HPLC Method for Simultaneous Determination of Six Isoflavones in Soybean Yoghurt

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HPLC Method for Simultaneous Determination of Six Isoflavones in Soybean Yoghurt

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Abstract. Objective: To establish an HPLC method for simultaneous determination of six isoflavones (daidzin, glycitin, genistin, daidzein, glycitein, and genistein) in soybean yoghurt, providing a reference for its development of quality standards and functional evaluation. **Methods:** The six kinds of isoflavones were measured on a C18 column (250 mm×4.6 mm, 5μm) with a gradient solvent system of acetonitrile - trifluoroacetic acid solution as mobile phase at a flow rate of 1mL·mL-1 and the column temperature was kept at 40°C. The detection wavelength was 255nm. **Results:** Excellent chromatographic separation and good linearity with peak areas were achieved for 6 kinds of soybean isoflavones composition. The experiment was reproducible. Within the studied concentration ranges, the RSD were less than 2.50% and the average recovery were between 94.10% and 99.70%. **Conclusion:** This HPLC method is simple, feasible, stable and reliable. More importantly, it can detect the content of six soybean isoflavones simultaneously.

1. Introduction

Soybean yoghurt is a well recognized healthy food due to its high content of vegetable protein and Bifidobacterium factors[1]. Because of this, the standard of soybean yoghurt and the evaluation of its health care function emphasized the viable counts of probiotic bacterium [2], and the value of active ingredients of flavonoid in soybean yoghurt has not been paid enough attention to. Isoflavones found in soybeans are nonsteroidal phytoestrogenic and antioxidative diphenolic compounds with potential roles in the prevention of chronic diseases, including hormone dependent cancer cardiovascular diseases, osteoporosis, and postmenopausal syndrome [3]. However, natural isoflavones found in soybean and ordinary soybean products generally exist in glycoside form, which is not easy to digest and absorb by human intestinal, thus limiting their biological activities. Previous studies have shown that soybean isoflavones can be transformed into free aglycones after fermentation by lactic acid bacteria, this transformation will benefit to the bioavailability of soybean isoflavones[4]. It's necessary to establish a detection method of isoflavones about soybean yoghurt, for the purpose of evaluating its health care effect and setting standards.

At present, the detection of Soybean isoflavones by HPLC mainly aimed at raw soybean or soybean meal[5], more than 20 isoflavones have been isolated and identified, and the common six are daidzin, glycitin, genistin, daidzein, glycitein, and genistein, three of them are glycosides, and the others are aglycones. Because the isoflavones in soybean are non-free and enclosed by plant cell wall, and the molecular polarity of soybean isoflavones is quite different between the the form of glycosides and aglycones, it is difficult to extract the two forms together and detect these six components simultaneously. Therefore, an integrated method for detecting isoflavones in soybean was proposed, the isoflavones were divided into two groups, and they were extracted and measured in different

conditions[6]. Nevertheless, the cell wall was ruptured in fermented soybean products, that makes the extraction of isoflavones readily. Furthermore, the hydrolysis of glycosides changes the composition of isoflavones [7], that makes it possible to detect soybean isoflavones by HPLC simultaneously . The objective of this paper is to establish an accurate, sensitive and reproducible HPLC method to detect these six isoflavones in soybean yoghurt simultaneously. The detection efficiency will improved compared with the traditional method.

2. Material and Methods

2.1. Samples

Reference substances of soybean isoflavone, Daidzein (MUST-14042616), Glycitein (MUST-14021311), Glycitin(MUST-14070311), Genistin(MUST-14060511), Genistein (MUST-14010611) and Daidzin (MUST-14031414), was purchased from Chengdu Manst Biotechnology Co., Ltd.

The other reagents are chromatographic grade acetonitrile (USA TEDIA), pure water (Watson's pure water, filtered by ultra-pure water meter before use), trifluoroacetic acid (Hangzhou Changqing Chemical Industry) and microporous filtration membrane (Qingdao Renhe Xing Experimental Technology Co., Ltd.).

The Soybean yoghurt was made in the food science laboratory of Zhejiang Chinese medical university according to the article[2]. The fermentation process comprises the following steps: 1) producing seed culture solution by Nord Dairy UHT milk Dupont Lactobacillus powder; 2) making soybean milk by soybeans obtained from a local grocerystore, and hydrolyzing the milk by pepsin;3) adding the starter to the pretreated soybean milk, then ferment to soybean yoghurt .

2.2. Instrument

The HPLC system was consisted by P230II reciprocating plunger pulsation-free pump, UV230II ultraviolet detector, EC2000 control software (Dalian Yilite Analytical Instrument Co., Ltd.) and AT-330 column oven (Aotesai Eensi Co., Ltd.). The rest were YXJ-2 high-speed centrifuge (Changzhou Jintan telecommunications and electrical appliances), Smart Direct-Q3 ultrapure water meter (Merck Millipore) and WD-9415C ultrasonic cleaner (Beijing Liuyi Instrument Factory).

2.3. HPLC Conditions

A RP-18 column (Dalian Yilite , 250 mm x 4.6 mm, 5 μm, Liaoning, China) was used for isoflavones, the isoflavones were analyzed at 255 nm at a flow rate of 1 mL/min using 70% acetonitrile + 0.05% trifluoroacetic acid (TFA) as the solvent A and HPLC grade water + 0.1 % TFA as solvent B. The column was maintained at 40℃ in a column oven. The gradient elution conditions were 10%-22% of A for 0-18min, 22%-22% of A for 18-25min, 22%-30% of A for 25-45 min, and 30%-10% of A for 45-50 min at a flow rate of 1 mL/min. The sample size was 20 uL.

2.4. Standard Curves

Stock solutions of isoflavone were prepared by weighing each reference substance precisely and dissolving them in 95% ethanol separately. The stock solutions were mixed and diluted proportionally and volumetrically to obtain a series of combined reference substance solutions, the were filtered by 0.45 um microporous membrane before injection.

Table 1. The concentration of isoflavones in combined reference substances.

Group	Daidzin	Glycitin	Genistin	Daidzein	Glycitein	Genistein
1	0.05	0.05	0.025	0.014	0.05	0.05
2	0.08	0.08	0.040	0.022	0.08	0.08
3	0.10	0.10	0.050	0.028	0.10	0.10
4	0.12	0.12	0.060	0.036	0.12	0.12
5	0.15	0.15	0.075	0.042	0.15	0.15

Firstly, the single reference stock was diluted 10 times by ethanol, and measuring by HPLC respectively, in order to obtain the appearance time of each isoflavone. Secondly, five combined reference solutions shown on Table 1 were measured by HPLC system too, and the peaks on chromatograms were identified depending on their appearance time obtained previously. Finally, six calibration curves were generated by plotting each peak area (mV•s) versus the concentration (mg•ml^{-1}) of each isoflavone in five combined reference substance solutions.

2.5. Sample Determination

The soybean yoghurt was prepared by literature method[2]. 0.5mL soybean yoghurt, 1.5mL water and 8mL ethanol were blended together and treated by ultrasonic for 10 minutes, then the mixture was centrifugated at 3500 rpm. After centrifugation the precipitate was discarded, and the solution was mingled with 2.5 ml n-hexane. The solution was separated by pipette, and the lower phase was loaded into a 25 mL measuring flask. 8 mL 80% ethanol was added into the upper phase and stood after agitation to purify it through layering. The two phases was separated again before merging the lower phase in measuring flask and metering to 25mL by 80% ethanol. This solution was filtrated by 0.45um membrane before injecting to HPLC system. The HPLC determination should be carried out for 5 times and the content of isoflavones was calculated.

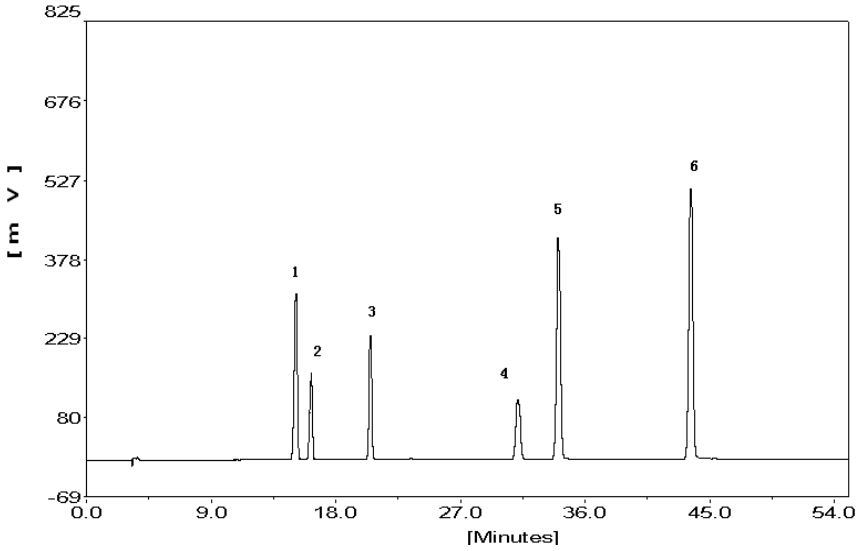
2.6. Methodological Investigation

The recovery experiment was carried out by mixing 5 mL combined reference substances of Group 4 on Table 1 (Daidzin, Glycitin, Glycitein, Genistein 0.12mg·mL-1 Genistin was 0.06 mg·mL-1, Daidzein was 0.036 mg·mL-1) with 25 ml soybean yoghurt. The same soybean yoghurt was measured for 5 times and calculated the relative standard deviation (RSD) to investigate its precision and repeatability.

3. Results and Discussion

3.1 Standard Curve

Figure 1 showed the chromatogram of the combined reference substances, six isoflavones were well separated on the HPLC condition with resolution capacity over 1.0. Table 2 showed the calibration curves of these isoflavones and their linear response ranges. The high R^2 value demonstrated the viability of the method used to detect isoflavones and the linear response for isoflavone could cover the normal levels of soybean yoghurt.



1. Daidzin 2. Glycitin 3. Genistin) 4. Daidzein 5. Glycitein 6. Genistein

Figure 1. HPLC chromatograms of combined reference substances.

Table 2. Standard curve equation of soybean isoflavones.

No.	Soybean isoflavone	Standard curve equation	R^2	Linearity range ($mg \cdot mL^{-1}$)
1	Daidzin	y = 54048x - 47.177	0.9980	0.050-0.150
2	Glycitin	y = 26271x + 41.013	0.9982	0.050-0.150
3	Genistin	y = 131808.2612x - 532.58	0.9964	0.025-0.075
4	Daidzein	y = 105915.9539x + 137.63	0.9996	0.014-0.042
5	Glycitein	y = 98969x + 62.083	0.9993	0.050-0.150
6	Genistein	y = 127467.3961x - 101.25	0.9984	0.050-0.150

3.2 Methodological Investigation

Table 3 showed the recovery rate of each isoflavone in this method, the average recovery of six soybean isoflavones was between 90 and 100%, which indicated that the extracting process of isoflavones were appropriate and accurate. Precision of each isoflavone was assessed by performing five replicate analyses and the RSDs of the standards were in the range of 1−2% (Table 4). The repetitive experiments had produced similar results, while the average coefficients of variation were less than 2.5% (Table 5). These methodological investigation demonstrated that the established extraction process and HPLC condition was accurate, specific and reproducible.

Table 3. Recovery test results.

Isoflavone	Daidzin	Glycitin	Genistin	Daidzein	Glycitein	Genistein
Average recovery	94.10%	94.73%	99.70%	95.43%	98.24%	96.23%
RSD	3.19%	3.63%	5.31%	3.76%	1.84%	2.11%

Table 4. Precision test results.

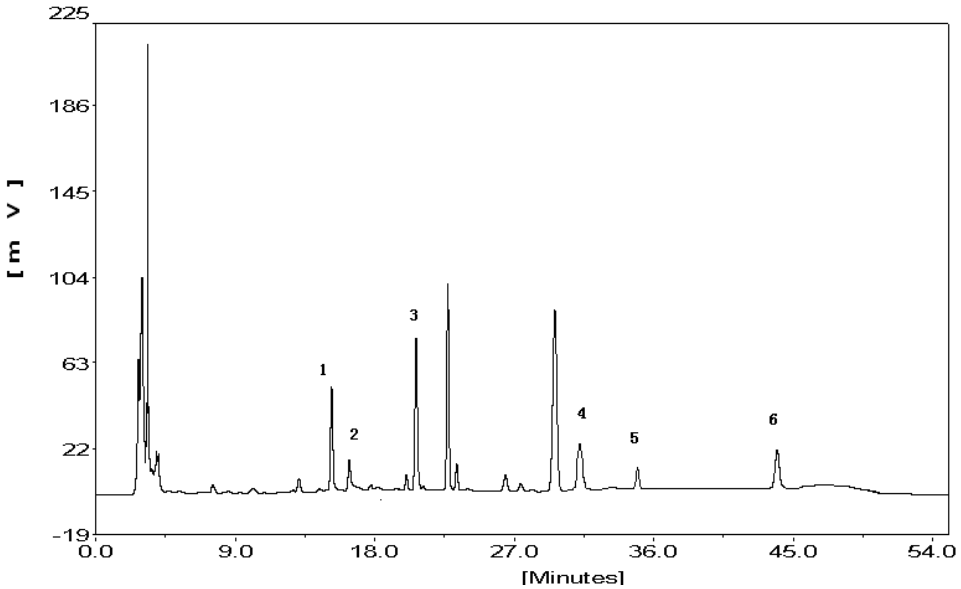
Isoflavone	Daidzin	Glycitin	Genistin	Daidzein	Glycitein	Genistein
RSD	1.90%	2.07%	2.27%	2.39%	1.75%	1.62%

Table 5. Repetitive experimental results.

Isoflavone	Daidzin	Glycitin	Genistin	Daidzein	Glycitein	Genistein
CV	1.95%	1.99%	2.11%	2.45%	1.96%	1.58%

3.3 Content of Isoflavones in Soybean Yoghurt

Under the above conditions, clean chromatograms were obtained for quantitative detection of isoflavones in soybean yoghurt, all of six main isoflavones can be determined in the HPLC chromatogram(Figure 2). Table 6 showed the actual contents of isoflavones in soybean yoghurt made in laboratory, this result illustrates the main existing forms of isoflavones were glycosides rather than aglycone, although the some glycosides had enzymatic hydrolysised to aglycone during fermentation[8].



1. Daidzin 2. Glycitin 3. Genistin 4. Daidzein 5. Glycitein 6. Genistein

Figure 2. HPLC chromatograms of isoflavones in soybean yoghurt.

Table 6. The isoflavones' content in soybean yoghurt

No.	Isoflavone	mg (in 100mL soybean yoghurt)
1	Daidzin	10.5±0.2
2	Glycitin	3.6±0.3
3	Genistin	10.4±0.2
4	Daidzein	6.0±0.1
5	Glycitein	0. 40±0.01
6	Genistein	1.5±0.1

4. Discussion

The processing of soybean yoghurt makes the extraction of isoflavones in soybean yoghurt easier than that in other non-fermented soybean products. Because the pretreatment of soybean milk has broken the cell wall of soybean and swelled its fiber, and the isoflavones become dissociative and dissolvable after 6 hours fermentation at 42°C. Besides, the lactic acid produced in fermentation is also conducive to the extraction of soybean isoflavones. However, some insoluble soybean proteins were hydrolyzed into water-soluble peptides by lactic acid bacteria during fermentation. The peptides is easy to block the chromatography column, or lead to broad peaks and tailing peaks. Therefore, trifluoroacetic acid (TFA) was added to the mobile phase to improve the peak shape and prevent irreversible adsorption of proteins and peptides on the silica-based packing materials.

According to the results, the composition of isoflavones in soybean yoghurt was different from unfermented soybean due to the glucosides was transfered into aglycones by glycosidase from lactobacillus. So the contents of daidzein, glycitein and genistein increased, while the daidzin, glycitin and genistin decreased compared with soybean raw materials.

5. Conclusion

A new method for simultaneous determination of Daidzin, Glycitin, Genistin, Daidzein, Glycitein and Genistein in soybean yoghurt by HPLC was established. Six isoflavones were measured on a C18 column (250 mm×4.6 mm, 5μm) with a gradient solvent system of acetonitrile - trifluoroacetic acid solution as mobile phase at a flow rate of 1mL•mL-1 and the column temperature was kept at 40°C. The detection wavelength was 255nm. The six soybean isoflavones components were well separated,

and the mass concentration and peak area of each component presented a good linear relationship within the range determined by this experiment, and the experimental reproducibility was good. The RSD of the six soybean isoflavones in the precision experiment was less than 2.5%, and the average sample recovery was also between 94.1% and 99.10%.

In short, this HPLC method is convenient to operate, possesses good recovery rate, precision and repeatability, and is capable for the determination of isoflavones in soybean yoghurt.

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