High Performance Liquid Chromatography (HPLC) Method for Determination of Isoflavones Content in Shade-Tolerant Soybean Dena I

Adawiah Daud¹², Hermin Sulistyarti*¹², Rurini Retnowati¹², Erliana Ginting³
¹Department of Chemistry, Faculty of Mathematics and Natural Sciences, Brawijaya University, Indonesia
²Research Center for Low Cost and Automated Method and Instrumentation Analysis (LCAMIA), Brawijaya University, Indonesia
³Indonesian Legumes and Tuber Crops Research Institute (ILETRI), Indonesia
*Corresponding author: hermin@ub.ac.id

Abstract. Soybeans (Glycine max (L.) Merrill) are the most common source of isoflavones in human foods which useful for health as preventions of cardiovascular disease, cancer, diabetes, hypertension, osteoporosis, and menopause. The amount of isoflavones in soybeans may differ significantly depends on both genetic and environmental factors. Among the four forms of isoflavones, the highest biological activity is indicated by isoflavones aglycones i.e., genistein, daidzein, and glycitein. Genistein and daidzein found as major compounds with significant amounts contained in soybeans. Indonesian Legumes and Tuber Crops Research Institute (ILETRI) cultivate a shade-tolerant soybeans variety called Dena I to overcome the production of soybeans in Indonesia. Although finding exist for Dena I variety, there are no firm data regarding the isoflavones content of this variety. In this research, high performance liquid chromatography (HPLC) method using a C-18 column with UV detection in optimum condition was performed to identify and quantify the isoflavones content in the extract of Dena I soybean. The satisfied separation was obtained using the mobile phase of acetonitrile-acetic acid 0.1% with the gradual increase of acetonitrile 15-30%, the flow rate of 1 mL/min, and detection wavelength of 260 nm. Isoflavone genistein in Dena I extract was eluted at the retention time (tR) of 24.696 min, the same way with the retention time (tR) of standard genistein of 24.490 min. The concentration of genistein was found 0.0524 mg/g Dena I soybean.

1. Introduction
Indonesia as a tropical country that producing soybeans (Glycine max (L.) Merrill), a sub-tropical plant that belongs to Leguminosae, starts many research on improving the nutritional quality of soybeans, such as cultivate a new variety of soybeans. The high consumption of soybean due to its appreciated nutritional and functional properties has brought about a growing interest in improving its cultivation and characteristic [1]. Soybeans need increase year to year whereas the production of soybean decreases in Indonesia. The decreasing of soybeans production was impacted by soybean harvest areas reduce. Indonesian Legumes and Tuber Crops Research Institute (ILETRI) cultivate a new soybean variety from plant breeding scheme which is shade-tolerant soybean, called Dena I. Dena I is one of an important...
variety of soybean to overcome soybean production in Indonesia because it is possible to cultivate in shade or semi-shady conditions between trees.

Plants produce secondary metabolites as a defense mechanism from biotic and abiotic stress. Isoflavones as secondary metabolites which are the major component in soybeans produce by plants through the synthesis of 2-hydroxyisoflavone synthase and classified into four groups: aglycones, glucosides, malonyl glucosides, and acetyl glucosides. These compounds are not synthesized by microorganisms, therefore, soybeans are the main source of isoflavone compounds in nature. Isoflavones in soybeans have some beneficial health effects such as cancer prevention agents, cardiovascular phytoestrogens agents, controlling menopausal symptoms, diabetes, diabetic wound healing agents, and anti-inflammatory agents [2]. Among the four forms of isoflavones, the highest biological activity is indicated by isoflavones aglycones i.e., genistein, daidzein, and glycitein because they can. The isoflavone aglycones were absorbed faster and in greater amounts than the glucosides in the human body. Genistein and daidzein was reported as major compounds with a significant amount contained in soybean [3].

Isoflavones content in soybeans depends on both genetic and environmental factors, including climate, planting location, crop year, planting dates within a given crop year, and storage conditions [4]. No firm data regarding the isoflavones content of Dena I variety although this variety had produced. There is a need of method to identify and quantify isoflavones in Dena I, therefore the results of this research may use as a reference to isoflavones content of Dena I soybean. High-performance liquid chromatography (HPLC) is the most commonly used procedure in the determination of isoflavones. The extraction of compounds from plant materials is one of the most important steps prior to their determination by HPLC. Some novel extraction methods of flavonoids have been developed e.g., ultrasound-assisted extraction (UAE) and microwave-assisted extraction (MAE) [5]. MAE needs the shortest time, however, this method uses high temperature and this may affect the stability of isoflavones. Ultrasound also exerts a mechanical effect, allowing greater penetration of solvent into the sample matrix, increasing the contact surface area between the solid and liquid phases. The extraction of bioactive compounds under ultrasound irradiation offers high efficiency in a shorter time. Additionally, the UAE is the cheapest technique and has the lowest instrumental requirements. Therefore, in this study ultrasound-assisted extraction (UAE) is used to extract isoflavones from Dena I soybean with methanol 80% as a solvent.

In this research, high performance liquid chromatography (HPLC) method using a C-18 column with UV detection in optimum condition was performed to identify and quantify the isoflavones content in the extract of Dena I soybean. Determination optimum conditions for isoflavones analysis using HPLC consist of optimization of operational and chemical conditions, including optimization of flow rate, UV detection wavelength, and mobile phase composition with isocratic and gradient elution techniques.

2. Material and Methods

2.1. Samples and chemicals
The chemical used involved HPLC-grade methanol purchased from Merck (Darmstadt, Germany), HPLC-grade acetonitrile purchased from Fulltime (China), pro-analysis acetic acid glacial 100% purchased from Merck (Darmstadt, Germany), genistein standard, and distilled water. Soybean seeds, Dena I, were obtained from Indonesian Legumes and Tuber Crops Research Institute Indonesia (ILETRI). The chemical structure of genistein shown in Figure 1.

2.2. Sample preparation
The plant material in this study was soybean seeds, Dena I. Certificates of the varieties were taken from Management Unit Seeds of ILETRI. Soybeans was ground, powders of soy were passed through the No.100 sieve before extraction. One gram of Dena I soybeans powder placed into 50 mL Erlenmeyer flask then added with 25 mL of 80% methanol as an extraction solvent in an ultrasonic bath for 1 hour.
The extract yield concentrated in a rotary evaporator RV 05 ST (IKA Werke, Staufen im Breisgau, Germany), centrifuged, filtered by a 0.45 μm Whatman microfilter transferred into a 5 mL vial bottle, and stored 4°C until the analysis.

2.3. HPLC optimization analysis
HPLC analysis of isoflavones was performed on a set of HPLC from Shimadzu (Kyoto, Japan), univerSil HS C18 (Forties Technologies, UK) (250 x 4.6 mm i.d., 5 μm particle size) as a stationary phase, and the mobile phase of acetonitrile and acetic acid 0.1%.

2.3.1. Optimization of flow rate and wavelength detection
The proposed HPLC method was optimized under the following conditions:
- Temperature: Room temperature
- Column: univerSil HS C18 (Forties Technologies, UK) (250 x 4.6 mm i.d., 5 μm particle size)
- Flow rate: 0.5 - 1.5 mL/min
- Detector: UV 249-262 nm
- Mobile phase: Acetonitrile and acetic acid 0.1%, 10-90% (isocratic elution) and acetonitrile (A)-acetic acid 0.1% (B), with gradual increase of acetonitrile 15-30% (gradient elution).

The optimum flow rate were determined by the degree of resolution (Rs) and analysis time, then the optimum detection wavelength were determined intensity from chromatogram peaks.

2.3.2. Optimization of mobile phase composition
Optimization of mobile phase composition was carried out under optimum flow rate and wavelength.

The composition of the mobile phase was optimized by changing acetonitrile-acetic acid 0.1% composition in isocratic elution technique from 10% to 90%, for gradient elution the composition of acetonitrile-acetic acid 0.1%, with a gradual increase of acetonitrile from 15-30% for 40 minutes.

The optimum mobile phase composition was determined by the degree of separation and chromatogram performance.

![Figure 1. Chemical structures of isoflavone genistein](image)

2.4. Analysis of Isoflavones from Dena I Extract
The identity of the isoflavones compound was acquired by comparison of retention time and UV–Vis spectra of corresponding standard genistein (GEN), due to limited availability of standard isoflavones. Additionally, mass spectrometer in the m/z range of 100–800 used for qualitative analysis to confirm the availability of isoflavone genistein in Dena I extract. Table 1 shows data for confirming of the identity of the investigated isoflavone compounds based on literature. Quantitative analysis of genistein in the methanol extract of Dena I soybean was done with the external standard method.
Table 1. Data for confirming the identity of isoflavones compound

| Isoflavones    | $\lambda$ (nm) | m/z  |
|----------------|----------------|------|
| Daidzein       | 249            | 255  |
| Daidzin        | 249            | 417  |
| Malonyldaidzin | 249            | 503  |
| Genistein      | 260            | 271  |
| Genistin       | 260            | 433  |
| Malonylgenistin| 260            | 519  |
| Glycitein      | 260            | 285  |
| Glycitin       | 260            | 477  |

3. Results and Discussion

3.1. Optimization of HPLC-UV method

The analysis of isoflavones content especially daidzein and genistein in soybean varieties produced by ILETRI is a new determining parameter in deciding which types of soybeans are best to be planted by farmers and produce good quality soybeans. Quantification of isoflavones is commonly carried out by HPLC on C-18 column, water, and methanol or acetonitrile containing small amounts of acid (formic acid, acetic acid, or trifluoroacetic acid) as mobile phase [4] to set the polarity of the stationary phase [5].

![Figure 2. Wavelength optimization on the chromatogram of isoflavone from Dena I extract. Conditions: mobile phase of acetonitrile:acetic acid 0.1 % (20:80); sample volume 2 μL; flow rate 1 mL/min.]
Optimization of wavelength carried out in order to obtain a suitable detection wavelength which gave high sensitivity to all isoflavones compound in the sample. Optimization of the wavelength was varied from 249 nm; 250 nm; 254 nm; 257 nm; 260 nm; and 262 nm in Dena I extract (Figure 2). From these optimizations the wavelength of 260 nm was chosen as optimum because give good sensitivity to all compounds in Dena I extract.

Optimization of flow rate carried out in order to obtain good separation and efficient analysis time. Flow rate 0.5 mL/min; 0.8 mL/min; 1.0 mL/min; 1.2 mL/min; and 1.5 mL/min applied in optimization flow rate in Dena I extract (Figure 3). From these optimization flow rate, 1.0 mL/min was chosen as optimum flow rate because give good separation to each peaks of compound and acceptable analysis time in Dena I extract.

Optimization mobile phase composition carried out with mobile phase composition variation. Wavelength and flow rate optimum was applied to Dena I extract under isocratic elution technique using a various composition of acetonitrile:acetic acid 0.1% (20:80; 25:75; 30:70; 40:60; 50:50; 60:40) (Figure 4). The variation of the mobile phase composition caused the changes pH value of the mobile phase. In this analysis pH value plays an important role in determining retention time and selectivity of the separation. Variation of composition of acetonitrile and acetic acid 0.1%, changes mobile phase pH value in composition as follow: acetonitrile:acetic acid 0.1%, 5:95; 10:90; 15:85; 20:80; 90:10; and 80:20, pH value 3.50; 3.60; 3.79; 3.84; 6.08, and 6.18, respectively. The chromatogram shows that good separation carried out when composition of acetic acid 0.1% more than the composition of acetonitrile and the pH value of mobile phase range 3.50-3.84. Figure 5 shows that separation of isoflavones with a mobile phase composition of acetonitrile: acetic acid 0.1% 20:80 in isocratic elution resulting 6 peaks, however the peaks still close to each other, this indicates that the separation of isoflavones compounds not good enough with this condition. Therefore, in order to improve the separation, the elution technique was changed into gradient elution.

Figure 3. Flow rate optimization on the chromatogram of isoflavone from Dena I extract. Conditions: mobile phase of acetonitrile:acetic acid 0.1 % (20:80); sample volume 2 μL; λ 260 nm.
Figure 4. Chromatogram of Dena I extract using isocratic elution with varying mobile phase composition. Conditions: acetonitrile:acetic acid 0.1 % (a) 20:80; (b) 25:75; (c) 30:70; (d) 40:60; (e) 50:50; (f) 60:40; sample volume 2 μL; 0.1 mL/min; λ=260 nm.

Figure 5. Chromatogram of Dena I extract using isocratic elution. Conditions: mobile phase acetonitrile:acetic acid 0.1 % (80:20); sample volume 2 μL, flow rate 1 mL/min, λ=260 nm.

The first mobile phase composition in gradient elution was GE-1, the gradient program was as follows: 10-17% for 0–5 min; 19-22% A for 5–15 min; and 23-25% A for 15–40 min. Figure 6 GE-1 shows 8 peaks on chromatogram; however peaks in the initial minutes have not separate well. The mobile phase composition for the second condition was GE-2, the gradient program was as follows: 10-17% for 0–5 min; 19-22% A for 5–15 min; and 26-30% for 15–40 min. The chromatogram under this composition was shown in Figure 6 GE-2. The separation of peaks in initial minutes was slightly improved. The improvement of separation from using GE-2 system was done by using a third mobile phase composition GE-3 (Figure 6 GE-3), the gradient program was as follows: 10-16% A for 0–3 min;
17-23% A for 3–12 min; 24-29% A for 12–17 min; 30-35% A for 17–23 min; 24-29% A for 23–30 min; and 17-23% for 30–40 min. This gradient elution (GE-3) showed excellent separation for all compounds (7 main peaks) in methanol extract of Dena I soybean C18 column, therefore GE-3 was selected as optimum condition for elution and used for further separation and determination of isoflavones in Dena I extract.

3.2. Analysis of Genistein
Qualitative analysis for determination of genistein in Dena I extract was done using three methods, (1) comparison of retention time of genistein (GEN) standard to the retention time of peaks in Dena I extract chromatogram; (2) based external standard method, and (3) based on UV and MS spectra. As shown in Figure 7, a peak with the retention time of 24.696 min from Dena I extract chromatogram has similar retention time to the retention time of genistein (GEN) standard peak of 24.490 min, it means that genistein available in Dena I extract. Based on comparison with a single standard, the concentration of genistein in the Dena I extract was found containing 0.0524 mg/g genistein.
Figure 7. Chromatogram of genistein (GEN) standard 20 ppm by HPLC optimum; Chromatogram of Dena I extract.

To prove that the retention time of 24.699 min was genistein, the extract of Dena I was also analyzed using MS spectrometer. Ion trap mass spectrometer was used to detect the occurrence of genistein which has a molecular weight of 271. The availability of genistein in Dena I was confirmed by the appearance of molecular ion peaks at m/z 542.90 as base peak, which represents the molecular weight dimers of the genistein.

4. Conclusion
The optimum HPLC conditions for analysis of isoflavones in shade-tolerant soybean, Dena I, obtained at a flow rate of 1 mL/min, wavelength at 260 nm, gradient elution program, GE-3. The gradient program was as follows: 10-16% A for 0–3 min; 17-23% A for 3–12 min; 24-29% A for 12–17 min; 30-35% A for 17–23 min; 24-29% A for 23–30 min; and 17-23% for 30–40 min. High performance liquid chromatography has proven that extract Dena I soybean contained one of isoflavone aglycone i.e genistein that has high biological activity. The concentration of isoflavone genistein was found 0.0524 mg/g Dena I soybean.

Acknowledgment
The authors would like to thank Department of Chemistry, University of Brawijaya, for the research facilities and conference, also Indonesian Legumes and Tuber Crops Research Institute (ILETRI) Malang for providing the sample, Dena I soybean.
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