A PRECISE AND ROBUST METHOD FOR QUANTIFYING ISOFLAVONES

***[1]Swathi K, Rajput Jamatsingh Darbarsingh[2]**

[1,2]Department of Analytical Chemistry, Malwanchal University, Indore, Madhya Pradesh, India

DOI: 10.31838/ecb/2023.12.si6.154

ABSTRACT

High Performance Liquid Chromatography (HPLC) was used in this study to establish precise robust and Stability Indicating method. Reversed-phase HPLC (RP-HPLC) with an end-capped Waters symmetry C18 column (250 × 4.6 mm, 5µm) was employed in this work for separation. Acetonitrile and 0.1% acetic acid made up the binary mobile phase (55:45). With a flow rate of 1.0 ml/min, a run time of 25 minutes, and an injection volume of 25 µl, an isocratic program was employed. The sample cooler and column Temperatures were ambient. At 254 nm, the analytes were detected. The developed method revealed that the sensitivity of the method, accuracy, precision, robustness and stability indicating characteristic parameters satisfy the method validation requirements.

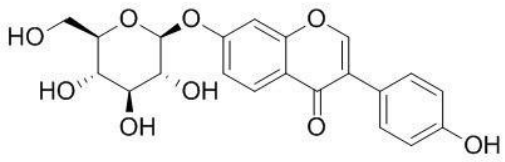
Keywords: Isoflavones, HPLC, Method development, Method Validation, Sensitivity of the method, Robustness, Precision, Accuracy and Stability Indicating Characteristics.

INTRODUCTION

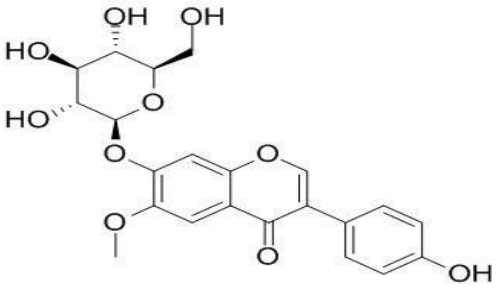
Isoflavones are plant-based compounds found almost exclusively in beans, like soybeans, that mimic the action of the hormone estrogen. As a result, soy isoflavones have been shown to reduce tumor cell proliferation and induce tumor cell apoptosis, as well as to be able to regulate hormone balance and reduce the risks of breast cancer, heart disease, and osteoporosis.

The structure of different isoflavones were presented below:

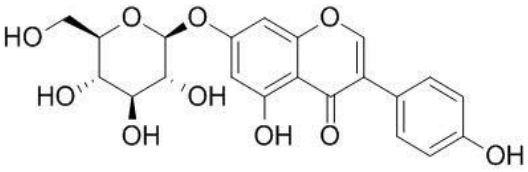
1) Daidzin: CAS No: 552-66-9



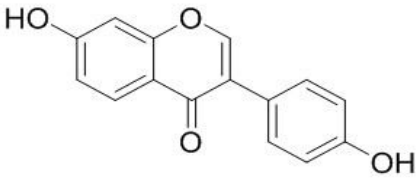
2) Glycitin: CAS No: 40246-10-4



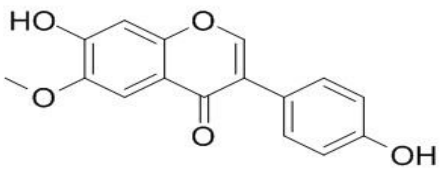
3) Genistin : CAS No: 529-59-9



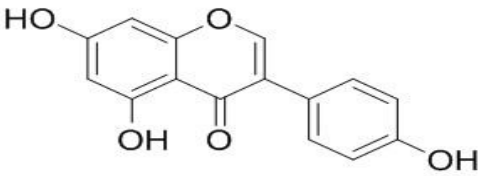
4) Daidzein: CAS No: 486-66-8



5) Glycitein: CAS No: 40957-83-3



6) Genistein: CAS No: 446-72-0



The major isoflavones in soybean are genistein and daidzein[2]. The phenylpropanoid pathway begins from the amino acid phenylalanine, and an intermediate of the pathway, naringenin, is sequentially converted into the isoflavone genistein by two legume-specific enzymes, isoflavone synthase, and a dehydratase. Similarly, another intermediate naringenin chalcone is converted to the isoflavone daidzein by sequential action of three legume-specific enzymes[3] chalcone reductase, type II chalcone isomerase, and isoflavone synthase.

Plants use isoflavones and their derivatives as phytoalexin compounds to ward off disease-causing pathogenic fungi and other microbes[4]. In addition, soybean uses isoflavones to stimulate soil-microbe rhizobium to form nitrogen-fixing root nodules[5]. Isoflavones are produced via a branch of the general phenyl propanoid pathway that produces flavonoid compounds in higher plants. Soybeans are the most common source of Isoflavones in human food.[6]

The objective of the study is to develop and validate RP-HPLC analytical method for quantifying Isoflavones. In these current study validation parameters like Sensitivity of the method, Robustness, Precision, Accuracy and Stability Indicating Characteristics were performed.

The objective of this study is to develop and validate following parameters like linearity for the determination of Isoflavones was published and reported by RP-HPLC

Method.[8]In continuation with the literature reported[9], following validation parameters like Sensitivity of the method, Robustness, Precision, Accuracy and Stability Indicating Characteristics were performed as per ICH guidelines[7,].

MATERIALS & METHOD

Materials The standard chemicals of Isoflavones were purchased from Sigma Aldrich. Soya isoflavone extract powder purchased from Herbanic India, U.P. The HPLC grade solvents, water for chromatography (LC-MS grade), methanol and acetonitrile chromatography grade were obtained from Merck (Darmstadt, Germany), acetic acid were also bought from Merck (Darmstadt, Germany).

Preparation of Standard solution

Quantitative analysis of Isoflavones standard was performed. Weighed and transfer each of

25 mg of Isofalvones standard into a 50 mL volumetric flak, added premix diluent of 80:20 (V/V) DMSO: Water diluent, sonicated for 5minutes to dissolve and dilute up to mark with diluent.

Transfer accurately 2 mL of each standard stock solution into a 25 mL volumetric flask and dilute to volume with diluent.

HPLC Instrumentation

All solutions of samples were subjected to RP-HPLC measurement using the condition as follows Waters symmetry C18 column (250 × 4.6 mm, 5µm) was used for the separation. The binary mobile phase consisted of Acetonitrile and 0.1% acetic acid (55:45). An isocratic program was used with a flow rate at 1.0 ml/min, Run time 25mins and the injection volume was 25µl.Column and Sample cooler Temp 25°C The analytes were detected by using Photo-diode 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. The procedure was validated in terms of Sensitivity of the method, Robustness, Precision, Accuracy and Stability Indicating Characteristics.

RESULTS AND DISCUSSION:

HPLC Condition Optimization

Quantitative analysis of Isoflavones 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 25 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 Isoflavones (40 µg/ml). Resolution, tailing factor and N plate were determined for different proportions of acetonitrile or methanol and the aqueous solvents. Different proportions of these solvents were evaluated.

System suitability results of standard soution[7]:

System suitability:

5. .

1. Resolution should be not less than 2.0.
2. % RSD for peak area of Isofalvones for five replicate injections of standard solution should be not more than 5.0.
3. USP Tailing should be not more than 2.0.
4. USP Plate count should be not less than 2000

Table 1: System Suitability of standard solution

Isofalvones	Retention Time	%RSD	USP Tailing	USP Plate count	USP Resolution
Daidzin	4.56	0.35	1.00	11254	NA
Glycitin	6.52	1.21	0.99	12015	8.0
Genistin	7.81	1.11	0.98	11085	4.5
Daidzein	9.79	1.85	0.98	8889	7.8
Glycitein	15.43	0.85	0.98	12411	22.3
Genistein	19.52	1.35	0.98	7659	18.6

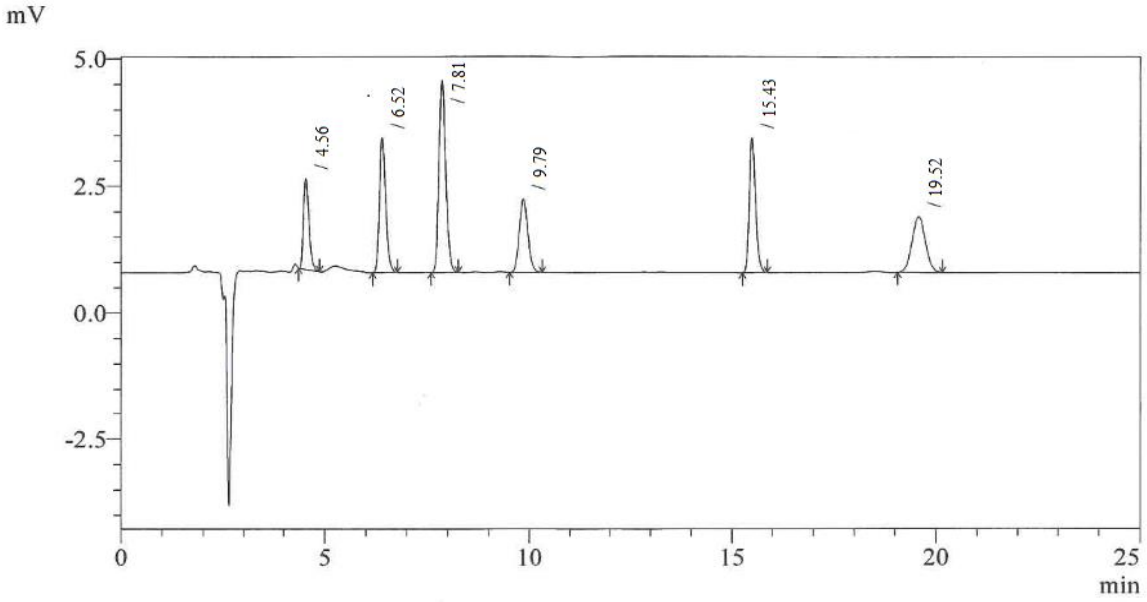


Figure: 2 System suitability of Isofalvones

The intended compounds are determined by calculating their separation or resolution (Rs) from other compounds.

Sensitivity[7]:

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. 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.

Table 2: LOD & LOQ Results

Isoflavone	LOD Areas	LOQ Areas	S/N Ratio	Resolution
Daidzin	11256	37279	12.21	NA
Glycitin	12145	35610	15.32	7.9
Genistin	11245	35822	14.25	4.3
Daidzein	10102	34819	16.21	7.6
Glycitein	12123	36111	13.25	21.9
Genistein	12952	40550	18.12	18.3

Stability of standard & test preparation[7]:

The intended compounds are determined by calculating their separation or resolution (Rs) from other compounds.

Table 3: Stability of standard & test preparation Initial

Isoflavone	Retention Time (Control)	Resolution (SST)
Daidzin	4.56	NA
Glycitin	6.52	8.0
Genistin	7.81	4.5
Daidzein	9.79	7.8
Glycitein	15.43	22.3
Genistein	19.52	18.6

Table 4: Stability of standard & test preparation Day-01

Isoflavone	Retention Time (Control)	Retention Time (Room temperature)-Day-1	RT Ratio	Retention Time (2-8 °C) Day-1	RT Ratio	Resolution (SST)
Daidzin	4.55	4.55	1.00	4.54	0.99	NA
Glycitin	6.53	6.53	1.00	6.53	1.00	7.8
Genistin	7.80	7.76	0.99	7.78	0.99	4.4
Daidzein	9.79	9.79	1.00	9.76	0.99	7.7
Glycitein	15.42	15.39	0.99	15.40	0.99	21.6
Genistein	19.51	19.48	0.99	19.45	0.99	18.1
	Acceptance criteria	RT Ratio should be between **0.98 to 1.02**				

Table 5: Stability of standard & test preparation Day-02

Isoflavone	Retention	Retention	RT Ratio	Retention Time (2-8 °C)	RT Ratio	Resolution (SST)

	Time (Control)	Time (Room temperature)-Day-2		Day-2		
Daidzin	4.55	4.57	1.00	4.53	0.99	NA
Glycitin	6.53	6.52	0.99	6.55	1.00	7.6
Genistin	7.80	7.81	1.00	7.84	1.00	4.3
Daidzein	9.79	9.80	1.00	9.83	1.00	7.5
Glycitein	15.42	15.43	1.00	15.45	1.00	20.9
Genistein	19.51	19.50	0.99	19.46	0.99	17.8
	Acceptance criteria	RT Ratio should be between **0.98 to 1.02**				

Robustness[7]:

Effect of Variation in low buffer [7]:

Design:

Analyzed system suitability preparations as per the methodology at low buffer (50%) and high buffer (55%).

Table: 6: System suitability comparisons Variation in buffer % change (Low Buffer)

Mobile Phase Composition ACN: Buffer (%)	Isoflavone	Retention Time	Resolution
50:50	Daidzin	4.75	NA
50:50	Glycitin	6.66	8.1
50:50	Genistin	7.95	4.7
50:50	Daidzein	9.66	7.9
50:50	Glycitein	15.35	22.8
50:50	Genistein	19.65	18.9

Acceptance criteria: Resolution should be NLT 2.0

Conclusion: The above results reveal that the method is robust at low buffer %.

Effect of Variation in high % buffer [7]:

Design:

Analyzed system suitability preparations as per the methodology at low buffer (50%) and high buffer (55%).

Table: 7: System suitability comparisons Variation in buffer % change (High Buffer)

Mobile Phase Composition ACN: Buffer (%)	Isoflavone	Retention Time	Resolution
45:55	Daidzin	4.95	NA
45:55	Glycitin	6.96	8.4
45:55	Genistin	8.05	4.9
45:55	Daidzein	9.92	8.2
45:55	Glycitein	15.75	23.2
45:55	Genistein	19.95	19.5

Acceptance criteria: Resolution should be NLT 2.0

Conclusion: The above results reveal that the method is robust at high buffer %.

Effect of Variation in Flow rate[7]:

Design:

Analyzed system suitability preparations as per the methodology at low column flow (0.8 mL/min) and high flow (1.2 mL/min).

Table: 8: System suitability comparison data of flow rate variation

Isoflavone	As Per method Retention Time	RT at flow (0.8 mL/min)	RT at flow (1.2 mL/min)	Resolution at flow (0.8 mL/min)	Resolution at flow (1.2 mL/min)
Daidzin	4.55	4.85	4.12	NA	NA
Glycitin	6.53	6.87	6.05	8.6	7.8
Genistin	7.80	8.12	7.16	5.2	4.2

Daidzein	9.79	9.99	9.11	8.2	7.5
Glycitein	15.42	15.68	14.98	22.5	21.9
Genistein	19.51	19.97	18.95	18.9	18.2

Acceptance criteria: Resolution should be NLT 2.0

Conclusion:

The above results reveal that the method is robust at flow between 0.8 mL/min and 1.2 mL/min.

Effect of Variation in Column Oven Temperature[7]:

Design:

Analyzed system suitability preparations as per the methodology at low column oven temperature (20°C) and high column temperature (30°C).

Table: Table: 9: System suitability comparison data of temperature variation

Isoflavone	**As Per method Retention Time**	**RT at 20°C**	**RT at 30°C**	**Resolution at 20°C**	**Resolution at 30°C**
Daidzin	4.55	4.60	4.30	NA	NA
Glycitin	6.53	6.57	6.36	7.7	8.2
Genistin	7.80	7.85	7.68	4.3	4.7
Daidzein	9.79	9.85	9.68	7.6	8.2
Glycitein	15.42	15.50	15.25	22.1	22.6
Genistein	19.51	19.65	19.15	18.3	18.9

Acceptance criteria: Resolution should be NLT 2.0

Conclusion: The above results reveal that the method is robust at column oven temperature between 20°C to 30°C variations.

Method Precision[7]:

The precision was evaluated by analysing six samples (spiked) during the same day. Precision is measured as relative standard deviation. This is done by making 6 replication samples and injecting them into the HPLC system, Method Precision was performed at LOQ (at 5% level) and 100% levels. The results are tabulated below.

Table: 10: LOQ (5%) Method precision Results

S.No.	Daidzin	Glycitin	Genistin	Daidzein	Glycitein	Genistein
01	37852	35302	34852	33142	36542	41255
02	36842	34055	35642	34143	34695	39425
03	38851	36542	37851	36551	37841	40211
04	37654	35484	35654	34587	35648	40454
05	35652	35441	35111	34781	37412	39412
06	36822	36541	35822	35712	34525	42541
Mean	37279	35561	35822	34819	36111	40550
%RSD	2.94	2.60	2.96	3.43	3.84	2.95

Table: 11: Method precision Results

S.No.	Daidzin	Glycitin	Genistin	Daidzein	Glycitein	Genistein
01	745852	754302	764802	758551	778541	815351
02	745862	745862	754822	760551	765484	845745
03	746852	751322	766802	759845	789487	825413
04	745652	754650	756654	745821	753365	841365

05	745852	754501	755614	755535	765484	815935
06	745822	754302	763302	745895	778545	832145
Mean	745982	752490	760333	754366	771818	829326
%RSD	0.06	0.46	0.69	0.90	1.66	1.54

Accuracy[7]:

The accuracy of the method was determined by application of the standard addition method. The total amount of each compound was calculated from the corresponding calibration plot and the percentage recovery of each compound was calculated. Accuracy was performed at LOQ (at 5% level), 50%, 100% and 150% levels.

Table: 12 Accuracy Results

%Accuracy	Daidzin	Glycitin	Genistin	Daidzein	Glycitein	Genistein
LOQ-01	98.51%	97.24%	96.94 %	99.15%	97.22%	98.26%
LOQ-02	97.36%	96.65%	97.36%	99.25%	98.81%	97.22%
LOQ-03	98.15%	97.11%	98.12%	98.16%	98.46%	99.35%
50%-01	98.32%	99.13%	98.65%	98.44%	98.16%	98.26%
50%-02	99.31%	98.66%	99.16%	99.10%	98.11%	98.83%
50%-03	99.15%	98.48%	99.16%	98.77%	98.77%	98.33%
100%-01	98.73%	99.10%	98.45%	98.68%	98.99%	99.13%
100%-02	98.66%	99.14%	98.33%	97.31%	98.23%	99.93%
100%-03	98.91%	98.30%	98.65%	98.46%	99.13%	98.30%
150%-01	99.23%	97.31%	98.74%	98.30%	98.43%	99.31%
150%-02	98.45%	99.01%	98.15%	98.71%	98.33%	98.11%
150%-03	98.29%	98.13%	98.33%	99.03%	98.76%	98.22%

Table: 13 STABILITY INDICATING CHARACTERSTICS

Parameter	Sample type	Acceptance criteria	Results

Forced degradation	a) Control sample b) Dry heat at 70°C at 24 hrs c) Acid stress with 0.1N HCl for 1 hr at room temperature d) Base stress with 0.1N NaOH for 30 min at room temperature e) Peroxide stress with 3% H_2O_2 for 1 hr at room temperature	System should meet the system suitability criteria.	Complies
		No interference of blank and other degradation impurities with Isoflavones in all degraded samples.	No interference of blank and other degradation impurities with Isoflavones in all degraded samples

Table: 14 Retention time Comparison Table for Interference of Blank and other degraded impurities with isoflavones

Name of Condition	Stress Condition	Interference at RT of (Yes/No)					
		Daidzin	Glycitin	Genistin	Daidzein	Glycitein	Genistein
Thermal	Stressed for about 24 Hours at 70 °C in Oven	No	No	No	No	No	No
Acid	Stressed with 0.1N HCl for 1 hr at room temperature.	No	No	No	No	No	No
Base	Stressed with 0.1N NaOH for 30 min at room temperature.	No	No	No	No	No	No
Peroxide	Stressed with 3% H_2O_2 for 1 hr at room temperature	No	No	No	No	No	No

CONCLUSION

HPLC has already found wide use in the laboratory, using a cost-effective technique, we have developed the first HPLC approach for simultaneous measurement of Isoflavones. Although the analytes have

different physicochemical properties, Developed HPLC method using reversed phase mode offered reliable and validated for the quantification of Isofalvones. The developed method proved as Accurate, Precise, stable, Robust and stability indicating for quantification of Isoflavones. The method can be used for regular and routine quality control analysis purpose.

REFERENCES

1. Gaspar, Alexandra; Matos, Maria João; Garrido, Jorge; Uriarte, Eugenio; Borges, Fernanda (2014). "Chromone: A Valid Scaffold in Medicinal Chemistry". Chemical Reviews. 114 (9): 4960–4992. doi:10.1021/cr400265z. PMID 24555663.
2. Jump up to:[a] [b] Cotrim, G. S.; Silva, D. M.; Graça, J. P.; Oliveira Junior, A.; Castro, C.; Zocolo, G. J.; Lannes, L. S.; Hoffmann-Campo, C. B. (2023). "Glycine max (L.) Merr. (Soybean) metabolome responses to potassium availability". Phytochemistry. 205: 113472. doi:10.1016/j.phytochem.2022.113472. ISSN 0031-9422. PMID 36270412. S2CID 253027906.
3. Boué, Stephen M.; Wiese, Thomas E.; Nehls, Suzanne; Burow, Matthew E.; Elliott, Steven; Carter-Wientjes, Carol H.; Shih, Betty Y.; McLachlan, John A.; Cleveland, Thomas E. (2003). "Evaluation of the Estrogenic Effects of Legume Extracts Containing Phytoestrogens". Journal of Agricultural and Food Chemistry. 51 (8): 2193–2199. Doi:10.1021/jf021114s. ISSN 0021-8561. PMID 12670155.
4. Luca SV, Macovei I, Bujor A, Trifan A (2020). "Bioactivity of dietary polyphenols: The role of metabolites". Critical Reviews in Food Science and Nutrition. 60 (4): 626–659. Doi:10.1080/10408398.2018.1546669. PMID 30614249. S2CID 58651581.
5. Kasparovska, J; Pecinkova, M; Dadakova, K; Krizova, L; Hadrova, S; Lexa, M; Lochman, J; Kasparovsky, T (2016). "Effects of Isoflavone-Enriched Feed on the Rumen Microbiota in Dairy Cows". PLOS ONE. 11 (4): e0154642. Bibcode: 2016PLoSO...1154642K. Doi:10.1

371/journal.pone.0154642. PMC 4 849651. PMID 27124615.

6. Mulsow K, Eidenschink J, MF Melzig MF. FT-IR method for the quantification of isoflavonol glycosides in nutritional supplements of soy (Glycine max (L.) Merr.). Sci Pharma 2015; 83:377-86. 15. Chang CC, Yang MH, Wen HM, Chern JC. Estimation of total flavonoid content in propolis by two complementary colometric methods. J Food Drug Anal 2002; 89:178-82. 16. Luthria DL, Biswas R, Natarajan S. Comparison of extraction solvents and techniques used for the assay of isoflavones from soybean. Food Chem 2007; 105:325-33.
7. ICH guidelines for validation of analytical procedures: text and methodology Q2 (R1) 2005.
8. Swathi K, Rajput Jamatsingh Darbarsingh Development and validation of Analytical method for quantifying Isoflavones. Eur. Chem. Bull. 2023, 12(Special Issue 1), 1431-1441.