Journal of Chromatographic Science 2013;51:430–435
doi:10.1093/chromsci/bms159 Advance Access publication October 7, 2012

Article

Simultaneous Quantification of Stemocurtisine, Stemocurtisinol and Stemofoline in *Stemona curtisii* (Stemonaceae) by TLC–Densitometric Method

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Received 26 April 2012; revised 28 August 2012

***Stemona curtisii* Hook. F. (Family Stemonaceae), a prominent species distributed in the south and southwest of Thailand, has widely been used as a natural pesticide and as treatment for head lice and skin diseases. This study developed a thin-layer chromatography (TLC)–densitometric method for the simultaneous quantification of major components—stemocurtisine, stemocurtisinol and stemofoline—in the extracts from the roots of *S. curtisii* collected from 10 locations in Thailand. Components were found in the ranges of 0.0353–0.1949, <0.0121–0.0859 and 0.0733–0.1689 percent dry weight, respectively. The method was validated for linearity, precision, accuracy, robustness, limit of detection and limit of quantitation. The linearity was found over the range of 40–320 ng/spot with a good correlation coefficient (*r* > 0.9866). Intra-day and inter-day precision showed a relative standard deviation of less than 6%. The accuracy of the method was determined by a recovery study, and the average recoveries were 100.4, 100.2 and 100.3% for stemocurtisine, stemocurtisinol and stemofoline, respectively. The proposed TLC–densitometric method was found to be simple, precise, specific and inexpensive, and can be used simultaneously for the routine quality control of raw materials of *S. curtisii* roots, extracts and their products, and also other products containing these markers.**

Introduction

Stemona plants have historically been used as natural pesticides and medicinal plants for the treatment of skin and respiratory diseases in many southeast Asian countries (1–3). A recent taxonomic revision indicates that *Stemona* in Thailand comprises 11 species (4). However, various *Stemona* species in Thailand have been known by the same vernacular name "Non Tai Yak" because of their similar root shapes (4, 5). The inconsistency and confusion regarding the proper *Stemona* plant materials have led to confusion in the quality control of authentic *Stemona* in agricultural and pharmaceutical products. Thus, the qualitative and quantitative analysis of active components in the raw material of *Stemona* are useful to identify their fingerprints.

S. curtisii Hook. F. is a prominent species and popular insecticidal herb distributed in the south and southwest of Thailand (4, 5). Its root contains a specific group of *Stemona* alkaloids, including stemofoline, 2′-hydroxystemofoline, oxyprotostemonine, dehydroprotostemonine, protostemonine, stemocochinine, stemocurtisine (pyridostemin), stemocurtisinol and oxystemokerrine (6–10). *Stemona* alkaloids promote potential biological activities such as insect toxicity (6, 8), nitric oxide inhibition (11) and increased chemosensitivity via *P*-glycoprotein-mediated multidrug resistance (12–14). The insect toxicity property of *S. curtisii* root extracts (6, 8), which does not harm humans (15, 16), popularizes biopesticide formulations in the markets. This has motivated the sale of self-made and local-made *S. curtisii* extracts as natural pesticides in many rural areas of Thailand. The local-made biopesticides are generally prepared from the roots of various *Stemona*. The problem of the variations of chemical constituents and effectiveness of the local-made preparations is always a concern. Therefore, it is necessary to use analytical tools for the quality control of the raw materials, extracts and biopesticide preparations prepared from *Stemona*.

Several analytical techniques, such as high-performance liquid chromatography (HPLC) (17) and capillary electrophoresis (18), were developed for the quantitative analysis of *S. curtisii* root extracts. However, a simple and rapid method for the simultaneous analysis of major constituents in *S. curtisii* is necessary and useful for routine work. Thin-layer chromatography (TLC) is widely used in the analysis of pharmaceuticals, botanicals, foodstuffs, and environmental and clinical samples (19). It is a preferred analytical tool for fingerprint analysis and quantification of marker compounds in herbal drugs because of its simplicity, accuracy and suitability for high-throughput screening. Therefore, this study was aimed to develop and validate a TLC–densitometric method for the qualitative and quantitative analysis of major components—stemocurtisine, stemocurtisinol, and stemofoline—in *S. curtisii* roots. These results may provide an alternative method for the quality assessment of raw materials, extracts and finished products containing these *Stemona* alkaloids.

Experimental

Materials and reagents

All reagents and solvents were of analytical grade. TLC was performed on pre-coated silica gel GF254 plates (Merck, Darmstadt, Germany). The roots of *S. curtisii* (SCR001–SCR002) were collected from Na Tha Wi, Songkhla province and Muang, Krabi province, respectively, and samples with VC numbers (VC002–VC004, VC006, VC007 and VC012–VC014) were obtained from a cultivated farm at the National Corn and Sorghum Research Center in Nakhon Ratchasima province, Thailand. All samples were collected between December, 2009 and March, 2010 (Table I). The samples were identified by Dr. Srunya Vajrodaya of the Faculty of Botany, Kasetsart University (Thailand) and the voucher specimens were deposited at the Department of

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Table I
Contents of Stemocurtisine, Stemocurtisinol, and Stemofoline in Dried Powder of *S. curtisii* Roots Collected from 10 Different Locations in Thailand

Sample/location (floristic region)*	Content (% w/w)		
	Stemocurtisine	Stemocurtisinol	Stemofoline
VC002 Songkhla, Hat Yai (PEN)	—	—	0.0773 ± 0.0052
VC003 Surat Thani, Kanchanadit (PEN)	—	—	0.1621 ± 0.0089
VC004 Nakhon Si Thammarat, Thung Song (PEN)	0.1002 ± 0.0036	0.0355 ± 0.0025	—
VC006 Krabi, Ao Luek (PEN)	—	—	0.1146 ± 0.0142
VC007 Prachuap Khiri Khan, Sam Roi Yot (SW)	0.1949 ± 0.0110	0.0442 ± 0.0010	—
VC012 Prachuap Khiri Khan, Bang Saphan Noi (SW)	0.1827 ± 0.0157	0.0859 ± 0.0152	—
VC013 Trang, Ratsada (PEN)	0.0657 ± 0.0099	< 0.0121	0.0756 ± 0.0066
VC014 Phattalung, Muang (PEN)	0.0353 ± 0.0044	< 0.0121	0.1139 ± 0.0071
SCR001 Songkhla, Na Tha Wi (PEN)	—	—	0.1689 ± 0.0101
SCR002 Krabi, Muang (PEN)	0.0497 ± 0.0031	< 0.0121	0.0733 ± 0.0017
Average	0.1048 ± 0.0687	0.0552 ± 0.0269	0.1122 ± 0.0404

*PEN: peninsular; SW: southwestern.

Pharmacognosy, Faculty of Pharmacy, Mahidol University (Bangkok, Thailand). Each sample was washed thoroughly with tap water, cut into small pieces and dried in a hot air oven at 50°C for 72 h. Each dried sample was ground into powder, passed through a sieve (60 mesh) and stored in an air-tight container and protected from light at room temperature (28–30°C) until use.

Pure stemocurtisine, stemocurtisinol and stemofoline, isolated and identified in a previous work (10), were used as standard compounds.

Apparatus

The spotting device was a Linomat 5 automatic sample spotter (CAMAG, Muttenz, Switzerland). A 100 µL syringe was used (Hamilton, Bonaduz, Switzerland). The TLC chamber was a glass twin-trough chamber (20 × 10 × 4 cm, CAMAG); the densitometer was a TLC scanner 3 linked to winCATS software (CAMAG); and the TLC plates were 20 × 10 cm with 0.2 mm layer thickness, precoated with silica gel 60 F254 (Cat. No. 1.05554.0001); Merck.

Preparation of standard solutions

Stock solutions of stemocurtisine, stemocurtisinol and stemofoline were prepared by dissolving each standard compound in methanol in a volumetric flask at a concentration of 1,000 µg/mL. The stock solution was further diluted with methanol to give five working standard solutions for analysis (concentrations of 4, 12, 20, 28 and 32 µg/mL).

Preparation of sample solutions

Each sample of *S. curtisii* roots was accurately weighed (150 mg) and exhaustively extracted (monitored by TLC) by sonication with methanol (3 × 7 mL) in an ultrasonic bath for each 30 min at ambient temperature (28–30°C). The methanol extracts were combined, filtered and concentrated under vacuum in a rotary evaporator. The concentrated extract was adjusted with methanol to a 10 mL volume in a volumetric flask. The solution was filtered using a 0.45 µm nylon membrane filter before application onto the TLC plate. Each sample was prepared and analyzed in triplicate.

Validation of the method

The analytical method was validated for linearity, precision, accuracy, limit of detection (LOD), limit of quantitation (LOQ) and robustness according to International Conference on Harmonization (ICH) guidelines (20).

Linearity

Linearity was determined by using the standard solution in methanol. Ten microliters at five concentration levels of each reference standard, individually prepared (4, 12, 20, 28 and 32 µg/mL) were spotted on the TLC plate to obtain the calibration range of 40–320 ng/spot. The calibration graphs were obtained by plotting the peak area versus the concentration of the standard solutions.

Precision

The precision was determined by analyzing 100, 180 and 260 ng/spot of each standard solution after the application by the proposed method onto a TLC plate on the same day for intra-day precision and on three different days for inter-day precision. The precision was expressed as percent relative standard deviation (RSD).

Accuracy

The accuracy of the method was tested by performing recovery studies at three levels of each standard (stemocurtisine,

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stemocurtisinol and stemofoline) added to the sample. The solutions were applied onto a TLC plate and analyzed by using the proposed method. Three determinations were performed for each concentration level of the standards. The recovery and average recovery were calculated as: recovery (%) = 100 × (amount found − original amount) / amount spiked.

LOD and LOQ

LOD and LOQ were determined by scanning the blank (methanol) spot and detecting the noise. The standard deviation (SD) was then calculated based on 20 blank peaks. LOD and LOQ were calculated using following equations: LOD = 3 SD/S; LOQ = 10 SD/S, where SD is the standard deviation of blank peaks and S is the mean slope of the regression line.

Robustness

The robustness of the method was evaluated by introducing small changes in certain chromatographic parameters at each standard concentration level of 180 ng/spot. The composition of mobile phase was changed slightly; e.g., 70:25:5:1, 70:25:4:1 and 55:45:5:1, v/v/v/v, for dichloromethane–ethyl acetate–methanol–ammonium hydroxide, respectively. The volume applied to the TLC plate was varied; e.g.. 5 µL of 36 µg/mL, 10 µL of 18 µg/mL and 20 µL of 9 µg/mL. The periods of time between spotting the standards onto the TLC plate and developing the plate (0 and 30 min), duration of TLC tank saturation (0, 30 and 60 min) and duration between TLC plate development and scanning (0, 30 and 60 min) was varied, and the RSD values of the peak areas were calculated for all variations.

Chromatographic conditions

The TLC plates were pre-washed with methanol and activated at 105–110°C for 15 min before use. The samples were spotted as 7 mm bands wide with a 100 µL Hamilton synringe at 10 mm from the bottom edge using a CAMAG Linomat 5 automatic sample spotter with a constant rate of 100 nL/s. Each sample solution (10–20 µL) was applied in triplicate. The mobile phase consisted of dichloromethane–ethyl acetate–methanol–ammonium hydroxide (70:25:5:1, v/v/v/v). Linear ascending

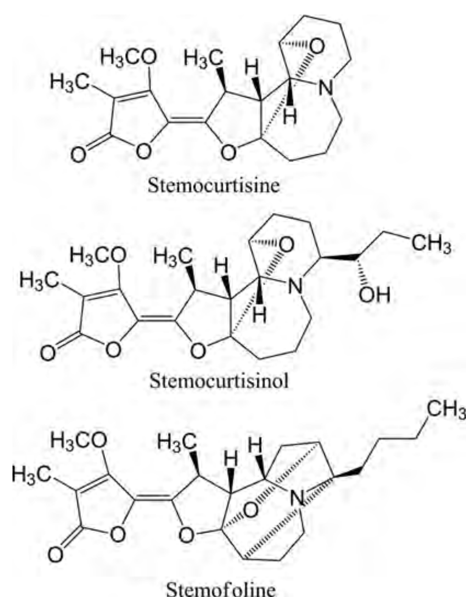

Figure 1. Structures of stemocurtisine, stemocurtisinol, and stemofoline.

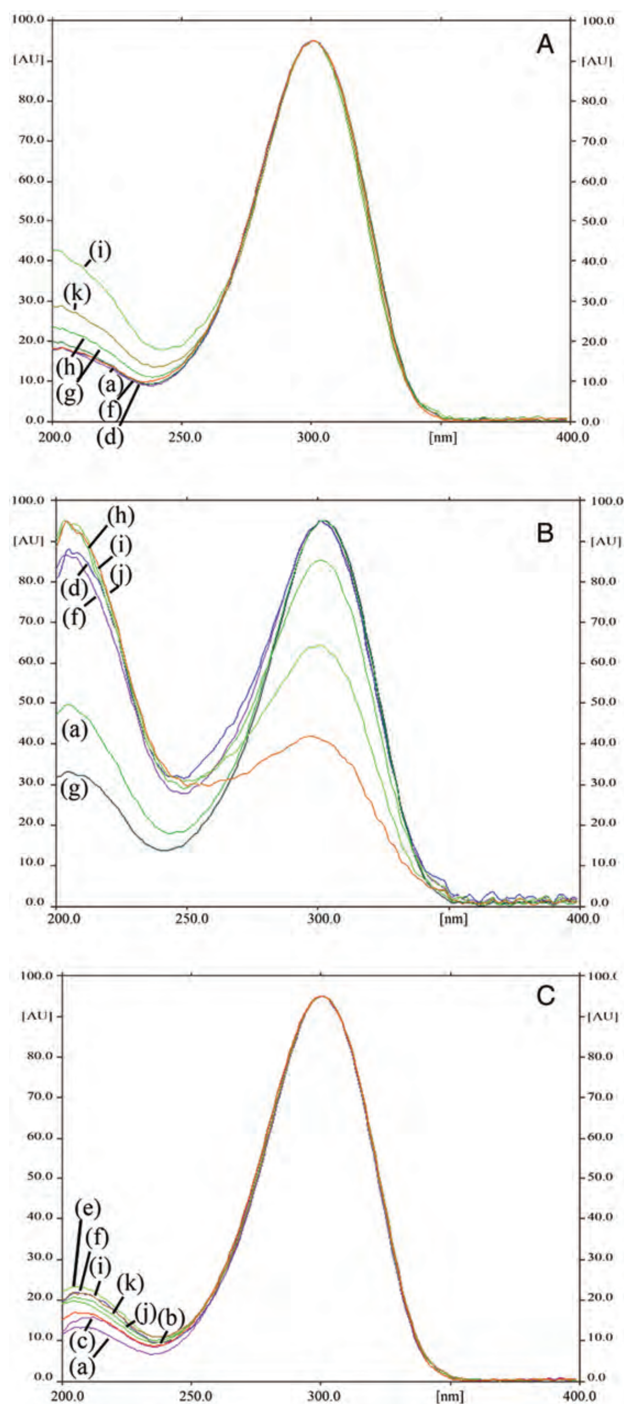

Figure 2. Overlay UV absorption spectra of standards in the *S. curtisii* root extracts: stemocurtisine (A); stemocurtisinol (B); stemofoline (C); spectra illustrating peak purities of: reference standard (a), sample VC002 (b), sample VC003 (c), sample VC004 (d), sample VC006 (e), sample VC007 (f), sample VC012 (g), sample VC013 (h), sample VC014 (i), sample SCR001 (j) and sample SCR002 (k).

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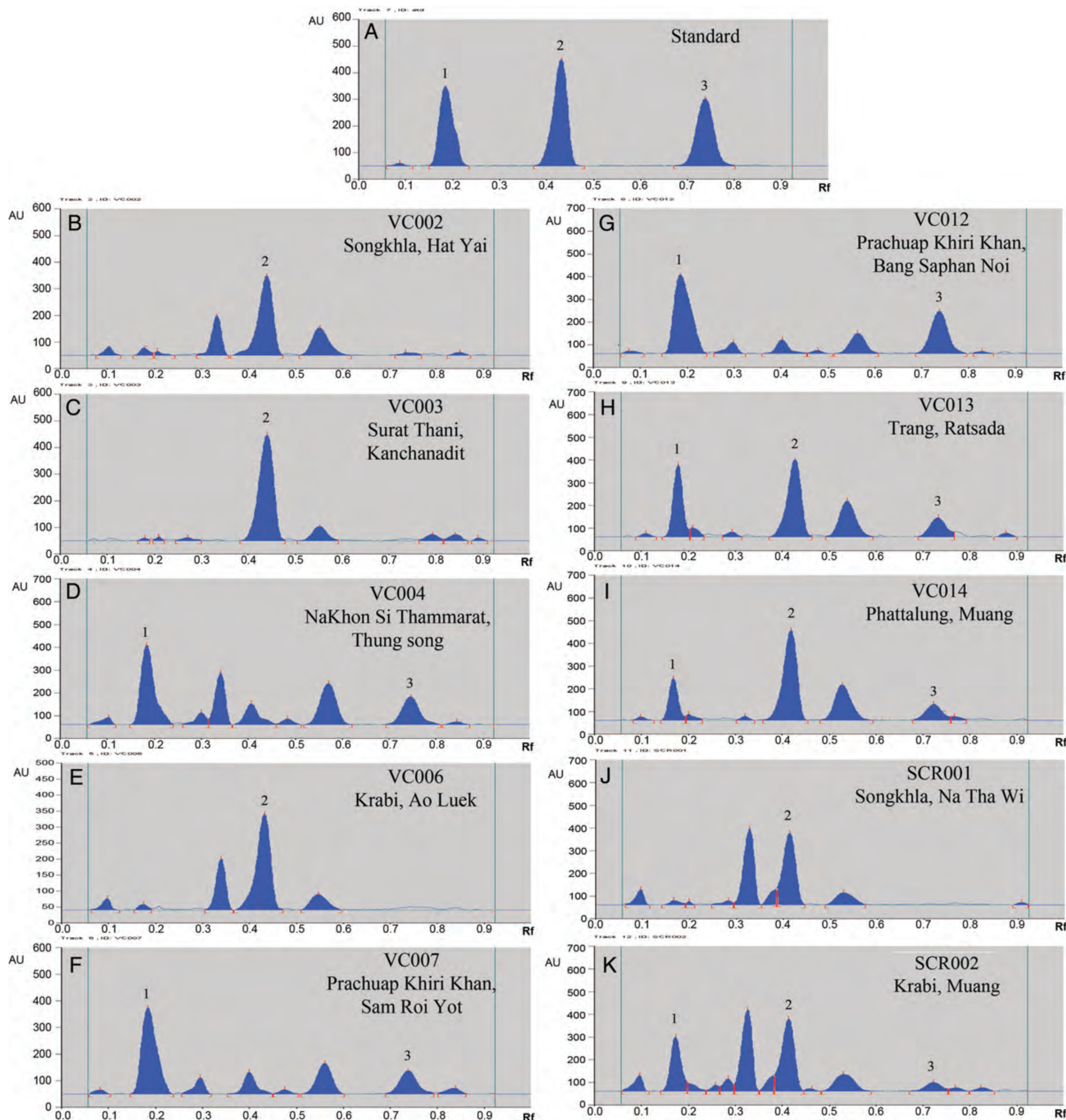

Figure 3. TLC densitograms of *S. curtisii* root extracts and standards: stemocurtisine, stemofoline and stemocurtisinol standards; peak 1, stemocurtisine (R_f 0.18); peak 2, stemofoline (R_f 0.43); peak 3, stemocurtisinol (R_f 0.73) (A); extracts of *S. curtisii* roots collected from 10 different locations of Thailand (B–K).

development was performed in a twin through-glass chamber pre-saturated with the mobile phase for 30 min at room temperature (28–30°C). The length of the chromatogram run was 80 mm and development time was approximately 14 min. Densitometric scanning was performed using a CAMAG TLC 3 scanner in the reflectance-absorbance mode at 300 nm controlled by winCATs software. The slit dimension was 6.00 × 0.45 mm with a scanning speed of 20 mm/s. A five points calibration was performed for each analysis by the proposed method. The amounts of stemocurtisine, stemocurtisinol and stemofoline were calculated via peak area using the calibration graph.

Results and Discussion

S. curtisii root extract is composed of many alkaloids and their related analogues. Stemocurtisine, stemocurtisinol and stemofoline (Figure 1) are major alkaloids in the root extracts, and these alkaloids were used as markers in TLC–densitometric

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Table II
Method Validation Parameters for the Quantitation of Stemocurtisine, Stemocurtisinol and Stemofoline by the Proposed TLC–Densitometric Method

	Results		
Parameter	Stemocurtisine	Stemocurtisinol	Stemofoline
Linear range (ng/spot)	44–350	41–327	40–319
Calibration equation*	$Y = 22.631X + 1226.28$	$Y = 31.030X + 1682.321$	$Y = 32.235X + 752.38$
Correlation coefficient (r)	0.9866	0.9967	0.9953
LOQ (ng)	11.70	15.13	12.55
LOD (ng)	3.51	4.53	3.77

*X is the amount of each standard in ng and Y is the peak area at 300 nm.

Table III
Intra-Day and Inter-Day Precision of Stemocurtisine, Stemocurtisinol and Stemofoline

Compounds	Concentration (ng/spot)	Intra-day precision* (%)	Inter-day precision* (%)
Stemocurtisine	109	3.99	2.81
	197	2.26	5.29
	284	3.75	3.21
Stemocurtisinol	102	3.63	3.96
	184	3.95	4.10
	266	3.71	2.62
Stemofoline	100	2.33	1.67
	179	2.99	2.79
	259	3.22	3.83

*Present as percent RSD ($n = 3$).

Table IV
Recovery Studies of Stemocurtisine, Stemocurtisinol and Stemofoline

Compound	Serial number	Theoretical value (ng)	Amount found* (ng)	Recovery* (%)
Stemocurtisine	1	148.36	150.49 ± 1.51	101.4 ± 1.0
	2	206.72	199.56 ± 1.15	96.45 ± 0.56
	3	221.51	228.79 ± 1.23	103.3 ± 0.6
	Average			100.4
Stemocurtisinol	1	84.79	82.12 ± 0.74	96.85 ± 0.88
	2	96.88	99.84 ± 2.38	103.0 ± 2.5
	3	132.22	133.06 ± 2.72	100.6 ± 2.1
	Average			100.2
Stemofoline	1	125.01	126.55 ± 2.31	101.2 ± 1.8
	2	169.98	170.52 ± 3.30	100.3 ± 1.9
	3	218.45	217.39 ± 2.53	99.51 ± 1.16
	Average			100.3

*Expressed as mean ± SD (n =3).

Table V
Robustness Studies of Stemocurtisine, Stemocurtisinol and Stemofoline

	RSD (%)		
Parameter	Stemocurtisine	Stemocurtisinol	Stemofoline
Mobile phase composition*	6.13	4.49	3.34
Time from spotting to chromatography*	1.29	2.42	2.61
Time from chromatography to scanning*	0.69	0.31	0.38
Presaturation time*	3.15	1.42	3.41
Applied volume†	1.57	4.78	2.52

*Performed in six determinations.
†Performed in four determinations.

analysis. For the chromatographic conditions, several trials were made using different solvent systems. Dichloromethane–ethyl acetate–methanol–ammonium hydroxide (70:25:5:1, v/v/v/v) offered the best separation of stemocurtisine, stemocurtisinol and stemofoline with R_f values of 0.18, 0.73 and 0.43, respectively. The identities of the bands of stemocurtisine, stemocurtisinol and stemofoline in the sample chromatograms were confirmed by overlaying their ultraviolet (UV) absorption spectra with those of each standard using the TLC 3 scanner (Figure 2). The wavelength of 300 nm was chosen for the analysis. Densitograms of stemocurtisine, stemocurtisinol and stemofoline reference standards and other constituents in the extracts of *S. curtisii* are shown in Figure 3.

The method was validated for its linearity, accuracy, precision, robustness, LOD and LOQ. The linear calibration graphs for stemocurtisine, stemocurtisinol and stemofoline were within the concentration ranges of 44–350, 41–327 and 40–319 ng/spot, with correlation coefficients (r) of 0.9866, 0.9967 and 0.9953, respectively (Table II). The inter-day and intra-day precisions of stemocurtisine, stemocurtisinol and stemofoline are shown in Table III. The results showed acceptable precision of the method, with RSD less than 6% (Table III). The average recoveries at three different levels of stemocurtisine, stemocurtisinol and stemofoline were 100.4, 100.2 and 100.3%, respectively (Table IV). These values indicated the accuracy of the method. For the robustness test, the standard deviation of peak areas was calculated for each parameter and the RSD was found to be less than 6% in each case (Table V). The change of mobile phase composition was the primary deviation of the method, the others showed less significance on the RSD of the method.

The validated method was used to determine the contents of stemocurtisine, stemocurtisinol and stemofoline in 10 extracts of *S. curtisii* roots collected from different parts of Thailand. The results showed a significant difference in the contents of major constituents in *S. curtisii* roots (Table I). The amounts of stemocurtisine, stemocurtisinol and stemofoline showed infraspecific variation from non-detectable to detectable quantities and ranged from 0.0497 to 0.1949, less than 0.0121 to 0.0859 and 0.0733 to 0.1689% dried weight, respectively (Figure 3, Table I). The developed TLC–densitometric method was accurate and precise for the qualitative and quantitative determination of stemocurtisine, stemocurtisinol and stemofoline in *S. curtisii* roots, and is suitable for the routine quantification of these marker compounds in *Stemona* raw materials, extracts and the products containing these compounds. This method was also simple and inexpensive. It can be used as an alternative method for the quantitative analysis of major compounds in the roots of *S. curtisii*. By using the proposed method, various *Stemona* samples could be qualitatively and quantitatively analyzed at the same time.

Conclusion

A TLC–densitometric method was developed and validated for the qualitative and quantitative analysis of stemocurtisine, stemocurtisinol and stemofoline which are major compounds in the extracts of *S. curtisii* roots collected from 10 locations in Thailand. The contents of stemocurtisine, stemocurtisinol and

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stemofoline in the roots of *S. curtisii* collected from various locations in Thailand were 0.0497–0.1949, <0.0121–0.0859 and 0.0733–0.1689 percent dry weight, respectively. These data can be useful for further standardization of *S. curtisii* raw materials and for finding good sources of the roots of this plant in Thailand. The method is simple, precise, specific and accurate, and can simultaneously analyze many samples. It can be used for quality control of raw materials, extracts and products containing these *Stemona* alkaloids.

Acknowledgments

This study is a part of a Ph.D. thesis of Mahidol University financially supported by Thailand Research Fund and Mahidol University (Royal Golden Jubilee Ph.D. Program Grant No. PHD/0139/2550). The project was also supported by the Office of the Higher Education Commission and Mahidol University under the National Research Universities Initiative. We thank Assoc. Prof. Dr. Srunya Vajrodaya and Miss Pajaree Inthachub for discussion and identification of the plant samples, and Mr. Panupon Khumsupan for his kind help on proofreading the manuscript.

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