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RSM-BASED OPTIMIZATION OF AN RP-HPLC METHOD, ANALYTICAL METHOD VALIDATION, AND CONTENT DETERMINATION OF DAIDZEIN IN SOY SAUCE

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Soybean is extensively consumed in Indonesia and is known to benefit human health. One of the soybean products is soy sauce. Soybean and its products are reported to contain isoflavone aglycone daidzein which can provide beneficial biological activities for humans. Hence, it is important to develop a suitable analytical method to analyze the daidzein content in soy sauce. The Box–Behnken design (BBD), a widely used response surface methodology (RSM), was performed to optimize the independent variables such as mobile phase composition of methanol and acetonitrile and flow rate condition. Analytical method validation including linearity and range, selectivity, accuracy, and precision was evaluated in this study. The optimized conditions for daidzein analysis were the mobile phase of methanol:water:acetonitrile (60:35:5 v/v/v), flow rate of 1.0 mL/minute, and detection wavelength at 251 nm. This condition met the requirements of the system suitability test with low variance for tailing factor, resolution, retention time, number of theoretical plates, and area under curve. The analytical method was successfully validated for several parameters including linearity and range, selectivity, accuracy, and precision. A reversed-phase high-performance liquid chromatography (RP-HPLC) method for analyzing daidzein in soy sauce can be optimized by implementing the Box-Behnken Design. This method met the analytical method validation acceptance criteria for linearity, range, selectivity, accuracy, and precision. According to the quantitative determination result, it was found that daidzein content in soy sauce sample was of 0.014 mg/g.

Keywords: *daidzein, response surface methodology, reverse phase HPLC, soy sauce, validation*

INTRODUCTION

Soybean (*Glycine max* L.) is known as a food material that contains high nutrients in Indonesia. Soybean is also reported to be beneficial for human health, such as reducing the risk of coronary heart disease and breast cancer [1]. Soybean and soybean products are reported to contain many bioactive compounds[2]. Isoflavones, one of the bioactive compounds group contained in soybean, are flavonoid subgroup with a 3-phenyl chromone structure[3]. The aglycone isoflavones in soybeans consist of daidzein, genistein, and glycitein[2]. As one of soybean products, soy sauce is quite popular in the Asian region including Indonesia and usually made through traditional fermentation processes [4].

Fermentation in the soy sauce manufacturing process is divided into two stages: the *koji* fermentation stage and the *moromi* fermentation stage. This fermentation process has an impact for isoflavone content in soy sauce. The concentration of isoflavone glycosides is very high in the *koji* fermentation stage, but gradually decreases and is replaced by aglycone isoflavones in the *moromi* fermentation stage. This is because the

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fermentation process involves the hydrolysis of isoflavone glycosides into aglycone isoflavones[4]. Daidzein (**Fig.** 1) as one of isoflavone aglycones is a non-steroidal phytoestrogen compound with potential antioxidant activity. Some biological activities of daidzein include cardioprotective, anticancer, and osteoporosis prevention effects[5].

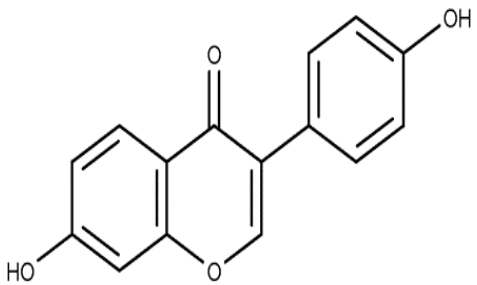

Fig. 1: Chemical structure of daidzein.

One of the analytical methods used for the separation of daidzein is a reversed-phase high-performance liquid chromatography (RP-HPLC). RP-HPLC was used due to its process automation, relatively fast, and efficient [6]. HPLC is a separation technique that employs a solid stationary phase and a liquid mobile phase [7]. The optimization of the analytical method using HPLC can be assisted by applying the Response Surface Methodology (RSM). Response surface methodology can be conducted in the optimization research equipped by a free statistical computation purpose namely R software[8]. The RSM model of the Box-Behnken Design (BBD) can be generated to assist the optimization [9].

Previous study from Yuliani et al. (2018) reported the extraction optimization to achieve genistein and daidzein from tempeh, a fermented product from soybean [10]. Other studies reported the evaluation of daidzein along with genistein and glycitein in soy milk products [8, 11]. However, there are still limited studies reporting the daidzein evaluation in soy sauce marketed in Indonesia. Since it was reported that daidzein has several benefits to human health, it is important to provide nutritional information related to the isoflavone aglycone in soy sauce.

The study aimed to implement the RSM-based optimization of RP-HPLC method along with the analytical method validation for determining the content of daidzein in soy sauce samples. In this study, methanol percentage, acetonitrile percentage, and flowrate were selected as independent variables or factors, whereas several chromatographic responses including resolution, retention time, tailing factor, and theoretical plate number were stated as dependent variables or responses.

MATERIAL AND METHODS

Material

Daidzein and genistein reference standards were bought from Sigma-Aldrich, Singapore. The solvents that utilized were liquid chromatography grade of methanol (Merck Millipore, Germany), ethyl acetate, acetonitrile (Merck Millipore, Germany), petroleum ether (Smart Lab, Indonesia), and redistilled water that prepared in Laboratory of Faculty of Pharmacy, Universitas Sanata Dharma, Indonesia. Sodium sulfate anhydrous was bought from Merck Millipore, Germany. Soy sauce with the brand of "ABC" was purchased from a local market in Sleman, Yogyakarta, Indonesia.

Instruments and Application

Instrumentation utilized in this study were HPLC system of Shimadzu® LC-2010 CHT (Japan) with UV/Vis detector and a computer, a C18 column of Hibar® 250-4,6 Purospher® STAR (Germany) RP-18 endcapped (5 µm), ultra-micro analytical balance RADWAG® (Poland) series of UYA 2.3Y (max: 2.1 g, min 0.01 mg), an Orbital Shaker, a system of Buchi Rotary Evaporator (UK), Retsch® T460 ultrasonicator (Germany), Gast® vacuum pump model DOA-P504-BN (USA), sterile syringe filter with a 0.2 µm pore size hydrophilic PTFE membrane (Merck Millipore, Germany), a set of Socorex® (Switzerland) micropipettes, and glasswares. R software version 4.1.3 and RStudio software version 4.1.3 with a rsm package were used to run the application of statistical technique and response surface.

Preparation of Standard and Sample Solution

Approximately 1.0 mg daidzein and 1.0 mg genistein were weighted and transferred into a different 5 mL volumetric flask. The stock solution for each standard with concentration 200 µg/mL was prepared by

diluting the content of each volumetric flask with methanol. The mixture solution containing daidzein and genistein standard was obtained by transferring 0.5 mL of both standard stock solutions into the same 5 mL volumetric flask, continued by dilution with methanol into the volume. The solution then filtered using a sterile syringe filter membrane (0.45 µm) before being injected into the HPLC system.

The sample preparation method was adapted with several modifications from the previous study[8]. Fifty milliliters of soy sauce and 100 mL petroleum ether were added into an Erlenmeyer with the ratio of sample and petroleum ether of 1:2 (v/v). Liquid-liquid extraction was carried out for 40 minutes (150 rpm) using orbital shaker and covered with an aluminium foil. Then petroleum ether was removed to obtain an extract of soy sauce.

Fifty milliliters of ethyl acetate were added into an Erlenmeyer that containing the extract which was prepared. Then followed by extraction process with orbital shaker for 90 minutes (150 rpm). Liquid-liquid extraction was carried out by adding 50 mL distilled water in separatory funnel. This process was repeated until three times. The filtrate of ethyl acetate was transferred into beaker glass. Sodium sulfate anhydrous was added to the solution followed by filtration process to obtain a yellow-coloured solution of ethyl acetate. Dry extract was obtained by evaporating the yellow-coloured solution using rotary evaporator. The obtained dry extract was diluted with 10 mL methanol.

Response Surface Methodology

RSM was applied in this study to optimize HPLC separation. Mobile phase used in this study were methanol, acetonitrile, and redistilled water. Box Behnken Design has been applied to analyze three independent variables such as the percentage of methanol (X1), the percentage of acetonitrile (X2), and flowrate (X3) with three different level each. Every independent variable was coded as -1, 0, +1 for low, medium, and high levels, respectively. The variables and their observed values for each level are shown in **Table 1**. HPLC separation responses including tailing factor (Y1), resolution (Y2), retention time (Y3), and theoretical plate number (Y4) opted as dependent variables. The BBD design used in this study was acquired through the application of this formula utilizing R software:

```
> library(rsm)
> bbd(3, randomize=TRUE)
```

BBD model with 16 runs that generated using R software was used to perform each run by HPLC system of Shimadzu LC-2010 CHT with UV/Vis detector and a C_{18} column of Hibar 250-4,6 Purospher STAR RP-18 uncapped (5 µm). Wavelength detection was set to 251 nm and the injection volume of each run was 10 µL.

Multiple Response Optimization

The results from 16 runs using BBD were documented for each response to construct RSM model. The impact of independent variables on each dependent variable was suited using the RSM function, applying a second-order model. The statistical analysis involved an analysis of variance (ANOVA) table to identify significant variations among the independent variables. Statistical analysis using R software also showed stationary points and eigenvalues that help the optimization process. Perspective plots for each dependent variable were also built by R software to visualize RSM model. Multiple response optimization was also performed since there were four different dependent variables with contrasting goals (maximum or minimum) for each variable. The desirability function was applied in this multiple-response optimization process. This computational optimization was done using R software.

Table 1: Levels of variables and their observed values

Variables	Low (-1)	Medium (0)	High (+1)
X1: Methanol (%)	60	65	70
X2: Acetonitrile (%)	5	10	15
X3: Flowrate (mL/min)	0.6	0.8	1.0

System Suitability Test

The system suitability test was conducted for both reference standard and soy sauce sample. A mixture solution containing all standards with a concentration of 20 µg/mL for daidzein and genistein each was injected to the system. The reference standard solution was injected to the system with injection volume of 10 µL and replicated six times.

The solution of sample was diluted by taking 1.0 mL sample solution and inserted to 10 mL volumetric flask. The daidzein reference standard solution was taken 2.5 mL with concentration of 20 µg/mL, inserted to the same volumetric flask and diluted with methanol to achieve a sample solution. The sample solution of 10 µL was injected to the system and replicated six times.

Analytical Method Validation

Selectivity. The standard daidzein solution with a concentration of 20 µg/mL was taken 0.5 mL and transferred into 5 mL volumetric flask, then methanol was added into the volume. The similar procedure was done to prepare a standard genistein solution of 20 µg/mL. After that, 2 mL of the extracted sample was taken and then transferred into 5 mL volumetric flask, added with methanol into the volume. Then 1.5 mL was taken from each volumetric flask, filtered with filter membrane, and transferred into different HPLC vial. The selectivity test was done by identifying the peak profile. The accepted standard for resolution value was more than 1.5 which demonstrate good peak separation.

Sensitivity. Sensitivity of the method was evaluated using the limit of detection (LOD) and limit of quantitation (LOQ) value. The LOD and LOQ were determined using standard deviation approach according to Miller & Miller [12]. The LOD and LOD can be calculated using the following formulas:

$LOD = y_B + 3s_B$ ..... (1)

$LOQ = y_B + 10s_B$ ..... (2)

where y_B is the analyte concentration giving a signal equal to the blank signal and s_B is standard deviation of the blank.

Linearity and range. Solutions for this test were prepared from stock solution at ten concentration levels (1–60 µg/mL). A calibration curve was created by plotting the concentration versus the peak area. The correlation coefficient, slope, and intercept were determined.

Accuracy and precision. Solutions for accuracy and precision test were obtained by transferring 100 µL (0.1 mL) of the sample solution combined with the standard daidzein solution at three different concentration levels (20, 35, and 50 µg/mL) into 10 mL volumetric flask for each concentration and diluted with methanol. Each solution was injected 10 µL into the HPLC system and wavelength detection at UV-251 nm. Accuracy and precision were evaluated by calculating the recovery percentage and Relative Standard Deviation (RSD) values at three concentration levels (low, medium, high) with three replicates for each concentration.

Content Determination of Daidzein

In this study, 2.0 mL of the sample solution was transferred into 10 mL volumetric flask, then diluted with methanol. The sample was filtered using 0.45 µm millipore filter membrane, then transferred to HPLC vial. Injection was replicated six times; the AUC value was recorded and the levels were calculated by plotting the AUC value against the standard curve. The average value of levels and its RSD were determined.

RESULTS AND DISCUSSION

Results

Response Surface Methodology

A set of sixteen runs of experiment were carried out with different combination of X1, X2, X3 using BBD with four central point's replication and generated into second order response surface methodology. These runs enable to collect responses data of dependent variables Y1, Y2, Y3, Y4. After successfully collect the responses data, the empirical models of RSM were generated using R software.

A predictive model has a significant effect on response if the p-value is <0.05 (5%) [8]. The independent variable can be stated to significantly affect the response if the multiple $R^2 \geq 0.8$ and adjusted $R^2 > 0.8$, with the difference between the multiple R^2 and adjusted R^2 values ≤ 0.2 [8]. The lack of fit value from the model obtained must >0.05 so it can be stated that the regression model is suitable (there is no lack of fit)[13]. Excluding the tailing factor, all equation models generated met the acceptable p-value ($p < 0.05$), both multiple R^2 and adjusted R^2, and lack of fit with $p > 0.05$.

Table 2: The Box Behnken Design and the results obtained from daidzein separation using RP-HPLC method**.**

Run	Factors			Responses			
	Methanol (%)	Acetonitrile (%)	Flow rate (mL/min)	Tailing factor	Resolution	Time retention	Theoretical plate number
1	65	10	0.80	1.30	2.13	4.76	4908
2	70	10	0.60	1.31	1.28	5.77	5367
3	70	15	0.80	0.00	1.72	4.07	5139
4	65	10	0.80	1.33	0.22	4.81	4901
5	60	10	0.60	1.34	3.27	7.13	5356
6	60	10	1.00	1.29	2.37	4.34	4338
7	60	5	0.80	1.29	4.38	6.51	5057
8	65	15	0.60	1.36	2.18	5.79	5758
9	65	5	1.00	1.29	3.14	4.44	4355
10	65	5	0.60	1.34	3.29	7.25	5419
11	65	10	0.80	1.32	0.70	4.82	5067
12	60	15	0.80	1.33	2.53	4.73	5147
13	70	5	0.80	1.32	0.90	4.89	5033
14	65	10	0.80	1.29	1.11	4.77	4728
15	65	15	1.00	1.29	0.72	3.47	4237
16	70	10	1.00	1.28	0.77	3.50	4157

Table 3: RSM analysis of tailing factor, resolution, retention time, and theoretical plate number.

Responses	p-value	Multiple R^2	Adjusted R^2	Lack of Fit	Stationary points (*eigen values)*		
					Methanol (%)	Acetonitrile (%)	Flow rate (mL/min)
Tailing factor	0.22	0.745	0.363	1.25e-04	63.97 (4.15)	8.56 (0.2e-03)	0.82 (-0.01)
Resolution	0.04	0.867	0.667	0.75	70.09 (10.48)	10.01 (0.04)	0.87 (0.01)
Retention time	1.67e-09	0.999	0.999	0.37	64.73 (7.23)	16.46 (0.01)	1.19 (0.2e-03)
Theoretical plate number	3.26e-04	0.977	0.941	0.81	59.53 (7.71)	6.081 (1.25)	0.43 (-3104.36)

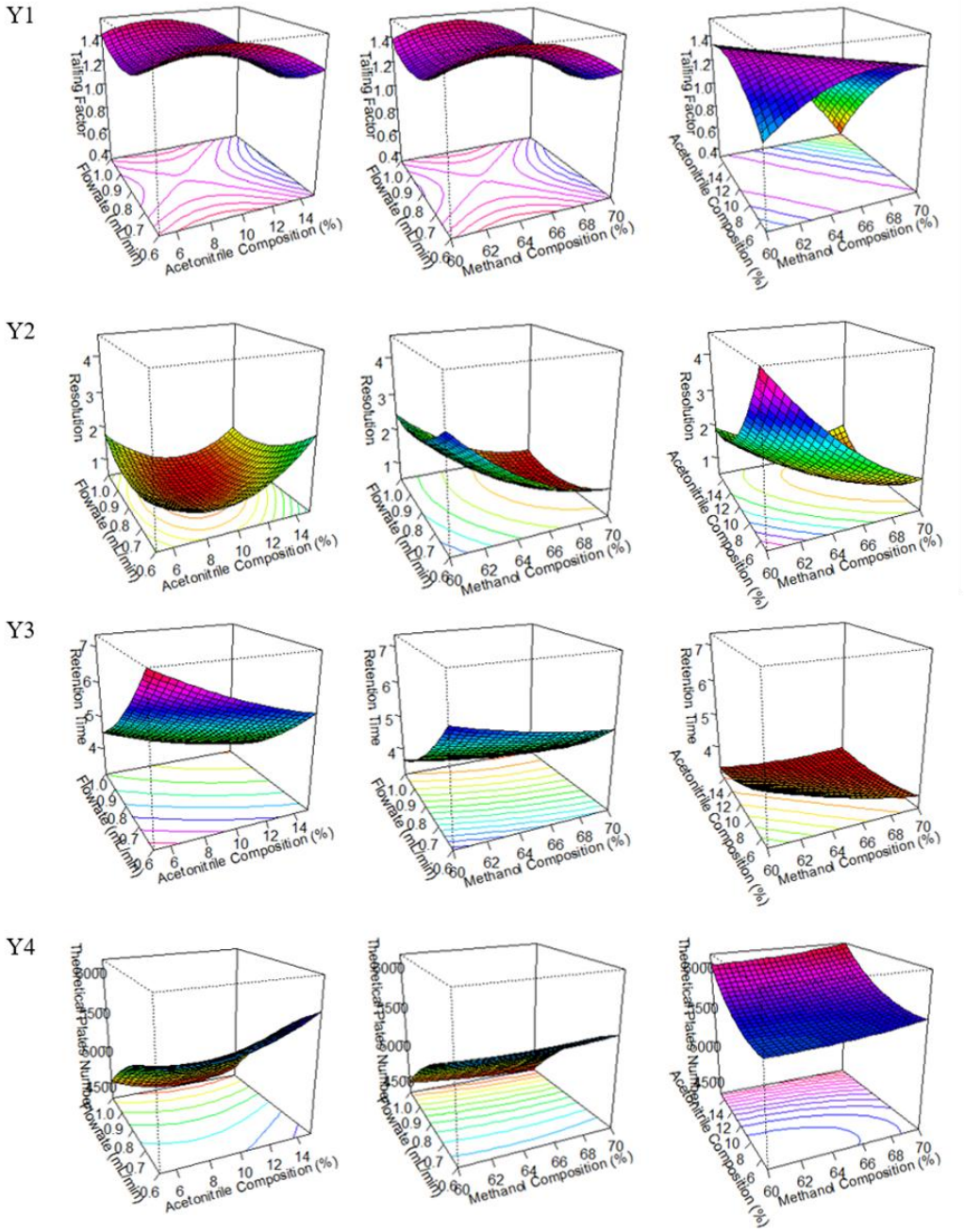

Fig. 2: The perspective plot of tailing factor (Y1), resolution (Y2), retention time (Y3), and theoretical plate number (Y4) for daidzein.

Stationary points and eigen values for each independent variable were generated by R software. The stationary points generated from the R application are not used further because in several cases the stationary points are estimates that cannot be applied to research[8]. This can be seen in the estimated flow rate on the number of theoretical plates response which was too slow and in the retention time response which was too fast. If the flow rate was too slow, it led to longer analytical times and potential band broadening. In the other hand, a faster flow rate increased the column pressure and potentially shortening the column's lifetime[8]. The eigenvalues characterized the pattern of the perspective contour plot. Positive eigenvalues represented an upward curve, while negative eigenvalues depicted a downward curve. Therefore, all negative eigenvalues denoted a maximum stable point, all positive eigenvalues signified a minimum stable point, and mixed-sign eigenvalues suggested a saddle-shaped stable point lifetime[8]. All the results of the perspective contour plot that were

successfully formed from each dependent variable can be seen in **Fig. 2**. It can be observed that responses of tailing factor (Y1), resolution (Y2), retention time (Y3), and theoretical plate number (Y4) for daidzein can be affected by independent variables of flowrate vs acetonitrile composition, flowrate vs methanol composition, and methanol composition vs acetonitrile composition. The maximum stable point resulted from the negative eigenvalue of the model, whereas the minimum stable point resulted from the positive eigenvalue. The mixed sign of eigenvalue (positive and negative) indicated the saddle profile of the stable point[8].

Daidzein can be separated using reversed-phase HPLC with C18 column because of the possibility of interaction between isoflavone aglycone daidzein with the silica-based column[10, 14]. Analysis of daidzein compound in soy sauce using reverse phase HPLC with a mixture of methanol, water, and acetonitrile as mobile phase. Methanol was chosen because it can interact with the isoflavone aglycone daidzein through hydrogen interactions where daidzein can act as a hydrogen donor or acceptor [8]. Acetonitrile (CH_3CN) has very good solubility in water and water has polar properties so that both can be used as mobile phases in reverse phase HPLC[15, 16].

Multiple Response Optimization

Three out of four responses that have the value of p-value, R-square, and lack of fit that meet the requirements was selected. The three response models chosen were resolution, retention time, and theoretical plate number and were chosen to build a predictive model for daidzein separation in HPLC. The purpose of retention time response is to obtain the lowest value, while the purpose of theoretical plate number and resolution response are to obtain the highest value. Therefore, it is necessary to perform multiple response optimization. Multiple response optimization was carried out by calculating the desirability function in R software. The desirability function has a value from 0 to 1. A value of 0 indicates the most unwanted response because the response is outside the range of the target value and a value of 1 indicates the most desirable response because the response is close to the target value [17, 18]. The individual desirability function will be calculated by entering the maximum desirability formula in R software with the target for each response as shown in **Table 4**.

Table 4

The individual desirability function will form the total desirability function which is the geometric mean of the individual desirability functions [18]. The value of the total desirability function was obtained for the three separation responses and this value will be used as a reference for determining the optimum condition for daidzein analysis using HPLC. This function was executed in software R using the following formula:

$$D = (d1 \; x \; d2 \; x \; d3)^{1/3} \quad \text{..... (3)}$$

where,
D = Total desirability
d1 = individual desirability retention time
d2 = individual desirability resolution
d3 = individual desirability theoretical plate number[18]

The total desirability value of each combination of the three independent variables is calculated using software R and the combination that has the best total desirability value is taken. Based on the results, the optimum conditions for daidzein analysis using HPLC were the mobile phase composition of methanol:water: acetonitrile (60 : 35 : 5 v/v/v) and a flow rate of 1 mL/minute with a total desirability value of 0.57. This optimum condition will then perform a system suitability test.

Table 4: Target of Multiple Response Optimization with Desirability Function.

Responses	Limit		Target
	Lower	Upper	
Retention time	3	7	Minimum
Resolution	1.5	5	Maximum
Theoretical plate number	4300	5000	Maximum

System Suitability Test

System suitability test is an inseparable part of the analysis when using high performance liquid chromatography method. This test aims to ensure that the chromatography system is adequate for carrying out the analysis and provides results that are close for each test [7]. The system suitability test was carried out by running six injections for both of the standard mixture of daidzein and genistein, also the soy sauce sample. Genistein standard solution was also injected in this study to evaluate the separation between two isoflavone aglycones. The results of parameters such as tailing factor, resolution, theoretical plate number, and retention time were evaluated. System suitability test results for both reference standard and soy sauce sample are presented in **Table 5**. Chromatogram profiles of system suitability test were presented in **Fig. 3**.

From the test result of the reference standard, all parameters met the acceptance criteria, such as the relative standard deviation of AUC and retention time ≤ 2%, resolution more than 2, tailing factor value ≤ 2, and the value of the number of theoretical plates more than 2000[19]. Parameters that meet the criteria indicate that the reverse phase HPLC system is adequate and can provide results that are close to each other.

From the test results of the soy sauce sample, almost all parameters met the acceptance criteria, such as the relative standard deviation of AUC and retention time ≤ 2%, resolution did not meet the acceptance criteria (Rs > 2), tailing factor value ≤ 2, and the value of the theoretical plate number more than 2000[19].

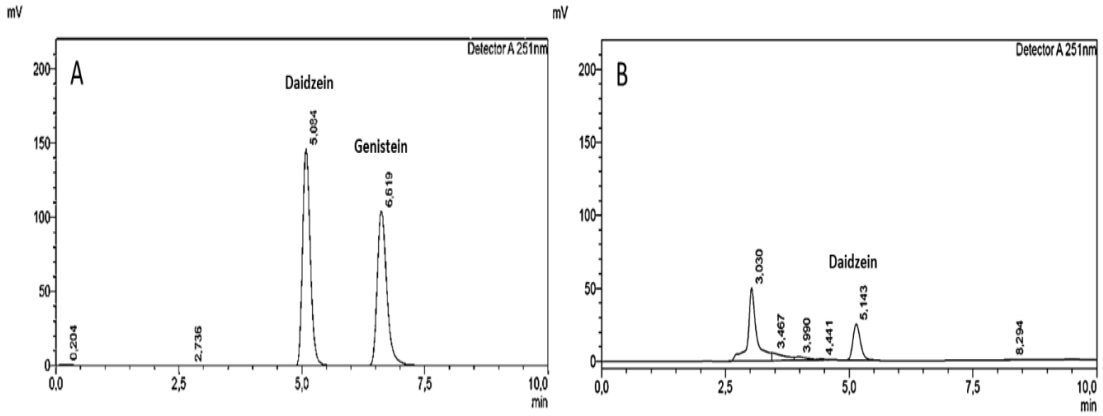

Fig. 3: HPLC chromatograms of mixture standard solution containing daidzein and genistein (A) and soy sauce sample solution containing daidzein (B). Mobile phase: methanol, water, acetonitrile (60:35:5). Flowrate: 1 mL/min. Column: C18 column of Hibar® 250-4,6 Purospher® STAR RP-18 endcapped (5 μm) at UV-251 nm. Volume injection: 10 μL.

Table 5: Results of system suitability test (n = 6)**.**

Solution	Tailing factor	Resolution	Theoretical plate number	Retention Time		Area	
				Mean	RSD (%)	Mean	RSD (%)
Standard solution	1.245	9.155	4894.167	5.090	0.151	1609658	0.418
Soy sauce sample solution	1.269	1.623	5409.833	5.181	0.534	268127	1.009

Analytical Method Validation

Selectivity

Selectivity is an indicator that shows the extent to which a method can measure certain analytes in a complex mixture without interference from other components in the mixture. Selectivity can be seen by separating adjacent peaks by calculating the resolution value (Rs) (Sahoo et al., 2018). Separation can be said to be selective if the value of $Rs \geq 1.5$[21]. This stage was carried out by injecting four different solutions, namely blank, daidzein standard, mixed daidzein and genistein standard, and soy sauce extract samples. The standard peaks produced are used to be compared with the peaks produced in the sample solution, so that it will be easy to find out the identity of the daidzein compound peak in the soy sauce extract sample. While the blank, is used to determine that the solvent used does not affect the reading of the desired compound. Chromatogram profiles of selectivity evaluation were presented in **Fig. 4**.

Linearity and range

The linearity range used in this test for daidzein were 1.01 - 60.44 µg/mL. Calibration curve equation of daidzein were y = y = 55139x + 4984.60 (r = 0.9978). These results showed that the method used in this test can provide a linear response to daidzein concentrations within the range.

Accuracy and precision

Accuracy and precision of the method was determined using standard addition method in three levels of concentration (low, medium, high) with three replications from each concentration. According to **Table 6**, the mean value of the recovery as the accuracy parameter at three concentration levels of daidzein are within the required range. Percentage of RSD as the precision parameter at three levels of daidzein were below the maximum limit of AOAC's requirement. These results show that this method produced highly precision and accuracy for determining daidzein in all concentration levels.

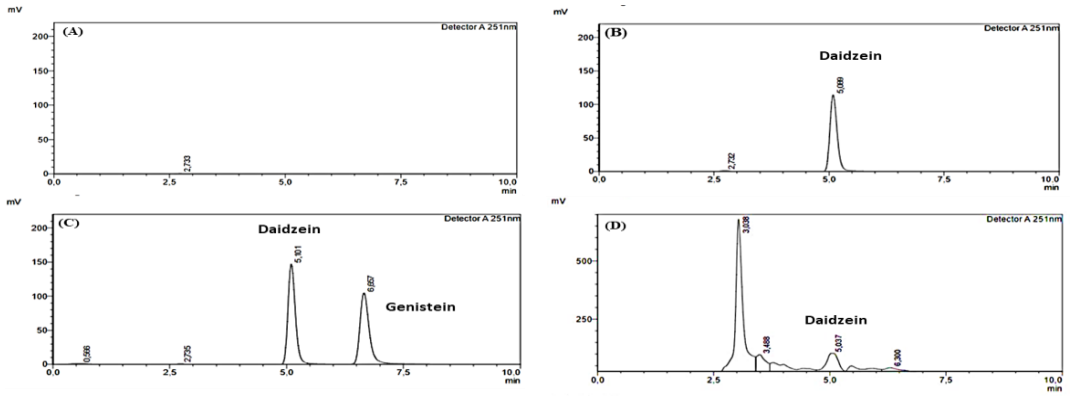

Fig. 4: Solvent blank (methanol) (A), HPLC separation profiles of daidzein standard (B), daidzein and genistein standard mixture (C), and soy sauce sample (D). Column: C18 column of Hibar® 250-4,6 Purospher® STAR RP-18 endcapped. Wavelength detection at 251 nm. Volume injection: 10 µL.

Table 6: Accuracy and Precision Result of Spiked Soy Sauce Samples (n=3).

Intraday					
Compounds	Level	Added amount (µg/mL)	Found amount (µg/mL)	Recovery (%)	RSD (%)
Daidzein	Low	20.15	21.58	107.09	0.13
	Medium	35.26	38.15	108.16	2.40
	High	50.37	56.16	111.50	0.08
Interday					
Compounds	Level	Added amount (µg/mL)	Found amount (µg/mL)	Recovery (%)	RSD (%)
Daidzein	Low	20.15	19.89	98.73	1.06
	Medium	35.26	39.05	110.40	0.69
	High	50.37	56.07	111.32	0.36

Sensivity

The LOD and LOQ were determined using standard deviation approach according to Miller & Miller[12]. LOD and LOQ values for daidzein were 0.16 and 0.47 µg/mL respectively.

Content Determination of Daidzein

The optimized and validated RP-HPLC method was applied to determine the daidzein content in soy sauce samples. From six replications of determination, it was found that the content of daidzein in soy sauce samples was 0.0014% (w/w) or 0.014 mg/g dry weight with the RSD value of 0.90. According to the results, it was found that the RSD value met the requirements of <5.30% [21]. The daidzein levels that tend to be obtained are lower than the results of previous study[22]. Witold et al. stated that daidzein content were 0.105 – 0.850 mg/g from dry weight. This variation is possibly occurred due to the variations in the types of soybeans, soy sauce processing, and fermentation.

Conclusions

In this study, an RP-HPLC method was successfully optimized by employing the RSM model to evaluate the content of daidzein in soy sauce samples. The optimized condition was applied in the analytical method validation stage. The developed RP-HPLC method met the requirements for validation variables including selectivity, linearity and range, sensitivity, accuracy, and precision. It was also possible to apply the RP-HPLC method for determining the content of daidzein in soy sauce samples.

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نشرة العلوم الصيدليـــة
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التحسين القائم على منهجية السطح الاستجابي لطريقة كروماتوغرافيا السائل عالية الأداء ذات الطور العكسي ، التحقق من صحة الطريقة التحليلية، وتحديد محتوي الدايدزين في صلصلة الصويا

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يُعد فول الصويا من الأطعمة التي تستهلك بشكل واسع في إندونيسيا ويُعرف بفوائده الصحية للإنسان. ومن بين منتجات فول الصويا هو صلصة الصويا. وتشير التقارير إلى أن فول الصويا ومنتجاته تحتوي على الدايدزين ، وهو أحد مركبات الأيزوفلافون التي توفر أنشطة بيولوجية مفيدة للبشر. لذلك، من المهم تطوير طريقة تحليلية مناسبة لتحليل محتوى الـ الدايدزين في صلصة الصويا. تم استخدام تصميم بوكس بهنكن، وهي منهجية شائعة للاسطح الاستجابي، لتحسين المتغيرات المستقلة مثل تكوين الطور المتحرك من الميثانول والأسيتونيتريل وظروف معدل التدفق. تم تقييم التحقق من صحة الطريقة التحليلية بما في ذلك الخطية والنطاق، الانتقائية، الدقة، والتكرارية في هذه الدراسة. كانت الشروط المثلى لتحليل الـ الدايدزين هي الطور المتحرك من الميثانول:الماء:الأسيتونيتريل (٦٠:٣٥:٥ v/v/v)، ومعدل تدفق ١.٠ مل/دقيقة، وطول موجة الكشف عند ٢٥١ نانومتر. حققت هذه الشروط متطلبات اختبار ملاءمة النظام مع تفاوت منخفض لعوامل التموج، الفصل، وقت الاحتفاظ، عدد الألواح النظرية، ومساحة تحت المنحنى. تم التحقق من صحة الطريقة التحليلية بنجاح لعدة معايير بما في ذلك الخطية والنطاق، الانتقائية، الدقة، والتكرارية. يمكن تحسين طريقة كروماتوغرافيا السائل عالية الأداء ذات الطور العكسي لتحليل الدايدزين في صلصة الصويا باستخدام تصميم بوكس بهنكن وحققت هذه الطريقة معايير قبول التحقق من صحة الطريقة التحليلية من حيث الخطية والنطاق، الانتقائية، الدقة، والتكرارية. وفقًا لنتائج التحليل الكمي، وُجد أن محتوى الدايدزين في عينة صلصة الصويا بلغ ٠.٠١٤ ملغم/غرام.