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Original Article

Exploring Factors Affecting the Quantitative Analysis of Vitexin, Imperatorin and Gallic Acid in Tri-Kaysornmas Formulation

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ABSTRACT

Background: The Tri-kaysornmas formula is one of the Thai traditional formulas in Thailand. Many phytochemical active compounds are found in this formula such as vitexin, imperatorin and gallic acid. **Objectives:** The current study explores the factors affecting the quantitative analysis of some active compounds in Tri-kaysornmas formulation. **Materials and Methods:** To quantify the content of these compounds, high-performance liquid chromatography was applied. A reverse-phase column, Hypersil ODS was used, and the absorbance detection was at 254 nm (imperatorin), 275 nm (gallic acid) and 340 nm (vitexin). Two factors including flow rate and acetonitrile composition in the HPLC system's mobile phase and 3 responses including capacity factor, tailing factor and number of theoretical plates were studied. Three level factorial designs with response surface methods were selected for this study. Experimental design with a 3-level flow rate from 0.8-1.2 mL/min and 3-level percentage acetonitrile from 10-15% were set. **Results:** For imperatorin, only capacity factor response could be explained and statistically related to and affected by the variable factors, flow rate and percentage of acetonitrile at *p*-value <0.0001 with the model showing significant linear effected. The model equations of gallic acid could predict all the responses (capacity factor, tailing factor and number of theoretical plates). From the ANOVA results all *p*-values were less than 0.05 (0.0012, <0.0001 and <0.0001 in respectively). From the ANOVA results of vitexin, the statistical analysis of RSM for capacity factor and number of theoretical plates performed a good-fitted model at a *p*-value less than 0.0001. **Conclusion:** it could be concluded that both flow rate and acetonitrile composition in the mobile phase affect the parameters including capacity factor, tailing factor and number of theoretical plates with different manner for each compound depending on the physicochemical properties. Therefore, the HPLC analysis of imperatorin, gallic acid and vitexin, the main active components in the Tri-karsornmas formula, should consider these factors to achieve high-quality separation and determination.

Keywords: Experimental design, HPLC, Response surface method, 3-level factorial design, Tri-kaysornmas formula.

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INTRODUCTION

Phytochemical constituents are commonly found in herbal plants.[1] The detection and quantitation of active compounds are crucial and related to the pharmacological activity of the herbal plant.[2,3] Some Thai traditional formulas are composed of many herbal plants such as Pikad Tri-Kaysornmas (TKM) containing 3 important parts from 3 herbal plants (*Aegle marmelos* fruit, *Nelumbo nucifera* stamen and *Jatropha multifida* bark).[4,5] Three active compounds, imperatorin, gallic acid and vitexin were found in this TKM and had many pharmacological activities.[6-15] Their structures are shown in Figure 1. Thus, the appropriate analytical method should be explored to determine these active compounds. Many analytical techniques, such as UV-visible spectroscopy, gas chromatography and High-Performance Liquid Chromatography (HPLC) are generally used for this aspect which depends on the physico-chemical properties of the compounds.[16] HPLC is one of the familiar techniques that can be used to determine the active constituents of herbal plants and traditional formulations.[17-19] Using HPLC to determine some active

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compounds in *Moringa oleifera, Piper nigrum* and *Wolffia globosa* was revealed, confirming the essential analytical method.[20-22]

Many factors can affect HPLC determination's analytical results, such as mobile phase flow rate and organic solvent composition in mobile phase solution. Three level factorial design and response surface method were implemented to use for the study. The experimental design and Response Surface Methodology (RSM) can predict how the values of measurable responses relate to and interact with a set of experimental factors that are believed to influence those responses. Moreover, it could be used for predicting the response value at various factor conditions. The 3-level design can be expressed as a 3^k factorial design, where k factors are evaluated at each of the 3 levels. These levels are typically referred to as low, intermediate and high and are represented by the digits -1, 0 and +1.[23] The simplest design is the 3^2 designs, which includes 2 factors, each evaluated at 3 levels. Nine treatment combinations for this design are illustrated in Figure 2. However, additional treatments in the middle can be added for more complete results. For this study, 2 factors including flow rate and percentage of acetonitrile with 3-level were varied and 3 responses (capacity factor, tailing factor and number of theoretical plates) were evaluated.

Hence, the primary aim of this study is to explore the factors that affect quantitative analysis. of some active compounds in Tri-kaysornmas formulation using an experimental design with response surface methodology.

MATERIALS AND METHODS

Materials

Imperatorin, gallic acid and vitexin were obtained from Sigma-Aldrich, St. Louis, MO. HPLC-grade acetonitrile and methanol's were sourced from Merck Ltd., Darmstadt, Germany. Analytical grade acetic acid was purchased from BDH, VWR International Ltd., USA.

Methods

Experimental design for HPLC condition

Three level factorial design for 2 factors which is the flow rate (0.8, 1.0, 1.2 mL/min) and percentage of acetonitrile in the mobile phase (10, 12.5 and 15%) were studied. The responses from the design were capacity factor, tailing factor and number of theoretical plates. Thirteen experiments were set as shown in Table 1. The factors and responses were evaluated for their interaction and optimization by Design Expert software (version 13) via response surface methodology.

This experimental design with response surface methodology reveals the influence of these 2 factors affecting the responses (capacity factor, tailing factor and number of theoretical plates). These 3 responses are of essential value concerning the effective analytical HPLC condition. The retention factor or capacity factor (k) assesses the degree of retention of an analyte on the chromatographic column. A high-capacity factor indicates that the compound of interest is retained for a significant duration and interacts extensively with the stationary phase. The useful capacity factor might be in the range of 2-10. The capacity factor is calculated from the ratio of the retention time of the compound of interest on the column to the retention time of a compound without interaction with the stationary phase.[24] The tailing factor serves as a key parameter in the system suitability testing of HPLC analysis. The U.S. Pharmacopeia (USP) additionally recommends the evaluation of the Tailing factor (T), which is defined as the ratio of the back portion to the front portion of a bisected peak, measured at 5% of its height. The ratio is computed by dividing the overall width by twice the front width. A tailing peak exhibits a front exceeding 1.0, whereas a fronting peak displays a front less than 1.0.[24] The Number of theoretical plates (N) indicates the peak dispersion on the HPLC column and reflects the column's performance. It serves as a key metric for assessing the performance and efficiency of chromatographic columns. It provides an indirect evaluation of peak width at a given retention time. The formula for calculating the number of theoretical plates is $N=16(t_R / W)^2$, where t_R represents retention time and W denotes peak width.[24]

RSM with experimental design by 3-level factorial design could be applied to optimize the parameters or factors of HPLC conditions and evaluate the effects for all factors and responses.[25,26] The model equation generated from the Analysis of Variance (ANOVA) can be explained as follows (eq. 1)

$$Y=a_0 + a_1X_1 + a_2X_2 + a_{12}X_1X_2 + a_{11}X_1^2 + a_{22}X_2^2 \quad (1)$$

Where Y is the response, a_0 is a constant, a_1-a_{22} are the regression coefficient calculated from the experimental results, X_1 and X_2 are the variables factors of these experiments representing the flow rate and acetonitrile composition in the mobile phase. represents the interaction term between factors. X_1^2 and X_2^2 are quadratic term of these factors.

Preparation of stock standard solutions

One mg of each reference standard, imperatorin, gallic acid and vitexin was weighed into vials and 1000 µL of methanol was added and completely dissolved until a homogeneous solution was obtained.

Preparation of a mixture solution of imperatorin, gallic acid and vitexin

The stock standard solution (1000 µg/mL) from the process above was pipetted by micropipette for 50 mL into a 5-mL volumetric flask, then diluted and adjusted to volume with the mixture solution of acetonitrile and 0.2% acetic acid solution (1:1). Then, a mixture of standard solutions was injected into the HPLC system.

HPLC analytical conditions

An HPLC instrument (Agilent Technologies Inc., Santa Clara, USA) and Hypersil ODS column (5 µm, 4.0x250 mm) were used in this study. The mobile phase solution comprised acetonitrile and 0.2 % acetic acid solution. The percentage of acetonitrile and flow rate of the mobile phase varied depending on the experimental design by 3-level factorial design using Design Expert software (version 13). Gradient elution condition was run within 60 min. A diode array detector was used, and the absorbance was detected at 254 nm for imperatorin, 275 nm for gallic acid and 340 nm for vitexin.

Statistical analysis

All data from each experiment were analyzed and evaluated using Design Expert, statistical software developed by Stat-Ease Inc. based in Minneapolis, Minnesota, USA. This software was employed to design the experiment set with a 3-level factorial design.

RESULTS

Experimental responses from the 3-level factorial design

According to the 3-level factorial design with RSM, 13 sets of experiments were evaluated. The relationship and interaction between 2 factors (flow rate and percentage of acetonitrile) and 3 responses were analyzed using RSM. The various responses obtained from all 13 experimental runs were incorporated into the design to prove the model fit. The investigated responses are shown in Table 2. The ANOVA from the statistical analysis of RSM for 3 responses is shown in Table 3. The perturbation plots performing the effect of these 2 factors (flow rate and percentage of acetonitrile in mobile phase) on the responses (capacity factor, tailing factor and theoretical plate) are shown in Figure 3. The relationship between the observed value response from the experiments of 13 runs and the predicted value from the model equation were plotted and shown in Figure 4. It was found that some responses could not fit and explain with the model equation.

For imperatorin, only capacity factor response could be explained and statistically related to and affected by the variable factors, flow rate and percentage of acetonitrile at p-value <0.0001 with the model showed significant linear effected to the equation 2 (eq.2). The model F-value of 296.80 implied the model was significant. Flow rate and percentage of acetonitrile in mobile phase were significant model terms which strongly effect to capacity factor.

However, the perturbation plot for the effect of these 2 factors to the capacity factor performed that flow rate had higher effect than percentage of acetonitrile which the curve showed steeper line. The perturbation graph is shown in Figure 3A. No interaction effect between 2 factors was found from the response surface method which no interaction term (X_1X_2) in the model equation, where X_1: flow rate; X_2: percentage of acetonitrile.

$$\text{Capacity factor} = 20 + 3.38\,X_1 + 0.35\,X_2 \quad \text{(eq.2)}$$

For gallic acid, the model equations (eq.3, 4 and 5) could predict all the responses (capacity factor, tailing factor and number of theoretical plate). From the ANOVA results in Table 3, the model F-value of each response (14.29, 46.42 and 39.55) implied that the model equation significantly fitted, and all p-values were less than 0.05 (0.0012, <0.0001 and <0.0001 in respectively). R^2 and adjusted R^2 were 0.7408, 0.6889 for capacity factor, 0.9028, 0.8833 for tailing factor and 0.9519, 0.9278 for number of theoretical plates which could performed linear relationship between variable factors and these responses. The equation expressed in coded variables could be used to make predictions about the response from these studies for given levels of each factor. These coded equations were useful for identifying the relative impact of the factors by comparing the factor coefficients. It was found that the coefficients of percentage acetonitrile performed higher

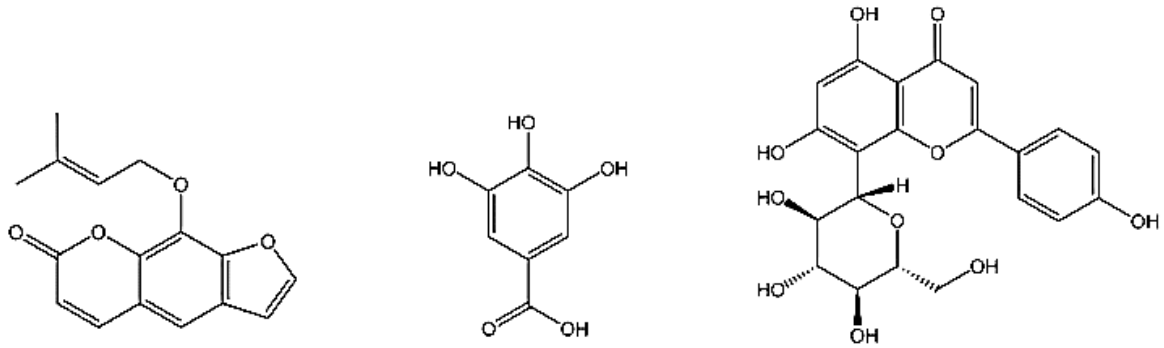

Figure 1: Chemical structure of imperatorin, gallic acid and vitexin.

Table 1: Experimental set from the 3-level factorial design of 2 variable factors.

Run number	Variable factors (code)		Variable factors	
	Flow rate (mL/min)	Percentage of acetonitrile (%)	Flow rate (mL/min)	Percentage of acetonitrile (%)
1	0	1	1.0	15.0
2	1	-1	1.2	10.0
3	0	0	1.0	12.5
4	0	0	1.0	12.5
5	0	-1	1.0	10.0
6	-1	0	0.8	12.5
7	0	0	1.0	12.5
8	-1	1	0.8	15.0
9	1	0	1.2	12.5
10	1	1	1.2	15.0
11	0	0	1.0	12.5
12	0	0	1.0	12.5
13	-1	-1	0.8	10.0

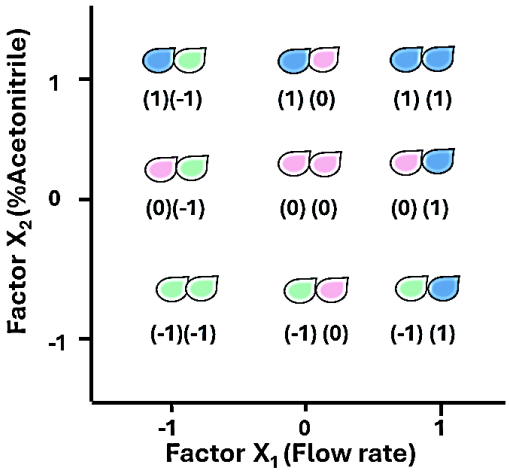

Figure 2: A 3^2 design with 2 factors at each of 3 levels (code -1,0,1).

influence on all responses than flow rate as shown from all equations from all responses (eq. 3,4 and 5). No interaction effect between 2 factors was found from the RSM which no interaction term (X_1X_2) was observed in the model equation. Only the model equation for the number of theoretical plates revealed the significant effect from quadratic terms (X_1^2 , X_2^2) while the other responses could not be observed.

$$\text{Capacity factor} = 0.2085 + 0.0067X_1 - 0.0567X_2 \quad \text{(eq.3)}$$

$$\text{Tailing factor} = 0.5746 - 0.0067X_1 - 0.2667X_2 \quad \text{(eq.4)}$$

$$\text{Number of theoretical plates} = 1919.48 - 111.17X_1 + 590.67X_2 - 134.19 + 546.31 \quad \text{(eq.5)}$$

The perturbation plot in Figure 3B demonstrates that the flow rate had a higher effect than a percentage of acetonitrile which the curve showed a steeper line that conformed to the explanation by the coefficient of each factor in the equations.

For vitexin, the relationship between variable factors and the responses was evaluated and the model equations were shown in equations 6 and 7 (eq. 6,7).

$$\text{Capacity factor} = 5.59 + 0.4217X_1 - 1.48X_2 - 0.3050 + 0.0957 - 0.2843 \quad \text{(eq. 6)}$$

$$\text{Number of theoretical plates} = 11816 - 2119.17X_1 - 7057.33X_2 + 1399.0 + 1110.47 + 888.97 \quad \text{(eq. 7)}$$

Table 2: **The responses from all 13 experimental designs by 3-level factorial design.**

Run number	Factors		Responses								
	FR	% Acn	Capacity factor			Tailing factor			Number of theoretical plates		
			Im	Ga	Vi	Im	Ga	Vi	Im	Ga	Vi
1	1.0	15.0	20.21	0.15	3.91	1.30	0.27	0.68	191368	3017	5714
2	1.2	10.0	23.33	0.31	7.74	1.25	0.87	0.85	306353	1542	17245
3	1.0	12.5	20.05	0.21	5.59	1.48	0.58	0.97	250841	1836	12186
4	1.0	12.5	20.03	0.21	5.54	1.44	0.61	0.84	278271	1906	11852
5	1.0	10.0	18.72	0.20	6.52	1.22	0.69	0.71	172729	2118	19302
6	0.8	12.5	16.70	0.20	5.19	0.65	0.64	0.85	134925	1952	14167
7	1.0	12.5	20.12	0.20	5.69	1.47	0.64	0.78	342559	1934	11921
8	0.8	15.0	16.90	0.15	3.75	1.19	0.24	0.79	257078	3133	7787
9	1.2	12.5	23.50	0.21	6.00	1.26	0.52	0.94	266000	1822	11292
10	1.2	15.0	23.59	0.16	4.00	1.28	0.32	0.79	290063	2751	5665
11	1.0	12.5	20.10	0.21	5.62	1.22	0.60	0.87	259930	1801	11627
12	1.0	12.5	20.20	0.21	5.66	1.21	0.62	0.90	245522	1917	11893
13	0.8	10.0	16.55	0.29	6.27	1.41	0.87	0.87	239616	1697	24963

FR: flow rate, Im: imperatorin, Ga: gallic acid, Vi: Vitexin, Acn: acetonitrile.

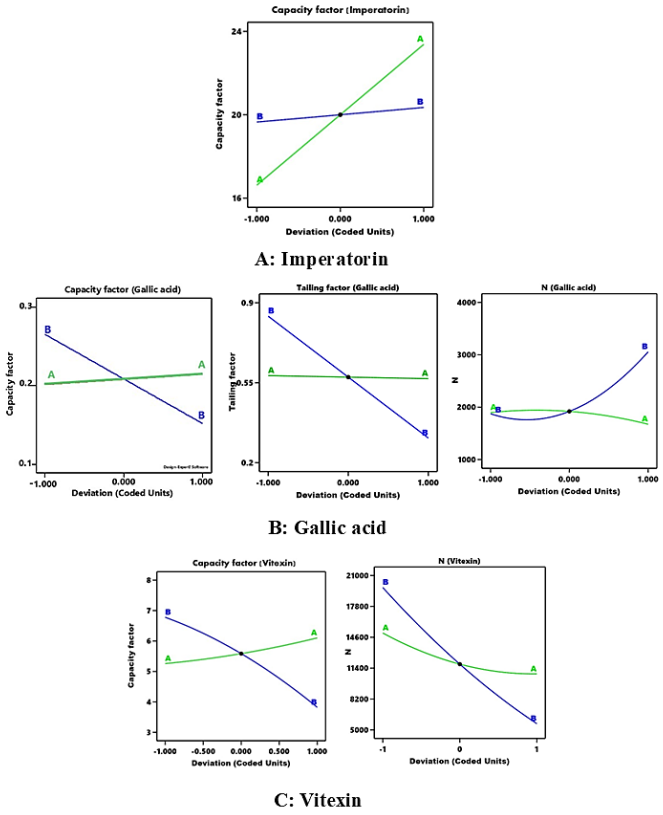

Figure 3: The perturbation plots performing the effect of these 2 factors (flow rate, line A and acetonitrile composition in mobile phase, line B) on the responses (capacity factor, tailing factor and theoretical plate) which A: Imperatorin; B: Gallic acid; C: Vitexin.

The ANOVA results from the statistical analysis of RSM for capacity factor and number of theoretical plates performed a good-fitted model at a *p*-value less than 0.0001 and an F-value of 139.89 and 241.28, respectively. Both responses R^2 and predicted R^2 were in reasonable agreement with the difference between the R^2 and predicted R^2 being less than 2 revealing the suitable fitted model. The observed value of both responses was plotted against the predicted value calculated from the model equation as shown in Figure 4C. Figure 3C shows which factors were important and influential on the capacity factor and number of theoretical plates, as observed from the perturbation graph (Figure 3). If the graph had a steeper slope, it indicated that the factor had a greater effect on the response. It was found that the percentage of acetonitrile has a steeper graph slope. Therefore, the percentage of acetonitrile had a greater impact on both responses compared to the flow rate.

DISCUSSION

From the results of all compounds, the chromatographic system used in this study was reversed-phase system. In this reversed-phase HPLC system, the stationary phase is non-polar. Increasing the polarity of the mobile phase can cause the hydrophobic (non-polar) sections of the analyte molecules to be increasingly repelled into the stationary phase. As a result, the analyte can be retained longer in the column. Many parameters are used for testing the performance of HPLC system for the analysis of compounds.[24] Capacity factor, tailing factor and number of theoretical plates are factors or parameters that are usually used for checking or approving the system suitable for HPLC analysis.[24] The capacity factor or retention factor

contributes to the resolution of the compound separation. The most effective and practical way to alter the retention factor of a peak is by adjusting the solvent strength of the HPLC mobile phase. In reversed-phase chromatography, this is typically done by changing the amount of organic solvent in the mixture solution of the mobile phase. Increasing the organic content in the mobile phase which altering the mobile phase composition could decrease the capacity factor.[24] Consistent with the results of this study, the percentage of acetonitrile significantly influenced the capacity factor of imperatorin, gallic acid and vitexin, as shown in equations 2, 3 and 6 and in the perturbation plot in Figure 3. However, the results for imperatorin differed from those for gallic acid and vitexin. When the percentage of acetonitrile increased, the capacity factor for imperatorin did not decrease. This can be explained by imperatorin's very high-capacity factor (16.55-23.59), indicating strong retention in the non-polar stationary phase. Thus, changing the composition of acetonitrile in the mobile phase from 10% to 15% did not significantly affect imperatorin, as it is strongly associated with the non-polar stationary phase.

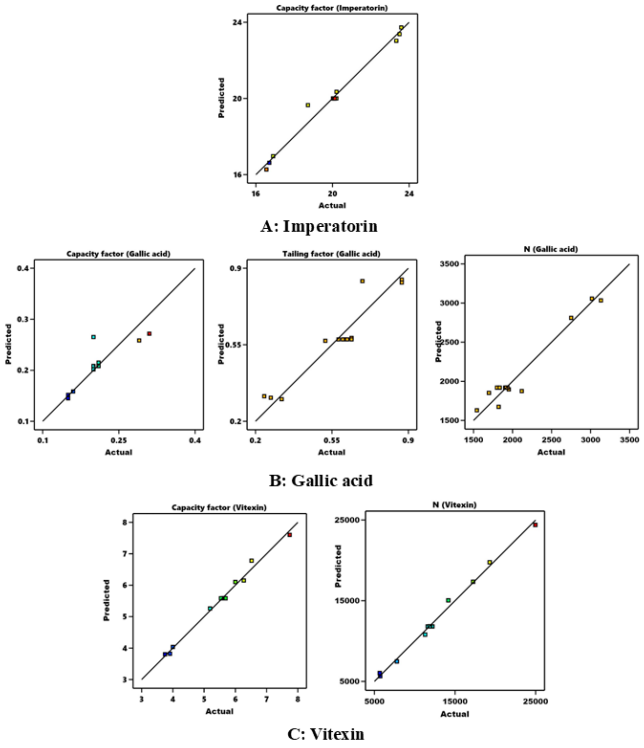

Figure 4: The relationship between the actual values from the experiments and the predicted values from the model equation of each response for imperatorin, gallic acid and vitexin.

The tailing factor is one parameter concerning the efficiency of HPLC analysis. Peak symmetry also affects column efficiency and, consequently, resolution. Tailing peaks are often caused by strongly adsorptive active sites. Ideally, HPLC peaks in the chromatogram would be shown in thin lines and the distribution isotherm of the compound between the mobile phase and stationary phase should be linear and give the Gaussian peak shape.[24] Results obtained in this research revealed only the model equation of gallic acid could explain the effect of flow rate and acetonitrile composition on the character of the tailing factor. Notably, the physicochemical properties, particularly the polarity of gallic acid, behaved differently from the other 2 compounds. Gallic acid eluted faster, spending less time in the stationary phase, with a retention time of 2.7 min, compared to 15.2 min for vitexin and 48.9 min for imperatorin. These factors alone could not fully explain the peak behavior of some compounds. Other factors or parameters, beyond flow rate and percentage of organic solvent, might play a key role in providing a clearer explanation.

Table 3: The ANOVA from the statistical analysis of RSM for capacity factor, tailing factor and number of theoretical plates.

Responses (*Yi*)	Model equation p-value	Model equation F-value	R^2	Adjusted R^2
Imperatorin				
Capacity factor (Y_1)	<0.0001*	296.80	0.9834	0.9801
Tailing factor (Y_2)	***	***	***	***
Number of theoretical plates (Y_3)	0.2541**	1.28	0.2397	0.0876
Gallic acid				
Capacity factor (Y_1)	0.0012*	14.29	0.7408	0.6889
Tailing factor (Y_2)	<0.0001*	46.42	0.9028	0.8833
Number of theoretical plates (Y_3)	<0.0001*	39.55	0.9519	0.9278
Vitexin				
Capacity factor (Y_1)	<0.0001*	139.89	0.9901	0.9830
Tailing factor (Y_2)	0.1566**	2.28	0.6193	0.3473
Number of theoretical plates (Y_3)	<0.0001*	241.28	0.9942	0.9901

*Significant at *p*-value <0.05. **Not significant at *p*-value >0.05. ***could not fit the model.

The number of theoretical plates is a parameter that measures the peak dispersion on the HPLC column and can reflect the column performance. Therefore, A column with more theoretical plates allows for greater equilibration and enhances the quality of separation.[24] The composition of acetonitrile in the mobile phase had a greater influence than the flow rate, as indicated by the coefficient in the model equation and the steeper slope in the perturbation plots in Figures 3B and 3C.

CONCLUSION

The results from this study provided not only information on which factor performed the higher influence on the responses. The model equations from experimental design with 3-level factorial design for all compounds with ANOVA and RSM could reveal the interaction between factors and the responses. Moreover, the model equation could be used for the optimization of the factors which could provide the needed responses. From the results of this study, it could be concluded that both flow rate and acetonitrile composition in the mobile phase affect the parameters including capacity factor, tailing factor and number of theoretical plates with different manner for each compound depending on the physicochemical properties. Thus, the HPLC analysis of imperatorin, gallic acid and vitexin which were the main active component in Tri-karsornmas formula should concern to these factors to get good quality separation and determination.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

HPLC: High-performance liquid chromatography; **RSM:** Response surface method; **TKM:** Tri-Kaysornmas formula; **nm:** Nanometer; **µL:** Microliter; **mL:** Milliliter; **N:** Number of theoretical plates; **T:** Tailing factor; **K:** Capacity factor; $\mathbf{t_R}$**:** Retention time; **Wb:** Peak width; **min:** Minute.

SUMMARY

This study aimed to explores the factors affecting the quantitative analysis of some active compounds in Tri-kaysornmas formulation. The tri-kaysornmas formula is one of the Thai traditional formulas in Thailand. Many phytochemical active compounds are found in this formula such as vitexin, imperatorin and gallic acid. High-performance liquid chromatography was used for the analysis of these compounds. A reverse-phase column, Hypersil ODS was used and the absorbance detection was at 254 nm (imperatorin), 275 nm (gallic acid) and 340 nm (vitexin). Two factors including flow rate and acetonitrile composition in the mobile phase of the HPLC system and 3 responses including capacity factor, tailing factor and number of theoretical plates were studied. Three level factorial designs with response surface methods were selected for this study. Experimental design with a 3-level flow rate from 0.8-1.2 mL/min and 3-level percentage acetonitrile from 10-15% were set. it could be concluded that both flow rate and percentage of acetonitrile in the mobile phase affect the parameters including capacity factor, tailing factor and number of theoretical plates with different manner for each compound depending on the physicochemical properties. These 3-level factorial designs with response surface methodology could be applied to this study and extended for HPLC determination of other active compounds in herbal formulations.

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