Development of tlc/densitometry for quantitative determination of flavonoid m from marigold (*Tagetes erectus* L.) leaf powder and its frap activity[a]

การพัฒนาวิธีวิเคราะห์ปริมาณสารฟลาโวนอยด์เอ็มในตัวอย่างใบดาวเรืองและฤทธิ์ต้านอนุมูลอิสระวิธี frap

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Abstract

The objective of this study was to develop quantitative analytical method for active antioxidant 'Flavonoid M', (5, 3', 4'-trihydroxyflavone 7-C-glucoside); in Marigold (*Tagetes erectus* L.) leaf by TLC/Densitometry. Parameters for analytical method according to APVMA (Australian Pesticides &Veterinary Medicine Authority) were determined for validity of the designed method. Application of developed method was used to determine the content of active flavonoid M in dried leaf powder and dried ethanol leaf extract. Ferric reduction power assay (FRAP) of ethanol extract and flavonoid M was also determined compared to standard 3-hydroxyflavone. Result showed that the developed analytical method had validation parameter conforming to APVMA criteria (2004). 1.0 g marigold leaf powder contains 7.59-12.04 mg flavonoid M and dried leaves extract contained 20.4 mg% w/w flavonoid M. For ferric reduction power assay (FRAP), flavonoid M had the slightly lower potency than standard 3-hydroxyflavone (EC_{50}: flavonoid M 42.46 μg/ml, 3-hyroxyflavone 30.37 μg/ml, ethanol leaf extract 74.96 μg/ml)

Keywords: Validation of analytical method, TLC/Densitometry, Flavonoid M, 5, 3', 4'-trihydroxyflavone 7-C-glucoside, Marigold (*Tagetes erectus* L.) leaf, Ferric reduction power Assay (FRAP)

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บทคัดย่อ

รายงานการวิจัยเป็นการพัฒนาวิธีวิเคราะห์ปริมาณสาร Flavonoid M (5, 3', 4'-trihydroxyflavone 7-C-glucoside) ที่แยกได้จากสารสกัดใบดาวเรืองโดยใช้เครื่องมือวิเคราะห์คือ TLC/Densitometer เกณฑ์ในการพิจารณาคุณภาพของวิธีวิเคราะห์ที่พัฒนาขึ้นยึดตาม APVMA criteria (2004) ซึ่งเป็นเอกสารแนะนำการพัฒนาวิธีวิเคราะห์สำหรับสารเคมีที่มีฤทธิ์ที่ได้มาจากผลผลิตทางการเกษตรหรือจากพวกปศุสัตว์ โดยรัฐบาลของประเทศออสเตรเลีย โดยมีการกำหนดหัวข้อทดสอบสำหรับวิธีวิเคราะห์ที่พัฒนาขึ้น ได้แก่ ความได้กลับคืนจากการวิเคราะห์ (Accuracy) ความแม่นยำของวิธีวิเคราะห์ (Precision) ความจำเพาะเจาะจงแก่สารที่จะวิเคราะห์ (Specificity) ช่วงความสัมพันธ์ที่เป็นเส้นตรงระหว่างปริมาณสารกับสัญญาณที่ได้จากเครื่องมือ (linearity & range) ค่าต่ำสุดที่วิเคราะห์ได้แม่นยำ (Limit of quantitation) ค่าต่ำสุดที่เครื่องน่าจะตรวจพบได้ (Limit of detection) ซึ่งนอกจากหัวข้อดังกล่าว การวิเคราะห์ที่พัฒนาขึ้นยังแสดง ข้อมูลด้านความไว (Sensitivity) ของเครื่องมือด้วยการใช้สารปริมาณน้อยให้เครื่องตรวจวัด ทำให้พบข้อมูลที่น่าสนใจอีกประการหนึ่งว่า การใช้ความสูงของ peak ที่ได้จากเครื่อง นั้นมีความแปรผันน้อยกว่าการใช้สัญญาณของพื้นที่ของ peak ผลการวิเคราะห์ข้อมูลสำหรับสาร Flavonoid M กับเครื่อง TLC/Densitometer พบว่า วิธีวิเคราะห์ที่พัฒนาขึ้นได้มาตรฐานตามเกณฑ์ของ APVMA2004 ที่กำหนดไว้ เมื่อนำวิธีที่พัฒนาขึ้นไปวิเคราะห์หาปริมาณ Flavonoid M จากสารสกัดแอลกอฮอล์เหลวจากส่วนใบของดาวเรือง และสารสกัดเหลวที่ทำให้แห้งที่เตรียมจากใบดาวเรือง พบว่าปริมาณ Flavonoid M จากทั้งสองแหล่งให้ผลไม่แตกต่างกันในทางสถิติที่ระดับนัยสำคัญ 0.05 (T-test: P=0.001, F-test: 0.021) วิธีที่พัฒนาขึ้นนี้จึงสามารถใช้ในการควบคุมปริมาณ Flavonoid M จากทั้งสารสกัดเอทานอลเหลวภายหลังสกัดได้จากพืชโดยตรง หรือจะวิเคราะห์ในรูปสารสกัดแห้ง ก็สามารถทำได้ รายงานยังได้ยืนยันว่าสาร Flavonoid M มีฤทธิ์เป็น antioxidant เหมือนสารฟลาโวนอยด์โดยทั่วไป โดยใช้วิธี FRAP method ผลการทดสอบพบว่าสาร Flavonoid M มีฤทธิ์รีดักชันเฟอริกไอออนได้ใกล้เคียงกับสารสังเคราะห์ 3-hydroxyflavone โดยทดสอบร่วมกับสารสกัดหยาบร่วมด้วย แม้จะยืนยันฤทธิ์ antioxidant เพียงวิธีการเดียว แต่วิธีการทดสอบฤทธิ์ FRAP ดังกล่าวเป็นวิธีใหม่ที่ทดสอบด้วย 96 well plate ที่พัฒนาขึ้นจากวิธีเดิมของ Ferreira ICFR นอกจากนี้ผลวิเคราะห์ยังยืนยันได้ว่า สาร Flavonoid M นั้นเป็นสารหลักที่ออกฤทธิ์ในสารสกัดเมื่อทดสอบด้วยวิธี FRAP เพราะสารดังกล่าว (ตัวเดียว) มีฤทธิ์แรงกว่าสารสกัดจากใบ (มีสารผสมหลายตัว) ถึง 1.76 เท่า

คำสำคัญ: การพัฒนาวิธีวิเคราะห์ปริมาณสารสำคัญ, TLC/Densitometry, Flavonoid M (5, 3', 4'-trihydroxyflavone 7-C-glucoside), ใบดาวเรือง (*Tagetes erectus* L.), Ferric reduction power Assay (FRAP)

Introduction

Concept about validation of analytical instrument was very important in pharmaceutical science due to the safety aspect not only in detection of toxic substances in environment, or in work place of the factory, but also in controlling the concentration of active substances (drug) of various pharmaceutical products. For development of analytical method, hierarchy of methodology (Taylor 1983) was suggested from technique (scientific principle providing compositional information), method (distinct adaptation of a technique for a selected measurement purpose), procedure (directions necessary to use a method) and protocol (a set of definitive directions). For known substances, a valid protocol could be searchable in United State Pharmacopeia (Authority of the United States Pharmacopeial Convention 2011), International Conference on Harmonization (ICH) or Food and Drug Administration (FDA) for analysis to follow (Green 1996), but for a new synthetic substance or active phypharmaceutical product (chemical found in plant material), analytical method of selected instrument should be developed and validated for validity of any new analytical procedure (Suntornsuk 2005).

APVMA (2004) recommended the acceptable validation parameters such as selectivity (ability to assess that compound unequivocally i.e. impurities), linearity (linear relationship between response and compound's concentration), range (80-120% of concentration in sample), limit of detection; LOD (the lowest amount of compound that can be detected) and limit of quantitation; LOQ (the amount of compound that can be determined with acceptable precision and accuracy under certain condition).

'Flavonoid M', (5, 3', 4'-trihydroxyflavone 7-C-glucoside); was a new substance occurencely found from leaf of marigold (*Tagetes erectus* L.) (Chanut 2013), and an active antioxidant principle by ferric reduction power assay, was selected for development for method of its content analysis by TLC/densitometric method. Necessary parameters of method validation for flavonoid M were determined and compared with acceptable value from APVMA guideline including content analysis this active in dried leaf powder and dried ethanol leaf extract of Marigold. This article also showed the evidence that Flavonoid M was the substance responsible for antioxidant activity (FRAP) for its leaf alcoholic extract.

Materials and methods

1. Validation of analytical method. Flavonoid M was prepared to be concentration of 1.0 μg/μl, and then triplicately diluted to be final concentration of 0.125, 0.250, 0.375, 0.500 and 0.625 μg/μl in 96 well plate using methanol AR as solvent.

1.1 Sensitivity. On activated 20x20 cm thin layer chromatography (TLC; Aluminium sheet; Merck, Germany); pre-developed with methanol AR, the five diluted concentrations of flavonoid M were duplicately spotted for 2 μl for each concentration on TLC plate. Then TLC was developed with dichloromethane: methanol: ethyl acetate (5: 0.95: 13.9) for 190 mm. The air-dried solvent TLC was analysis for measuring absorbance at 360 nm by Scanner (CAMAG densitometry) for all detectable spots on the plate.

1.2 Linearity, range, precision, accuracy, LOD and LOQ. The second 20x20 cm TLC plate was used for APVMA parameters. Five prepared concentrations of flavonoid M (0.125, 0.250, 0.375, 0.500 and 0.625 μg/μl) were applied 9μl volume (triplicately for each concentration) on TLC plate. The TLC was developed with the same solvent system as described in 1.1. The air-dried solvent TLC was measured for absorbance at 360 nm for all detectable spots on the plate. The detected signal was used as data for accuracy, linearity, range, precision LOD and LOQ.

2. Content determination of flavonoid M

2.1 Content in dried leaf powder. Dried and cut Marigold leaf was weighed for 2.0 g in 25 ml Erlenmeyer flask for three samples, then extracted with 20 ml methanol AR in ultrasonic bath at 40°C for 0.25 hr. Clarified leaf solution was obtained by filtered with whatman no.1 under reduced pressure, then 100 μl of three prepared solutions were transferred into 96 well plate, two wells for each prepared solution. The third 10x10 cm TLC plate was used and spotted by 9 μl of 0.250 μg/μl flavonoid M solution and 9 μl of three leaf sample solution (2 spots for each sample). The solvent system as described in 1.1 was used for developing plate for 90 mm. The air-dried solvent TLC was measured for absorbance at 360 nm for all detectable spots on the plate, then content of active was calculated.

2.2 Content in dried ethanol leaf extract. Dried and cut Marigold leaf was weighed for 2.0 g in 25 ml Erlenmeyer flask for three samples, then extracted with 3x10

ml ethanol AR in ultrasonic bath at room temperature for 0.1 hr. Three clarified leaf solutions of each sample, obtained by filtering with whatman no.1 under reduced pressure, were combined into one known-weight evaporating disc and evaporated to dryness on water bath (95°C). Three dried ethanol leaf extracts were reweighed for their yields. For content analysis, the extract solution of each sample was prepared at 2.0 mg in 1.0 ml methanol AR (using ultrasonic bath for clear solution). 100 µl of each prepared extract solution was triplicately transferred into 96 well plate and adjusted to final 200 µl volume. On prepared 10x10 cm TLC plate, 9 µl of nine diluted extract solutions (from three samples) were spotted compared to 9 µl of flavonoid M (3 spots). After developing plate and scanning for absorbance at 360 nm, the content of active was calculated.

2.3 Comparing of flavonoid M between method 2.1 and 2.2. Flavonoid M content (mg) in 1.0 Marigold dried leaf powder from method 2.1 and 2.2 was calculated and statistically compared by F-test and T-test by Microsoft Excel 2003 Software.

3. Antioxidant activity by ferric reducing power assay (FRAP). The ethanol leaf extract of Marigold, flavonoid M and standard 3-hydroxyflavone were prepared for three different concentrations (12.5, 25.0 and 50 µg/ml) by 40% ethanol AR (200 µl final volume). A solution of ferric chloride (0.1% w/v) was mixed with the testing sample (100 µl: 100 µl) in 96 well plate triplicately. All mixtures were incubated at 42°C for 1,000 sec. After incubation, 50 µl of each mixture was transferred and mixed with 150 µl trichloroacetic acid (26% w/v) in another plate. At last 66 µl of mixture solution was mixed with 66 µl of 0.2 M Sodium phosphate buffer (Helmenstine 2012) and 66 µ of potassium ferric cyanate (1% w/v) in the third 96 well plate. All mixture on the third plate was measured for absorbance at 700 nm by microplate reader compared to mixture of 66 µl $FeSO_4.7H_2O$ (0.125, 0.100, 0.075, 0.050 and 0.025 mM), 66 µl distilled water and 66 µl potassium ferric cyanate (1% w/v). Percentage of production of $FeSO_4.7H_2O$ and EC_{50} of leaf extract, flavonoid M and standard 3-hydroxyflavone were calculated.

Results

1. Validation of analytical method.

1.1 Sensitivity. From all ten spots of five concentrations, only seven spots (R_f: 0.14-0.16) were detectable into two different signal types; max. height and area by instrument. Noticeably, the standard deviation of max height (2.0338) was much lower than area (21.6225) so max height was selected for flavonoid content analysis.

1.2 Linearity, range, precision, accuracy, LOD and LOQ. Crude data form flavonoid absorbance was not shown. All validated parameters according to APVMA (2004) were shown in figure 1 and table 1.

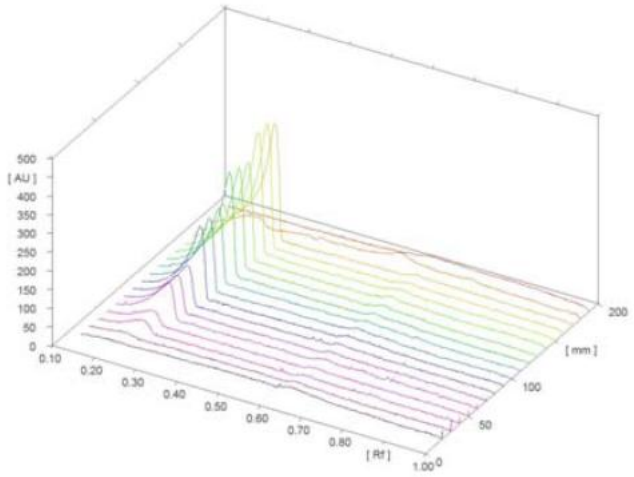

Figure 1 Absorbance at 366 nm of 9 µl 'Flavonoid M' at 0.125 – 0.625 µg/µl

Table 1 Validated parameters of TLC/densitometer for flavonoid M

Parameters	Results	APVMA 2004	Conformity
Accuracy (shown by %Recovery)	90.42% (88.76-91.42%)	90-110% (≥1% active)	Pass
Precision (shown by %RSD)	1.93% (0.66-5.39%)	≤ 5% (1.0 up to 10% active)	Pass
Specificity	R_f value 0.15	Present	Pass
Limit of detection	0.0115 µg/µl	Not required	Addition
Limit of quantitation	0.0347 µg/µl	Not required	Addition
Linearity (shown by least square analysis)	R = 0.9970	R > 0.99	Pass
Range of linearity *(compared to lower level)	84.5 – 118.4%*	80-120%	Pass

2. Content determination of flavonoid M. Raw data for flavonoid content determination were not shown. Calculated content of flavonoid M from method 2.1 and 2.2, statistical analysis for the difference between these two methods were shown in table 2.

Table 2 Determination of flavonoid M content by developed method

Method Analysis	mg Flavonoid M in 1.0 g dried leaf powder	Mean (mg)	Difference analysis (p>0.05)	
			F-test/T-test	Interpretation
2.1	12.888, 11.956, 12.944, 10.381	12.04	F-test: 0.021	No difference
2.2	7.738, 7.582, 7.492	7.60	T-test: 0.001	No difference

3. Antioxidant activity by ferric reducing power assay (FRAP). All three test samples; Marigold leaf extract, flavonoid M and 3-hydroxyflavone (standard) could produce ferrous ion from ferric ion and this FRAP activity of these test samples had linear relationship (R^2 > 0.90) with their concentrations, so effective concentration at 50% could be calculated as shown in table 3. When comparing of FRAP potency by EC_{50} value of three test samples, it could order as 3-hydroxyflavone (1-fold) > flavonoid M (1.40-fold) > leaf ethanol extract (2.47-fold).

Table 3 FRAP activity of leaf extract and flavonoid M compared to 3-hydroxyflavone

Test sample	Concentration (μg/ml)	% Production of $FeSO_4.7H_2O$	Linear coefficient	EC_{50} (μg/ml)
Leaf extract	12.4	0.54	R^2 = 0.9996	74.96
	24.8	9.81		
	46.9	29.97		
Flavonoid M	12.6	1.63	R^2 = 0.9794	42.46
	25.2	14.44		
	50.4	65.94		
3-Hydroxyflavone	12.2	4.90	R^2 = 0.9596	30.37
	25.2	21.52		
	49.6	111.44		

Conclusion

In this article, we tried to develop analytical method for flavonoid M; the new compound isolated from leaf Marigold, and result showed that newly developed method by TLC/CAMAG densitometry had validated parameter conformed to APVMA 2004 (for active range 1-10%). From content analysis, two different methods were designed and result showed that content of flavonoid M by whether directly analysis from liquid extract (method 2.1) or analysis from dried, prepared extract (method 2.2) was not significantly different. We also showed the activity of flavonoid M by FRAP method to confirm that this compound also contained antioxidant property comparable to standard 3-hydroxyflavone. So it could be inferred that Flavonoid M was responsible for antioxidant (by FRAP method) of the leaf extract due to its more potent (1.76-fold) than the original leaf extract. For validation method of Flavonoid M, three procedures guided by" Basic Education Commission (OBEC)" were performed; 1) study factors, 2) analyze factors used in research and 3) study the best practices (Wipaporn 2012). It was evidently that Thai GMP certificated companies were not developed their analytical method by themselves (Piyarosv 2008). So this article might activate Thai drug companies for more interested in development of drug's analytical method by their own.

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