Original article

# Optimization of extraction total flavonoids and marker compounds from *Passiflora foetida* L. using response surface methodology

***Nguyen Huu Tien*, Dang Tran Minh Xuan, Tran Huu Dung***
*Faculty of Pharmacy, Hue University of Medicine and Pharmacy, Hue University*

**Abstract**

**Background:** Currently, a wide range of products derived from *Passiflora foetida* are commercially available; however, the lack of standardized extraction procedures has led to inconsistencies in product quality. **Objectives:** To develop an optimized extraction process for total flavonoid content and marker compounds from *Passiflora foetida* using a conventional extraction method. **Materials and methods**: The material studied is the leaves and stems of *Passiflora foetida*. The extraction conditions for vitexin, isovitexin, and total flavonoid content from *Passiflora foetida* were investigated and optimized using response surface methodology. **Results:** The optimal extraction conditions were determined using a central composite design, with 56% ethanol concentration, a solvent-to-solid ratio of 17.54 mL/g, and a soaking time of 3 hours. Under these conditions, the extraction yields were 0.0644 ± 0.0005% for vitexin, 0.00180 ± 0.00003% for isovitexin, and 1.385 ± 0.056% for total flavonoids. **Conclusion:** An optimized extraction process for bioactive compounds from *P. foetida* using percolation has been successfully developed.

**Keywords***: Passiflora foetida, vitexin, isovitexin, flavonoid.*

## 1. INTRODUCTION

*Passiflora foetida* L. is a commonly used medicinal herb in traditional Vietnamese medicine, primarily applied as a sedative for treating insomnia, anxiety and nervous exhaustion [1]. Recent research has demonstrated that extracts from *P. foetida* exhibit various promising biological activities, including antidiarrheal, antiulcer, analgesic, antidepressant, anti-inflammatory, antihypertensive, hepatoprotective, anticancer and antibacterial effects [2].

Flavonoids are the major class of bioactive constituents in this plant [3], among which vitexin and isovitexin have received particular attention for their anti-inflammatory, antioxidant, anticancer, and neuroprotective activities [4]. These compounds are frequently used as chemical markers in quantitative analysis; however, their contents are strongly influenced by extraction conditions such as solvent composition, temperature, duration, solvent-to-material ratio, and storage.

In recent years, products derived from *P. foetida*, including tea infusions, capsules, and liquid extracts used to support sleep, have become increasingly prevalent. However, the absence of standardized and systematically controlled extraction methods, often characterized by labor-intensive procedures, has resulted in inconsistency in product quality. This underscores the pressing necessity for a rigorously validated extraction protocol that optimizes the yield of active phytochemicals while maintaining both the safety and pharmacological consistency of the resulting extract.

Therefore, the present research was carried out to establish a standardized, efficient, and practical extraction protocol applicable to the development of *P. foetida*-based herbal preparations, with the aim of enhancing both product quality and therapeutic effectiveness for consumers.

## 2.MATERIALS AND METHODS

### 2.1.Materials

#### *2.1.1 Plant material*

Leaves and stems of *Passiflora foetida* L. (Passifloraceae) were collected from Hue, Vietnam, in August 2024. The plant material was dried, ground into powder and sieved through a 0.71 mm sieve.

#### *2.1.2. Chemicals and Reagents*

Reference standards vitexin (purity 98%, batch No. PRF10102941) and isovitexin (purity 98%, batch No. PRF20111321) were purchased from Chengdu Biopurify Phytochemicals Ltd. (China). Rutin (purity 98%) were purchased from Bomei Bio. HPLC-grade methanol and acetonitrile were procured from Merck (Germany). Absolute ethanol (99.5%) was obtained from Cemaco (Vietnam). Ultrapure water was obtained from an ultrapure water system, Altotoc UF, Avidity, UK.

#### *2.1.3. Equipment*

The high-performance liquid chromatography

**Corresponding Author: Nguyen Huu Tien.*
*E-mail: nhtien@huemed-univ.edu.vn; nhtien@hueuni.edu.vn*
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(HPLC) system was equipped with a SHIMADZU SPD-M20A photodiode array (PDA) detector (Japan). Absorbance measurements were performed using a Jasco V730 UV-Vis spectrophotometer (Japan). Ultrasonic cleaning was performed using an Elmasonic S100H ultrasonic bath (Germany). Sample centrifugation utilized a Hermle Z326K refrigerated centrifuge (Germany). Analytical weighing was carried out on a Sartorius Quintix 125D-1S balance (Germany). Micropipettes with volumes of 100 µL, 200 µL and 1000 µL were obtained from Labnet (USA). In addition, precise glassware was used throughout the experiments.

**2.2.Methods**

***2.2.1. Determination of vitexin (VTX) and isovitexin (ISV) content***

The simultaneous quantification of VTX and ISV in *P. foetida* extract was performed following the method previously described by DTM. Xuan [5], with minor adjustments.

Five grams of powdered sample were extracted with 50 mL of 50% ethanol by shaking for 5 minutes followed by 30 minutes of sonication (in a bath). The extract was filtered through filter paper and a 0.45 µm membrane before HPLC analysis.

Standard stock solutions of VTX and ISV at 1000 µg/mL were prepared by dissolving 10 mg of each standard in 10 mL of solvents and sonicated to ensure complete dissolution. Specifically, VTX was dissolved in a 1:1 (v/v) mixture of methanol and dimethylformamide, while ISV was dissolved in methanol. A mixed standard solution was then prepared by combining 1 mL of VTX and 0.5 mL of ISV stocks, diluted to 10 mL with methanol.

Chromatographic separation was carried out on an InertSustain™ C18 column (4.6 × 250 mm, 5 µm) with a ZORBAX Eclipse XDB C18 guard cartridge (4.6 × 12.5 mm, 5 µm). The mobile phase consisted of acetonitrile (A), methanol (B) and 2% aqueous acetic acid (C) with a gradient elution program: 16.8% A : 2% B : 81.2% C from 0 to 6 minutes, 21% A : 2% B : 77% C at 20 minutes, returning to 16.8% A : 2% B : 81.2% C from 21 to 30 minutes. The flow rate was maintained at 1.0 mL/min with an injection volume of 15 µL. Detection was performed using a photodiode array (PDA) detector set at a wavelength of 337 nm.

***2.2.2. Determination of total flavonoid content (TFC)***

Total flavonoid content was determined by a colorimetric method based on the formation of stable complexes between flavonoids and 2% aluminum chloride ($AlCl_3$), which exhibit maximum absorbance at 400 nm [6]. Rutin was used as the standard compound, and results were expressed as rutin equivalents.

A series of rutin standard solutions ranging from 12.5 to 50 µg/mL were prepared in 50% ethanol. Each standard solution was mixed with an equal volume of 2% $AlCl_3$ solution, incubated in the dark at room temperature for 10 minutes and then measured for absorbance at 400 nm to generate a calibration curve. Sample extracts were diluted appropriately with 50% ethanol, reacted under the same conditions with $AlCl_3$ and their absorbance measured at 400 nm.

***2.2.3. Extraction process development for Passiflora foetida concentrated extract***

*2.2.3.1. Preliminary screening of extraction methods and factors*

Two extraction techniques, exhaustive percolation and reflux extraction, were preliminarily evaluated based on their efficiency to extract VTX, ISV and TFC from *P. foetida* powder (~20 g).

For exhaustive percolation, approximately 20 g of powdered material was pre-moistened with extraction solvent and soaked for 2 hours, followed by extraction under various conditions. The extract was collected, brought to volume and filtered through a 0.45 µm membrane. The parameters investigated included ethanol concentration (30%, 50%, 70% and 90%), liquid-to-solid ratio (6, 12, 16 and 20 mL/g) and soaking time (2, 4, 6 and 24 hours).

For reflux extraction, approximately 20 g of powdered material was extracted under different conditions, filtered through a 0.45 µm membranes. Factors studied were ethanol concentration (30%, 50%, 70%), liquid-to-solid ratio (6, 10, 12, 20, 30, 50 mL/g), extraction temperature (50°C, 60°C, 70°C) and extraction time (1, 2, 3 hours).

The extraction methods were compared by evaluating the yields of VTX, ISV and TFC under preliminary optimized conditions. Statistical significance of differences between the two methods was assessed using a t-test, with p-values less than 0.05 considered significant. This analysis aimed to identify the most efficient extraction technique for subsequent optimization.

*2.2.3.2. Optimization by response surface methodology using central composite design (CCD)*

The chosen extraction method from the preliminary study was further optimized by RSM with CCD using Design Expert 13 software. Independent variables were coded at five levels (-1, 0, +1, with axial points at $-\alpha$ and $+\alpha$). Based on preliminary results, selected key factors were modeled to

maximize extraction yields.

The dependent variables in this study were the extraction yields expressed as percentages of VTX ($X_1$), ISV ($X_2$) and TFC ($X_3$). Upon determining the optimal extraction conditions that maximize these responses, the experiments were performed in triplicate to validate the model predictions.

**2.2.4. Data processing**

Data analysis was conducted using Microsoft Excel 2016. Statistical significance was defined at a threshold of $p < 0.05$. The optimization of extraction parameters was carried out with Design-Expert software (version 13.0, Stat-Ease Inc., Minnesota, USA).

## 3.RESULTS

### 3.1. Preliminary screening of extraction methods

Using the exhaustive percolation method, the effects of ethanol concentration (30%, 50%, 70%, and 90%), liquid-to-solid ratio (6, 12, 16, and 20 mL/g), and soaking time (2, 4, 6, and 24 hours) on the extraction yields of VTX, ISV and TFC were investigated.

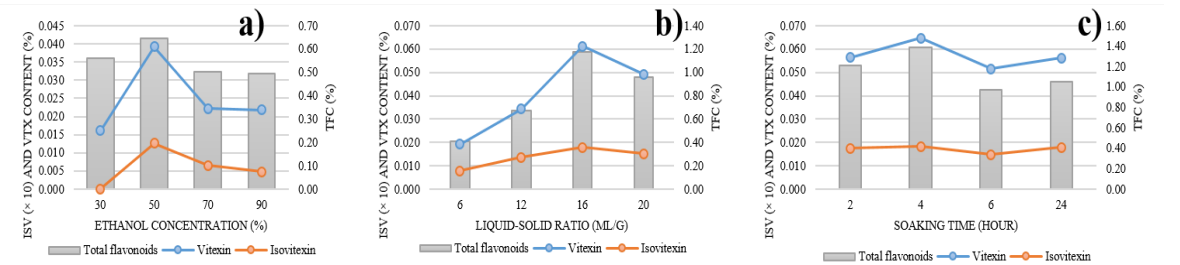



**Figure 1.** Investigation of exhaustive percolation parameters (ethanol concentration (a); solvent-to-solid ratio (b); cold-soaking time (c)) (n=3)

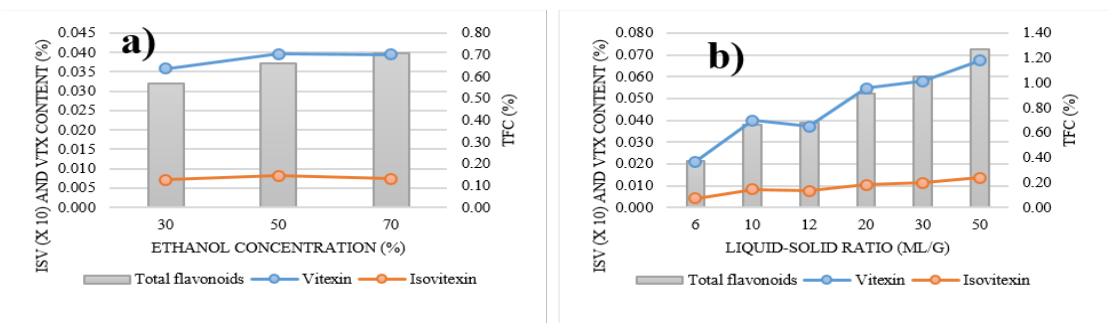
Results presented in Figure 1 demonstrate the influence of these factors on extraction efficiency. Among the ethanol concentrations tested, 50% ethanol yielded the highest contents of VTX (0.03922%), ISV (0.00127%), and TFC (0.64631%). ISV was not detected at 30% ethanol, suggesting that lower solvent polarity is insufficient for its extraction. Increasing ethanol concentration beyond 50% did not enhance yields and in some cases reduced them, indicating an optimal solvent polarity range.

The extraction efficiency increased with the liquid-to-solid ratio up to 16 mL/g, where the yields of VTX, ISV, and TFC achieved their highest yields. Ratios exceeding this threshold showed no significant improvement, indicating a saturation point in extraction capacity.

Regarding soaking time, maximum extraction yields were observed at 4 hours, with extended durations resulting in only marginal changes, suggesting that equilibrium was attained.

Consequently, the optimal preliminary maceration conditions were determined to be 50% ethanol, a solvent-to-solid ratio of 16 mL/g, and 4 hours of cold soaking, yielding optimized levels of 0.06129% VTX, 0.00179% ISV, and 1.17441% TFC.

Using the reflux extraction method, the effects of ethanol concentration (30%, 50%, and 70%), solvent-to-solid ratio (6, 10, 12, 20, 30, and 50 mL/g), extraction temperature (50°C, 60°C, and 70°C), and extraction time (1, 2, and 3 hours) on the yields of VTX, ISV and TFC were evaluated.



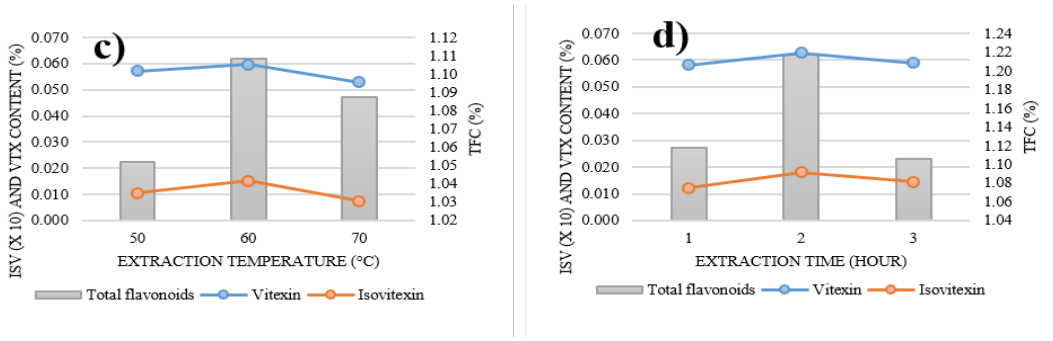



**Figure 2.** Investigation of reflux extraction parameters (ethanol concentration (a); liquid-to-solid ratio (b); extraction temperature (c) and extraction time (d)) (n=3)

Results shown in Figure 2 indicate that 50% ethanol provided the highest yields of VTX and ISV while 70% ethanol yielded a higher TFC. Since the focus was on optimizing VTX and ISV 50% ethanol was selected for further experiments.

Increasing the solvent-to-solid ratio generally enhanced extraction efficiency with yields peaking at a ratio of 30 mL/g. Further increase to 50 mL/g showed continued improvements but 30 mL/g was chosen for subsequent tests considering efficiency and solvent usage.

Extraction temperature optimization revealed the highest yields of VTX, ISV and TFC at 60°C. Therefore 60°C was selected as the optimal temperature.

Extraction time trials demonstrated that 2 hours of reflux extraction produced the maximum yields of VTX ISV and TFC with longer durations showing no significant benefit.

Consequently, the optimal preliminary reflux extraction conditions were determined to be 50% ethanol, a solvent-to-solid ratio of 30 mL/g, an extraction temperature of 60°C, and an extraction time of 2 hours, yielding optimized levels of 0.06736% VTX, 0.00136% ISV, and 1.26622% TFC.

The study compared exhaustive percolation and reflux extraction methods, each performed in triplicate under preliminary optimal conditions (Figure 3). The results showed that exhaustive percolation yielded higher average contents of VTX (0.06768%) and TFC (1.37747%) compared to reflux extraction (VTX 0.05993%, TFC 1.20917%). These differences were statistically significant ($p < 0.05$). In contrast, the ISV content exhibited minimal variation between the two methods, with 0.00180% for exhaustive percolation and 0.00177% for reflux extraction, and no significant difference was found ($p > 0.05$) based on t-test analysis.

Based on these findings, exhaustive percolation was selected as the preferred extraction method for further optimization due to its superior efficiency in extracting compounds.

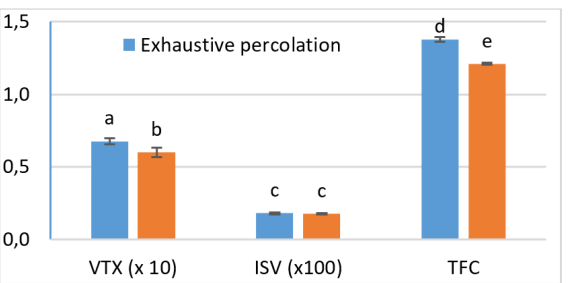



**Figure 3.** Comparison of extraction efficiency between reflux extraction and exhaustive percolation methods. Different letters indicate significant differences ($p < 0.05$).

### 3.2. Optimization of compounds extraction using RSM

This study utilized RSM with CCD to thoroughly examine the interactions and effects of different factors in the extraction process. The objective was to optimize extraction conditions for VTX, ISV and TFC from *Passiflora foetida*.

The independent variables considered in the study included ethanol concentration (A), liquid-to-solid ratio (B) and soaking time (C), while the dependent variables were the extraction yields of compounds. Based on the findings from prior single-factor experiments, the coded values and levels of these independent variables are summarized in Table 1.

**Table 1** Independent variable codes and levels in experimental design for compounds extraction optimization

| Variables | Coded levels of variables | | |
|---|---|---|---|
| | -1 | 0 | +1 |
| Ethanol concentration (%) (A) | 40 | 50 | 60 |
| Liquid-solid ratio (mL/g) (B) | 12 | 16 | 20 |
| Soaking time (hrs) (C) | 2 | 4 | 6 |

A total of 17 experiments were conducted, with the corresponding results presented in Table 2.

**Table 2.** Design and response surface methodology results of VTX, ISV and FLA extraction optimization

| No. | A | B | C | VTX content (x$10^{-1}$ %) $X_1$ | ISV content (x$10^{-2}$ %) $X_2$ | TFC (%) $X_3$ |
|---|---|---|---|---|---|---|
| 1 | 50 | 16 | 7.364 | 0.6038 | 0.172 | 1.34400 |
| 2 | 40 | 20 | 2 | 0.6328 | 0.145 | 1.38494 |
| 3 | 50 | 16 | 0.636 | 0.6291 | 0.173 | 1.38806 |
| 4 | 60 | 12 | 2 | 0.6262 | 0.186 | 1.38134 |
| 5 | 33.182 | 16 | 4 | 0.6229 | 0.142 | 1.29418 |
| 6 | 66.818 | 16 | 4 | 0.6252 | 0.174 | 1.44064 |
| 7 | 50 | 16 | 4 | 0.6440 | 0.182 | 1.38543 |
| 8 | 40 | 12 | 6 | 0.6150 | 0.168 | 1.22152 |
| 9 | 50 | 16 | 4 | 0.6454 | 0.180 | 1.38576 |
| 10 | 50 | 22.727 | 4 | 0.6337 | 0.169 | 1.46209 |
| 11 | 60 | 12 | 6 | 0.6075 | 0.171 | 1.33497 |
| 12 | 40 | 12 | 2 | 0.6284 | 0.170 | 1.26104 |
| 13 | 50 | 16 | 4 | 0.6445 | 0.182 | 1.40615 |
| 14 | 50 | 9.273 | 4 | 0.6282 | 0.181 | 1.27553 |
| 15 | 60 | 20 | 2 | 0.6339 | 0.166 | 1.47409 |
| 16 | 40 | 20 | 6 | 0.6231 | 0.163 | 1.32858 |
| 17 | 60 | 20 | 6 | 0.6131 | 0.171 | 1.39452 |

Analysis of variance (ANOVA) for the VTX extraction model (Table S1 in the Supplementary Material) indicates that the overall model is highly significant, with a p-value less than 0.0001 and a lack-of-fit p-value of 0.0563, which exceeds the 0.05 threshold - confirming an adequate fit to the experimental data. Furthermore, the independent factors B and C, along with the quadratic terms $A^2$, $B^2$, and $C^2$, all have p-values below 0.05, suggesting statistically significant effects on VTX yield at the 95% confidence level.

The model also demonstrates excellent predictive performance and linearity, as reflected by the high coefficients of determination: $R^2$ = 0.9814, adjusted $R^2$ = 0.9575, and predicted $R^2$ = 0.8580. These results support the model's reliability for predicting optimal extraction conditions for VTX. The regression equation describing the relationship between the independent variables and VTX content (denoted as $X_1$) is given by:

$X_1$ = 0,033853 + 0,000755×A + 0,001012×B + 0,002083×C + 0,00000025×AB – 0,000010×AC + 0,0000025×BC – 0,0000073×$A^2$ – 0,000030×$B^2$ – 0,000249×$C^2$ (Eq. 1).

Analysis of variance (ANOVA) for the ISV extraction model (Table S2 in the Supplementary Material)

reveals that the model is statistically significant, with a p-value less than 0.05 and a lack-of-fit p-value of 0.0910, which exceeds the 0.05 threshold - indicating that the model fits the experimental data well. Notably, the independent variables A and B, the interaction terms AC and BC, as well as the quadratic components $A^2$, $B^2$, and $C^2$ all have p-values below 0.05, demonstrating significant influence on ISV yield at the 95% confidence level.

The model shows strong linearity and predictive capability, as evidenced by high coefficients of determination: $R^2$ = 0.9687, adjusted $R^2$ = 0.9284, and predicted $R^2$ = 0.7682. These values confirm that the model can be reliably applied to forecast optimal extraction conditions for ISV. The regression equation describing the relationship between the independent variables and ISV content (denoted as $X_2$) is given by:

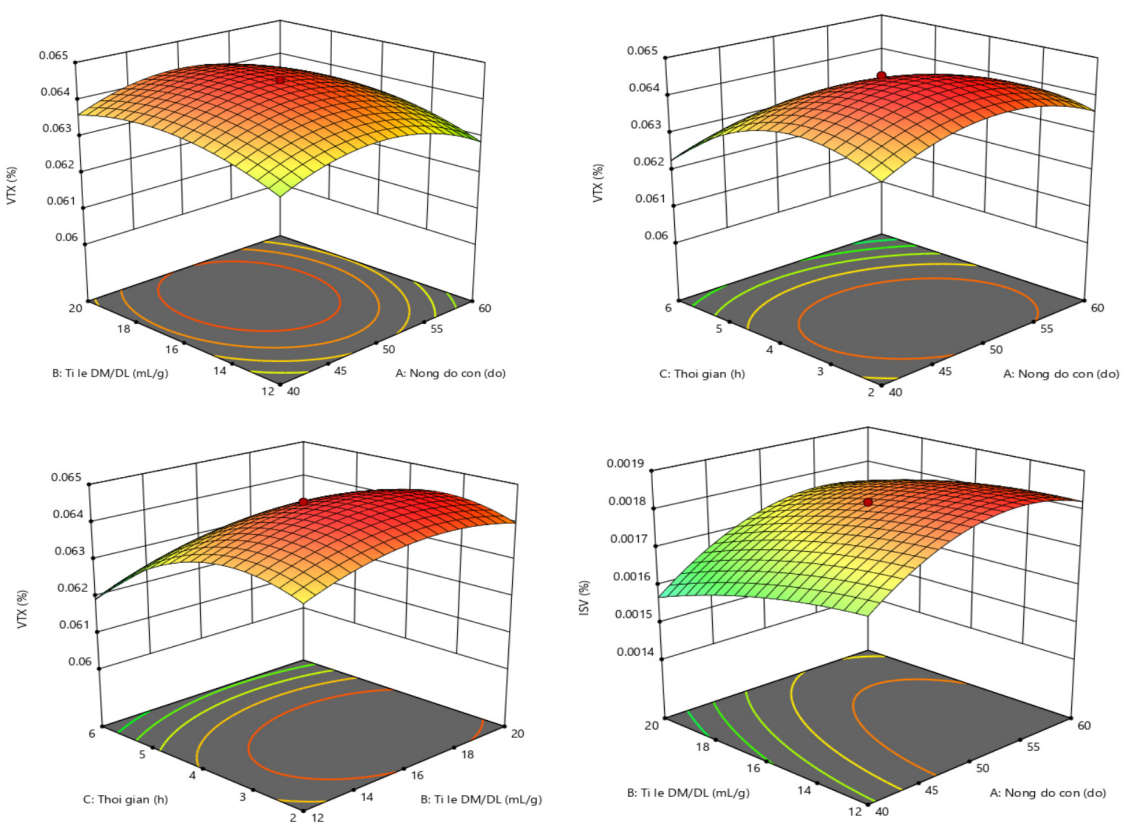
$X_2$ = - 0,000603 + 0,000092×A – 0,0000077×B + 0,000046×C + 0,00000031×AB – 0,0000016×AC + 0,0000063×BC – 0,00000083×$A^2$ – 0,0000014×$B^2$ – 0,0000079×$C^2$ (Eq. 2)

Analysis of variance (ANOVA) for the TFC extraction model (Table S3 in the Supplementary Material) indicates that the model is highly significant, with a p-value below 0.0001 and a lack-of-fit p-value of 0.1994—well above the 0.05 threshold - demonstrating an adequate fit to the experimental data. The p-values of all three independent variables (A, B, and C) are less than 0.05, confirming that these factors exert statistically significant effects on TFC yield at the 95% confidence level.

The model also exhibits strong linearity and predictive accuracy, as reflected by high determination coefficients: $R^2$ = 0.9096, adjusted $R^2$ = 0.8887, and predicted $R^2$ = 0.8568. These indicators support the model's suitability for predicting optimal extraction conditions for TFC. The regression equation describing the relationship between the independent variables and TFC (denoted as $X_3$) is given by:

$X_3$ = 0,969165 + 0,004651×A + 0,012759×B – 0,010834×C (Eq. 3).

To observe the interactive effects of the conditions on extraction efficiency, the response surface model illustrating the interaction of each pair of condition while keeping the remaining conditions constant was performed in Figure 4.



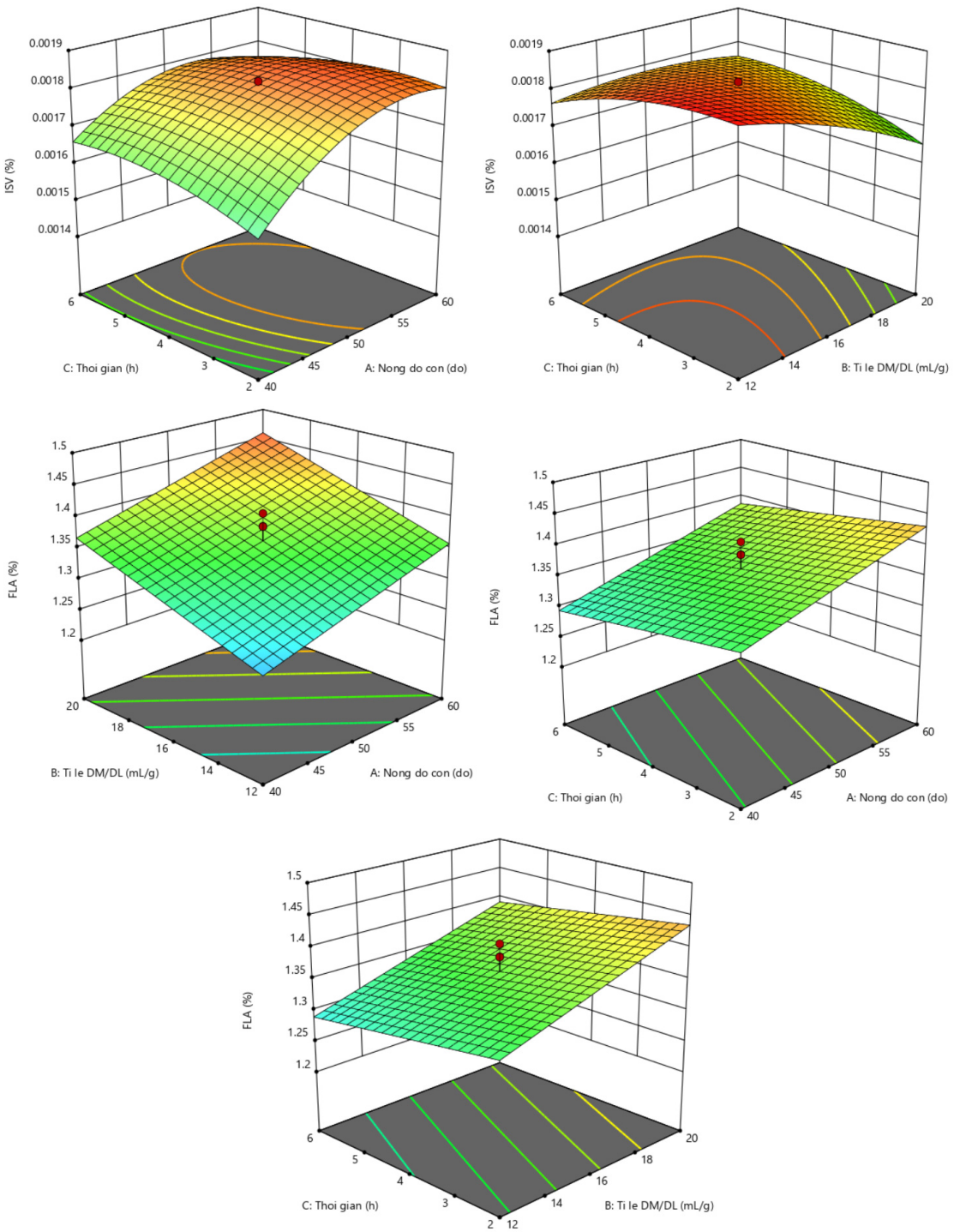



**Figure 4.** The response surface model illustrates the interactive effects of the conditions on the VTX content (a, b, c), ISV content (d, e, f) and TFC (g, h, i) extracted from *P. foetida*.

Based on data obtained from 17 experimental runs designed using the CCD, the optimal extraction conditions were determined to be 56.003% ethanol concentration, a solvent-to-solid ratio of 17.538 mL/g, and a soaking duration of 3.033 hours. To accommodate the technical constraints of laboratory equipment during the validation process, these parameters were slightly adjusted to practical

values: 56% ethanol, 17.54 mL/g solvent-to-solid ratio, and 3 hours of soaking time.

Under these adjusted conditions, the experimental extraction efficiencies achieved were 0.0644 ± 0.0005% for VTX, 0.00180 ± 0.00003% for ISV, and 1.385 ± 0.056% for TFC. The statistical comparison of the contents of the compounds using the t-test indicated no significant differences between the predicted and observed values for VTX, ISV, and TFC, with a p-value > 0.05 at a 95% confidence level. This confirms the reliability and accuracy of the optimized extraction model.

**4. DISCUSSION**

With the aim of applying the herbal extract preparation process on a larger scale, the study selected classical extraction methods that are scalable and compatible with practical production conditions. Two methods were investigated: percolation and reflux extraction.

Methanol, ethanol and acetone are suitable for flavonoid extraction [7]. However, to ensure safety and green extraction, ethanol was selected as the solvent for investigation [8]. Since solvent polarity varies with ethanol concentration, various levels were tested to optimize flavonoid yield. VTX and ISV, moderately polar compounds, showed highest extraction efficiency at 50% ethanol in both percolation and reflux methods. However, TFC peaked at 50% in percolation, but at 70% in reflux, indicating that optimal ethanol concentration may vary with extraction conditions and equipment.

The liquid-to-solid ratio significantly influences extraction yield. While increasing this ratio initially enhances extraction yield, it may later decrease due to impurity extraction or oxidation [9], [10], [11], [12]. In percolation, a 16 mL/g ratio offers optimal yield by lowering viscosity and sustaining concentration gradients. In reflux extraction, higher ratios improve VTX, ISV, and TFC contents by enhancing diffusion through greater concentration differentials.

In the percolation method, extraction time depends on soaking time and the amount of solvent used. In particular, cold soaking time plays an important role and affect on extraction efficiency through the effective contact time between the medicinal material and the solvent. Cold soaking facilitates the diffusion of active compounds into the solvent due to concentration gradient differences. The optimal cold soaking time is the time at which the active compounds have dissolved into the solvent to saturation [13]. The study results show that exhaustive percolation provides superior extraction efficiency compared to reflux extraction in terms of VTX and TFC content, with statistically significant differences. Additionally, reflux extraction has lower extraction efficiency and consumes more energy, as it requires maintaining a continuous high temperature and recycling the solvent, leading to increased energy costs throughout the extraction process. Therefore, the exhaustive percolation process will continue to be optimized to improve extraction efficiency.

Conventional optimization strategies in analytical chemistry typically involve examining the influence of a single experimental factor at a time while maintaining all other conditions constant. This one-factor-at-a-time (OFAT) approach, although simple, fails to account for possible interactions between variables. As a result, it may not accurately capture the comprehensive influence of all parameters on the response. Moreover, the requirement for multiple individual experiments makes the process time-consuming and resource-intensive, increasing the consumption of solvents, reagents, and labor costs [14].

In contrast, RSM offers a more efficient alternative by simultaneously assessing the primary and interaction effects of multiple variables, thus enabling a deeper understanding of the relationships between experimental factors and responses [15]. Among RSM, the CCD includes a greater number of runs and extends to axial points beyond the factorial space, enhancing prediction accuracy compared to the BBD. Therefore, this research adopted the CCD-based RSM approach to optimize the extraction conditions for *P. foetida*.

The suitability of the regression model and its predictive equation was assessed using statistical analysis, specifically the Fisher F-test and lack-of-fit test, with a significance threshold (α) of 0.05. A p-value less than α indicates that the model is statistically significant, with smaller values suggesting better fit. For the lack-of-fit test, a p-value greater than 0.05 confirms the model's adequacy, with higher values reflecting better alignment with the experimental data [16]. Additionally, the coefficient of determination ($R^2$) was used to evaluate the agreement between observed and predicted results, where an $R^2$ above 0.8 is considered indicative of a reliable model [17]. Variables showing p-values below 0.05—whether linear, quadratic, or interaction terms—were interpreted as having significant impacts on the response.

Compared to a previous study that optimized the extraction of total flavonoids from P. foetida using

ultrasound methods [18], this study not only focused on the total flavonoid content but also included marker compounds such as VTX and ISV, which provides a more comprehensive and reliable analysis. Additionally, the study employed percolation and reflux extraction methods with simpler equipment, which could be more practical for scaling up to pilot-scale research and applications.

## 5. CONCLUSION

*P. foetida* was extracted using the percolation method, employing a CCD design to optimize the extraction process. The optimal parameters were as follows: ethanol concentration of 56%, liquid-to-solid ratio of 17.54 mL/g and soaking time of 3 hours. Under optimal conditions: the experiments yielded an extraction efficiency of 0.0644 ± 0.0005% for VTX, 0.00180 ± 0.00003% for ISV and 1.385 ± 0.056% for TFC. From these results, further research can be conducted to establish quality standards for products derived from *P. foetida*.

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