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RESEARCH ARTICLE

Validation and determination of Daidzein in *Aspergillus oryzae* using thin-layer chromatography-densitometry

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Abstract

Background: Daidzin (glycosidic isoflavone) bioconversion into daidzein (aglyconic isoflavone) increases its bioactivity. This bioconversion can be carried out using fermentation, using a fungus that has been widely used for making tempeh in Indonesia, *Aspergillus oryzae*. **Objective:** This study aimed to validate the method of analysis used and to determine the daidzein content in *A. oryzae*-fermented edamame using TLC densitometry. **Method:** Daidzein content determination was carried out on non-fermented (D0) and fermented edamame on days one to four (D1-D4) using the TLC densitometry method. The validation method of analysis was done previously using linearity, the limit of detection (LOD), the limit of quantification (LOQ), selectivity, specificity, precision, and accuracy parameters. **Result:** The TLC densitometry method used met all the validation method of analysis parameters, except for the selectivity. The daidzein content in edamame from D0 to D4 were 0.0433 ± 0.00221; 0.0336 ± 0.00698; 0.0276 ± 0.00039; 0.0219 ± 0.00041; 0.1002 ± 0.00924 % w/w, respectively. **Conclusion:** The highest daidzein level was obtained in day four of *A. oryzae*-fermented edamame.

Introduction

The life expectancy of Indonesian women is increasing, from 73.33 years in 2010 to 73.55 years in 2016, resulting in an increase in the number of menopausal women (Badan Pusat Statistik, 2021). Menopausal women often experience uncomfortable symptoms i.e., hot flushes, insomnia, and vaginal dryness (Bruce & Rymer, 2009). They are seeking ways to decrease those symptoms, 76.1% are undergoing therapies including taking dietary soy and isoflavone supplements with 22.9% proportion. Among women using therapies, 89-100% found it beneficial (Newton *et al.*, 2002).

Soybean contains several natural isoflavones, and so does edamame, the vegetable soybean (Mentreddy *et. al*, 2002). Soybean isoflavones are found mostly in the glycosidic form. Glycosidic isoflavone is more bioactive than glycosidic ones. Fermentation can remove the glycosidic group of glycosidic isoflavone resulting in a glycosidic isoflavone (Barnes, 2010).

This research was conducted to determine the daidzein (the major glycosidic isoflavone in soybeans) content in *Aspergillus oryzae*-fermented edamame using the thin-layer chromatography (TLC) method. *A. oryzae* was chosen because it is the common fungus used to make fermented soybean products, including tempeh and soy sauce (Yoneya, 2003). The validation method of analysis was done prior to the analysis since the determination of daidzein content in *A. oryzae*-fermented edamame has never been done before.

Method

Material

The SPMI edamame variety used in this study was obtained from PT. Mitra Tani Dua Tujuh and Jember . It was cultivated in Jember and harvested at 63rd-68th days. Other materials used were daidzein standard (Sigma), potato dextrose agar (BD Difco), Tween 80, TLC plate silica gel 60 F254 (Merck), toluene (Smart Lab Indonesia), ethyl acetate (Smart Lab Indonesia), acetone (Smart Lab Indonesia), n-hexane (Smart Lab Indonesia), ethanol 70%, methanol (Smart Lab Indonesia), and aqua dest.

Fermentation using A. oryzae

The edamame was peeled, washed, and sterilised using an autoclave. The fermentation was done on the cooled edamame using 10 ml of 10^6 *A. oryzae*'s spore/ml per 500 g edamame, then incubated for one to four days (D1-D4) at 30^0C 95% RH until solid-state fermentation was obtained (Lee, Hung, & Chou, 2008).

Extraction

The non-fermented (D0) and fermented edamame (D1-D4) were dried at 60^0C for 30 hours in the oven, pulverised, and sifted. The defatting was done using 1:5 of n-hexane for three hours in the soxhlet apparatus. The defatted edamame was then air-dried overnight and extracted using ultrasonication and 1:6 of sample: 70% ethanol for one hour, followed by deposition using a centrifuge at 2,600 rpm for ten minutes (Miao, Qi, & Zhao, 2005). The extraction was repeated three times on the residue. The filtrate was collected and concentrated using a rotary evaporator until a thick extract was obtained (Luthria, Biswas, & Natarajan, 2007). The extract yield was calculated using the following formula:

$$\text{Yield} = \frac{\text{Extract weight}}{\text{Edamame weight}} \times 100\%$$

Analytical condition optimisation

The optimisation of the analytical condition was done prior to the validation method of analysis. The optimisation was done on solvent, stationary phase, eluent, λ_{max}, and sample concentration. The optimisation on the solvent was done by comparing the following eluent: n-hexane – ethyl acetate – formic acid (1:5:0.05), n-hexane – ethyl acetate – water (2:5:0.25), n-hexane – ethyl acetate (3:5), and n-hexane – ethyl acetate – acetate acid (2:5:0.15). λ_{max} was determined by wavelength scanning on the analyte at 200-400 nm. While the sample concentration optimisation was done by using three sample concentrations and comparing them for the biggest N value (theoretical plate number) and smallest H value (height equivalent of a theoretical plate).

Validation method of analysis

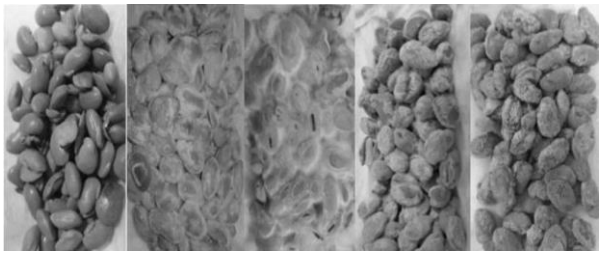
The validation method of analysis on daidzein determination using TLC was done on linearity, LOD, LOQ, selectivity, specificity, precision, and accuracy. Those parameters were calculated using the validation method of the analysis programme. The selectivity was determined using the resolution (Rs) parameter, it was calculated based on the separation between the daidzein peak and two other peaks, i.e., genistein and glycitein on the sample chromatogram. The resolution was calculated by dividing the distance between two spots by the sum of the two spot radii. The parameters used to justify whether the method is valid or not are those from AOAC International (AOAC International, 2012).

Determination of daidzein content in non-fermented and A. oryzae-fermented edamame

The determination of daidzein content in non-fermented and *A. oryzae*-fermented edamame was done using the validated method of analysis. The data were analyzed using ANOVA followed by LSD post hoc ($\alpha = 0.01$).

Result

Mycelium growth was observed from D1 fermentation (Figure 1). It was originally whiteish (D1 and D2 fermentation), then turned brownish on D3 to D4 fermentation where a more compact mass was formed.



D0 D1 D2 D3 D4

Figure 1: Morphological characteristic of non-fermented (D0) and *A. oryzae*-fermented (D1-D4) edamame

Extraction

The extract obtained from each sample is shown in Table I.

Table I: Extract obtained from edamame

Extract	Yield (% w/w)	Colour
D0	9.690	Light yellow
D1	17.185	Brownish yellow
D2	23.749	Dark brown
D3	12.570	Dark brown
D4	20.531	Dark brown

Analytical condition optimisation

The optimum condition of analysis is shown in Table II.

Table II: Optimum condition of analysis using TLC method

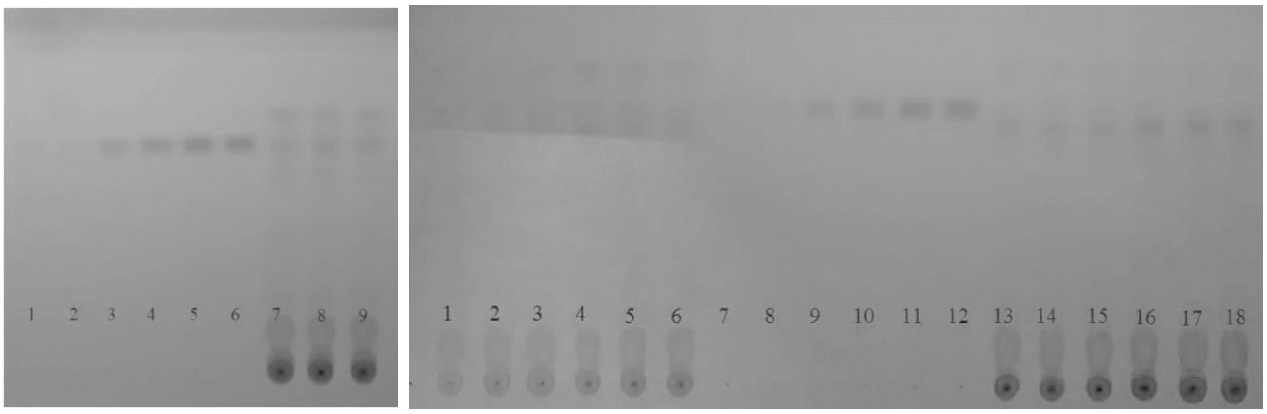
Analytical condition	Optimum condition of analysis
Solvent	Methanol p.a.
Stationary phase	Silica gel 60 F_{254}
Eluent	n-hexane : ethyl acetate : acetic acid (2:5:0.15)
λ_{max}	273 nm
Sample concentration	80 mg/mL

The validation method of analysis

The standard curve obtained from the linearity test was y=15.169x – 234.522 (the sample concentrations used were six points ranging from ten to 100 µg/ml) with r value (correlation coefficient) of 0.999, Vx0 (the coefficient of variation of the regression function) = 3.068%, and Xp = 55.675. The LOD and LOQ value was 23.694 ng and 71.089 ng, respectively. The selectivity test resulted in the Rs value of daidzein-glycitein of 0.128, and in the Rs value of daidzein-genistein of 0.198. The purity and identity test for specificity resulted in both r(s,m) and r(m,e) values of 0.99. The precision parameter calculated from the six samples used was repeatability (4.467%) and intermediate precision (4.121%). While the recovery was used as an accuracy parameter (99.015%). It was calculated using the standard addition method.

Determination of daidzein content in A. oryzae-fermented edamame

The TLC profile of daidzein content determination in non-fermented and *A. oryzae*-fermented edamame is as shown in Figure 2. While the calculation on daidzein content is shown in Figure 3. The daidzein content in non-fermented and D1 of fermentation using *A. oryzae* was the same, but it was increasing significantly on day two to day four of fermentation and reached its peak on day four of fermentation.

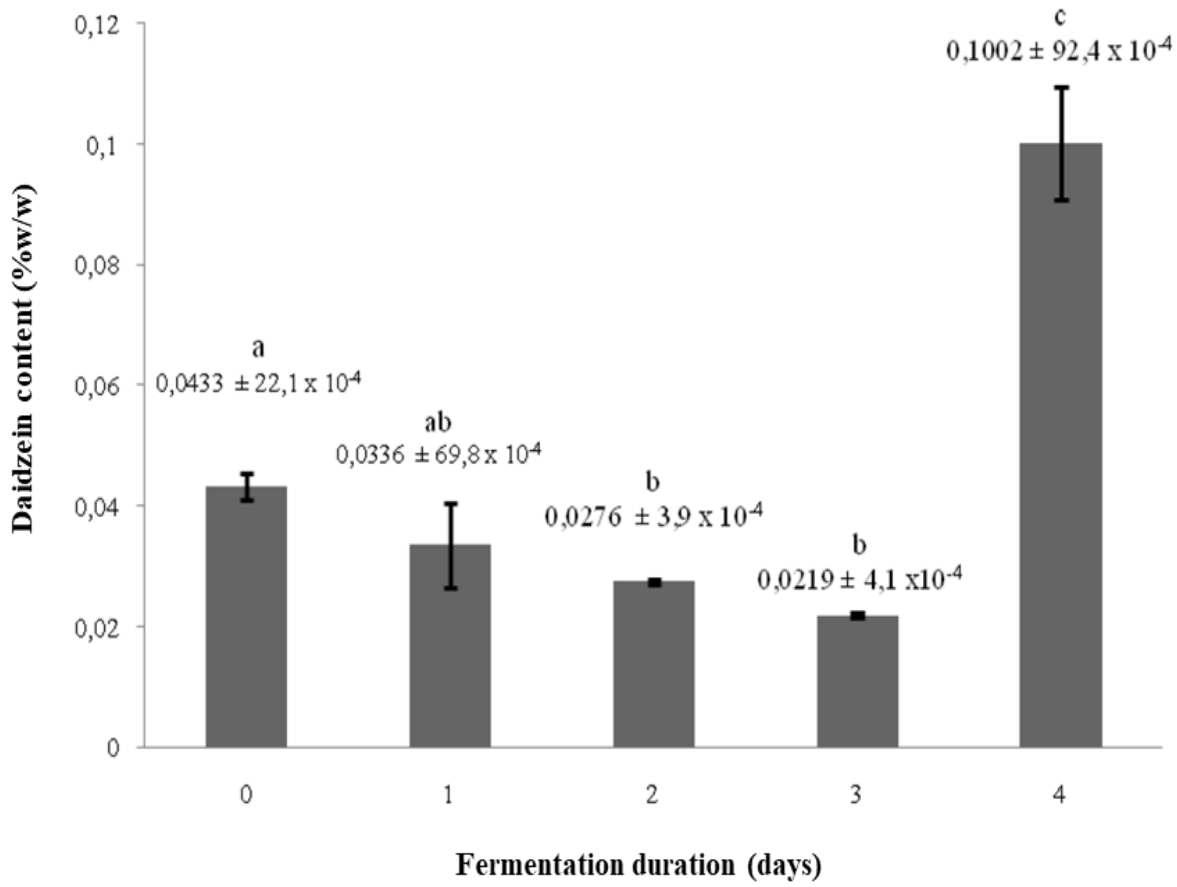


(1) Daidzein 10 µg/ml; (2) Daidzein 30 µg/ml; (3) Daidzein 50 µg/ml; (4) Daidzein 70 µg/ml; (5) Daidzein 90 µg/ml; (6) Daidzein 100 µg/ml; (7) D0 sample replication 1; (8) D0 sample replication 2; (9) D0 sample replication

(1) D1 sample replication 1; (2) D1 sample replication 2; (3) D1 sample replication 3; (4) D2 sample replication 1; (5) D2 sample replication 2; (6) D2 sample replication 3; (7) Daidzein 10 µg/ml; (8) Daidzein 30 µg/ml; (9) Daidzein 50 µg/ml; (10) Daidzein 70 µg/ml; (11) Daidzein 90 µg/ml; (12) Daidzein 100 µg/ml; (13) D3 sample replication 1; (14) D3 sample replication 2; (15) D3 sample replication 3; (16) D4 sample replication 1; (17) D4 sample replication 2; (18) D4 sample replication 3

(a) Non-fermented edamame (b) *A. oryzae*-fermented edamame

Figure 2: TLC profile of non-fermented (a) and *A. oryzae*-fermented (b) edamame under UV 254 nm



The data are shown as mean ± SD (n=3). Different annotation shows a significant difference (LSD, $p < 0.01$)

Figure 3: Daidzein content in non-fermented and *A. oryzae*-fermented edamame

Discussion

This research was conducted to mimic the process of making tempeh, one of the Indonesian traditional foods rich in isoflavones (Mani & Ming, 2017). However, we used edamame instead of soybean as the original source. The growth phase in edamame fermentation occurs at zero to 30 hours of fermentation, this phase is characterised by the growth of mycelia on the surface of the seeds. The next phase is the transition phase which occurs at 30-50 hours of fermentation, this phase is characterised by the presence of a specific odour such as the typical smell of tempeh (Dwinanto, 2011).

The extracts obtained from non-fermented and *A. oryzae*-fermented edamame are shown in Table I. The highest yield was obtained on the second day of fermentation (D2), which was 23.749 % w/w. The large yield indicates the quantity of compound in the extract but is not directly correlated to its activity. Extract yield only describes the total number of compounds taken, but does not represent the concentration of one particular compound (Nuri *et al.*, 2020).

The validation method of analysis fulfilled all the criteria from AOAC International (2012), except for the specificity. To be considered specific, the Rs value should be > 1.5 (AOAC International, 2012). There is no literature pinpointing the Rs value of daidzein separation. The resolution value that did not meet the acceptance range may be due to the close proximity of the Log P values of the three components i.e., daidzein 2.5, genistein 2.7, and glycitein 2.4, making the separation of these three components difficult (National Center for Biotechnology Information, 2004a; National Center for Biotechnology Information, 2004b; National Center for Biotechnology Information, 2005).

Interestingly, the fermentation using *A. oryzae* on edamame fermentation made the daidzein content decrease on the first three days of fermentation, then drastically increased on the fourth day of fermentation using *A. oryzae* (Figure 3). The daidzein content then significantly increased on day four, with the value of

0.1002 ± 0.0092 % w/w. These findings are contradictive with previous research stating that fermentation would increase aglyconic isoflavone over time (Chun *et al.*, 2007; Chun, Kim, & Kim, 2008). Further research needs to be done to determine the exact mechanism of these phenomena.

Conclusion

The method of daidzein analysis fulfilled all the validation parameters, except for the selectivity. The highest daidzein content was that on the fourth day of fermentation

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