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Research Article

Validation Assay of Total Flavonoids Content in *Ipomoea batatas* L., as Rutin Equivalent, by Using Thin Layer Spectrophotodensitometry

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ABSTRACT

Total flavonoid content (TFC), as rutin equivalent is the most determinant parameter for the antioxidant potency of herbal medicine. The dry spot of the 2,2-diphenylpicryl-1-hydrazyl (DPPH) colorimetric complex reaction has been used to quantify the scavenging activity of herbal medicine potential antioxidant. The *Ipomoea batatas* L is potential antioxidant herbal medicine, has rich flavonoid and has high potency for anti-diabetic and dyslipidemia effects. Aim of this study was to develop a densitometry alternative method for TFC estimation. The precision instrument of both rutin and extract dry spots were ranged 0.4-1.3%. The repeatability and intermediary of Al-flavonoid dry spot were 2.8 – 5.8 % and 3.3 - 4.9%, respectively. These relative high RSD values were donated by variability of the complex reaction time. On the other hand the relative process standard deviation were lower the 5% and the regression coefficients of linear calibration curve were 0.961 – 0.986, with linear range measurement of 10 – 75 ng/spot. The TFC densitometry determination method obtained similar result to the UV spectrophotometer. The densitometer delivered more sensitive method then UV spectrophotometer.

Keywords: Total flavonoid content; densitometry assay; *Ipomoea batatas* L.; comparison test.

INTRODUCTION

Ipomoea batatas L. has reported to have pharmacological effects as anti hyperglycemia[1]. The Anthocyanin was presumed as active compound of these effects. The total flavonoid content (TFC) is the determinate parameter for evaluating food or herbal medicine samples. The Al-flavonoid complex reaction in wet media is one analytical method to determinate the TFC on sample. This method firstly is proposed by Christ and Mueller (1960) and has been used for systematic flavonoids identification in both aquatic solution or on thin layer chromatography (TLC) spot-staining[2]. The Al-flavonoid complex introduced similar UV spectra between solution and TLC media[2]. The spectrophoto-densitometric dry spot analysis method has been developed to quantify the 2,2-diphenylpicryl-1-hydrazyl (DPPH) scavenging activity of herbal medicine potential antioxidant without any chromatographic development[3]. This introduces an alternative method to quantify the dry spot of Al-flavonoid complex reaction on TLC plat in propose to estimate TFC of herbal medicine.

The colorimetric reaction between $AlCl_3$ forms complexes with the C-4 keto group and either the C-3 or C-5 hydroxyl group of flavones and flavonols, and also with the ortho-dihydroxyl groups in the A- B- ring of flavonoids. This complex reaction is usually applied in presence of acid or acetate solution and the absorbance measure after 2-60 minutes incubation at 404-430 nm[2]. The quercetin, galangin, and rutin were used as the standard compounds to predict TFC of sample.

This research is intended to develop and validate a simple, precise and low cost analytical method for determination of total flavonoids contain in *Ipomoea batatas L*, as rutin equivalents, based on thin layer densitometry. It focused on the parameters validation based on ICH harmonization.

MATERIALS AND METHODS

Materials

All chemicals were used of analytical grade, such as: methanol (Malincord-China); ethanol, AlCl3, sodium acetate, and Al-TLC si GF254 plate (Merck-Germany); and rutin (Sigma Aldrich-Germany); the purple sweet potatoes tuber from Karangasem – Bali.

Preparation of Standard Solutions and Samples

Rutin (10 mg) was accurately weighed, transferred into 25 mL volumetric flask and dissolved in 25 mL of absolute ethanol. This solution was further diluted with absolute ethanol into final concentration of 10, 15, 25, 50, and 75 µg/mL.

The 10 µL of each rutin solutions were mixed w 86 µL of absolute ethanol, 2 µL of 10% AlCl3 in absolute ethanol, and 2 µL of 1 M sodium acetate in absolute ethanol. The colorimetric reaction was incubated for 30 minutes before spotted onto the TLC-plate. The each level concentration of Al-rutin complex reaction was conducted in six plicate.

The 150 µL of each rutin solution in ethanol was mixed with 450 µL ethanol, 30 µL of 10% AlCl3, 30 µL of 1M sodium acetate, and 840 µL of distillate water. Each of mixture reaction was done in six replicate and there absorbance were measured at 413 nm by using 300 µL micro cuvette.

The 200 g of purple sweet potatoes tumber was extracted with 400 mL of 70% ethanol for 24 hours. The filtered extract was dried with help of vacuum rotary evaporator. The 200 mg dried extract was diluted with 25 mL of absolute ethanol. The Al-flavonoid complex reaction for the thin layer densitometry and UV-spectrophotometery determination was conducted in same manner as the Al-rutin complex reaction. Rutin was used to make the calibration curve.

Validation method

The method was validated in accordance with ICH Q2 (R1) guidelines for method validation. The method was validated for linearity, accuracy, intra-day precision, the decision limit (CCα), the capability of detection (CCβ), and the quantitation limit (QL).

Thin layer densitometry analysis

The 10 µLof Al-rutin complex reaction after 30 mintes incubation time was applied to the TLC plate with semi auto sample applicator (Camag, Muttenz, Switzerland) as band length of 3.5 mm, the start position x axis was 10 mm, the distance between tracks was 6.2 mm, the first application position y axis was 10 mm, shifting up by 10 mm increments with every new run of mixture reaction. The spot was scanned by absorption mode at both 396 and 413 nm. The scanner was set for maximum light optimization with slit length of 80% of band length spot [4], slit dimension 3 x 0.5 mm, scanning speed 20 mm/second, and data resolution 100 µm/step. The in-situ spectrum of spot was recorded in range of 250 – 500 nm with slit dimension 4 x 0.30 mm, resolution optimize optical system, scanning speed 100 nm/second, data resolution 1 nm/step, and automatic reference spectrum. The validation method was done as well as on the spectrophotometer method. The 10 µL extract of Al-flavonoid complex reaction of purple sweet potatoes tuber was spotted onto the plat in the same way as Al-rutin compex reaction.

Table 1: Validation result of Al-rutin complex-densitometry determination method

Concentration	Precision Instrument (Mean area, RSD, %)			
Rutin (100 ng/spot, n=3)	4826.8 (0.5)			
Sample (40 ng/spot, n=6)	10781.9 (1.1)			
Sample (80 ng/spot, n=6)	14994.2 (0.4)			
Sample (120 ng/spot, n=6)	20212.9 (1.3)			
Concentration	Precision			
	Day 1 (n=6)	Day 2 (n=6)	Day 3 (n=6)	Intra-Day (n=18)
	Mean area (RSD,%)	Mean area (RSD,%)	Mean area (RSD,%)	Mean area (RSD,%)
10 ng/spot	779.5 (4.1)	759.4 (4.8)	787.3 (5.8)	775.4 (4.9)
75 ng/spot	3293.3 (2.4)	3289.6 (2.8)	3269.5 (4.7)	3284.1 (3.3)
Linear regression	Calibration curve (level concentrations=5, n=6)			
	Day 1	Day 2	Day 3	Intra-Day
Linear equation	y = 596.2 + 37.1x	y = 369.2 + 36.5x	y = 394.0 + 37.0x	y = 453.2 + 36.8x
r	0.981	0.961	0.981	0.961
The relative process standard deviation	0.22 %	0.50 %	0.33 %	1.16%
CCα (95% one side, ng/spot)	5.41	7.91	5.47	7.72
CCβ (95% one side, ng/spot)	10.83	15.83	10.94	15.45
QL (ng/spot)	16.24	23.74	16.42	23.17
Trueness (n=3, ng/spot)	Accuracy (n=3)			
	Absolute bias (ng/spot)	Relative bias (%)	Recovery (%)	
20	-1.36 ± 1.36	-6.82 ± 6.78	93.18 ± 6.78	
30	2.73 ± 0.49	9.09 ± 1.63	109.09 ± 1.63	
40	-1.36 ± 1.16	-3.43 ± 2.90	96.59 ± 2.90	

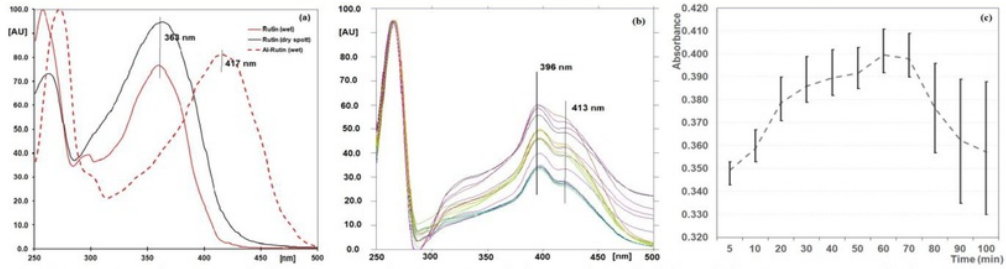

Figure 1: UV-spectra of rutin and Al-rutin complex in wet and dry medium (a-b) and the time course of Al-rutin complexe – UV absorbance (c).

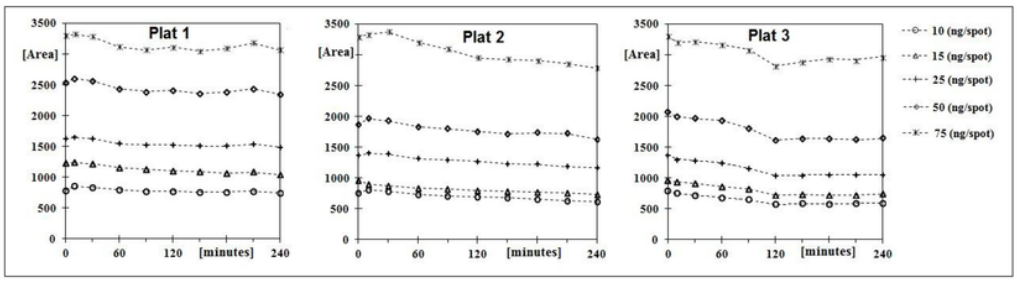

Figure 2: The stability of Al-rutin complex on the TLC plates

RESULTS AND DISCUSSION

Fig.1. presented UV-spectra of rutin and Al-rutin on wet and dry media. The similarity of rutin spectra between both media was 92.03 % and they presented same maximum peak at 363 nm. The λ_{max} of Al-rutin was observed in 417 at wet media (with 54 nm bathochromic shift) and 396 nm (with 33 nm bathochromic shift) at dry media. The Al-flavonoid complex reaction on TLC plat has been used for qualitative identification of the flavonoid[2]. The in-situ UV spectra indicated the quantitative assay of flavonoid content, as rutin equivalent, is possible.

The time course of Al-rutin complex-UV absorbance described in Figure 1.(c). The absorbance intensity increased from first time reaction and reached maximum at 60 minute, afterward the absorbance elapsed gradually. The absorbance was relative stabile between 20-50 minutes. This range time was used for TFC determination. The maximum and minimum absorbance gaps was 0.010 at first 5 minutes, then widened to 0.02 from 10 to 70 minutes. These gaps continued to expand to 0.058 in the 100th minute. The reaction time has been reported as an influenced factor, to form the Al-flavonoid complex[5,6]. The solvent media also contributed errors on the TFC determination[5]. The reaction time between 20 and 50 minutes delivered relative constant of absorbance and the constant gaps of absorption measurement. This range time provided minimum quantification error.

The precision instrument-RSD values of densitometry method were ranged from 0.4 – 1.3%. Area measurement of both rutin and sample without complex reaction were precise. The repeatability and intermediary precision of Al-rutin complex was ranged between 2.8 – 5.8 % and 3.3 - 4.9%, respectively. While no statistic different was observed on the intermediary precision. The p-values for the intermediary ANOVA were 0.456 for 10 ng/spot and 0.945 for 75 ng/spot, respectively. The inter-day precision-RSD values of UV-spectrophotometer method were ranged between 3.26 and 4.25. These high repeatability and intermediary RSD-values may be induced by the time reaction of Al-flavonoid complex formation.

The linear range measurement of densitometry method was 10-75 ng/spot, with the relative process standard deviation was ranged 0.22 – 0.5%. This described, that the method delivered god precision measurement responses among concentration data sets. The decision limit of densitometry was 5.41-7.91 ng/spot and the detection limit was 16.24 -23.74 ng/spot. This indicated the sensitivity of the method was adequate.

Table 2: The Verification results of Al-Rutin complex UV spectrophotometry method

Concentration	Precision (n=6, mean absorption, RSD, %)		
10 µg/mL	0.040 (4.12)		
25 µg/mL	0.080 (4.25)		
75 µg/mL	0.245 (3.26)		
Linear regression	Calibration curve (level concentrations=5, n=6)		
function	y = 0.032 + 0.0037x		
r	0.994		
Precision process	4.58		
CCα (95% one side, µg/mL)	3.33		
CCβ (95% one side, µg/mL)	6.66		
QL (µg/mL)	10.00		
Trueness (n=3, ng/spot)	Accuracy (n=3)		
	Absolute bias (µg/mL)	Relative bias (%)	Recovery (%)
20 µg/mL	0.067	0.333	100.3
30 µg/mL	0.017	0.061	100.1
40 µg/mL	0.917	2.290	102.3

The area of Al-rutin complex spots on TLC tended to decrease for 4 hours stored in room temperature (see Fig. 2). The p-values of pair t-test of the peak areas among concentration data sets between the initial spotting time and after 4 hours of storage were 0.504 for plate 1, 0.224 for plate 2 and 0.216 for plate 3, respectively. These showed there were significant different. The Al-flavonoid complex on the dry media was relative stabile.

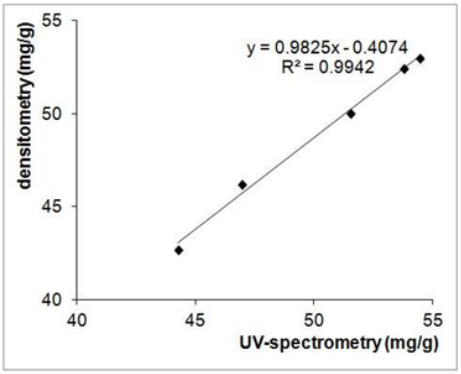

Figure 3: The comparison test of TFC determination

The TFC determination results of 5 samples *Ipomoea batatas L*. extract by using both methods are presented in the Fig. 3. The comparison test of TFC determination with both methods governed linear regression slope of 0.9825 and the R^2 of 0.9942. The both methods delivered adequate similar results.

CONCLUSION

The TFC densitometry determination method obtained similar result to the UV spectrophotometer. The densitometry delivered more sensitive method then UV spectrophotometer.

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