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Journal of Medicinal Materials, 2024, Vol. 29, No. 2 (pp. 98 - 106)

OPTIMIZATION OF PRESSURIZED SOLVENT EXTRACTION USING RESPONSE SURFACE METHODOLOGY AND HPLC ANALYSIS OF VITEXIN FROM *PASSIFLORA FOETIDA* L.

Nguyen Thi Kim Anh[1], Do Thi Thuy Linh[1], Le Xuan Duy[1], Nguyen Phu Quang[1], Phan Thanh Thuy[1], Bui Thi Thuy Luyen[2, *]

[1]National Institute of Medicinal Material (NIMM), Hanoi, Vietnam;

[2]Department of Pharmaceutical Chemistry Technology and Extraction, Faculty of Pharmaceutical Chemistry and Technology, Hanoi University of Pharmacy, Hanoi, Vietnam

*Corresponding author: luyenbtt@hup.edu.vn

(Received November 15th, 2023)

Summary

Optimization of Pressurized Solvent Extraction Using Response Surface Methodology and HPLC Analysis of Vitexin from *Passiflora Foetida* L.

The study aims to optimize the automated sample preparation step using accelerated solvent extraction (ASE) and response surface methodology (RSM) and integrate it with a simple, quick, precise, and accurate high performance liquid chromatography (HPLC) technique for vitexin measurement in *P. foetida*. The RSM results demonstrated that the optimal conditions for vitexin extraction were an extraction temperature of 150.59°C, an extraction time of 19.16 min, and a solvent volume of 140.91 mL; The actual values at the optimal conditions according to the model showed the maximum value of the objective function of vitexin yield was 11.18 ± 0.15 (mg/g). The validated HPLC method was as follows: A mixture of acetonitrile-0.1% formic acid solution with the elution gradients were: 0 - 40 min, 15% acetonitrile; 40 - 45 min, 100% acetonitrile. Other running conditions included the detection wavelength (342 nm), the flow rate (1 mL/min), the injection volume (20 µL), and the column temperature (40°C).

Keywords: *Passiflora foetida* L., *Vitexin, Accelerated solvent extraction (ASE), High performance liquid chromatography (HPLC), Response surface methodology (RSM).*

1. Introduction

With over 500 species, *Passiflora* is the most common genus in the Passifloraceae family [1]. *Passiflora foetida* L. (*P. foetida*) is a plant native to South America that has extended to many tropical locations, including Vietnam regions. The plant has been exploited as an ingredient for traditional medicine related to its bioactive action like antiproliferative, sedative, anti-anxiety, antibacterial, antispasmodic, emetic, dressing for wounds, and antiulcer [2]. In Vietnam, dry leaves are consumed in the form of tea to treat sleep disorders. The major phytochemical constituents of *P. foetida* are chrysoeriol, apigenin, luteolin, kaempferol, isoschaftoside, 2"-xylosylvitexin, isovitexin, and vitexin. Vitexin also has been chosen as the marker for quantitation of *P. foetida* while was shown to have powerful hypotensive, anti-inflammatory, and anti-spasmodic effects [3],[4],[5].

Numerous approaches, including spectroscopic and chromatographic methods, have been used to measure the levels of vitexin in various plants [4],[6],[7],[8],[9]. Among them, high performance liquid chromatography (HPLC) is overwhelmingly the most popular method for determining vitexin. The high precision of HPLC primarily results from its automated processes, except for the sample preparation phase. Even though manual sample preparation methods have been in use for years, a significant number of analytical chemists continue to depend on them, consuming a substantial portion of the overall analysis time. In fact, sample preparation alone

accounts for two-thirds of the total time, surpassing the time required for data gathering, analysis, and management [10]. Undoubtedly, streamlining or automating the sample extraction phase would lead to time savings and enhance the capacity for analyzing samples. Accelerated solvent extraction (ASE) emerges as a promising technique for sample preparation, as it incorporates elevated temperatures and pressures alongside liquid solvents. This combination effectively minimizes extraction time, reduces solvent usage, enhances extraction efficiency, and elevates the quality of the obtained extract [11],[12].

The conventional approach of adjusting one factor at a time while keeping all others constant has various drawbacks, such as its labor-intensive and time-consuming process. An alternative method for enhancing extraction yields is Response Surface Methodology (RSM), a statistical experimental design. RSM involves utilizing mathematical and statistical techniques to identify an optimal functional relationship between a desired response and specific process variables. When combined with the subcritical water extraction technique, RSM enables the development of optimal extraction procedures that are beneficial for both research purposes and industrial applications [13],[14],[15].

The goal of our study is to improve the automated sample preparation step utilizing the ASE system and RSM technique to combine with a simple, quick, precise, and accurate HPLC method for the measurement of vitexin in *P. foetida* and quantification of vitexin content of *P. foetida* in Vietnam region.

2. Materials and methods

2.1. Materials

P. foetida was collected in April 2022 in Thanh Hoa, Nam Dinh, Hanoi and Ha Nam Province, Vietnam. Samples were collected and identified by the Medicinal Material Resource Center, National Institute of Medicinal Materials. The samples were stored at the Department of Extraction Technology, National of Medicinal Material. Stems and leaves of *P. foetida* were dried until moisture content was less than 5% at 55^{0}C, crushed, and sieved through a 2mm sieve to obtain samples of uniform size, then sealed in vacuum bags and stored at -5^{o}C before proceeding to the next steps. The value of moisture content was kept under 10% before further experiments.

2.2. Chemicals and standards

Acetonitrile and formic acid were obtained from Merck (Darmstadt, Germany) and were of analytical grades. Standard vitexin (>95% pure) was obtained from Sigma-Aldrich Chemie (Steinheim, Germany) with batch number BCCG5744.

2.3. Preparation of standard solution

A vitexin stock solution was prepared with methanol to give a series of working standard solutions with concentrations of 5, 10, 25, 50, 100, 200, and 300 µg/mL.

2.4. Sample preparation procedure

The pressured liquid extraction procedure utilized an ASE 350 System (Dionex, Sunnyvale, CA, USA) equipped with a stainless-steel extraction cell. Approximately 1 g of the *P. foetida* sample was mixed uniformly with an equal weight of diatomaceous earth before being placed into the extraction cell. To prevent the powder from entering the extraction bottle, a frit and a filter (Dionex) were positioned at the end of the cell. The extraction cells were organized in a cell tray, and the samples were extracted with ethanol 60% under specific conditions. Subsequently, the obtained extracts were transferred to collection bottles and evaporated to dryness using a rotary evaporator set at 50°C. The resulting dried extract was diluted in 50 mL of methanol and filtered through a 0.45 µm filter in preparation for HPLC analysis.

2.5. HPLC analysis and validation

The HPLC was performed using the Shimadzu SPD-20A system (Shimadzu Co., Ltd., Kyoto, Japan) with a C_{18} column (250 mm × 4.6 mm, 5 µm). According to the literature [7],[16],[17] and our preliminary test, the optimum condition has been described as follows: The mixture of acetonitrile-0.1% formic acid solution was used as the mobile phase. The elution mode was a binary, high-pressure gradient system, and the elution gradients were: 0-40 min, 15% acetonitrile; 40-45 min, 100% acetonitrile. Other running conditions included the detection wavelength (336nm), the flow rate (1 mL/min), the injection volume (20 µL), and the column temperature (40°C).

The optimized HPLC condition was established by comparing the resolution retention time, and asymmetry factor of analyzed peaks in each chromatogram after testing.

The (mg/g) content of vitexin in the test sample (calculated according to dried herbs) is determined using the following formula:

$$X\left(\frac{mg}{g}\right) = \frac{C \times V \times P}{m^*(100-a)} \qquad (1)$$

C: the concentration of the analyzed compound in the sample solution from the calibration curve

equation (µg/mL); V: volume of the sample solution (mL); m: weight of standard taken to prepare the sample solution (mg); a: the moisture of the test sample (%); P: purity of standard (%).

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

2.6. Experimental design

In conjunction with the Box-Wilson central composite design (CCD), the response surface methodology was employed to estimate the effect of 3 parameters (extraction temperature, extraction time, and solvent volume) on the vitexin yield of the extraction process [18]. The procedure for constructing the 15-experiment orthogonal design matrix with the mathematical-statistical treatments and determining optimal conditions was executed using Design-Expert 11.0.0 software. The range of extraction temperature, extraction time, solvent volume, and the central point were selected based on preliminary experimental results. Firstly, we conducted a single-factor investigation of three factors by individually varying one factor while keeping other variables at a fixed value. The obtained sets of values by single factor investigation would then be used in Central Composite Design to produce 15 sets of experiment conditions with a coefficient of α = 1.215 (Table 5 and Table 6). Estimation results would be tested with ANOVA analysis to confirm model validity. Then, optimal conditions were calculated from the final model and verified by an actual experiment attempted. The dependent variables as a function of independent variables were expressed using the following second-orderpolynomial equation:

$$\hat{Y} = b_0 + \sum_{j=1}^{k} b_j X_j + \sum_{u,j=1}^{k} b_{uj} X_u X_j + \sum_{j=1}^{k} b_{jj} X_j^2 \quad (3)$$

where Y is the predicted response; b_0 is the intercept coefficient; b_j is the linear coefficient; b_{jj} is the square coefficient; b_{uj} is the interaction coefficient; X_u and X_j are the coded independent variables, terms of $X_u X_j$ and X_j^2 are the interaction and quadratic terms, respectively.

3. Results and discussions

3.1. Analysis method validation

The detection wavelength for quantification of vitexin was set at 336 nm, based on the maximum absorption observed in the UV-VIS spectrum. This wavelength choice was consistent with Jang's report [17].

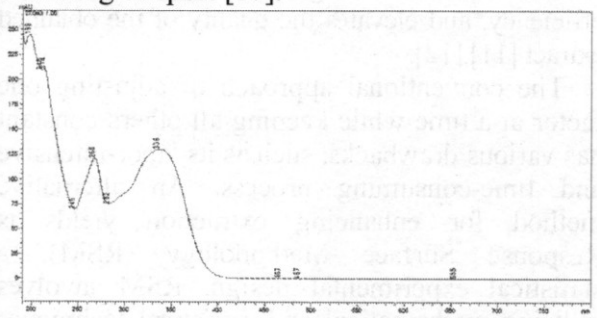


Fig.1. UV spectrum of vitexin

Calibration curve:

Different concentrations of vitexin standard solutions were analyzed to validate the linearity criteria. The result in Fig. 3 showed satisfactory linearity in the concentration ranges of 5 - 300 µg/mL.

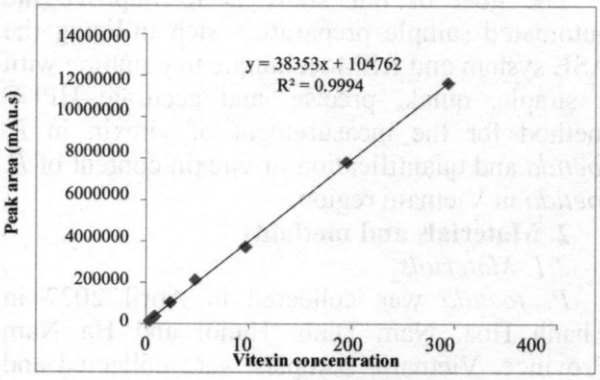


Fig.3. The calibration curves of vitexin

Table 1. Results of validation of the vitexin quantification process

Validation Criteria	Request	Result
Specificity	- The blank sample has no peak at retention time - The retention time and UV spectrum of the Vitexin peak in the test sample must be the same as the retention time and UV spectrum of the Vitexin peak in the standard sample.	Pass
System suitability	Peak area: RSD <2 %(n=6)	RSD = 0.654
	Retention time: RSD < 1% (n=6)	RSD = 0.281
Accuracy	98.0 – 102.0 %	99.732%
Precision	RSD ≤ 2%	RSD=1.35%
The limits of detection (LOD) and limit of quantification (LOQ):	RSDSpic at LOD ≤ 10% LOQ = LOD X 3.3.	LOD: 0.18 µg/mL LOQ: 0.63 µg/mL

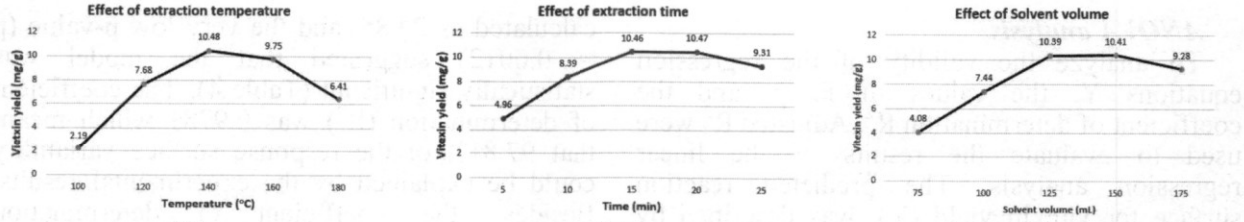
The typical calibration curve equations were $y = 38353x + 104762$ for vitexin ($R^2 = 0.9994$), where y was the area of peak analyzed and x was its concentration (µg/mL). Linear correlation coefficient (R^2) values were above 0.999, as well as each calculated concentration of each calibration point was within ±15% of the true value. Therefore, it can be concluded that this developed method conforms the linearity.

3.2. Single factor analysis

To determine the optimal conditions for extracting vitexin, three factors were examined: extraction time (in minutes), extraction temperature (in °C), and solvent volume (in mL). During the optimization process, one factor was altered while the remaining factors were held constant at predetermined values.

As can be seen in Fig. 4A, the influence of temperature (°C) on vitexin concentration (mg/g) was observed. The temperature was adjusted from 100°C to 180°C, while other extraction variables were set as follows: a solvent volume of 125 mL and extraction time of 15 min. The vitexin yield increased with the temperature up to 140°C and began to drop off, and the maximum concentration value was 10.48 mg/g at 140°C. As the temperature reached 180°C, additional phenolic compounds present in the *P. foetida* extract started to be released from the cellulose plant cell wall. Some of these compounds could potentially affect the detection of vitexin. This experiment aligns with the findings of Juntachote, who observed that higher extraction temperatures facilitate the extraction of phenolic compounds by reducing solvent viscosity and surface tension. This, in turn, enhances the mass transfer of phenolic compounds [19]. However, exposure to elevated temperatures for specific durations can result in a reduction in extraction yield, as the high temperature induces oxidation and degradation of the desired compounds [20]. Therefore, we chose the extraction temperature of 140°C as the base level for further experiments.

Fig. 4B demonstrates the impact of extraction time (in minutes) on the vitexin yield extraction (mg/g). Throughout this series of experiments, the duration was varied from 5 minutes to 25 minutes while maintaining a constant solvent volume of 125 mL and a temperature of 140°C. As the extraction time increased from 5 to 15 minutes, the yield of vitexin steadily rose from 4.96 mg/g to 10.46 mg/g at the 15-minute mark. However, there was a reduction to 9.31 mg/g at 25 minutes. These results indicate that the extraction yield of vitexin increases with longer extraction times within a specific range. It can be seen that initially, increasing the extraction time allowed the solutes to better diffuse into the solvent. However, at a certain time, an equilibrium would be established and even increasing extraction time would not produce any perceptible modification in extraction yield [15]. In contrast, flavonoid or polyphenol compounds were less stable to heat and easily degraded when extraction time was prolonged at high temperatures, which led to a decrease in total flavonoid content. Based on the obtained results, we selected the extraction time of 15 min as the base level for the subsequent experiments.



(A) (B) (C)

Fig.4. Effect of parameters on vitexin yield (A. Temperature (^{0}C) B. Time (min) C. Solvent volume (mL)

Fig. 4C illustrates the extraction yield of vitexin extraction with varying solvent volumes ranging from 75 mL to 175 mL in the third experiment. The extraction temperature and duration were kept constant at 140°C and 15 minutes, respectively. The extraction yields of vitexin showed an increase from 4.08 mg/g to 10.39 mg/g as the solvent volume was raised from 75 mL to 125 mL. These findings align with Pan et al. [16], who reported a similar enhancement in the extraction of polyphenolic compounds with an increased sample-to-solvent ratio. Augmenting the ratio of sample to solvent enlarges the overall extraction surface area,

facilitating the transfer of solutes from the matrix to the solvent. In the present study, the vitexin content did not change significantly when the solvent volume was increased to 150 mL. This could be attributed to the depletion of extractable compounds, where the additional solvent failed to extract any additional vitexin from the sample. Besides, continuing to increase the amount of solvent to 175 mL, the vitexin yield tends to decrease to 9.28 mg/g. Based on the obtained results, to meet the economic efficiency, we chose the solvent volume of 125 mL as the base level for the experiments.

3.3. Predicted model and statistical analysi model setting

Based on the results of the single-factor experiment in section 3.2, we set up the variation range and experimental levels of 3 independent variables including extraction temperature (°C), extraction time (min), and solvent volume (mL) (Table 2). Table 3 included 15 different sets of experimental conditions designed according to the Box-Willson model. The results of the target function Y - Vitexin yield (mg/g) were calculated. These values were the basis for evaluating the compatibility of the model with the experiment.

Table 2. Independent variables and their corresponding levels

Independent Variables	Codes	Available range (Δ)	Levels				
			-α	-1	0	1	+α
Z_1: Temperature (°C)	A	20	115.7	120	140	160	164.3
Z_2: Extraction time (min)	B	5	8.93	10	15	20	21.07
Z_3: Solvent volume (mL)	C	25	94.63	100	125	150	155.37

(*Coefficient α = 1.215*)

Table 3. Experimental design and response values

Run	A	B	C	Y - Vitexin yield (mg/g)	
				Actual value	Predicted values
1	-1	-1	-1	4.99 ± 0.11	4.72
2	+1	-1	-1	5.55 ± 0.09	5.69
3	-1	+1	-1	6.04 ± 0.12	6.45
4	+1	+1	-1	7.82 ± 0.14	7.87
5	-1	-1	+1	5.68 ± 0.11	5.66
6	+1	-1	+1	8.80 ± 0.17	8.41
7	-1	+1	+1	7.89 ± 0.12	7.77
8	+1	+1	+1	10.65 ± 0.13	10.96
9	-1.215	0	0	6.72 ± 0.07	6.73
10	+1.215	0	0	9.34 ± 0.14	9.26
11	0	-1.215	0	7.27 ± 0.09	7.73
12	0	+1.215	0	10.87 ± 0.17	10.33
13	0	0	-1.215	8.15 ± 0.12	7.89
14	0	0	+1.215	10.16 ± 0.13	10.34
15	0	0	0	10.06 ± 0.11	10.19

ANOVA analysis

To analyze the validity of the regression equations Y, the values of F, p, and the coefficient of determination R^2, Adjusted R^2 were used to evaluate the results of the linear regression analysis. The predicted reaction surface for vitexin yield (Y), was described by independent variables expressed by quadratic polynomial equations such as mathematical equation number (4). The ANOVA analysis of the quadratic regression model demonstrated that the model was significant, and fit sufficiently with the experiment data. The F-value for Y was calculated as 24.85, and the very low p-value (p = 0.0012) suggested that the model was statistically significant (Table 4). The coefficient of determination (R^2) was 0.9781 which meant that 97.81% of the response surface variability could be explained by the experimental results. Besides, the coefficient of determination adjustment (Adj R^2) was 0.9388 and Adeq Precision was 15.8683 which were both very large. Based on the above-analyzed data, we could conclude that the objective function Y - Vitexin yield was regressive and compatible with the real tests [21],[22].

Table 4. Regression coefficients of second-degree polynomial models forvitexin yield (Y)

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	51.94	9	5.77	24.85	0.0012*	**Significant**
A-Temperature	11.88	1	11.88	51.14	0.0008*	

Source	Sum of Squares	df	Mean Square	F-value	p-value	
B-Time	12.60	1	12.60	54.23	0.0007*	
C-Solvent volume	11.14	1	11.14	47.96	0.0010*	
AB	0.096	1	0.096	0.41	0.5486^{NS}	
AC	1.56	1	1.56	6.74	0.0485*	
BC	0.069	1	0.069	0.29	0.6075^{NS}	
A^2	9.62	1	9.62	41.44	0.0013*	
B^2	2.67	1	2.67	11.49	0.0195*	
C^2	2.31	1	2.31	9.93	0.0253*	
Residual	1.16	5	0.2323			
Cor total	53.10	14				
R^2	0.9781					
Adjusted R^2	0.9388					
Adeq Precition	15.8683					

** $p<0.05$ (significant); NS = not significant*

From experimental data and the established mathematical model, after removing the non-significant coefficients (p-value > 0.05), the regression equation was determined corresponding to the vitexin yield (Y) as follows:

$$Y = 10.19 + 1.04A + 1.07B + 1.01C + 0.44AC - 1.48A^2 - 0.78B^2 - 0.72C^2 \quad (4)$$

Response Surface Analysis

Based on the experimental 2nd-order polynomial model, the experimental data were analyzed by response surface methodology using Design-Expert 11.0.0 software. The X and Y axes of the three-dimensional response surface represented two extraction factors, and the Z axis was of vitexin yield. The response surfaces were characterized in Fig. 5.

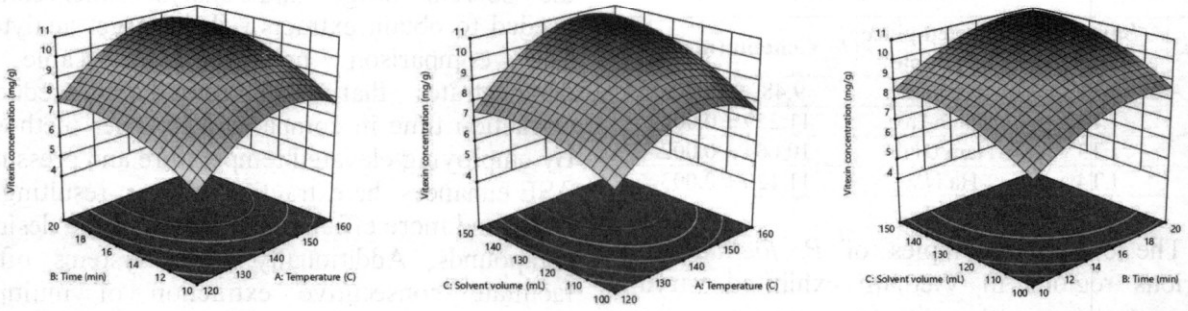


Fig.5. Response surface of vitexin yield (Y)

The response surfaces presented the simultaneous influence of two factors on the value of the objective functions. In Fig. 5, three response surfaces show the pairs of simultaneous interactions of 2 out of 3 extraction factors on vitexin yield. The red and yellow areas on the response surfaces were the optimal region, which contained the largest set of values. Based on the response surfaces, the best variable value range of each extraction factor was determined as follows: extraction temperature from 145 - 155°C, extraction time from 18 - 20 minutes, and solvent volume from 135 - 145 mL. Then the vitexin yield reached the maximum value in the range of 10 - 11 (mg/g). In addition, the positive influence of the pairs of factors of extraction temperature/extraction time, solvent volume/extraction time, and extraction temperature/solvent volume on the Y objective function was equivalent, corresponding to the surface area of the optimal region in the response surface.

Model optimization and verification

The model was optimized with the goal that function Y has the maximum value. The priority of the objective function Y (vitexin yield) was selected at level 5. The predicted values at the optimal conditions according to the model showed the maximum value of the objective function of vitexin yield was 11.26 (mg/g). At optimal conditions, the value of extraction factors was: extraction temperature 150.59°C, extraction time 19.16 minutes, and solvent volume 140.91 mL.

Table 5. The result for optimal extraction conditions

Optimal conditions			Target functions	Experimentalvalue** (mg/g)	Predicted value (mg/g)
A	B	C			
150.59 (°C)	19.16 (min)	140.91 (mL)	Y - vitexin yield	11.18 ± 0.15	11.26

** *Mean value of three experiments (n = 3)*

After a batch of tripled experiments with the optimal conditions, the value of the objective function Y was obtained as shown in Table 5. The results in Table 8 showed that the model for the vitexin extraction from *P. foetida* by the ASE method was confirmed when the predicted and experimental values of the objective functions had a difference of less than 5%. It can be seen that the ASE extraction method had the potential for extracting the active ingredient vitexin from *P. foetida* with the ability to thoroughly and selectively extract it. Short extraction times increased economic efficiency interms of energy.

3.4. Application of the method in the quantification of vitexin in some samples of P. foetida medicinal herbs

Table 6. Content of vitexin in some samples applied with the analysis process

No.	Sample name	Collected place of sample	Content (mg/g)
1	LT1	Ha Nam	9.489 ± 0.005
2	LT2	Thanh Hoa	11.259 ± 0.003
3	LT3	Nam Dinh	10.064 ± 0.002
4	LT4	Ha Noi	11.120 ± 0.003

The collected samples of *P. foetida* from various regions in Vietnam exhibited varying concentrations of vitexin, ranging from approximately 9.489 mg/g to 11.259 mg/g (Table 6). Notably, the samples obtained from Thanh Hoa province (Code LT2) showed the highest concentration of about 11.259 mg/g while the lowest concentration was observed in a sample with approximately 9.489 mg/g. These variations in vitexin concentrations can be attributed to the distinct farming methods, cultivars, and climates in each region of Vietnam, influencing the medicinal samples' vitexin levels.

4. Discussion

The primary objective of sample preparation is to extract the target analyte(s) from the sample matrix, enabling their measurement using the chosen analytical technique, such as LC-MS/MS or GC-MS. However, achieving effective sample preparation comes with numerous challenges, including complicated, time-consuming, and labor-intensive workflows, the potential for introducing errors, increased costs, and concerns about maintaining analyte recovery. Unfortunately, many analytical chemists still resort to using traditional soxhlet apparatus, which has been in use for over 50 years since the first gas chromatograph instruments were introduced. These conventional extraction methods suffer from several drawbacks and result in high expenses for laboratories. The traditional techniques often consume several hours per sample, utilize hundreds of milliliters of solvents for each sample, and require substantial labor and user intervention, thereby prolonging the overall sample preparation process.

ASE systems are ideal for analytical laboratories seeking to enhance the efficiency of their extraction process and significantly reduce the solvent usage and analyst intervention needed to obtain extracts rich in target analytes. The comparison presented in Table 7 demonstrates that ASE remarkably reduces extraction time in comparison to other methods. By employing elevated temperature and pressure, ASE enhances the extraction process, resulting in faster and more efficient extraction of the desired compounds. Additionally, ASE systems often facilitate consecutive extraction of multiple samples in a batch, thereby increasing sample throughput and saving time in the analytical workflow. ASE systems are suitable for any analytical laboratory aiming to optimize their extraction process and minimize the need for extensive solvent consumption and manual intervention, while obtaining extracts with high levels of the desired analytes. Furthermore, the extracts obtained through ASE are generally compatible with various downstream analytical techniques, including chromatography (HPLC, GC), spectroscopy (UV-Vis, FTIR), and mass spectrometry (MS). Furthermore, the natural products industry holds a keen interest in refining the extraction of botanical resources to foster the creation of premium-grade commodities. With this purpose RSM emerges as a formidable technique for investigating the relationship among variables, thereby enhancing processes or

elevating products where multiple factors can impact the resulting outcomes. The RSM model devised within the scope of this study facilitates the identification of optimal parameters, offering applicability not solely within laboratory trials but also holding promise for potential implementation on an industrial magnitude [23],[24].

Table 7. Comparison of time and vitexin yield with reported sample extraction methods.

Extraction Method	Extraction Time (min)	Yield of vitexin(mg/g)	Reference
Shaking Extraction	240	0.554	Zafari, S., & Sharifi, M. (2020)
Sonication Extraction	60	0.463	Zafari, S., & Sharifi, M. (2020)
Ultrasonic Circulating Extraction	28	0.96	Chen, F., Zhang, Q., Liu, J., Gu, H., & Yang, L. (2017)
Aqueous Extraction	240	0.386	Nasma, A., Aishath, N., Azilah,A., & Sulaiman, A. Z. (2018)
Accelerated SolventExtraction	19.16	11.18	This study

Vitexin, the primary flavonoid found in Passiflora plants, particularly *Passiflora foetida* L., serves as a crucial marker for ensuring the quality control of *Passiflora foetida* L.. As a result, numerous analytical protocols have been established to analyze and assess vitexin content in *Passiflora foetida* L. samples. In order to achieve a rapid, convenient, and automated protocol, the accelerated solvent extraction technique has been implemented for sample preparation, which is a crucial aspect influencing the success of the analysis. This analytical process provides valuable insights for assessing the quality standards of medicinal herbs derived from *Passiflora foetida* L., thereby contributing to the evaluation of these herbs used in medicine and available in the market.

5. Conclusion

In conclusion, our study aimed to enhance the automated sample extraction process by integrating the ASE system with optimized conditions using the RSM technique, alongside a simple, rapid, precise, and accurate HPLC technique for quantifying vitexin in *Passiflora foetida* L. This combined approach offers several advantages, including improved efficiency, reduced extraction time, and enhanced analytical performance. By optimizing the sample extraction step and employing HPLC for vitexin measurement, our study contributes to advancing the analytical methodology for assessing vitexin content *Passiflora foetida* L. This advancement holds value for quality control and standardization of medicinal herbs derived from this plant species.

Acknowledgments: *This study was funded by the National Institute of Medicinal Material on the project "A study on the extraction process of a standardized extract containing 10% of flavonoid concentration from Passiflora foetida* L.*". We acknowledge the study participants for their gracious help.*

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Journal of Medicinal Materials, 2024, Vol. 29, No. 2 (pp. 106 - 112)

A QUANTITATIVE METHOD FOR DETERMINING CALYCOSIN-7-*O*-*β*-D-GLUCOSIDE AND FREE ASTRAGALOSIDE IV IN RAW AND HONEY-PROCESSED *ASTRAGALI RADIX* BY HPLC-DAD

Nguyen Thi Ha Ly, Nguyen Thi Hang, Pham Thi Hien, Nguyen Thi Thu, Vu Thi Diep, Tran Thi Huyen Trang, Le Thanh Nghi, Do Thi Ha*

National Institute of Medicinal Materials (NIMM), Hanoi, Vietnam

*Corresponding author: hado.nimms@gmail.com

(Received January 03st, 2024)

Summary

A Quantitative Method for Determining Calycosin-7-*O*-*β*-D-Glucoside and Free Astragaloside IV in Raw and Honey-Processed *Astragali radix* by HPLC-DAD

An HPLC-DAD method was developed to quantify calycosin-7-*O*-*β*-D-glucoside and free astragaloside IV in raw and honey-processed *Astragali Radix* samples. The HPLC analysis was performed on an Agilent C_{18} column (4.6 × 250 mm, 5 µm particle size), with a mobile phase of acetonitrile-0.2% formic acid, the flow rate of 1.0 mL/min, and detection wavelengths of 260 nm for calycosin-7-*O*-*β*-D-glucoside and 203 nm for astragaloside IV. All calibration curves displayed a good linear relationship (R^2=0.999). The method was fully validated concerning the specificity, linearity, accuracy, and recovery according to the ICH guideline. The raw *Astragali Radix* samples had varying levels of calycosin-7-*O*-*β*-D-glucoside and free astragaloside IV, ranging from 0.016 - 0.032 % and 0.016-0.066 %, respectively. In the honey-processed *Astragali Radix* samples, the contents of free astragaloside IV and calycosin-7-*O*-*β*-D-glucoside were 0.018 - 0.052 % and 0.010 - 0.022 %, respectively.

Keywords: *Astragali Radix, HPLC-DAD, Calycosin-7-O-β-D-glucoside, Free astragaloside IV.*

1. Introduction

The dried root of *Astragalus membranaceous* (Fisch.) Bge. var. *mongolicus* (Bge.) Hsiao. is known as *Radix Astragali.* In Chinese traditional medicine, this plant has been utilized as a tonic, analgesic, and antisudorific [1],[2]. It has been shown to have well-researched and reviewed hepatoprotective, antioxidative, antiviral, antihypertensive, and immunostimulant properties [1],[2],[3]. Additionally, honey-processed *Astragali Radix* is a product of *Astragali Radix* mixed with honey by a traditional Chinese medicine processing method, which strengthens the tonic effect. Phytochemical studies have shown that *Astragali Radix* contains various active compounds, including isoflavonoids, saponins, and polysaccharides [1],[2],[3]. Two components (calycosin-7-*O*-*β*-D-glucoside and astragaloside IV) have been regarded as the main active constituents and were