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# Optimization of genistein and daidzein extraction from a tempeh-fermented product of soybean

[Optimización de la extracción de genisteína y daidzeína de un producto tempeh-fermentado de soja]

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**Abstract**

*Context*: Genistein and daidzein, major isoflavone aglycone compounds contained in tempeh, became an interesting subject since their activities as cancer prevention, cardiovascular, and wound healing agents. It is important to develop an efficient extraction method to obtained genistein and daidzein from tempeh.

*Aims*: To optimize extraction process of the two compounds from dried tempeh.

*Methods*: The full factorial design was used to analyze the effect of combining factors affecting the extraction process. Genistein and daidzein analysis have been performed by using a C18 column of Phenomenex® (250 x 4.6 mm, 5 µm) and a mobile phase containing methanol:distilled water (70:30). The detection was carried out at 261 nm with a flow rate of 0.6 mL/min in the isocratic reverse-phase HPLC system.

*Results*: The analytical method was validated according to AOAC guidelines including the parameters of selectivity, linearity, accuracy, precision, limit of detection, and limit of quantification.

*Conclusions*: The optimal extraction condition was achieved at the ethanol concentration of 96%, the particle size of 0.6 mm and extraction time of 270 minutes. Total amount obtained from the extraction method was 26.03 mg% and 19.42 mg% for genistein and daidzein, respectively.

*Keywords*: extract; factorial design; isoflavones; validation.

**Resumen**

*Contexto*: Genisteína y daidzeína, principales compuestos de agliconas de isoflavonas contenidos en el tempeh, se convirtieron en un tema interesante debido a sus actividades como prevención del cáncer, agentes cardiovasculares y cicatrizantes. Es importante desarrollar un método de extracción eficiente para obtener genisteína y daidzeína del tempeh.

*Objetivos*: Optimizar el proceso de extracción de los dos compuestos del tempeh seco.

*Métodos*: El diseño factorial completo se utilizó para analizar el efecto de la combinación de factores que afectan el proceso de extracción. El análisis de genisteína y daidzeína se ha realizado utilizando una columna C18 de Phenomenex® (250 x 4,6 mm, 5 µm) y una fase móvil que contenía metanol:agua destilada (70:30). La detección se llevó a cabo a 261 nm con un caudal de 0,6 mL/min en el sistema de HPLC de fase inversa isocrático.

*Resultados*: El método analítico se validó de acuerdo con las directrices de la AOAC, incluidos los parámetros de selectividad, linealidad, exactitud, precisión, límite de detección y límite de cuantificación.

*Conclusiones*: La condición de extracción óptima se logró a la concentración de etanol del 96%, el tamaño de partícula de 0,6 mm y el tiempo de extracción de 270 minutos. La cantidad total obtenida del método de extracción fue de 26.03 mg% y 19.42 mg% para genisteína y daidzeína, respectivamente.

*Palabras Clave*: extracto; diseño factorial; isoflavonas; validación.

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## INTRODUCTION

In recent years, the interest in soybean and soybean product was increased. Soybean, a natural isoflavones sources, was reported in many studies since it has some benefits for health (Sirotkin and Harrath, 2014). Isoflavones are classified into four groups: aglycones, glucosides, malonyl glucosides and acetyl glucosides (Lakshmi et al., 2013). Genistein and daidzein, isoflavone aglycones found in soybean, was reported as major compounds with significant amount contained in soybean (Rostagno et al., 2007; Kuligowski et al., 2017). Genistein and daidzein played important roles such as cancer prevention agents (Ju et al., 2002; Hess and Igal, 2011; Yuliani et al., 2016), cardiovascular phytoestrogens agent (Lissin and Cooke, 2000), controlling menopausal symptoms (Hirose et al., 2016; Khaodhiar et al., 2018), diabetes (Gilbert and Liu, 2013; El-Kordy et al., 2015; Liu et al., 2016), diabetic wound healing agents (Miyazaki et al., 2002; Park et al., 2011; Tie et al., 2013; Eo et al., 2016) and anti-inflammatory agents (Song et al., 2003; Valsecchi et al., 2011; Eo et al., 2015). Tempeh, a traditional food from Indonesia, was a fermented product from soybean (Yuliani et al., 2016). An increase of isoflavones content in soybean could occur after the fermentation process (Chaiyasut et al., 2010).

Extraction is one of the crucial stages in order to discover and develop drugs from natural resources. It was stated that the different extraction process contributed to the extraction efficiency of the active ingredient from the solid matrix (Cho et al., 2009; Jyoti et al., 2015). Previous studies described some factors affecting the effectiveness of extraction process, such as particle size of solids, solvents, extraction time, temperature, and liquid to solid ratio (Spigno and De Faveri, 2007; Spigno et al., 2007).

Many studies of genistein and daidzein extraction have been done before (Yoshiara et al., 2012) performed an optimal extraction of daidzein, genistein, and glycitein using water and acetone (1:1) (Yoshiara et al., 2012). Lakshmi et al. (2013) studied the extraction process condition of soy flour isoflavones, and stated that optimum condition of ethanol concentration, extraction time, and temperature were 78% ethanol, 105 minutes extraction time, and 44°C, respectively. Cho et al. (2009) performed a liquid-liquid extraction method to concentrate the isoflavones extract from 11.6 mg/g up to 229 mg/g.

In the previous study, conventional extraction optimization has been developed using only one factor as variable at a time while other factors were kept constant. Possible interaction between some factors could not be evaluated. However, it was a time-consuming and expensive approach as an optimization method (Banik and Pandey, 2008). Effects of combining factors in the extraction process of genistein and daidzein from tempeh can be studied simultaneously using an experimental model to increase its effectiveness. Employing a full factorial design, an experimental model was conducted for this purpose (Bolton and Bon, 2010). The response surface methodology technique was also performed to serve visual illustration of several independent variables or factors on extraction process and to determine the optimum region of extraction model (Banik and Pandey, 2008). Notably, the response surface methodology has been successfully performed to optimize the extraction process of isoflavones from soybean (Cho et al., 2009; Yoshiara et al., 2012).

The objective of this study was to optimize the factors that influence extraction of genistein and daidzein from tempeh, i.e., concentration, the particle size of solid (dried tempeh) and extraction time. The full factorial design was used to analyze the effect of combining factors, i.e., particle size of solid, ethanol concentration as solvent and extraction time from dried tempeh. The effect of individual factor, interaction between factors influencing the extraction, and the model describing combining effects of the factors on extraction were constructed by the $2^3$ full factorial design.

## MATERIAL AND METHODS

### Chemicals

All the chemicals used were of analytical grade and procured from genistein and daidzein (Sigma-Aldrich), methanol gradient grade for liquid chromatography, ethyl acetate, petroleum ether (E. Merck), redistilled water, and tempeh with the brand of "Muchlar" obtained from a traditional market in Yogyakarta Indonesia. The chosen tempeh was controlled with the fermentation time of three days.

## Instrumentation

The instruments used in this study were ultra-micro analytical balance RADWAG® series of UYA 2.3Y (max: 2.1 g, min 0.8 mg), a system of Shimadzu® LC-2010HT No. C21255111004 LP with UV/Vis detector, Retsch® T460 ultrasonicator, membrane filter holder of Whatman® (capacity of 300 mL) Cat. No. 1960-004, organic solvent membrane filter of Whatman® (0.5 µm pore size, 47 mm diameter); inorganic solvent membrane filter of Whatman® (0.45 µm pore size, 47 mm diameter), Millipore syringe filter (0.20 µm pore size, 25 mm diameter), and Socorex® micropipettes (1-10 µL, 10-100 µL, and 100-1000 µL). Design Expert™ 10.0.6.0 software.

### *Chromatographic conditions*

The system of Shimadzu LC-2010 CHT with LabSolution software and UV-Vis detector were developed. This chromatographic system was developed from Yuliani et al. (2016) with the flow rate and stop time modification. A $C_{18}$ column of Luna type Phenomenex (250 x 4,6 mm, 5 µm) was used. An isocratic elution system was developed with the composition of mobile phase was methanol-water (70:30), and mobile phase flow rate was 0.6 mL/minute. Chromatographic separation was performed at a detection wavelength of 261 nm, the injection volume of 10 µL, and stop time setting at 15 minutes.

### *Extraction process*

The tempeh was cut into small pieces and dried in the oven at 50°C for 24 hours. The dried tempeh was blended to produce tempeh powder in a certain particle size. A hundred grams of each particle size of tempeh powder was mixed with 200 mL petroleum ether and macerated in the beaker glass. The maceration process was performed on the shaker at 155 rpm for 24 hours. The filtrate was removed. The wet tempeh powder was dried using the oven at 50°C for 24 hours. Fifty grams of the dried powder with certain particle size was poured into the 500 mL beaker glass, added with 150 mL of solvent, and macerated using shaker at 155 rpm for certain time.

After maceration, the yellow filtrate and the powder were separated. The yellow filtrate was evaporated using a rotary evaporator until 10% of initial volume has been achieved. The produced filtrate was poured into the dish and dried to produce a constant weight of solids. The extraction factors optimized in this study were ethanol concentration, particle size of solids, and extraction time. The $2^3$ full factorial design model to optimize the genistein and daidzein extraction process from tempeh was presented in Table 1.

**Table 1.** The $2^3$ full factorial design for optimizing extraction process

| | $X_1$ | $X_2$ | $X_3$ | Ethanol concentration (%) | Particle size (mm) | Extraction time (min) |
|---|---|---|---|---|---|---|
| 1 | -1 | -1 | -1 | 70 | 0.6 | 90 |
| a | 1 | -1 | -1 | 96 | 0.6 | 90 |
| b | -1 | 1 | -1 | 70 | 1.2 | 90 |
| ab | 1 | 1 | -1 | 96 | 1.2 | 90 |
| c | -1 | -1 | 1 | 70 | 0.6 | 270 |
| ac | 1 | -1 | 1 | 96 | 0.6 | 270 |
| bc | -1 | 1 | 1 | 70 | 1.2 | 270 |
| abc | 1 | 1 | 1 | 96 | 1.2 | 270 |

Note: $X_1$=ethanol concentration; $X_2$=particle size; $X_3$=extraction time.

*Analytical method validation*

The analytical method for determining the content of genistein and daidzein was validated for selectivity, linearity and range, precision, accuracy detection limit, and quantitation limit, according to the AOAC guidelines (AOAC, 2012).

*Sample preparation*

One gram of solids extract was weighed and extracted using liquid-liquid extraction method. The liquid-liquid extraction method was performed using solvents of ethyl acetate and water of 15 mL for each cycle. The extraction method was repeated three times. The ethyl acetate fraction was collected and dried. A constant weight solid of extraction results was transferred into a 10 mL volumetric flask and diluted to volume with methanol. Fifty microliters of the solution were transferred into a microtube and diluted to 1.0 mL with methanol. This sample preparation method was replicated seven times. All the replicated sample solutions were sonicated for 10 minutes and filtered using Millipore syringe filter before injection.

## Experiment design for extraction

The effect of ethanol concentration (A; 70% - 96%), particle size (B; 0.6 mm – 1.2 mm) and extraction time (C; 90 min – 270 min) toward genistein and daidzein concentration in ethanolic extract of tempeh were analyzed by the $2^3$ full factorial design. The experimental design was conducted using eight experiments and three replicated for each experiment. The achieved data of genistein and daidzein concentration were fitted with the equation model as follows:

$$Y = b_o + b_1X_1 + b_2X_2 + b_{12}X_1X_2 + b_3X_3 + b_{13}X_1X_3 + b_{23}X_2X_3 + b_{123}X_1X_2X_3 \quad (1)$$

where Y was concentration of genistein and daidzein, $b_o$ was the intercept, $b_i$ was regression coefficient for linear regression, $b_i$, $b_{ij}$, $b_{ijk}$ were regression coefficients computed from the responses, Xi was the levels of factors. Design Expert™ 10.0.6.0 software was used to perform analysis of variance (ANOVA) and provide regression analysis.

The effect of three factors (ethanol concentration, particle size, and extraction time) of genistein and daidzein extraction from tempeh was analyzed at two levels of the experiment, high level denoted by (+) and low level denoted by (-) as mentioned in Table 1. The variables, which were significant at 5% ($p < 0.05$) from regression analysis were considered the significant impact to the extraction process of genistein and daidzein from tempeh.

Optimization of the process was analyzed using Design Expert™ 10.0.6.0 software with selected factors, which have a significant level of p-value. The contour plots were drawn using Design Expert™ 10.0.6.0 software. The overlying plot was defined using the optimal region achieved from contour plots of genistein and daidzein concentration.

## Statistical analysis

The Design Expert™ 10.0.6.0 software was used to perform analysis of variance (ANOVA) and provide regression analysis. The effect of three factors (ethanol concentration, particle size, and extraction time) of genistein and daidzein extraction from tempeh was analyzed at two levels of the experiment, high level denoted by (+) and low level denoted by (-) as mentioned in Table 1. The variables, which were significant at 5% ($p < 0.05$) from regression analysis were considered the significant impact to the extraction process of genistein and daidzein from tempeh.

The $2^3$ full factorial design was applied to optimize the three factors selected, i.e., ethanol concentration, the particle size of dried tempeh and extraction time. Eight experiments were carried out from the design, each of experiment replicated three times, and the concentration of genistein and daidzein were used for statistical analysis. The results of each experiment were presented in Table 2.

# RESULTS

## Analytical method validation

*Selectivity*

Selectivity test was conducted on both the mixed standard and the sample solution containing genistein and daidzein. Selectivity was determined by determining the resolution value indicates the value of each peak separation of the compounds. Representative chromatogram of the mixed stand-

ard and the sample solution were presented in Fig. 1. Resolution of genistein and daidzein were 3.186 and 1.759, respectively.

*Linearity and range*

The AUC data versus concentration of genistein and daidzein were treated by linear correlation coefficient. Calibration curve equation of genistein and daidzein were obtained y=120567x-124744 (r=0.999) and y=127910x-179548 (r=0.999), respectively. This method was linear in the range of 4.11–14.38 µg/mL and 5.07–17.75 µg/mL for genistein and daidzein, respectively.

**Table 2.** Correlation between levels of extraction factor toward genistein and daidzein concentrations.

| Run | Levels of extraction factor | | | Genistein (mg%) | Daidzein (mg%) |
|---|---|---|---|---|---|
| | $X_1$ | $X_2$ | $X_3$ | | |
| 1 | 1 | 1 | -1 | 7.26 | 4.27 |
| 2 | 1 | -1 | 1 | 25.12 | 18.84 |
| 3 | 1 | 1 | -1 | 7.66 | 4.46 |
| 4 | -1 | -1 | 1 | 11.93 | 9.25 |
| 5 | -1 | 1 | -1 | 11.04 | 11.17 |
| 6 | 1 | -1 | 1 | 25.04 | 18.44 |
| 7 | 1 | -1 | -1 | 12.13 | 10.29 |
| 8 | -1 | 1 | 1 | 11.15 | 9.98 |
| 9 | -1 | 1 | 1 | 10.46 | 9.98 |
| 10 | 1 | -1 | -1 | 14.53 | 10.52 |
| 11 | 1 | -1 | 1 | 27.92 | 21.38 |
| 12 | 1 | 1 | 1 | 14.92 | 11.29 |
| 13 | -1 | -1 | -1 | 8.66 | 7.43 |
| 14 | -1 | 1 | -1 | 11.34 | 11.35 |
| 15 | -1 | -1 | 1 | 12.36 | 10.67 |
| 16 | -1 | -1 | -1 | 8.67 | 7.47 |
| 17 | -1 | -1 | -1 | 9.97 | 7.94 |
| 18 | -1 | -1 | 1 | 12.62 | 9.2 |
| 19 | 1 | 1 | 1 | 14.91 | 11.26 |
| 20 | -1 | 1 | 1 | 10.22 | 9.75 |
| 21 | -1 | 1 | -1 | 10.92 | 11.1 |
| 22 | 1 | -1 | -1 | 14.49 | 10.4 |
| 23 | 1 | 1 | -1 | 7.02 | 4.16 |
| 24 | 1 | 1 | 1 | 14.33 | 11.84 |

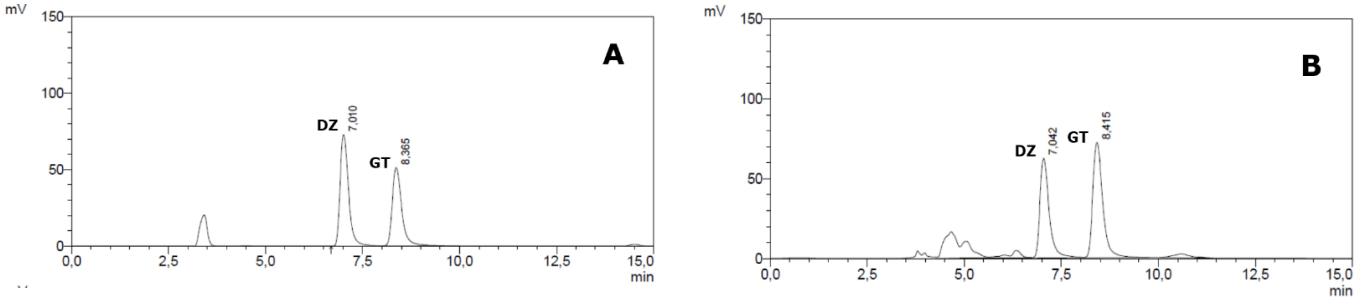


**Figure 1.** Representative chromatogram of the mixed standard **(A)** and the sample solution **(B)** containing genistein (GT) and daidzein (DZ). Column: Phenomenex® C18 column (250 x 4,6 mm, 5 µm). Mobile phase: methanol-water (70:30). Flow rate: 0.6 mL/min. Detection at 261 nm.

*Precision and accuracy test*

Precision and accuracy test of samples were performed by the standard addition method. The three levels of addition concentration levels consisted of the low, medium, and high has been added to the sample matrix of tempeh as shown in Table 3. The calculated accuracy at three concentration levels of genistein and daidzein were within the required range of 80-115% (AOAC, 2012). Percentage of RSD as the precision parameter at three concentration levels of genistein and daidzein were below the maximum limit of AOAC requirements for RSD% which was 6% (AOAC, 2012). These results showed that this method produced highly precision and accuracy for determining genistein and daidzein in all concentration levels not only for intraday evaluation but also for interday evaluation.

*Determination of detection limit and quantitation limit*

The detection limits of genistein and daidzein were 0.501 and 0.796 µg/mL, respectively. Quantitation limit of genistein and daidzein were 1.668 and 2.653 µg/mL, respectively. The results were obtained by the calculation using standard deviation approach.

*Effects of ethanol concentration, the particle size of dried tempeh and extraction time on genistein and daidzein concentration*

The equation models were developed by applying multiple regression analysis on the experimental data of genistein and daidzein concentration. The results were presented as the equation model (2) for genistein and the equation model (3) for daidzein as follows:

$$Y = 13.11 + 2.33X_1 - 2.18X_2 + 2.80X_3 - 2.25\ X_1X_2 + 2.13\ X_1X_3 - 1.07\ X_2X_3 \quad (2)$$

$$Y = 10.52 + 0.91\ X_1 - 1.30\ X_2 + 2.14\ X_3 - 2.25X_1X_2 + 1.94\ X_1X_3 - 0.67\ X_2X_3 \quad (3)$$

The results of analysis of variance (ANOVA) showed that the developed models were valid for genistein and daidzein model as presented in Table 4. The model of F-value for genistein and daidzein were 161.98 and 147.76, respectively, implying that the two models were significant. The value of Q2 indicated the internal validation of response with no outlier data.

## DISCUSSION

In this study, the analytical method validation was performed to determine genistein and daidzein in tempeh. This work proves that the method is selective, linear, accurate, precise, and sensitive for the assay of genistein and daidzein in tempeh. The valid method has been used to determine both genistein and daidzein in the matrix of tempeh extract produced by each extraction factor.

Among the three factors, the extraction time was found as the highest effect on the concentration of genistein that extracted from tempeh. It provided the highest linear coefficient (2.80) followed by ethanol concentration (2.33) while particle size of dried tempeh provided negative effect. The interaction of ethanol concentration and extraction time also showed a significant positive effect on genistein concentration. A positive effect implied that the increase of extraction time would increase the obtained amount of genistein from tempeh. The

particle size of dried tempeh showed a negative effect of -2.18 on the extraction process of genistein. It means that the decrease of particle size will increase the obtained amount of genistein from tempeh.

**Table 3.** Evaluation of intraday and interday for accuracy and precision (n=7).

| | Concentration level | Analyte concentration (µg/mL) | SD | RSD (%) | Accuracy (%) |
|---|---|---|---|---|---|
| *Intraday* | | | | | |
| Genistein | Low | 8.337 | 0.326 | 3.915 | 101.624 |
| | Medium | 10.590 | 0.288 | 2.715 | 112.779 |
| | High | 12.942 | 0.366 | 2.831 | 97.671 |
| Daidzein | Low | 8.096 | 0.319 | 3.942 | 101.549 |
| | Medium | 10.381 | 0.218 | 2.097 | 103.856 |
| | High | 12.425 | 0.570 | 4.586 | 103.334 |
| *Interday* | | | | | |
| Genistein | Low | 8.714 | 0.274 | 3.143 | 106.362 |
| | Medium | 10.319 | 0.185 | 1.789 | 111.392 |
| | High | 11.763 | 0.328 | 2.792 | 105.506 |
| Daidzein | Low | 8.574 | 0.248 | 2.888 | 107.552 |
| | Medium | 11.085 | 0.184 | 1.658 | 110.900 |
| | High | 11.863 | 0.399 | 3.363 | 98.607 |

**Table 4**. Regression coefficients of the multiple linear regression model for the determination of genistein and daidzein in tempeh.

| Factor | Genistein regression coefficient | Daidzein regression coefficient |
|---|---|---|
| $b_0$ | 13.11* | 10.52* |
| *Linear* | | |
| $b_1$ | 2.33* | 0.91* |
| $b_2$ | -2.18* | -1.30* |
| $b_3$ | 2.80* | 2.14* |
| *Multiple linear* | | |
| $b_1b_2$ | -2.25* | -2.25* |
| $b_1b_3$ | 2.13* | 1.94* |
| $b_2b_3$ | -1.07* | -0.67* |
| *Regression* | | |
| F-value | 161.98 | 147.76 |
| $R^2$ | 0.9828 | 0.9807 |
| Adj. $R^2$ | 0.9767 | 0.9738 |
| $Q^2$ | 0.9657 | 0.9615 |

*Significant at 5% level ($p < 0.05$).

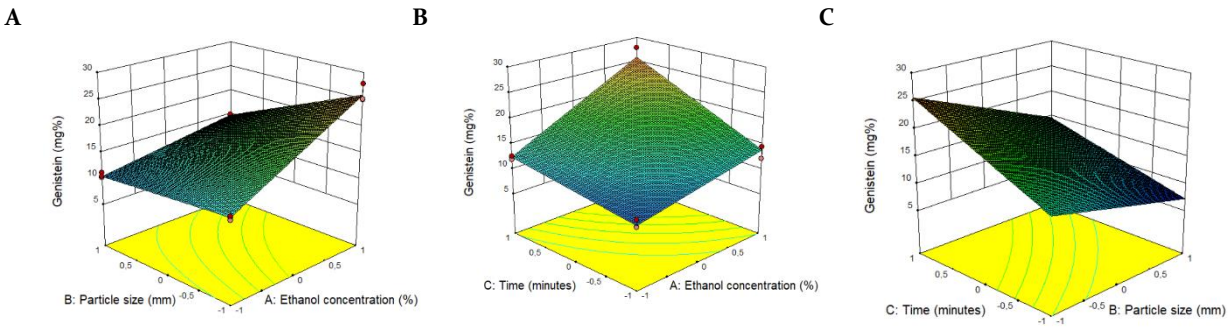


**Figure 2.** Response surface and contour plots for the effects of factors on genistein concentration extracted from dried tempeh, such as effect of particle size and ethanol concentration **(A)**, effect of extraction time and ethanol concentration **(B)**, and effect of extraction time and particle size **(C)**.

The three-dimensional plots (contour plots) that represented the effects of ethanol concentration, the particle size of dried tempeh and the extraction time were produced by equation (2) and were presented in Fig. 2. The plots were produced by each pair of factors, and the third factor was taken in the highest response. Fig. 2A represented the effect of particle size of dried tempeh and ethanol concentration. At the low level of ethanol concentration, reducing particle size did not change the obtained amount of genistein significantly. In contrast, at the high level of ethanol concentration, reducing particle size would increase the obtained amount of genistein. Similarly, with the effect of particle size, increasing ethanol concentration would increase the obtained amount of genistein at a low level of particle size. The maximum response was achieved at the ethanol concentration of 96% and particle size of 0.6 mm. The effect of extraction time and ethanol concentration was presented in Fig. 2B. At the high level of both factors of the extraction time and ethanol concentration, increasing the level of factors would increase the obtained amount of genistein that extracted from dried tempeh. The maximum concentration of genistein was obtained at the ethanol concentration at 96% and the extraction time of 270 minutes. Fig. 2C showed the effect of the extraction time and the particle size of dried tempeh. At a high level of the extraction time, reducing particle size would significantly increase the obtained amount of genistein. Reducing particle size will increase the contact area between solid particle and solvent. It will raise the value of coefficient diffusion of genistein. The maximum concentration of genistein was 26.03 mg% that achieved at the ethanol concentration of 96%, a particle size of 0.6 mm and the extraction time of 270 minutes.

The extraction time was found as the highest effect on the extraction process of daidzein from tempeh followed by ethanol concentration. Extraction time has a positive value (2.14), it was implied that increased extraction time would increase the obtained amount of daidzein. On the other hand, the particle size of dried tempeh has a negative value (-1.30), it was implied that decreased particle size would increase the daidzein concentration. The interaction between the ethanol concentration and extraction time has a positive value.

Fig. 3 presented the three-dimensional contour plots for the effects of the ethanol concentration, particle size of dried tempeh and extraction time on the obtained amount daidzein extracted from tempeh. The contour plots were developed by the equation (3). The plots were produced by each pair of factors, and the third factor was taken in the highest response of daidzein concentration. Fig. 3A showed the effect of the ethanol concentration and the particle size of dried tempeh. At the low level of ethanol concentration, reducing particle size did not increase the obtained amount of daidzein that extracted from tempeh, but at a high level of the ethanol concentration, reducing particle size would significantly increase the obtained amount of daidzein. The effect of ethanol concentration has the positive value of 0.91, increasing ethanol concentration will increase the obtained amount of daidzein. On the other hands, at a low level of particle size, increasing the ethanol concentration would significantly increase the daidzein concentration. The effect of the extraction time and the ethanol concen-

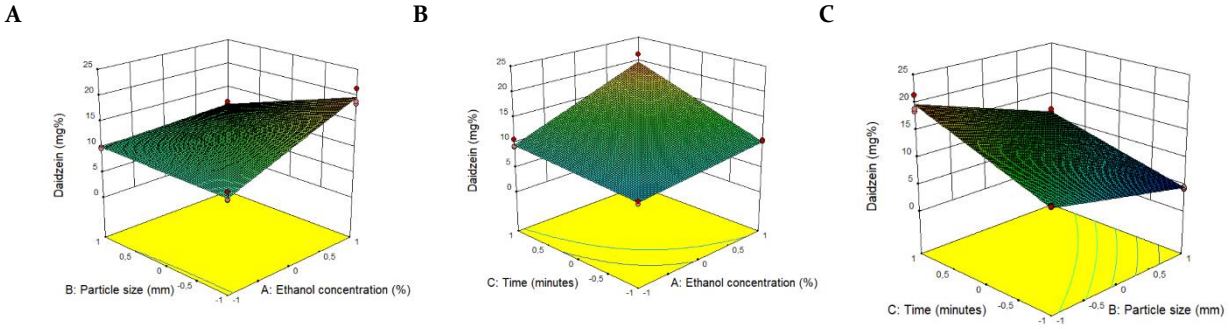


**Figure 3.** Response surface and contour plots for the effects of factors on daidzein concentration extracted from dried tempeh, such as the effect of particle size and ethanol concentration (**A**), the effect of extraction time and ethanol concentration (**B**), and effect of extraction time and particle size (**C**).

tration was presented in Fig. 3B. Both factors of the extraction time and ethanol concentration were significantly affected the obtained amount of daidzein at a high level of factors. Increasing the extraction time and the ethanol concentration would increase the obtained amount of daidzein. At a low level of both factors of the extraction time and ethanol concentration, the obtained amount of daidzein did not significantly increase. Fig. 3C illustrated the effects of the extraction time and the particle size of the dried extract on the obtained amount of daidzein. At low level and high level of particle size (0.6 and 1.2 mm respectively) increasing the extraction time would significantly increase the obtained amount of daidzein. The maximum concentration of daidzein was achieved at the ethanol concentration of 96%, a particle size of 0.6 mm and the extraction time of 270 minutes that produced 19.42 mg% daidzein.

The process of extraction would produce 20 mg% genistein if the ethanol concentration more than 87% and a particle size less than 0.9 mm at the extraction time of 270 minutes (Fig. 4). Daidzein 15 mg% can be achieved by the extraction process at the ethanol concentration more than 85% and a particle size less than 0.93 mm at the extraction time of 270 minutes. The yellow region represented the condition of extraction process that produced minimal 20 mg% of genistein and minimal 15 mg% of daidzein. Hence, the yellow region was defined as the optimal condition for extraction of genistein and daidzein from tempeh. However, the scale-up and further process development should be done in the near future to provide tempeh extract with the optimized amount of genistein and daidzein. It was valuable to perform the drying process using the spray drier instrument since it was important to provide the dried tempeh extract with the much better solid quality.

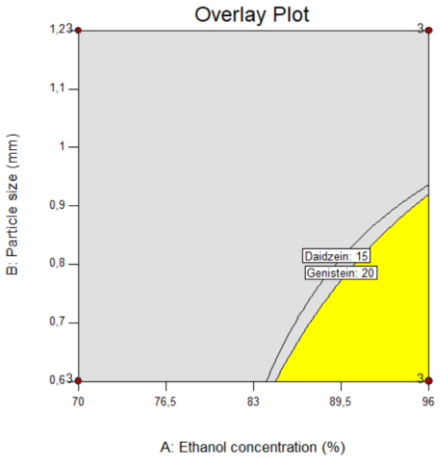


**Figure 4.** Overlay plot from genistein contour plots and daidzein contour plots. The extraction time was held on 270 minutes.

## CONCLUSIONS

The models for extraction genistein and daidzein were fit with a significant probability of F-value ($p < 0.0001$) to predict genistein and daidzein that extracted from dried tempeh. The optimal extraction condition was achieved at the ethanol concentra-

tion of 96%, a particle size of 0.6 mm and extraction time of 270 minutes. It produced 26.03 mg% genistein and 19.42 mg% daidzein that extracted from dried tempeh.

## CONFLICT OF INTEREST

The authors declare no conflict of interest.

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| Concepts or ideas | X | | | |
| Design | X | | | |
| Definition of intellectual content | X | | | |
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