

# BBD-driven optimization of an RP-HPLC method for simultaneous analysis of two major isoflavone aglycones in Tofu

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**ABSTRACT:** Tofu, one of soy food products widely consumed in Indonesia, has been reported as a source of natural protein. It is important to evaluate the contents of genistein and daidzein to provide information according to the nutrient information on soy food products. A reversed-phase high performance liquid chromatography (RP-HPLC) was developed in this study in order to analyze genistein and daidzein content in tofu samples simultaneously. Employing a Box-Behnken design (BBD) for designing the experiment, the response surface methodology (RSM) was then applied to optimize chromatographic conditions including methanol composition, flowrate, and column temperature. Separation response such as retention time, resolution, and tailing factor were evaluated to build an optimization model followed by analyzing the desirability. It was found that the model predicted a composite desirability of 0.9778 can be obtained by applying the methanol composition of 60%, flowrate of 0.80 mL.min<sup>-1</sup>, and column temperature of 50°C. The optimized HPLC conditions met the acceptance criteria for retention time and area deviations, resolution, tailing factor, and theoretical plates number.

**KEYWORDS:** daidzein; genistein; experimental design; tofu.

## 1. INTRODUCTION

Soy food products were widely consumed as functional food mostly in Asian countries. The research interest in soy food products increased since several health benefits have been reported such as agent of breast cancer, prostate cancer, gastrointestinal cancer, endometrial and ovarian cancer, antidiabetics, reproductive health, cardiovascular disease, immunomodulation, thyroid function, and renal function [1]. The biological activities of soy foods have been linked to the content of isoflavone aglycones content in the soybeans (*Glycine max*). Two major isoflavone aglycones in soybeans namely genistein and daidzein were widely reported to have potential pharmacologic activity [2,3]. The previous study reported that contents of genistein and daidzein were estimated at about 50% and 40%, respectively, compared to the total soy isoflavones [4].

Tofu, one of soy food products prepared by coagulating soymilk followed by pressing the obtained curds into solid blocks, was well-known as a traditional food in Indonesia [5,6]. Tofu became more popular in Indonesia as a natural protein source and is widely produced due to the increasing demand in several areas in Indonesia [7,8]. However, there was limited publication reporting the content of both genistein and daidzein in tofu. It has become more important since the need for information regarding the intake levels of soybeans food products as well as the nutrients contents should be reported to consumers [9].

Reversed phase high-performance liquid chromatography (RP-HPLC) was reported in several studies to analyze the content of analytes in the mixture matrix [10,11]. Previous studies on analyzing soybean products were performed by RP-HPLC [12,13]. However, the appropriate RP-HPLC conditions should be evaluated to achieve the good separation between analytes. Response surface methodology (RSM), an experimental design for optimization purposes, can be applied according to the Box-Behnken design (BBD) for natural product research [14]. RP-HPLC conditions can be optimized computationally to predict the

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desirable conditions for compounds separation. The desirability functions generated from the desirability analysis stage can be employed to enhance the prediction quality of experimental design research [15]. This study aimed to develop an RP-HPLC method aided by the RSM for obtaining appropriate chromatographic condition for simultaneous analysis of genistein and daidzein in tofu samples. Experimental factors including methanol composition, flowrate, and column temperature were evaluated in this study in order to achieve several responses namely retention time, resolution, and tailing factor.

## 2. RESULTS

The experimental design for optimization of independent variables and dependent variables of genistein and daidzein separation using RP-HPLC was presented in Table 1.

**Table 1.** The BBD for optimization of independent variables and experimental dependent variables of genistein and daidzein separation using RP-HPLC

Run	Independent variables			Dependent variables					
				Genistein			Daidzein		
	X1	X2	X2	Y1g	Y2g	Y3g	Y1d	Y2d	Y3d
1	60	0.6	40	13.288	5.774	1.297	9.913	1.381	1.348
2	80	0.6	40	6.195	1.724	1.484	5.671	0.006	1.455
3	60	1.0	40	7.538	6.027	1.267	5.704	7.172	1.260
4	80	1.0	40	3.654	1.582	1.356	3.356	1.206	1.385
5	60	0.8	30	10.165	6.140	0.975	7.557	1.130	1.057
6	80	0.8	30	4.649	1.780	1.274	4.239	0.144	1.315
7	60	0.8	50	7.902	4.595	1.047	6.219	4.578	1.164
8	80	0.8	50	4.224	1.254	0	3.942	2.359	0
9	70	0.6	30	8.406	3.859	1.206	6.999	6.134	1.259
10	70	1.0	30	5.055	3.614	1.185	4.205	4.998	1.230
11	70	0.6	50	7.241	2.745	1.184	6.304	2.741	1.247
12	70	1.0	50	4.180	2.395	1.212	3.667	1.533	1.265
13	70	0.8	40	5.660	3.019	1.223	4.852	1.214	1.259
14	70	0.8	40	5.687	3.051	1.211	4.872	2.758	1.252
15	70	0.8	40	5.697	3.062	1.207	4.868	1.528	1.248
16	70	0.8	40	5.715	3.046	1.212	4.896	1.484	1.242

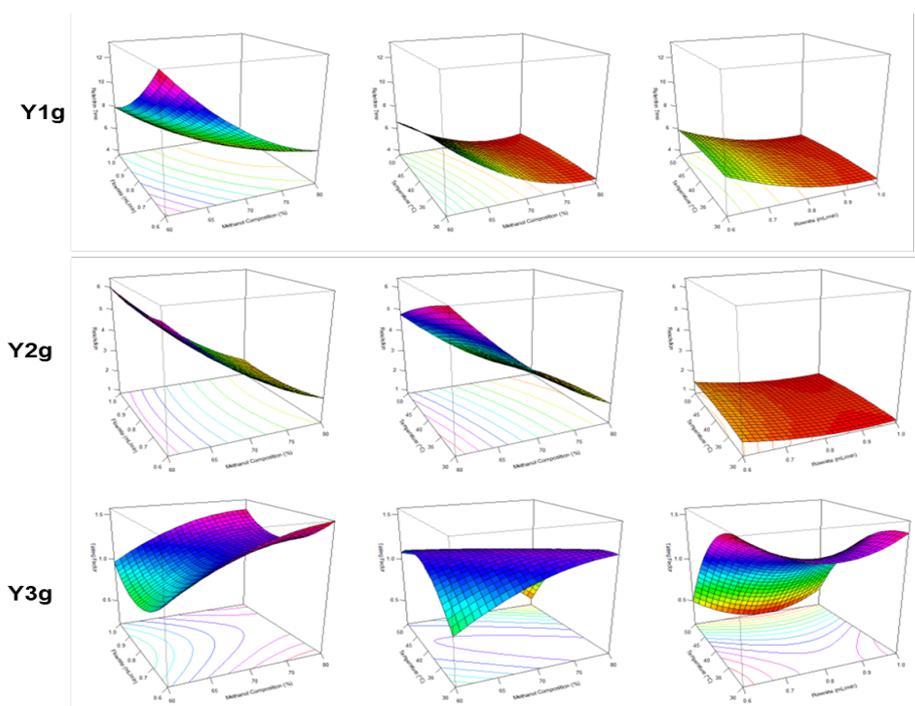
Notes: X1: methanol composition (%); X2: flowrate (mL.min<sup>-1</sup>); column temperature (°C); Y1g: retention time of genistein (min); Y2g: resolution of genistein; Y3g: tailing factor of genistein; Y1d: retention time of daidzein (min); Y2d: resolution of daidzein; Y3d: tailing factor of daidzein.

Sixteen experimental runs were carried out and observed. Retention time, resolution, and tailing factor of genistein and daidzein were evaluated. Each response for each analyte was modelled to obtain RSM model equations along with RSM properties such as multiple R<sup>2</sup>, adjusted R<sup>2</sup>, and p-value. The RSM model equations of retention time, resolution, and tailing factor for genistein and daidzein were presented in Table 2.

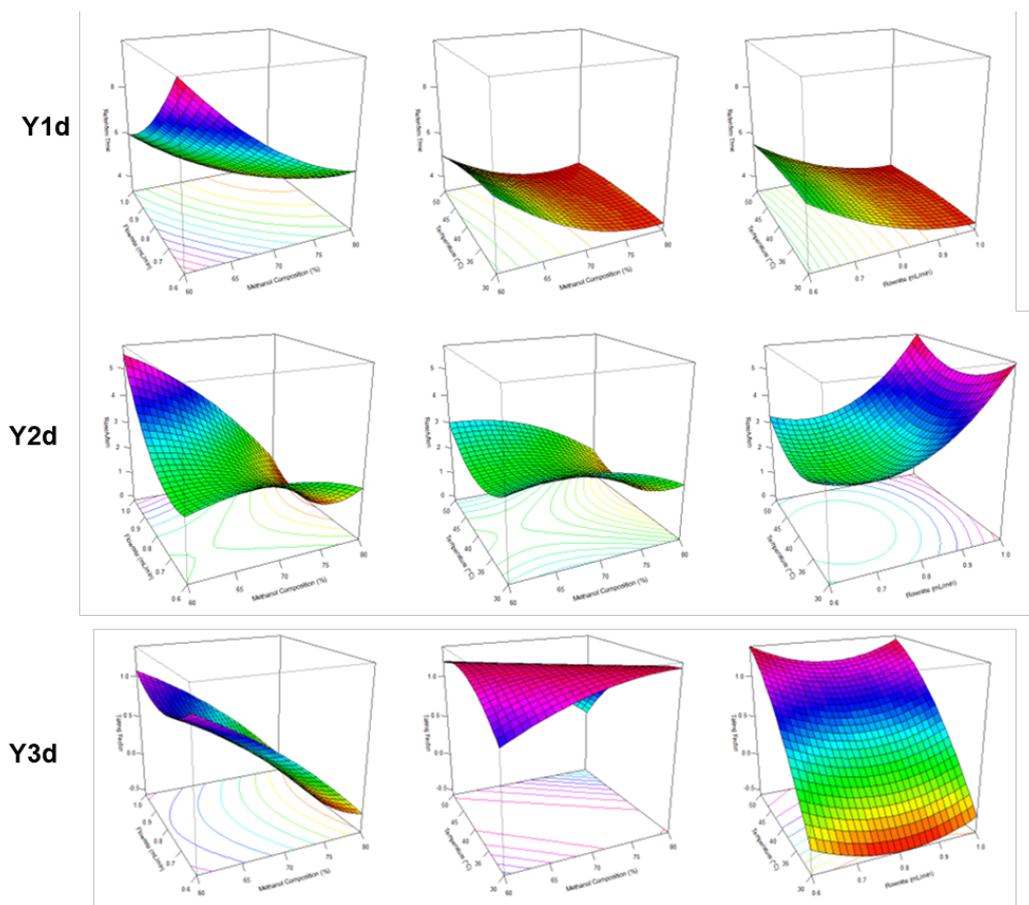
**Table 2.** The RSM model equations of retention time, resolution, and tailing factor for genistein and daidzein

Responses	RSM model equations	Multiple R <sup>2</sup>	Adjusted R <sup>2</sup>	p-value
<b>Genistein</b>				
Retention	$Y1g = 139.127 - 2.502X_1 - 68.008X_2 - 0.249X_3 + 0.401X_1X_2 + 0.005X_1X_3 + 0.036X_2X_3 + 0.012X_1^2 + 18.306X_2^2 - 0.002X_3^2$	0.9905	0.9761	2.274e-05
Resolution	$Y2g = 50.369 - 0.980X_1 - 5.186X_2 - 0.133X_3 - 0.049X_1X_2 - 0.003X_1X_3 - 0.013X_2X_3 + 0.005X_1^2 + 5.541X_2^2 - 0.001X_3^2$	0.9959	0.9898	1.794e-06
Tailing factor	$Y3g = -13.633 + 0.303X_1 - 9.692X_2 + 0.433X_3 - 0.012X_1X_2 - 0.003X_1X_3 + 0.006X_2X_3 - 0.001X_1^2 + 6.381X_2^2 - 0.003X_3^2$	0.8013	0.5032	0.1207
<b>Daidzein</b>				
Retention	$Y1d = 87.237 - 1.485X_1 - 46.699X_2 - 0.134X_3 + 0.237X_1X_2 + 0.003X_1X_3 + 0.020X_2X_3 + 0.007X_1^2 + 13.669X_2^2 - 0.001X_3^2$	0.9931	0.9826	8.813e-06
Resolution	$Y2d = -25.156 + 1.223X_1 - 6.444X_2 - 0.479X_3 - 0.574X_1X_2 - 0.003X_1X_3 - 0.009X_2X_3 - 0.006X_1^2 + 31.175X_2^2 - 0.009X_3^2$	0.4908	-0.2731	0.7358
Tailing factor	$Y3d = -13.358 + 0.310X_1 - 10.058X_2 + 0.421X_3 + 0.002X_1X_2 - 0.004X_1X_3 + 0.006X_2X_3 - 0.001X_1^2 + 5.975X_2^2 - 0.002X_3^2$	0.7811	0.4528	0.1519

According to the results, RSM model of Y1g and Y2g were analysed further for generating desirability function. Response surface plot of retention time (Y1g), resolution (Y2g), and tailing factor (Y3g) for genistein were depicted in Figure 1. Response surface plot of retention time (Y1d), resolution (Y2d), and tailing factor (Y3d) for daidzein were depicted in Figure 2.



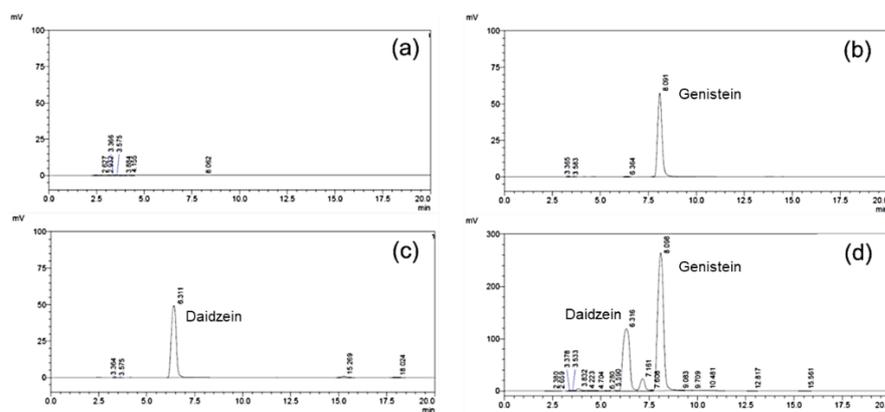
**Figure 1.** Response surface plot of retention time (Y1g), resolution (Y2g), and tailing factor (Y3g) for genistein



**Figure 2.** Response surface plot of retention time (Y1d), resolution (Y2d), and tailing factor (Y3d) for daidzein

The desirability functions have been generated using R statistical software with the package of ‘rsm’. Genistein retention time was set for a target value of 8 minutes, with the lower and upper value estimation of 6.5 and 10 minutes, respectively. Genistein resolution was set for a maximum value of 4.5, with a lower value estimation of 1.5. Daidzein retention time were set for a minimum value of 6.5 minutes, with the upper value estimation of 9 minutes.

The recommended conditions obtained from the RSM model followed by the desirability function were set in the HPLC system and applied for evaluating the solvent of methanol, genistein standard, daidzein standard, and tofu sample containing genistein and daidzein (Figure 3).



**Figure 3.** HPLC chromatograms of solvent (a), standard of genistein (b), standard of daidzein (c), and tofu sample containing genistein and daidzein (d). Mobile phase: methanol-water (60:40 v/v). Flowrate: 0.80 mL/min. Column: C<sub>18</sub> column of Hibar® 250-4,6 Purospher® STAR RP-18 endcapped (5 µm). Column temperature: 50°C. Wavelength detection at 260 nm. Volume injection: 10 µL.

**Table 3.** Results of system suitability test (n=6)

Analytes	Retention time		Area		Resolution	Tailing factor	Theoretical plates number
	Mean	RSD (%)	Mean	RSD (%)			
<i>Standards</i>							
Genistein	8.075	0.817	904670.8	0.734	4.403	1.201	5761.747
Daidzein	6.364	0.777	900766.0	0.915	4.664	1.220	3919.482
<i>Samples (tofu)</i>							
Genistein	8.047	0.713	5589872	0.910	4.185	1.255	5475.179
Daidzein	6.367	0.826	2823239	0.972	4.621	1.188	4007.568

Separation profiles of genistein and daidzein both in standard and sample solutions were observed. Six replications of standard solution and sample solution were injected into the HPLC system. Results of system suitability test were presented in Table 3.

### 3. DISCUSSION

#### 3.1. Experimental design

In this study, an optimization of the RP-HPLC condition for separating genistein and daidzein in tofu was performed. The experimental design was developed according to the BBD model with the implementation of RSM. Three independent variables or factors including methanol composition, flowrate, and column temperature were observed in this study. Retention time, resolution, and tailing factor of genistein and daidzein were stated as the dependent variables or responses. The BBD model was generated using R statistical software. A model of BBD using three factors, three levels, and four central points was successfully generated. This model was applied to optimize the RP-HPLC conditions followed by response observation to generate RSM model for each response.

#### 3.2. RSM observation

The RSM model for genistein was successfully generated for retention time (Y1g), resolution (Y2g), and tailing factor (Y3g). The quality of the RSM model of genistein can be evaluated according to multiple determination coefficient ( $R^2$ ), adjusted  $R^2$ , and p-value. It can be stated that experimental factors have significantly affected the responses only if the multiple  $R^2 \geq 0.8$  and adjusted  $R^2 > 0.8$ . Furthermore, the minimum difference between multiple  $R^2$  and the adjusted  $R^2$  (less than 0.2) indicates that the second-order polynomial models satisfactorily fit the actual data [16]. The p-value of the model indicates a good predictive model with a value of  $\leq 0.05$  [17]. It was found that the multiple  $R^2$  and adjusted  $R^2$  for Y1g were 0.9905 and 0.9761 (p-value =  $2.274 \times 10^{-5}$ ), respectively. The multiple  $R^2$  and adjusted  $R^2$  for Y2g were 0.9959 and 0.9898 (p-value =  $1.794 \times 10^{-6}$ ), respectively.

Similar to the genistein models, the RSM model for daidzein was successfully generated for retention time (Y1d), resolution (Y2d), and tailing factor (Y3d). It was found that the multiple  $R^2$  and adjusted  $R^2$  for Y1d were 0.9931 and 0.9826 (p-value =  $8.813 \times 10^{-5}$ ), respectively. Since only the retention time model of daidzein met the requirements of multiple  $R^2$ , adjusted  $R^2$ , and p-value; this RSM model was analysed further for generating desirability function.

#### 3.3. Desirability analysis

RSM can be developed along with the desirability analysis to obtain the selected condition for optimization purposes. Multiple responses of RSM with the significant model can be selected for desirability consideration. In this study, genistein retention time, genistein resolution, and daidzein retention time were chosen and applied in the desirability analysis.

The composite desirability was calculated computationally. It was found that the model predicted a total desirability of 0.9778 can be achieved by applying the methanol composition of 60%, flowrate of  $0.80 \text{ mL} \cdot \text{min}^{-1}$ , and column temperature of  $50^\circ\text{C}$ . Desirability values resulting from the desirability functions lie between 0 and 1. The desirability value of 0 corresponds to the undesirable response obtained from the predictive factors. On the other hand, the desirability value of 1 corresponds to the most expected responses [18,19].

#### 3.4. System suitability test

HPLC separation properties including retention time, area, resolution, tailing factor, and theoretical plates number were evaluated to ensure the appropriateness of the analytical method. According to the results, it can be found that the optimized HPLC conditions met the acceptance criteria for the system suitability test with minimum RSD of retention time and area ( $RSD < 1.0\%$ ), resolution of more than 4.0 ( $R_s > 2.0$ ), tailing factor of less than 2.0 ( $TF \leq 2.0$ ), and theoretical plates number of more than 3900 ( $N > 2000$ ) [20].

#### 4. CONCLUSION

An analytical method of RP-HPLC for simultaneously separating genistein and daidzein has been successfully developed. Optimization has been performed by applying the response surface methodology of the Box-Behnken design. The desirability functions have been successfully generated to strengthen the quality of the RSM. It was found that the optimized HPLC conditions were methanol composition of 60%, flowrate of 0.80 mL.min<sup>-1</sup>, and column temperature of 50°C. These conditions were set for the HPLC system followed by the system suitability test. Several separation properties such as retention time, area, resolution, tailing factor, and theoretical plates number were reported to meet the acceptance criteria of the system suitability test.

However, the optimized analytical method can be developed further. In the future, it is recommended to perform the analytical method validation to empirically demonstrate if the method is appropriate to be applied for the intended purposes.

#### 5. MATERIALS AND METHODS

##### 5.1. Materials

Tofu sample was purchased from the local market in Yogyakarta, Indonesia. Reference standards of genistein and daidzein were purchased from Sigma-Aldrich. Solvents of methanol gradient grade for liquid chromatography (Merckmillipore), ethyl acetate, petroleum ether (Smart Lab), and redistilled water (PT. Ikapharmindo Putramas) were used in this study. Anhydrous Na<sub>2</sub>SO<sub>4</sub> was purchased from Merckmillipore.

##### 5.2. Instrumentation and Software

A system of HPLC Shimadzu® LC-2010 CHT with UV/Vis detector accompanied with a C18 column of Hibar® 250-4,6 Purospher® STAR RP-18 endcapped (5 µm) was used in this study. Other instrumentation were listed as follow: a system of Buchi Rotary Evaporator ultra-micro analytical balance RADWAG® series of UYA 2.3Y (max: 2.1 g, min 0.01 mg), Gast® vacuum pump, Retsch® T460 ultrasonicator, sterile syringe filter with a 0.2 µm pore size hydrophilic PTFE membrane (Merckmillipore), and a set of Socorex® micropipettes. The R statistical software version 4.2.0 along with R Studio software version 2022.12.0 Build 353 were exploited in this study. The R software package namely 'rsm' was installed and applied to carry out statistical analysis of RSM and desirability analysis.

##### 5.3. Methods

###### 5.3.1. Standard and sample preparation

An accurate weight of 5.0 mg for each genistein and daidzein standard were transferred into 10 mL volumetric flask. Genistein and daidzein standards of each volumetric flask were diluted in methanol into the volume. These solutions were filtered using sterile syringe filter membrane before injection into HPLC system.

Preparation of tofu sample was applied using a modification from Yuliani et al. (2016) [2]. One kg of tofu was mixed and macerated in 50 mL petroleum ether for 40 minutes at 150 rpm and the petroleum ether was subsequently removed. The obtained residue and hydrophilic phase were fractionated using ethyl acetate and water. The fraction of ethyl acetate was separated and filtered to eliminate the solid residue. The ethyl acetate fraction was added with sodium sulfate anhydrous in order to remove the water. The obtained yellowish solution was subsequently proceeded using rotary evaporator to remove the solvent. The remained residue was diluted using methanol. This solution was filtered using sterile syringe filter membrane before injection into HPLC system.

###### 5.3.2. Experimental design

The BBD of using three factors, three levels, and four central points was developed. The percentage of methanol (X1), flowrate (X2), and column temperature (X3) was selected as factors (independent variables).

On the other hand, separation properties such as retention time (Y1), resolution (Y2), and tailing factor (Y3) were stated as the responses (dependent variables). Observational independent variables along with the experimental levels were presented in Table 4.

**Table 4.** Observational independent variables to build the BBD model

Variables	Levels		
	Low	Medium	High
X1: methanol composition (%)	60	70	80
X2: flowrate (mL.min <sup>-1</sup> )	0.6	0.8	1.0
X3: column temperature (°C)	30	40	50

Note: \* cross validation was performed using leave one out technique

Sixteen experimental runs will be achieved since the number of experiments can be calculated using formula  $2k(k-1)+C_p$ , where k is the number of factors and  $C_p$  is the number of central points. These runs were executed using the RP-HPLC system at 260 nm UV detection and volume injection of 10  $\mu$ L.

### 5.3.3. RSM observation

Sixteen BBD runs generated from the software were executed and observed. All responses for each compound were recorded and listed to build the RSM model. All factors and responses were exploited for generating the second-order polynomial models. The estimated coefficients of the RSM model were considered to obtain the following formula:

$$Y = \beta_0 + \beta_1X_1 + \beta_2X_2 + \beta_3X_3 + \beta_{12}X_1X_2 + \beta_{13}X_1X_3 + \beta_{23}X_2X_3 + \beta_{11}X_1^2 + \beta_{22}X_2^2 + \beta_{33}X_3^2 \dots (1)$$

where, Y is the predicted response,  $X_1$ ,  $X_2$ ,  $X_3$  are the independent variables,  $\beta_0$  is the intercept,  $\beta_1$ ,  $\beta_2$ ,  $\beta_3$  are the linear effect,  $\beta_{12}$ ,  $\beta_{13}$ ,  $\beta_{23}$  are the interaction effect, and  $\beta_1^2$ ,  $\beta_2^2$ ,  $\beta_3^2$  are the quadratic effect.

After achieving all functions for each compound, significant models were selected for generating the desirability function in the desirability analysis stage of the research. The perspective plots for each response were also depicted to visualize the RSM models.

### 5.3.4. Desirability analysis

The desirability function has been generated according to the previous study [21]. Each response can be set for minimum, maximum, or specific target value along with upper and lower value estimation.

### 5.3.5. System suitability test

The system suitability test was performed by injecting standards and samples solution containing genistein and daidzein. These solutions were filtered using sterile syringe filter membrane before injection into HPLC system. These solutions were injected in six replications.

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