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VALIDATION OF STABILITY INDICATING CHROMATOGRAPHIC METHOD FOR THE DETERMINATION OF VITEXIN IN *PASSIFLORA FOETIDA* LEAF EXTRACT

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KEYWORDS: Vitexin, HPLC, Method development, Method validation

INTRODUCTION

Vitexin is a C-glycosyl flavonoid (5, 7, 4-trihydroxyflavone-8-glucoside, Figure 1) found in *Passiflora* species[1]. It has various pharmacological activities such as hypotensive and anti-inflammatory action[2]. Recently, vitexin was shown to protect against myocardial ischemia/reperfusion injury in rat hearts[3] and possess neuroprotective effects[4].

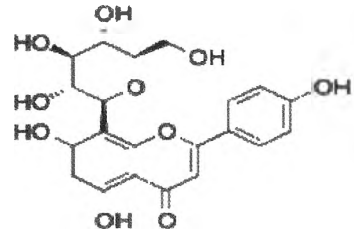


Figure 1 Chemical structure of vitexin

There are chromatographic methods available for analysis of flavonoids in *Passiflora* species[5-6]. However, our preliminary work suggested that the established method required some modification in order to characterize the content of vitexin in *Passiflora foetida* leaf extract. The aim of this study was to develop and validate high performance liquid chromatographic (HPLC) method for the determination of vitexin in *Passiflora foetida* leaf extract.

MATERIALS AND METHODS

Materials: Vitexin (Lot BCBF7415V, 96.1% purity, Fluka, Buchs, Switzerland), methanol (HPLC grade, Burdick & Jackson, Korea), acetonitrile (HPLC grade, RCI Labscan, Bangkok, Thailand), sulfuric acid (Mallinkrodt, Phillipsburg, USA), Ultra pure water.

Method and chromatographic condition:

Instruments and analytical column

HPLC system consisted of Waters model Alliance 2695 equipped with a photodiode array detector model 2998 (Water Corporation, Micromass UK Ltd., Manchester, UK). The chromatographic method was developed using ACE® C-18 columns (250 mm length x 4.6 mm diameter, 5 μm particle size, Advanced Chromatography Technologies, Aberdeen, Scotland), operating at 45°C.

Chromatographic conditions

The mobile phase consisted of 0.06% sulfuric acid, methanol and acetonitrile of which gradients were shown in Table 1. A mobile phase flow rate of 1 mL/min and an injection volume of 20 μL were applied. A photodiode array detector was used with the monitoring wavelength set at 350 nm.

Table 1 Mobile phase compositions

Time (min)	0.06% Sulfuric acid in water	Methanol	Acetonitrile
0	75	13.5	11.5
16	70	17	13
31	60	18	22
41	75	13	12
60	75	13	12

Preparation of standard solution: Vitexin reference standard solution was prepared at a final concentration of 0.1 mg/mL in methanol

Method validation

***Acid- induced degradation*:** Vitexin reference standard of 10 mg was accurately weighed into a 10 mL volumetric flask. Then, 1N HCl solution was added to dissolve vitexin and adjust to volume. After that the solution was heated at 80°C in water bath for 7 days. One milliliter of the heated solution was then pipetted into a 10 mL volumetric flask and diluted to volume with methanol. The final vitexin concentration was 0.1 mg/mL.

***Base-induced degradation*:** Vitexin reference standard of 10 mg was accurately weighed into a 10 mL volumetric flask. Then, 1N NaOH solution was added to dissolve vitexin and adjusted to volume. After that the solution was heated at 80 °C in water bath for 5 h. One milliliter of the heated solution was pipetted into a 10 mL volumetric flask and diluted to volume with methanol. The final vitexin concentration was 0.1 mg/mL.

***Hydrolysis degradation*:** Vitexin reference standard of 10 mg was accurately weighed into a 10 mL volumetric flask. Then, vitexin was dissolved with water and adjusted to volume. After that the solution was heated at 80 °C in water bath for 7 days. One milliliter of the heated solution was then pipetted into a 10 mL volumetric flask and diluted to volume with methanol. The final vitexin concentration was 0.1 mg/mL.

***Oxidative degradation*:** Vitexin reference standard of 10 mg was accurately weighed into a 10 mL volumetric flask. Then, vitexin was dissolved with 30% H_2O_2 solution and adjusted to volume. After that the solution was heated at 80 °C in water bath for 5 h. One milliliter of the heated solution was then pipetted into a 10 mL volumetric flask and diluted to volume with methanol. The final vitexin concentration was 0.1 mg/mL.

***Photo degradation*:** Vitexin reference standard of 10 mg was accurately weighed into a 10 ml volumetric flask and kept in a photostability chamber for 7 days at 30 °C. Then, vitexin was dissolved with methanol and adjusted to volume. One milliliter of the solution was then pipetted into a 10 mL volumetric flask and diluted to volume with methanol. The final vitexin concentration was 0.1 mg/mL.

The method was validated by evaluation of selectivity, linearity, precision, accuracy. Robustness of the method was evaluated using different batches of HPLC column.

RESULTS

The HPLC method able to analyse the total flavonoids in *Passiflora edulis* fruit pulp[5] was not suitable to characterize vitexin in the *Passiflora foetida* leaf extract. Modification of the analysis method was made with an ACE® C-18 column. Our initial method development using gradient of 1% acetic acid (solvent A) and methanol (solvent B) could not resolve the constitute patterns of the extract (data not shown). The improvement of resolving vitexin chromatogram was achieved by replacing 1% acetic acid with 0.06% sulfuric acid; nonetheless, the change in an acid modifier prolonged the analysis time. Acetonitrile was therefore added to the mobile phase as an organic modifier to shorten the analysis time. The vitexin peak in the *Passiflora* leaf extract chromatogram was separated successfully by using the gradient of a mobile phase comprising 0.06% sulfuric acid in water, methanol and acetonitrile. By comparing the leaf extract chromatogram to the vitexin standard chromatogram [Figure 3 (a)], the apparent retention time of vitexin was around 10 min as shown in Figure 2.

Vitexin was not significantly degraded after heat exposure at 80 °C for 7 days in either 1 N HCl or aqueous solution [Figure 3 (b), (d)]. It also appeared stable after exposure to UV light for 7 days [Figure 3 (f)]. Degradation of vitexin, however, was detected upon exposure to 1 N NaOH and to 30% H_2O_2 at 80 °C for 5 h. The selectivity of the method was supported by the purity of the vitexin peak shown by evaluating purity of the vitexin peak using UV spectrum of vitexin standard as a reference spectrum. Range, linearity, precision, accuracy and robustness of the analytical method were reported in Table 2.

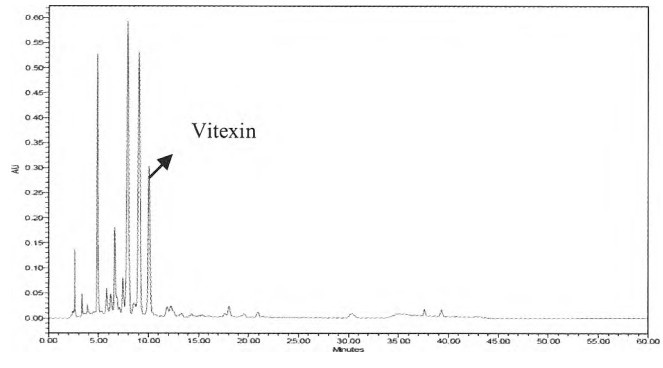
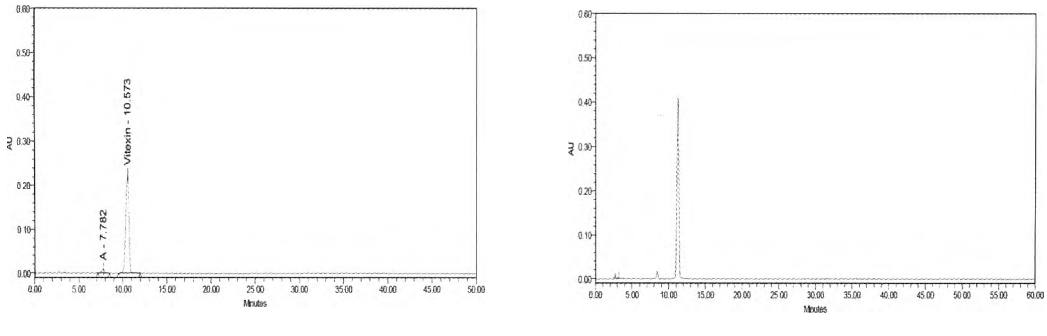


Figure 2 Chromatogram of *Passiflora foetida* leaf extract



(a) (b)

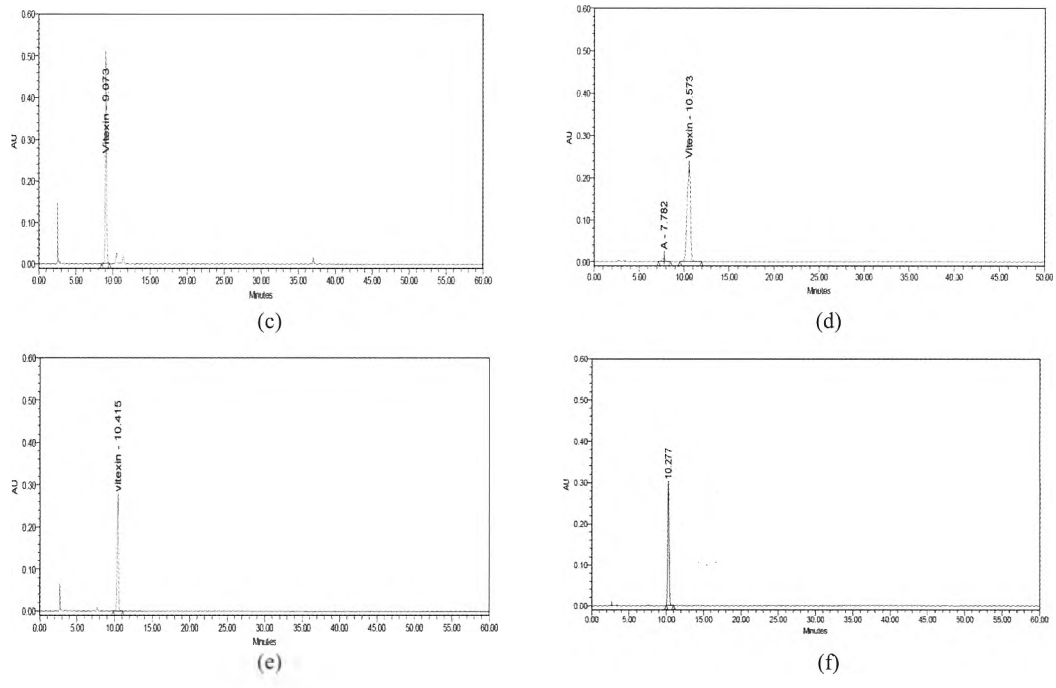


Figure 3 Chromatograms of vitexin (a) and vitexin after exposure to 1 N HCl for 7 days (b), 1 N NaOH for 5 h (c), water for 7 days (d), 30 % H_2O_2 for 5 h (e) and UV light for 7 days (f)

Table 2 Validation data of the developed HPLC method

Parameter	Result
Range	0.05-0.75 mg/mL
Linearity	$R^2 = 0.999$
Recovery (%, n = 3)	
- Concentration at 0.05 mg/mL	98.3
- Concentration at 0.5 mg/mL	99.2
- Concentration at 0.1 mg/mL	89.4
Precision (n=6, 0.5 mg/mL)	
-Repeatability	RSD= 0.13%
-Intermediate precision	RSD= 0.42%
Robustness :	
Column No.1(Batch V10-2985)	
-Retention time:	10.10
-Tailing factor:	1.02
Column No.2 (Batch V10-2768)	
-Retention time:	10.12
-Tailing factor:	1.10

DISCUSSION

In this study, *Passiflora foetida* leaf extract was characterized using vitexin as a marker. The analytical method was successfully developed. The validation results are shown in Table 2. A linear response of vitexin at the concentration between 0.05-0.75 mg/mL was obtained. The method was shown good results of recovery and precision and found to be robust on changing the column batches.

CONCLUSION

The stability indicating assay method was developed and validated to determine vitexin in *Passiflora foetida* leaf extract.

ACKNOWLEDGEMENTS

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