Journal of Applied Pharmaceutical Science Vol. 13(09), pp 048-057, September, 2023
Available online at http://www.japsonline.com
DOI: 10.7324/JAPS.2023.137317
ISSN 2231-3354

Factors influencing high-performance liquid chromatography for piperine determination in traditional Thai formulas

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ARTICLE INFO

Received on: 07/03/2023
Accepted on: 10/08/2023
Available Online: 04/09/2023

Key words:
Thai traditional formula, *Piper nigrum*, piperine, Box–Behnken design, response surface methodology, HPLC, system suitability testing.

ABSTRACT

Piperine, the major bioactive compound, was found in *Piper nigrum*. This present investigation aimed to find the factors that can affect high-performance liquid chromatography (HPLC) conditions for the evaluation of piperine content from Thai herbal medicines. Box–Behnken design for the response surface methodology was selected to assess the interaction between three factors (% acetonitrile in the mobile phase, flow rate, and detection wavelength) and five outputs (retention time, peak area, theoretical plate, tailing factor, and capacity factor). The optimal HPLC system was found to be a mobile phase containing 35% acetonitrile with a flow rate of 1.2 ml/minute at wavelength 254 nm. The evolved HPLC condition was subjected to system suitability and robustness testing. The results indicated a slight change to the acetonitrile ratio affected the retention time, peak area, tailing factor, and capacity factor. This HPLC method was reliable and was applied to determine the piperine constituent in seven Thai herbal recipes, ranging from 0.256 ± 0.064 to 22.284 ± 0.802 mg/g extract. Overall, the experimental design was very helpful in studying the factors that affect the HPLC conditions and robustness of this method, which was discovered to be uncomplicated to carry out and acceptable for the exploration of piperine in Thai herbal medicines.

INTRODUCTION

Thai traditional herbs have been used by local healers throughout history (Srichaikul *et al*., 2012), and more than 50 Thai traditional herbal formulas are listed in the National List of Essential Medicines (Thaenkham *et al*., 2017). *Piper nigrum* L. (black pepper) belongs to the Piperaceae family, which is a highly valuable plant and an essential ingredient in various traditional formulas, including in Ayurvedic literature (Zarai *et al*., 2013). The important pharmacological properties of *P. nigrum* include antimicrobial, antioxidant, anticancer, antidiabetic, anti-inflammatory, analgesic, anticonvulsant, and neuroprotective activities (Takooree *et al*., 2019). The major alkaloid of this plant is piperine (Shityakov *et al*., 2019). Piperine displays antioxidant, anticarcinogenic, anti-inflammatory, antiulcer, antithyroid, and antimicrobial activities (Gorgani *et al*., 2017). In addition, piperine promotes the bioavailability of some drugs by diminution of metabolism and decreases blood cholesterol levels (Duangjai *et al*., 2013).

Although researchers have previously reported on the establishment of high-performance liquid chromatography (HPLC) procedures for the estimation of piperine content (Hazra *et al*., 2019; Jana *et al*., 2021; Kamal *et al*., 2012; Khismatrao *et al*., 2018; Setyaningsih *et al*., 2021; Shrestha *et al*., 2020; Upadhyay *et al*., 2013), they have not employed systematic statistical optimization of those methods for use in herbal formulas. HPLC optimization methods are complicated procedures involving the simultaneous monitoring of the variation of parameters such as solvent system, pH, buffer concentration, flow rate, column temperature, and detector to achieve effective system optimization

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(Kazusaki *et al*., 2012; Kumar *et al*., 2015). After this, the HPLC method needs to be properly adjusted and modified before being used for analysis.

Factorial experimental design has been used for assessing and optimizing various processes, particularly in analytical research in which methods can be influenced by multiple factors (Sahu *et al*., 2018). Response surface methodology (RSM) was used to optimize the process when variable factors and interactions affect the observed response (Yolmeh and Jafari, 2017). Applying Box–Behnken design (BBD) to RSM is performed for the appropriate parameters collectively to obtain the maximum useful information from the fewest possible experiments, thereby minimizing cost and maximizing profit (Silva *et al*., 2017). Some studies revealed the application of RSM and BBD to optimize some extraction methods in herbal extract (Ngamkhae *et al*., 2022). This current study examined the factors that can affect HPLC analysis using RSM with BBD, followed by system suitability testing and robustness testing before application to determine piperine content in seven Thai traditional herbal formulas containing *P. nigrum*.

MATERIAL AND METHODS

Chemicals and reagents

Piperine was purchased from Sigma-Aldrich, St. Louis, MO. Acetonitrile and n-propanol at HPLC grade were purchased from Merck Ltd., Darmstadt, Germany. Formic acid and methanol (analytical reagent grade) were purchased from BDH (VWR International Ltd, USA). Seven Thai traditional formulas that contain *P. nigrum* in the formulation were purchased from herb shops in Khon Kaen province, Thailand.

Methods

Preparation of standard piperine solutions

Piperine stock solution (1,000 µg/ml) was prepared in methanol and diluted to the concentrations of 5, 10, 25, 50, 75, and 100 µg/ml as a working solution.

Preparation of sample solution

Dried powders of traditional Thai formulas (10 g in each formula) were macerated with 30 ml of methanol, sonicated for 30 minutes, and filtered through a 0.45 µm nylon syringe filter. All extracts were mixed with methanol at the ratio of 1:100 and kept at −20°C prior to analysis.

Experimental design for HPLC condition

The HPLC condition was established using factorial design RSM with BBD (Box and Behnken, 1960). This method was applied to find the important factors affecting piperine elution in the HPLC system. Three factors, flow rate, detection wavelength, and acetonitrile ratio, were differed at three levels each (Table 1) and monitored for five observed responses: retention time, peak area, number of theoretical plates, tailing factor, and capacity factor. The above responses were optimized using Design Expert software (version 13) via multiple response algorithms. The individual parameters with three levels give rise to the 17 experimental designs presented in Table 2. RSM with BBD statistical testing was applied to optimize the parameters and evaluate quadratic effects for all factors and responses. The linear polynomial equation generated from analysis of variance (ANOVA) is described as follows (Eq. 1):

$$Y = b_0 + b_1x_1 + b_2x_2 + b_3x_3 + b_{12}x_1x_2 + b_{13}x_1x_3 + b_{23}x_2x_3 + b_{11}x^2{}_1 + b_{22}x^2{}_2 + b_{33}x^2{}_3 \quad (1)$$

where Y is the observed response, b_0 is a constant, and b_1–b_{33} are the regression coefficients computed from the observed experiment, x_1, x_2, and x_3 are the coded values of independent variables representing the percentage of acetonitrile, wavelength, and flow rate, respectively.

Table 1. Variables and levels used in BBD.

Factors	Low (−1)	Medium (0)	High (+1)
Flow rate (ml/minute)	0.8	1	1.2
Wavelength (nm)	252	254	256
Percentage of acetonitrile (%)	25	30	35

Table 2. BBD for testing three variables at three levels of HPLC conditions.

Runs	x_1 Flow rate (ml/minute)	x_2 Wavelength (nm)	x_3 Acetonitrile (%)
1	+1	0	+1
2	0	−1	−1
3	+1	+1	0
4	−1	0	−1
5	0	0	0
6	0	0	0
7	−1	0	+1
8	0	0	0
9	+1	0	−1
10	0	0	0
11	0	+1	−1
12	+1	−1	0
13	−1	−1	0
14	0	0	0
15	−1	+1	0
16	0	+1	+1
17	0	−1	+1

Robustness testing

In pharmaceutical analysis, robustness testing is becoming a more important procedure in validation investigations of analytical experiments. Robustness testing determines the capacity of a system to remain unaffected by small changes in method parameters (Ferreira *et al*., 2017). Variations of flow rate, detection wavelength, and percentage of acetonitrile in the mobile phase were considered in this study. The tailing factor of the major piperine peak was one important chromatographic criterion used as a method response for robustness testing. This experimental design was also applied to assess the robustness of the developed method via an alternative approach in which factors were investigated simultaneously.

System suitability parameters

System suitability is regarded as the performance qualification of various analytical procedures. System suitability parameters are established for a specific procedure depending on the type of procedure being validated (Tiryaki *et al*., 2009). Six replicates of the piperine standards were injected and analyzed using the optimized method. The precision of the retention time, peak area, number of theoretical plates, tailing factor, and capacity factor responses were analyzed and calculated after injection (Bose, 2014). The following definitions of these parameters and the equations for calculating them are taken from the International Conference on Harmonisation of Technical Requirments for Registration of Pharmaceutical for Human Use (ICH) guideline (ICH-Q2 (R1), 2005) and the Center for Drug Evaluation and Research (CDER) guideline (CDER, 1994).

Retention time

The retention time is an easy-to-identify parameter. The condition of the column can influence this parameter, differences between lots of the mobile phase, and variations in ambient temperature (Tiryaki *et al*., 2009). For the analysis, the variation of retention time is defined in terms of relative standard deviation.

Peak area

The area under the peak represents the size and area of the component peak and is proportional to the amount of the component found in a sample. The peak area is measured and calculated automatically by the HPLC operating system (Vanbel and Schoenmakers, 2009).

Number of theoretical plates

The theoretical plate number is used to determine column efficiency (Bose, 2014). The plate number changes depending on the type of analysis carried out. This parameter is calculated by the following equation:

$$N = 16\,(t_R / t_W)^2 = L / H \quad (2)$$

where N is the number of theoretical plates, t_R is the retention time of the interested peak, t_W is the peak width measured at the baseline, L is the length of the column, and H is the height equivalent of the theoretical plate.

Tailing factor

The column efficiency depends not only on the number of theoretical plates but also on the tailing factor. The tailing factor is also called the symmetry factor. This parameter demonstrates the symmetry of the peak shape (Bose, 2014). The tailing factor is calculated by the following equation:

$$T = W_x / 2f \quad (3)$$

where T is the tailing factor, W_x is the width of the peak at either 5% (0.05) or 10% (0.10) from the baseline of the peak height, and f is the distance between the peak maximum and the peak front at W_x.

Capacity factor

The capacity factor is a measurement of the retention of the analyte with respect to the void volume or elution time of the nonretained components (Bose, 2014). The capacity factor is calculated by the following equation:

$$k' = (t_R - t_O) / t_O, \quad (4)$$

where k' is the capacity factor, t_R is the retention time of the interested peak, and t_O is the elution time of the void volume or nonretained components.

Chromatographic conditions for determination of piperine in Thai herbal formulas

This study was performed using a Shimadzu HPLC system consisting of a solvent delivery pump (LC-20AD), a UV-Vis detector (SPD-20A), and a manual injector with a 20 µl loop (Shimadzu USA Manufacturing Inc., Japan). Separation was performed on a C_{18} column (Hypersil ODS, 5 µm, 4.0 × 250 mm, Agilent Technologies Inc., Santa Clara, CA) with an isocratic system. The suitable mobile phase consisted of 1% formic acid: propanol: acetonitrile in the ratio of 55: 10: 35, v/v, with a flow rate of 1.2 ml/minute and wavelength at 254 nm.

Statistical analysis

The analysis of each experimental set was carried out in triplicate. Design Expert® (version 13), Stat-Ease Inc., Minneapolis, MN, statistical software was used for designing the optimized conditions of the HPLC method.

RESULTS AND DISCUSSION

Experimental design for HPLC condition

According to the BBD, 17 experimental runs were conducted for 3 variable factors at 3 levels each and 5 responses. Further investigation evaluated the relationship between the observed responses and independent factors using RSM. Based on these values, a quadratic model was selected for the retention time, peak area, number of theoretical plates, and capacity factor responses. In this study, three independent factors, flow rate (x_1), wavelength (x_2), and %acetonitrile (x_3) were varied during trial runs. The qualitative responses were retention time (Y_1), peak area (Y_2), number of theoretical plates (Y_3), tailing factor (Y_4), and capacity factor (Y_5). The investigated responses were calculated and are shown in Table 3. Based on the predicted residual error sum of squares (PRESS) values, a quadratic model was selected to fit all responses. PRESS provides a summary measure of model fitting, which is calculated from the sums of the squares of the prediction residuals for those observations (Xu, 2017). An ANOVA of the response in the quadratic model was performed, and the results are shown in Table 4. A statistically significant model of responses found that Y_1, Y_2, Y_4, and Y_5 showed p values less than 0.05 with low coefficients of variation (% CV). The ANOVA results for Y_1, Y_2, and Y_4 also showed high adjusted R^2 values indicating a good relationship between the experiment and the fitted model. For adequate precision, a ratio of the predicted values at the design points to the average prediction error greater than 4 indicates adequate model discrimination (Moradi *et al*., 2016). In this investigation, the adequate precision of all responses was found to be more than 4 and therefore considered satisfactory.

From the lack-of-fit results, the F-values of Y_2 (4.67) and Y_4 (1.62) were not significant (p values = 0.0852 and 0.3180, respectively), indicating the fit was good with a satisfactory model

Table 3. Observed responses of BBD testing of three variables at three levels.

Runs	Factors			Responses				
	X_1 Flow rate	X_2 Wavelength	X_3 Acetonitrile	Y_1 Retention time	Y_2 Peak area	Y_3 Theoretical plate	Y_4 Tailing factor	Y_5 Capacity factor
1	1.2	254	35	11.684	450,636.000	5,044.423	1.017	4.433
2	1	252	25	46.448	165,263.667	12,063.188	1.366	22.224
3	1.2	256	30	18.098	399,542.333	5,578.500	1.056	8.049
4	0.8	254	25	38.952	469,990.333	3,436.690	1.412	18.476
5	1	254	30	21.491	450,120.333	4,744.527	1.099	9.746
6	1	254	30	21.623	461,913.667	3,816.918	1.111	9.812
7	0.8	254	35	16.228	553,032.667	4,364.804	0.991	7.114
8	1	254	30	21.607	444,670.333	4,795.651	1.086	9.803
9	1.2	254	25	26.393	378,850.333	2,986.407	1.317	12.196
10	1	254	30	21.272	427,283.333	4,307.913	1.087	9.636
11	1	256	25	31.368	421,259.667	2,897.285	1.525	14.684
12	1.2	252	30	17.664	354,441.333	4,008.178	1.048	7.832
13	0.8	252	30	26.435	503,835.667	4,567.895	1.095	12.217
14	1	254	30	21.368	397,008.333	4,230.615	1.034	9.684
15	0.8	256	30	26.482	447,672.667	4,478.791	1.111	12.241
16	1	256	35	12.990	396,342.667	3,717.919	1.014	5.495
17	1	252	35	13.078	402,770.333	3,184.800	1.026	5.539

Table 4. The ANOVA results of the response surface model for retention time, peak area, theoretical plate, tailing factor, and capacity factor.

Source	Y_1 Retention time		Y_2 Peak area		Y_3 Theoretical plate		Y_4 Tailing factor		Y_5 Capacity factor	
	F	*p*	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*
Model	19.06	0.0004*	6.35	0.0118*	1.06	0.4804	40.96	<0.0001*	19.06	0.0004*
x_1	20.10	0.0029*	11.68	0.0112*	0.018	0.8969	3.33	0.1108	20.10	0.0029*
x_2	3.52	0.1026	4.35	0.0756	1.56	0.2519	3.36	0.1094	3.52	0.1026
x_3	132.36	<0.0001*	10.31	0.0148*	0.7843	0.4052	283.98	<0.0001*	132.36	<0.0001*
x_1x_2	0.0049	0.9462	1.57	0.2509	0.1679	0.6942	0.0156	0.9042	0.0049	0.9462
x_1x_3	1.69	0.2344	0.0194	0.8933	0.0779	0.7883	3.37	0.1089	1.69	0.2344
x_2x_3	7.35	0.0302*	10.52	0.0142*	5.74	0.0478*	6.72	0.0358*	7.35	0.0302*
x^2_1	0.6445	0.4485	7.35	0.0301*	0.3875	0.5533	2.8	0.1379	0.6445	0.4485
x^2_2	1.74	0.2283	10.3	0.0149*	0.8200	0.3953	1.75	0.228	1.74	0.2283
x^2_3	4.07	0.0834	1.81	0.2205	0.0383	0.8503	63.51	<0.0001*	4.07	0.0834
Lack of fit	772.91	<0.0001	4.67	0.0852	57.51	0.0010	1.62	0.3180	772.91	<0.0001
%CV	11.98		9.65		44.00		2.89		13.12	
R^2	0.9608		0.8909		0.5768		0.9814		0.9608	
Adjusted R^2	0.9104		0.7505		0.0327		0.9574		0.9104	

* Significant at *p* value less than 0.05.

(Bisht *et al*., 2012). As shown in Table 4, the linear parameter x_1 was significant at the level of $p < 0.05$ for Y_1 and Y_2, and the linear parameter x_3 was significant for Y_1, Y_2, and Y_4 at the level of $p < 0.001$, 0.05, and 0.001, respectively. The quadratic parameters x^2_1 and x^2_2 were significant for Y_2 at the level of $p < 0.05$, while the quadratic parameter x^2_3 was significant for Y_4 at the level of $p < 0.0001$. Moreover, the interaction parameter x_2x_3 was significant for Y_1, Y_2, Y_3, and Y_4 at the level of $p < 0.05$. For overall testing, the significant regression and nonsignificant lack-of-fit results revealed that the regression equation was suitable to show the relationship between the response values (Y) and independent variables, except for Y_3, with an R^2 value less than 0.75. The second-order regression equations (Eqs. 5–9) were fitted as follows:

Retention time:

$$Y_1 = 21.47 - 4.38X_1 - 1.84X_2 - 11.25X_3 + 0.097X_1X_2 + 1.8X_1X_3 + 3.75X_2X_3 - 1.08X^2_1 + 1.78X^2_2 + 2.72X^{23}. \quad (5)$$

Peak area:

$$Y_2 = 436{,}200 - 48{,}882.67x_1 + 29{,}813.29x_2 + 45{,}927.21x_3 + 25{,}316x_1x_2 - 2{,}814.17x_1x_3 - 65{,}605.92x_2x_3 + 53{,}446.02x^2_1 - 63{,}272.22x^2_2 - 26{,}517.89x^2_3. \quad (6)$$

Theoretical plate:

$$Y_3 = 4{,}379.12 + 96.17x_1 - 893.95x_2 - 633.95x_3 - 414.86x_1x_2 + 282.48x_1x_3 + 2{,}424.76x_2x_3 - 614.25x^2_1 + 893.47x^2_2 + 193.21x^2_3. \quad (7)$$

Tailing factor:

$$Y_4 = 1.08 - 0.0213x_1 + 0.0214x_2 - 0.1965x_3 - 0.0021x_1x_2 + 0.0303x_1x_3 - 0.0428x_2x_3 - 0.0269x^2_1 + 0.0212x^2_2 + 0.1281x^2_3. \quad (8)$$

Capacity factor:

$$Y_5 = 9.74 - 2.19x_1 - 0.9178x_2 - 5.62x_3 + 0.0484x_1x_2 + 0.8996x_1x_3 + 1.87x_2x_3 - 0.5410x^2_1 + 0.8897x^2_2 + 1.36x^2_3. \quad (9)$$

The response data values from the BBD were substituted into each model for evaluation using the coefficient of determination (R^2), which represents the proportion of the model that can explain the variation in the responses (Tan *et al*., 2010). The R^2 values for retention time, peak area, number of theoretical plates, tailing factor, and capacity factor were 0.9608, 0.8909, 0.5768, 0.9814, and 0.9608, respectively. Accordingly, Y_1, Y_2, Y_4, and Y_5 gave sufficiently high R^2 values to conclude that these developed models satisfactorily described the system's behavior within the range of the operating parameters (Box and Behnken, 1960), while Y_3 did not. This might reveal that the variable factors selected in this design (flow rate, detection wavelength, and percentage acetonitrile in mobile phase) were not appropriate and potentially affected the number of theoretical plates in this experiment. The number of theoretical plates value represents an equilibrated partitioning of the solute between the stationary and mobile phases, which is used to indicate column efficiency (Barth, 2018). The major factors that affect the theoretical plate response are the particle size of the stationary phase, injection volume, dead volume of interconnecting tubing, and column length and temperature. Mobile phase composition and flow rate show only weak-to-medium effects on the number of theoretical plates response (Maneenet *et al*., 2019).

To examine how the variable factors affect the different responses, a perturbation plot can be used to compare all factors at a particular point in the design space. Each response factor was plotted by changing one factor over its range while the other factors were kept constant (Fig. 1). A response is considered sensitive to a factor if the slope of the line in the perturbation plot is steep and curved, and if the line is flat, the response is considered insensitive to change by that factor. In our study, line C in Figure 1a represents the change in retention time as the percentage of acetonitrile in the mobile phase is varied. It shows a steep slope and curved shape, which indicates that this factor affected this response. Similarly, flow rate appeared to affect peak area (line A, Fig. 1b) and the percentage of acetonitrile in the mobile phase affected the tailing factor and capacity factor (line C, Fig. 1d and e).

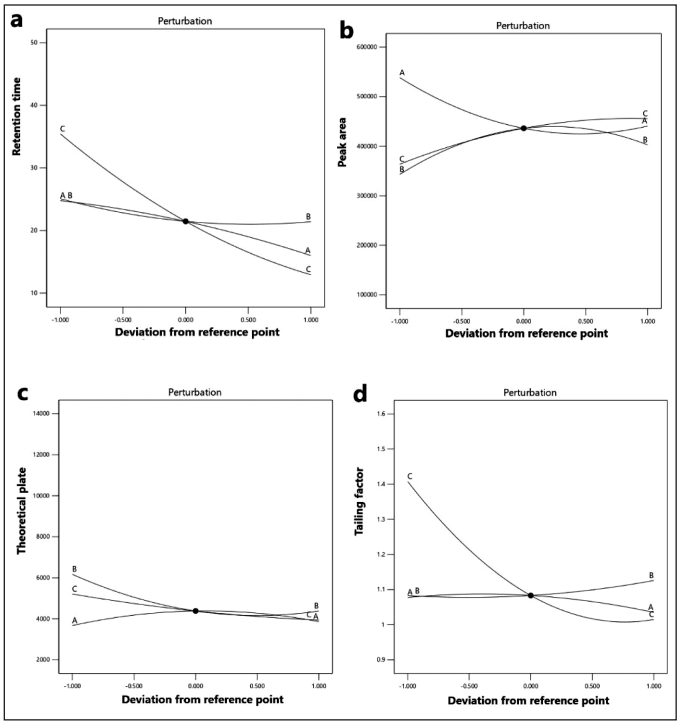

Figure 1. Perturbation plots showing the effect of various factors [lines: A: flow rate (0.8, 1.0, and 1.2 ml/minute), B: wavelength (250, 252, and 254 nm), and C: percentage acetonitrile (25%, 30%, and 35%)] on responses (a: retention time, b: peak area, c: theoretical plate, d: tailing factor, and e: capacity factor).

Further investigation using RSM evaluated the interaction between the independent factors and dependent responses. Three-dimensional (3D) response surface plots of the effect of flow rate, detection wavelength, and percentage acetonitrile of the HPLC system on the retention time, peak area, number of theoretical plates, tailing factor, and capacity factor responses are shown in Figure 2. The 3D response surface plots present dependent interactions between two variable factors while the third factor is kept constant. For example, Figure 2A–C indicates that decreasing the percentage of acetonitrile in the mobile phase substantially reduced retention time, while changes in flow rate showed a small effect and changes in detection wavelength showed no effect. Figure 2D–F indicates that increasing the percentage of acetonitrile and the flow rate had a medium effect on the piperine peak area. The percentage of acetonitrile showed a small effect on the number of theoretical plates response (Fig. 2G–I) and the tailing factor increased with increased flow rate and detection wavelength and reduced with a decreased percentage of acetonitrile (Fig. 2J–L). Figure 2M–O indicates that increased acetonitrile percentage and higher detection wavelength showed an effect on the capacity factor of the column.

Therefore, the appropriate HPLC system for the determination of piperine content was 35% acetonitrile in the mobile phase at a flow rate of 1.20 ml/minute with detection at a wavelength of 254 nm. These HPLC conditions gave the shortest retention time (11.68 minutes) and highest peak area (450,636), with optimum theoretical plate (5,044), tailing factor (1.017), and capacity factor (4.433) values. These results confirmed that the response model adequately reflected the expected optimization of

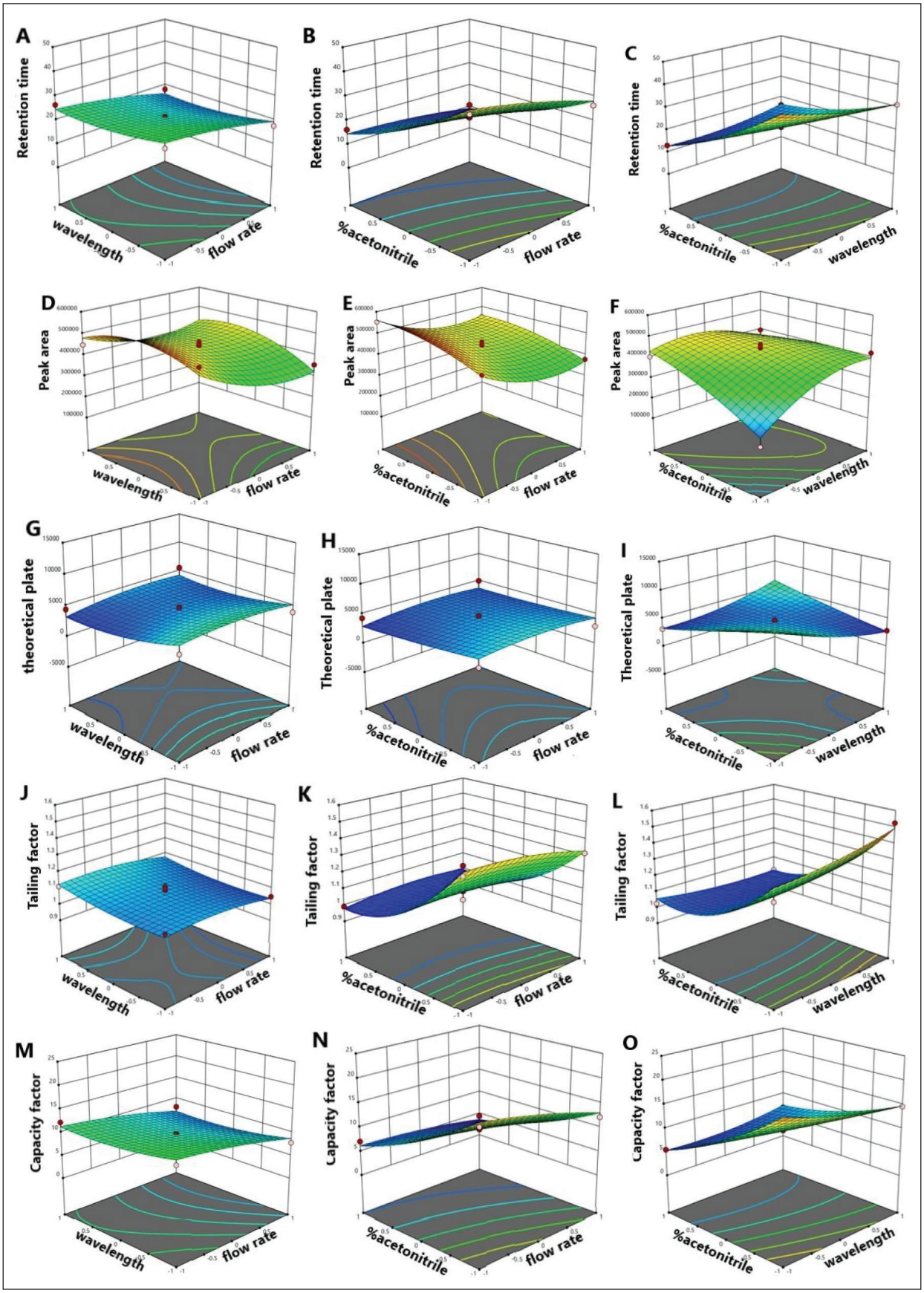

Figure 2. 3D response surface plot expressing interaction effects of various factors on responses. ABC: retention time; DEF: peak area; GHI: theoretical plate; JKL: tailing factor; MNO: capacity factor.

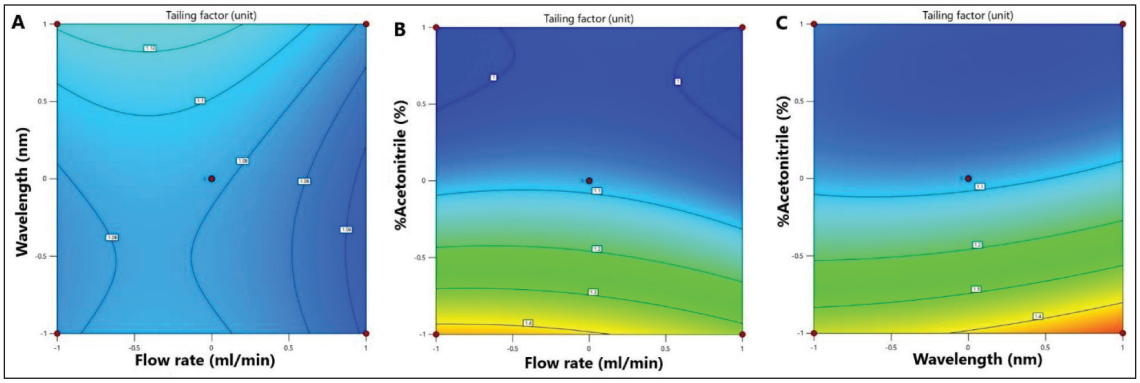

Figure 3. Response surface contour graphs showing the effect of flow rate, wavelength, and percentage acetonitrile on the tailing factor response. A: wavelength and flow rate; B: percentage acetonitrile and flow rate; and C: percentage acetonitrile and wavelength.

HPLC conditions, and the model was satisfactory and accurate. A study by Maneenet *et al*. (2019) previously determined piperine content in Kleeb Bua Daeng Thai traditional formula by this HPLC condition. The previous research was about Kleeb Bua Daeng, a Thai traditional herbal formula that ameliorated unpredictable chronic mild stress-induced cognitive impairment in ICR mice and revealed using HPLC for the determination of active compounds in some Thai formula and validating of the HPLC method, including the parameters of accuracy, precision, linearity, limit of detection, and limit of quantitation. However, there are many Thai herbal formulas in which one of the compositions in this formula contains *P. nigrum* (one of the compositions in the Kleeb Bua Daeng formula), and the main active compound in *P. nigrum* is piperine. Thus, the optimization for the condition of the HPLC method to analyze piperine should be developed from the previous study to improve the quality and potential of this HPLC method for the determination of piperine and applied to the other herbal formula containing *P. nigrum*. The previous study did not reveal some parameters, such as robustness testing, which is important and could improve the systematic development of HPLC conditions for the analysis and expand using this analytical method to determine the other herbal formulas.

Robustness testing

Robustness is an important parameter in method validation criteria, but robustness testing for piperine analysis has never previously been reported in the former study (Maneenet *et al*., 2019). The experimental design with the Box–Behnken method employed in this study could be applied to investigate the robustness of the developed HPLC method. The tailing factor was chosen as the response for robustness testing. From the tailing factor quadratic second-order regression equation (Eq. 8), the percentage of acetonitrile in the mobile phase (x_3) had the highest coefficient, indicating that it had the highest influence on the tailing factor (Y_4). Thus, large changes in the percentage of acetonitrile in the mobile phase could significantly affect the tailing factor. However, the variation in the tailing factor for all variable changes was less than 2, which is within the acceptance criteria for the tailing factor. Contour plots are two-dimensional representations of the function of varying two factors at a time while holding other factors at specified levels (Kumar *et al*., 2016). In this study, contour graphs were generated by plotting the tailing factor response against two varying factors while the third factor was held constant at the proposed optimum level. The percentage of acetonitrile in the mobile phase notably affected the tailing factor, as shown in Figure 3B and C.

Table 5. System suitability results from the HPLC system.

Parameter	Average	SD	%RSD
Retention time	10.86	0.02	0.21
Peak area	450,636.00	9,661.82	2.14
Theoretical plates	5,044.423	1,153.77	22.87
Tailing factor	1.02	0.09	8.58
Capacity factor	4.43	0.00	0.22

System suitability testing

System suitability testing assesses validated chromatographic systems before commencing sample analysis. According to the ICH and CDER, the system suitability testing represents the minimum acceptable system performance levels. The results of system suitability parameters are shown in Table 5. The value for the number of theoretical plates was 5,044.42, indicating good column efficiency and separation of piperine (acceptable theoretical plate limit > 2,000). For the tailing factor, the value was 1.017 indicating high efficacy of piperine quantitation (acceptable tailing factor limit ≤ 2). The capacity factor value was 4.433. The capacity factor represents how much the sample interacts with the chromatography material and should be between 2 and 8 (Tiryaki *et al*., 2009). Thus, the system suitability parameters were acceptable, and the method was found to be suitable (Table 5).

Chromatographic results of piperine content in Thai herbal formulas

Representative chromatograms of the seven traditional Thai formulas using the developed and optimized HPLC method are shown in Figure 4. Samples were analyzed in triplicate. The amount of piperine in the formulas ranged from 0.256 ± 0.064 to 22.284 ± 0.802 mg/g extract, as shown in Table 6. Variation in piperine content between products reflects the different compositions of *P. nigrum* in each formulation. The highest piperine content was found in product formula E, which shows the highest composition of *P. nigrum* on the product label (Fig. 4).

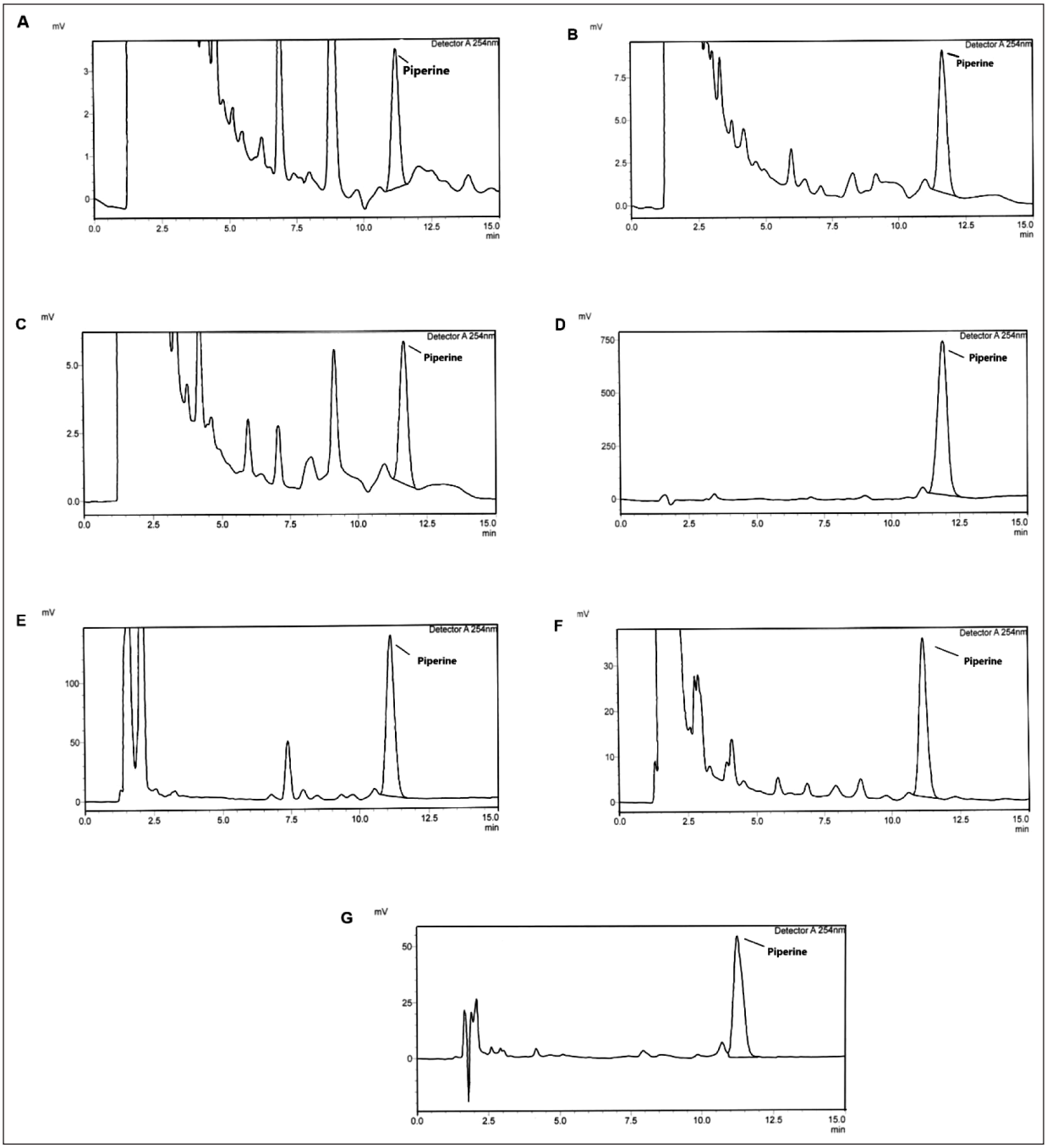

Figure 4. Chromatograms of piperine in seven traditional Thai herbal formulas (A–G).

Table 6. The content of piperine in seven traditional Thai herbal formulas.

Thai traditional herbal formula	Amount of piperine (mg/g extract)
Formula A	0.256 ± 0.064
Formula B	1.033 ± 0.176
Formula C	0.400 ± 0.089
Formula D	16.892 ± 4.601
Formula E	22.284 ± 0.802
Formula F	6.862 ± 0.170
Formula G	8.899 ± 0.141

These results confirm the usefulness of this HPLC condition for the analysis of piperine in herbal formulas.

CONCLUSION

An HPLC method was developed for the determination of piperine content in traditional Thai herbal formulas. A BBD was adopted to optimize the HPLC method. RSM was applied to assess interactions between factors (flow rate, detection wavelength, and percentage of acetonitrile in the mobile phase) and the observed responses (retention time, peak area, number of theoretical plates, tailing factor, and capacity factor). The percentage of acetonitrile in the mobile phase and flow rate showed the most impact on

retention time, peak area, and tailing factor, while detection wavelength showed only a small effect on these responses. System suitability testing of the analytical method showed it to be consistent, and robustness testing showed the method to be resistant to small changes in the analytical conditions. Therefore, the HPLC procedure was appropriated for the analysis of piperine from Thai herbal medicines and could potentially be applied to determine piperine content in other formulations containing *P. nigrum*.

ACKNOWLEDGMENTS

This work was supported by the Fundamental Fund (Basic Research Fund) of Khon Kaen University and funding support from the National Science, Research and Innovation Fund, Thailand. The authors thank Dr. Glenn Borlace for English language assistance through the Publication Clinic of Khon Kaen University.

AUTHOR CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work. All the authors are eligible to be an author as per the international committee of medical journal editors (ICMJE) requirements/guidelines.

CONFLICTS OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

ETHICAL APPROVALS

This study does not involve experiments on animals or human subjects.

DATA AVAILABILITY

All data generated and analyzed are included in this research article.

PUBLISHER'S NOTE

This journal remains neutral regarding jurisdictional claims in published institutional affiliation.

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How to cite this article:
Chulikhit Y, Maneenet J, Monthakantirat O, Khamphukdee C, Chotritthirong Y, Limsakul S, Boonyarat C, Daodee S. Factors influencing high-performance liquid chromatography for piperine determination in traditional Thai formulas. J Appl Pharm Sci, 2023; 13(09):048–057.