysis

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ABSTRACT

Usnea siamensis Wainio, a Thai herbal drug that used to treat diseases in folk medicine as bitter tonic, carminative, antidiarrhea, antidysentery and anti-tumor. The quantitative analyses of active component, (+)-usnic acid in *U. siamensis* samples from 15 locations were done by thin layer chromatography using chloroform and methanol (9:1) as mobile phase. The usnic acid contents were analyzed by TLC-densitometry performed with winCATS software and TLC image analysis performed with ImageJ software. The regression lines of both methods were polynomial in the range of 0.2-1.0 mg/spot and correlation coefficients were 0.9981 and 0.9994 respectively. The precisions calculated by the %RSD of repeatability and intermediate precision, were between 10.25-19.39 and 8.49-11.50 %RSD respectively. The average recoveries were between 83.77-100.45 and 99.17-120.49 % respectively. LOD and LOQ were 0.02, 0.06 mg and 0.04, 0.12 mg respectively. The robustness of mobile phase variation was 0.803 and 1.094 %RSD of peak area respectively. The usnic acid contents in *U. siamensis* were 2.32 ± 0.29 and 2.26 ± 0.25 g/100g of dried crude drug respectively. It was found that usnic acid contents by two methods were not statistically significantly different ($P = 0.256$). TLC image analysis is valid to be used instead of TLC scanner for quantitation of usnic acid in *U. siamensis*.

Keywords: Usnic acid, *Usnea siamensis*, TLC-densitometry, TLC image analysis

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INTRODUCTION

Since the World Health Organization recommends member countries to use folk medicines and herbal medicines in primary health care projects, herbal drugs have been popular and developmental in many countries. The herbal medicinal drugs are used as therapeutic and raw materials for the pharmaceutical industry. In Thailand, the herbal medicinal products have been used for years and years. Furthermore herbal medicinal drugs have been popular in the last few years, which are used to treat and alleviate the diseases. The herbal medicinal drugs are not only used in folk medicines but also widely used in the hospital and commercial market [1,2]. *Usnea siamensis* Wainio (Figure 1), in Thai is called "Foi-lom". It is fruticose lichen, up to 5 m long, grayish-green strands hanging from the branches of trees. *Usnea siamensis* comprises of dibenzofuran derivative, (+)-usnic acid (Figure 2) as a major constituent [3]. Usnic acid has been shown its biological and physiological activities such as antimicrobial, antiparasitic, antimitotic, antiproliferative, anti-inflammatory, analgesic and antipyretic activities [4,5]. Thin layer chromatography (TLC) is widely used because of its easy, rapid, inexpensive and useful method for chemical identification [6]. Combination of TLC with densitometry and image analysis offer quantitative analysis of medicinal plant component. Densitometer is fixed wavelength to measure the difference in absorbance or fluorescence signal between a separated zone and the empty plate background [7]. Image analysis method is estimated by comparing the spot color intensity with the plate color background [8]. The peak area data of the unknowns are compared with data from calibration standards chromatographed on the same plate. This study provided scientific information to compare usnic acid content in *Usnea siamensis* by TLC-densitometry and TLC image analysis.



Figure 1. Crude drug of *Usnea siamensis* (cm)

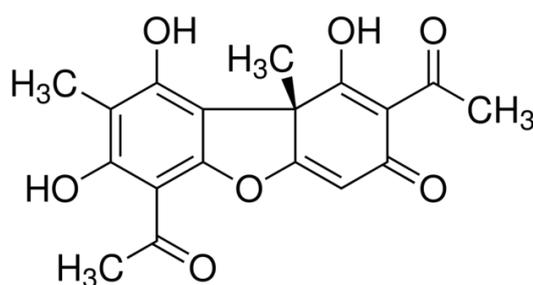


Figure 2. (+)-Usnic acid

MATERIALS AND METHODS

Materials and chemicals

Dried *Usnea siamensis* crude drug from 15 various locations throughout Thailand were authenticated by Assoc. Prof. Nijsiri Ruangrunsi, Ph.D. Voucher specimens were deposited at College of Public Health Sciences, Chulalongkorn University. Plant material was pulverized coarsely before use. Standard usnic acid was purchased from Sigma-Aldrich, USA. The chemicals used were of analytical grade.

Standard usnic acid solutions

The stock solution of standard usnic acid (1 mg/ml) was prepared in 10% methanol in dichloromethane. The stock solution was appropriately diluted to obtain the concentrations of 0.2, 0.4, 0.6, 0.8, 1.0 mg/ml. These solutions were stored with tightly sealed caps at 4 °C.

Benzene extracts of *Usnea siamensis*

Usnea siamensis (20.0 g) were exhaustively extracted with benzene by soxhlet apparatus. The extract was filtered, evaporated to dryness under reduced pressure at 50 °C and weighed. One milligram of the extract was dissolved with 1 ml of 10% methanol in dichloromethane for TLC analysis.

TLC-densitometry

Three microliters of *Usnea siamensis* benzene extract and 3.0 µl of standard usnic acid solutions were applied on the silica gel 60 F₂₅₄ 20 × 10 cm TLC plate using CAMAG Linomat 5. Sample band was set at 10.0 mm while distance between bands was 8.9 mm. The TLC plate was developed using a mixture of chloroform and methanol (9:1) as mobile solvent. After development, the plate was scanned by CAMAG TLC Scanner 3 at the wavelength of 293 nm (maximum absorbance of usnic acid) and expressed as chromatographic peak by winCATS software. The contents of usnic acid in *Usnea siamensis* extracts were determined based on the calibration curve of usnic acid performed in the same TLC plate. The tests were done in triplicate.

TLC image analysis by ImageJ software

Developed TLC plate was further observed under short wave (254 nm) ultraviolet light in Spectroline® UV viewing cabinet. The photos were taken using digital camera and saved as JPEG files with C mode ISO 80. The color intensity of usnic acid band was transformed to chromatographic peak by ImageJ software. The contents of usnic acid in *Usnea siamensis* extracts were determined based on the calibration curve of usnic acid performed in the same TLC plate. The tests were done in triplicate.

Method validation

According to the ICH guideline [9], specificity, accuracy, precision, limits of detection (LOD), limits of quantification (LOQ), range and robustness of usnic acid quantitative analysis in *Usnea siamensis* were validated.

Data analysis

Usnic acid content in each *Usnea siamensis* crude drug was calculated based on each extract yield. The contents between two analytical methods were statistically compared using paired student *t*-test.

RESULTS AND DISCUSSION

Benzene extraction of 15 *Usnea siamensis* crude drugs yielded $4.81 \pm 0.71\%$ by dry weight. Thin layer chromatography using densitometer and image analysis for quantitative analysis of usnic acid were validated in terms of the specificity, accuracy, precision, LOD, LOQ, range and robustness. Absorption spectrum of standard usnic acid showed the maximum absorbance at the wavelength of 293 nm. The absorption spectra of usnic acid in all samples and standard usnic acid were identical which represented the method specificity. Recovery was determined to evaluate the accuracy of the method. The data were obtained by spiking known concentrations of standard usnic acid (0.30, 0.50, 0.70 mg/spot) into *Usnea siamensis* extract to get the low, medium, high value and the results were presented in Table 1. The recovery values were within acceptable limits (83.77-100.45% for TLC-densitometry and 99.17-120.49% for TLC image analysis). The repeatability was determined by analyzing the sample solution (3 concentrations \times 3 replicates) on the same day, the intermediate precision was determined in 3 different days (Table 2, 3). The LOD and LOQ of TLC-densitometry and TLC image analysis were calculated by standard deviation of *y*-intercept of calibration curve and were found to be 0.02, 0.06 mg/spot and 0.04, 0.12 mg/spot respectively. The calibration curve of TLC-densitometry and TLC image analysis was found in range of 0.20-1.00 mg/spot with the regression coefficient (R^2) of 0.9981 and 0.9994 (Figure 3, 4). The robustness of TLC-densitometry and TLC image analysis performed by varying the mobile phase ratio expressed %RSD of 0.80% and 1.10% (Table 4). The usnic acid contents in dried *Usnea siamensis* crude drugs by TLC-densitometry and TLC image analysis in this study were 2.32 ± 0.53 and 2.26 ± 0.25 % by weight. Marcano *et al.* reported the usnic acid content of 2.7% in *Usnea laevis* by petroleum ether extraction and crystallization [10]. Cansaran *et al.* reported 0.22-6.49 % of usnic acid contents in acetone extracts of *Usnea* spp. other than *Usnea siamensis* determined by HPLC [11]. TLC image analysis was previously shown its quantitative compatibility with HPLC in medicinal plant research [12, 13].

The comparison of usnic acid content between TLC-densitometry and TLC image analysis (Table 5) were statistically tested using paired student *t*-test. It was found that the usnic acid content by two methods were not significantly different ($t = 1.183$, $P = 0.256$). TLC-densitometric method measured the difference in absorbance or fluorescence signal between a compound band and surrounding plate background. The advantage of TLC-densitometry is high accuracy, low operating cost (compared to other instrumental methods

e.g. HPLC), minimum sample clean up, using a small sample and less analysis time. Whereas, TLC image analysis used CCD camera to capture the image of TLC chromatogram and interpret the intensity of color of compound band and contrast background to chromatographic peak by ImageJ software. The advantage of TLC image analysis is low cost, simple equipment need, quick and easy experiment and available free software.

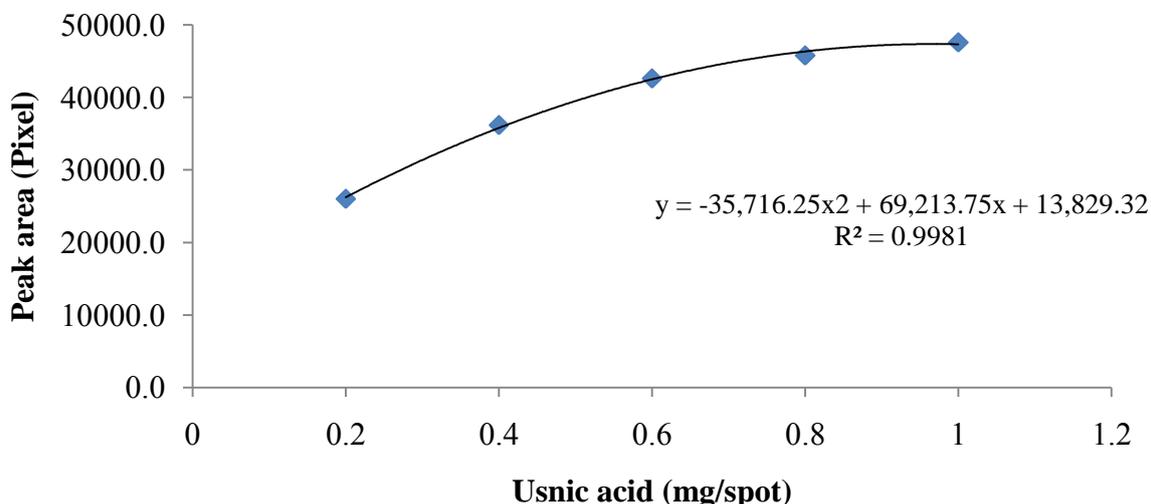


Figure 3. The calibration curve of usnic acid in *Usnea siamensis* by TLC-densitometric method

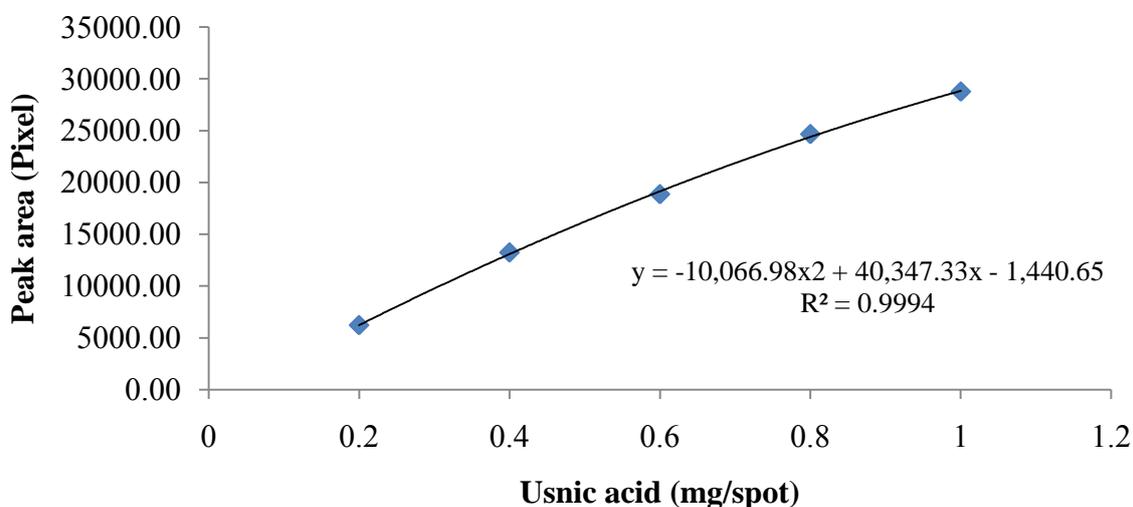


Figure 4. The calibration curve of usnic acid in *Usnea siamensis* by TLC image analysis method

Although the calibration curves of usnic acid by both methods were polynomial, the correlation coefficients were shown to be more than 0.99 in range of 0.2-1.0 mg/spot. The methods were demonstrated their validity for usnic acid quantitation in *Usnea siamensis*. TLC image analysis has higher limits of detection and quantitation of usnic acid than TLC densitometry and seems to be less robust but the results of usnic acid content obtained by both methods were not statistically significantly different. Therefore, TLC image analysis can be used instead of TLC densitometry in small laboratory with limited budget.

Table 1. Accuracy of usnic acid quantitation in *Usnea siamensis* by TLC-densitometry and TLC image analysis (each sample was done in triplicate)

Usnic acid added (mg)	TLC-densitometry		TLC image analysis	
	Usnic acid found (mg)	% Recovery	Usnic acid found (mg)	% Recovery
0.0	0.440	-	0.266	-
0.3	0.747	100.45	0.682	120.49
0.5	0.837	88.63	0.850	110.97
0.7	0.958	83.77	0.958	99.17
Average		90.95 ± 8.58		110.21 ± 10.68

Table 2. Repeatability of quantitation of usnic acid in *Usnea siamensis* by TLC-densitometry and TLC image analysis (each sample was done in triplicate)

Usnic acid level	TLC-densitometry		TLC image analysis	
	Amount detection	%RSD	Amount detection	%RSD
1	0.44 ± 0.05	10.70	0.27 ± 0.07	24.51
2	0.75 ± 0.03	4.06	0.58 ± 0.01	1.59
3	0.84 ± 0.12	13.80	0.85 ± 0.04	4.69
4	0.96 ± 0.12	12.45	0.96 ± 0.03	3.18
Average		10.25 ± 4.32		8.49 ± 10.76

Table 3. Intermediate precision of quantitation of usnic acid in *Usnea siamensis* by TLC-densitometry and TLC image analysis (each sample was done in triplicate)

Usnic acid level	TLC-densitometry		TLC image analysis	
	Amount detection	%RSD	Amount detection	%RSD
1	0.39 ± 0.08	20.13	0.27 ± 0.05	18.20
2	0.64 ± 0.11	17.29	0.66 ± 0.04	6.43
3	0.75 ± 0.15	19.37	0.82 ± 0.08	9.61
4	0.80 ± 0.17	20.77	0.88 ± 0.10	11.76
Average		19.39 ± 1.51		11.50 ± 4.98

Table 4. Robustness of quantitation of usnic acid in *Usnea siamensis* by TLC-densitometry and TLC image analysis

Mobile phase ratio (v/v)		Usnic acid peak area	
Chloroform	Methanol	TLC-densitometry	TLC image analysis
8.8	1.2	42647.0	19383.8
8.9	1.1	42323.9	19707.0
9.0	1.0	42617.2	19732.4
9.1	0.9	41802.0	19368.3
9.2	0.8	42306.7	19834.0
Mean		42339.4	19605.1
SD		339.8	214.5
%RSD		0.803	1.094

Table 5. The comparison of usnic acid contents between TLC-densitometry and TLC image analysis

Source	% Usnic acid content	
	TLC-densitometry	TLC image analysis
1	2.3995	2.7959
2	1.9829	1.9727
3	3.0855	2.7158
4	1.8886	1.9032
5	2.3995	2.1688
6	1.9397	1.6765
7	1.7605	1.7278
8	2.2556	2.0588
9	3.5749	3.3328
10	2.8988	2.8723
11	1.9777	2.3588
12	2.7184	2.5596
13	2.2913	2.2095
14	1.7347	1.6739
15	1.9941	1.8937
Average	2.3267 ± 0.2853	2.2613 ± 0.2487

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