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Original Article

# DEVELOPMENT AND VALIDATION FOR FREE AGLYCONES DAIDZEIN AND GENISTEIN IN SOYBEANS (*GLYCINE MAX* (L.) MERR.) USING RP HPLC METHOD

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**ABSTRACT**

**Objective:** The aim of this research was to develop a validation method for free aglycones analysis especially daidzein and genistein in soybean (*Glycine max* (L.) Merr.) using High Performance Liquid Chromatography (HPLC).

**Methods:** In this study, reversed-phase HPLC (RP-HPLC) equipped with an endcapped Sun Fire TM C-18 column (150 mm x 4.6 mm, 5 µm) was used for the separation. The binary mobile phase consisted of methanol and 0.1% acetid acid (53:47). An isocratic program was used with a flow rate at 1.0 ml/min and the injection volume was 10 µl. The analytes were detected by using Photo-diode array (PDA) at 254 nm and the samples in various soybean varieties from Balitkabi Malang, Indonesia.

**Results:** The developed method showed that the parameters of system suitability and selectivity meet the requirements of method validation. The accuracy values of recoveries for daidzein 83.0%-100.95% and genistein 80.07%-108.79%, and the precision was calculated %RSD ≤ 2% respectively, linearity of the method was the r2values ranged from 0,9989-0,9991 and all the parameters meet the acceptable criteria of validated method. The Limit of detection (LoD) and Limit of quantitation (LoQ) indicated a good sensitive method, with LoDs values obtained, were 0.05192 µg/ml and 0,0600 µg/ml for daidzein and genistein, respectively. Meanwhile, the LoQs values was 0.1731 µg/ml and 0.2000 µg/ml for daidzein and genistein, respectively.

**Conclusion:** A simple, accurate, precise and being specific HPLC method coupled to PDA for the analysis of daidzein and genistein in various soybeans varieties were developed and validated.

**Keywords:** Daidzein, Genistein, Free aglycones, HPLC, Soybean varieties


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## INTRODUCTION

Soybean (*Glycine max* (L.) Merr) is one of the essential food sources consumed by many Asian people including Indonesia. Isoflavones (plant secondary metabolites of the family flavonoids) are present in high concentrations in soybean and soy products [1]. The content of isoflavones in soybean will play important role in nutrients value of soybean. Therefore, the farmers will cultivate and plant soybean varieties containing a high concentration of isoflavones. Isoflavones have estrogenic effects, genistein is able to prevent osteoporosis [2]. Daidzein and genistein at doses of 0.2-20 µM were able to inhibit ovarian endometrioma cell proliferation in endometriosis in mice [3]. Soybeans are one of the biggest sources of isoflavones compared to nuts and meat products [4], and the best sources of isoflavones are in soybeans. Isoflavone can be found both forms, aglycone and glycoside form. However, an isoflavone in aglycone form such as daidzein and genistein, is the more effective than glycoside form, because it has good biological activity and also easily to be absorbed [5].

Isoflavones in soybeans consisted of malonil-glycosides, acetyl-glycosides, glycosides, and aglycones. Among the four forms of isoflavones, the highest biological activity is shown by aglycone isoflavones, especially genistein (5,7,4'-trihydroxy isoflavone), daidzein (7,4'-dihydroxy isoflavone) and glyciteine (6-methoxy-7,4'-dihydroxy isoflavone) [6]. Genistein and daidzein can prevent osteoponia in postmenopausal women [7]. In the field of food, daidzein enriched animal feed was able to improve the quality of beef [8]. However, soybean types, geographical locations, and processing methods have a major influence on the active substance content in soybeans [9].

Several analytical methods have been used for analysis of daidzein and genistein, namely high-performance liquid chromatography (HPLC) [10-12], Raman spectroscopy [13], FTIR spectroscopy and [14] polarimetry [15]. The most widely used analytical technique is reversed phase HPLC using C18 column with the mobile phase of acetonitrile, methanol [10, 11, 16]. Analysis of daidzein and genistein in soybeans requires a more efficient, accurate method and simple sample preparation. A lot of research used gradient elution systems [10, 16-19], but this method can be optimized by the isocratic system [11]. For this reason, this research was intended to develop an analytical method using HPLC for the analysis free aglycones, especially daidzein and genistein in various soybean varieties.

## MATERIALS AND METHODS

### Materials

The standard chemicals of daidzein and genistein were purchased from Sigma (Aldrich, USA). The HPLC grade solvents, water for chromatography (LC-MS grade), metanol and acetonitrile chromatography grade were obtained from E. Merck KgaA (Darmstadt, Germany), metanol pro analysis (analytical reagent grade) and acetic acid were also bought from Merck KgaA (Darmstadt, Germany). Soybean with various varieties was obtained from Indonesian Legumes and Tuber Crops Research Institute (Balitkabi), jalan raya Kendal Payak Km 8 Malang 65162, Indonesia.

### Preparation of working solution

Quantitative analysis of daidzein and genistein standard was performed by weighing an approximately of 1.0 g sample accurately and then dissolved with mobile phase. The solution was filtered using 0.45 µm membranefilter. The working solutions containing concentrations of daidzein and genistein were prepared by dilution using mobile phase, and 20 µl of the filtered solution was injected in HPLC system.

### HPLC instrumentation

All solutions of samples were subjected to RP-HPLC measurement using the condition as follows: column of *Sun Fire* TM C-18 (150 mm x 4.6 mm, 5 µm), the mobile phase a mixture of methanol and 0.1% acetid acid (53:47) and delivered isocratically with flow rate at 1.0

ml/min. The analytes were detected by using Photodiode array (PDA) at 254 nm.

**Validation of the analytical method**

Validation of the established method was performed based on parameters as suggested by ICH guideline [20]. The procedure was validated in terms of specificity, precision, accuracy, linearity, and sensitivity.

**Specificity**

Method specificity is the ability of an analytical method to measure the intended analyte precisely and specifically in the presence of other components in the sample. The intended compounds are determined by calculating their separation or resolution (Rs) from other compounds.

**Precision**

The intraprecision was determined by instrumental precision in both intraday precision (repeatability) and interday precision (intermediate precision). Precision was expressed in terms of calculated as relative standard deviation (RSD in percent). The intra-day precision was evaluated by analysing six samples during the same day. Precision is measured as relative standard deviation. This is done by making 6 replication samples at µg/ml and injecting them into the HPLC system.

**Accuracy**

The accuracy of the method was determined by application of the standard addition method. Three Levels of concentrations for daidzein and genistein were added into the sample and analyzed in triplicate as describe above. The total amount of each compound was calculated from the corresponding calibration plot and the percentage recovery of the each compound was calculated.

**Linearity**

For the linearity test, a standard solution containing daidzein and genistein (100 µg/ml) was prepared. Aliquots of the solution (2, 3, 4, 5, 6, 7, 8, 9 dan 10 µg/ml) were injected into the HPLC equipment. The linear regression equation and correlation coefficients were calculated for each daidzein and genistein using Microsoft Exel® (Microsoft Corp., USA). Linearity is a measure of the ability of the analytical method to relate the response to the levels of analytes on the calibration curve to form a straight line.

**Sensitivity**

The sensitivity of the analytical method was expressed by the limit of detection (LoD) and the limit of Quantification Limit (LoQ). For determination of LoD and LoQ, diluted standard solutions were injected into the HPLC equipment, at decreasing concentrations (1.4-0.4 µg/ml). LoD was defined as the concentration for which a signal to noise ratio of 3:1 was obtained, while LoQ was determined based on the signal to noise ratio of 10:1.

**Quantitative analysis of daidzein and gensitein in soybean**

Approximately of 2.5 g sample was massed into 100 ml amber vials and added with 25 ml methanol 50% for maceration. Finally, the supernatants were filtered through 0.45 µm syringe filter (PVDF Acrodisc LC) and transferred into a glass vial for HPLC analysis. Daidzein and genistein concentrations were calculated as mg of isoflavones per 100 g of soybean.

**RESULTS AND DISCUSSION**

**HPLC condition optimization**

Quantitative analysis of daidzein and genistein was performed by HPLC using PDA detector. The PDA detector is able to provide a collection of chromatograms simultaneously at different wavelengths in one running. Using PDA detector, UV spectra from 200–400 nm were online recorded for peak identification. The PDA detection was performed at 254 nm, The injection volume was 20 µl. Optimization of mobile phase composition and flow rate used for analysis affected the retention time (Rt) and resolution. The chromatographic parameters were initially evaluated for a standard solution of daidzein and genistein (5 µg/ml). Resolution, tailing factor and N plate were determined for different proportions of acetonitrile or methanol and the aqueous solvents. Different proportions of this solvents were evaluated and the results showed that acetonitrile and water as the mobile phase give unfavorable resolution results. The mobile phase of methanol-water has shown a good resolution for two compounds of daidzein and genistein. The separation of aglycones was evaluated in different mobile phase compositions. The mobile phase obtained from the optimization was a mixture of methanol: 0.1% acetid acid (53:47) with a flow rate of 1.0 ml/min. Isabela *et al.* [11] also used with similar mixture of the mobile phase, but with a different composition. The RSD values for peak area and peak height obtained meets the requirements of maximum RSD ≤ 2% [21]. The chromatogram of soybean samples containing-daidzein and genistein were shown in fig. 1, 2 and 3.

**System suitability test (SST)**

System suitability test was carried out by injecting daidzein and genistein standard solutions at a concentration of 5 µg/ml with six-time replicates. The results obtained showed that the conditions used for the determination of the levels of daidzein and genistein in the sample had good system suitability based on retention time, peak area and peak height requirements, % RSD ≤ 2% [24] for the analysis of daidzein and genistein, The values of SST in table 1.

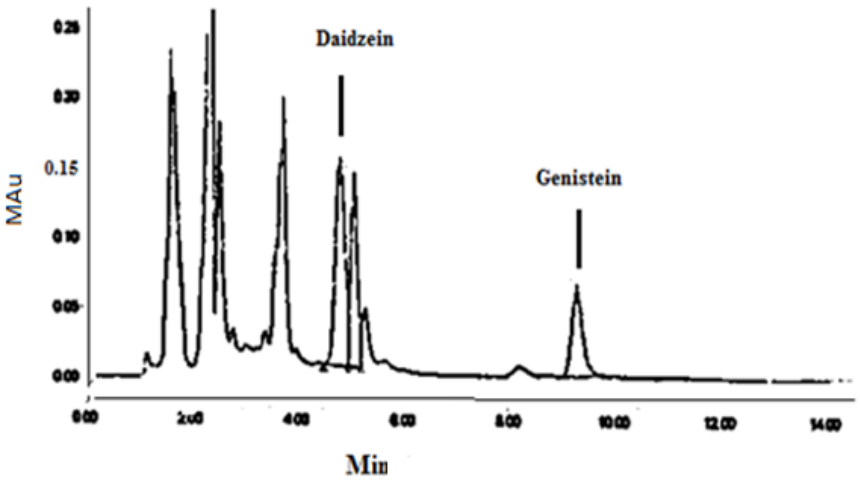


**Fig. 1: Chromatogram of soybean samples obtained by HPLC using sun fire TMC-18 C18 coloumn (150 mm x 4.6 mm, 5 µm). The mobile phase used was acetoniril: 0.1% acetic acid (35: 65), flow rate of 0.8 ml/min**

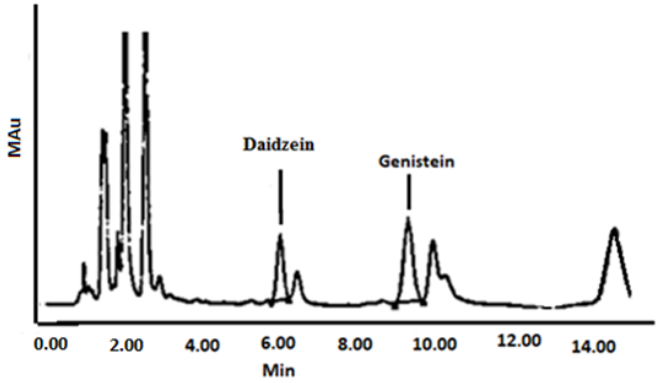


**Fig. 2: Chromatogram of soybean samples obtained by HPLC with the Sun Fire TMC-18 C18 coloumn (150 mm x 4.6 mm, 5 µm). The mobile phase used was methanol: 0.1% acetic acid (53:47), flow rate of 1.0 ml/min**

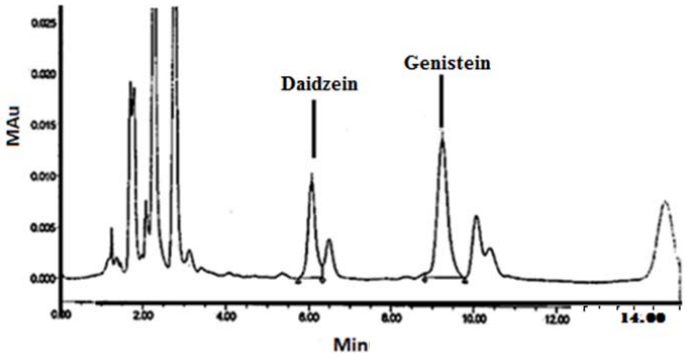


**Fig. 3: Chromatogram of the sample obtained by HPLC with Sun Fire TMC-18 C18 coloumn (150 mm x 4.6 mm, 5 µm). The mobile phase of methanol: 0.1% acetic acid (53:47), flow rate of 1.0 ml/min. Spiking was performed with 1 ppm of daidzein and genistein, respectively**

**Table 1: System suitability obtained for analysis of daidzein and genistein using HPLC with optimum condition**

| No | Daidzein | | | | | Genistein | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Rt/min | Area | Height | N | Rs | Rt/min | Area | Height | N | Rs |
| 1 | 6.519 | 152010 | 13585 | 8222 | 10.11 | 10.114 | 175071 | 10623 | 9025 | 10.21 |
| 2 | 6.507 | 152024 | 13588 | 8204 | 10.09 | 10.088 | 174429 | 10652 | 9044 | 10.22 |
| 3 | 6.467 | 152138 | 13618 | 8188 | 10.00 | 10.002 | 175971 | 10755 | 8790 | 10.13 |
| 4 | 6.67 | 153055 | 13735 | 8829 | 10.24 | 10.237 | 176726 | 10811 | 9543 | 10.25 |
| 5 | 6.391 | 152504 | 13896 | 8304 | 9.86 | 9.857 | 175190 | 10931 | 9112 | 10.13 |
| 6 | 6.342 | 151478 | 13957 | 8325 | 9.766 | 9.766 | 174835 | 10960 | 9028 | 10.08 |
| RSD (%) | 1.76 | 0.35 | 1.18 | | | 1.74 | 0.47 | 1.29 | | |

The accuracy of the method was investigated by means of recovery experiment with the addition standard of three-level was expressed calculated as the concentration recovery percentage. A mean recovery (n=9) of concentration obtained for daidzein 83.0%-100.95% and genistein 80.07%-108.79%. The acceptable value of recovery for biological compound analysis was 80 - 115% [23]. The precision of HPLC was evaluated by repeatability and intermediate precision at different days of analysis. The relative standard deviation (RSD) values were used for precision evaluation. In the intra-day precision analysis (n=6), the mean contents of daidzein was level 1 =0.33; 1.00; 0.67, level 2 = 0.99; 0.67; 1.24 and level 3 = 0.29; 1.41; 0.32. For genistein was level 1 =1.29; 1.11; and 1.43, level 2 = 1.29; 0.28; 1.43 and level 3 = 0.35; 0.70; 1.99. The obtained % RSD was less than 6.00%, so that the precision of the proposed method meet the required value as described in method validation guideline. [23].

Linearity is a measure of the ability of the analytical method to correlate the response with the analyte levels on the calibration curve to form a straight line. Linearity is also the ability of a method to obtain a test result that is directly proportional to the concentration of analytes in the given range. The linear regressions obtained were shown in fig. 4. Linear calibrations were obtained for daidzein and genistein with the correlation of determination in the range of 0,9989–0,9991 for calibrations, linearity is achieved when the coefficient of determination ($r^2$) is ≥0.997 [24].

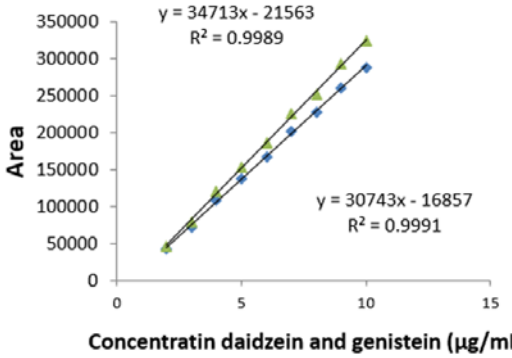


**Fig. 4: The relationship between the concentration of daidzein and genistein (x-axis) and peak area (y-axis)**

The sensitivity of the analytical method was expressed by the limit of detection (LoD) and limit of quantitation. LoD values obtained were of 0.05192 µg/ml and 0.0600 µg/ml for daidzein and genistein, respectively. Meanwhile, LoQ values obtained were 0.1731 µg/ml and 0.2000 µg/ml for daidzein and genistein, respectively. Based on validation parameters evaluated, it can be concluded that the HPLC method was valid for the quantitative analysis of daidzein and genistein in soybean samples. The validated HPLC method was used for analysis of soybean samples, and the results were compiled in table 2.

**Table 2: The contents of daidzein and genistein in several cultivars of soybean**

| No | Soybean cultivars | Daidzein (mg/100 g) | Genistein (mg/100g) |
|---|---|---|---|
| 1 | Burangrang | 20.32±0.39* | 15.07±0,66* |
| 2 | Dieng | 24.12±1.48* | 15.38±1.73* |
| 3 | Demas | 29.40±0.99** | 23.65±2.30** |
| 4 | Devon 1 | 19.11±0.30* | 17.04±0.34* |
| 5 | Dega | 13.75±0.43** | 12.82±1.07** |
| 6 | Detam 1 | 10.49±0.26* | 12.49±1.01* |
| 7 | Gema | 11.06±0.41* | 15.76±1.03* |
| 8 | Gepak Kuning | 18.00±0.37** | 17.29±1.07** |
| 9 | Gumintir | 26.39±1.74* | 23.29±1.03** |
| 10 | Kawi | 15.38±0.42** | 16.19±1.16** |
| 11 | Malika | 18.17±0.82** | 17.33±0.67** |
| 12 | Merbabu | 42.65±0.87* | 33.32±1.03* |
| 13 | Mahameru | 18.11±0.67* | 15.09±0.40* |
| 14 | Rajabasa | 26.32±0.99* | 22.43±5.76* |
| 15 | Rinjani | 22.49±0.39* | 19.71±1.72* |
| 16 | Roung | 26.92±2.08** | 24.50±1.25** |
| 17 | Pandermanan | 2.70±0.21* | 2.85±0.23* |

The results for daidzein and genistein contents in several cultivars of soybean in replication n=5 (*) and n=6(**)

## CONCLUSION

HPLC method using reversed phase mode offered reliable and validated techniques for the quantification of isoflavones free aglycones (daidzein and genistein) in soybean samples. The developed method is a simple method and could be applied for routine quality control of daidzein and genistein in various of soybeans varieties.

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## AUTHORS CONTRIBUTIONS

ES-performed research activities and prepared manuscript. EL,-, SM, and SR designed research analyzed data and made critical thinking on a manuscript.

## CONFLICTS OF INTERESTS

All authors have none to declare

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