

DEVELOPMENT AND VALIDATION OF UV-SPECTROPHOTOMETRIC METHOD FOR ESTIMATION OF DAIDZEIN IN SOY DRY EXTRACTS

Dr. Sachin Bhusari*, Akshay Lokhande and Pravin Wakte

University Department of Chemical Technology, Dr. Babasaheb Ambedkar Marathwada University, Aurangabad, Maharashtra, India.

Corresponding Author: Dr. Sachin Bhusari

University Department of Chemical Technology, Dr. Babasaheb Ambedkar Marathwada University, Aurangabad, Maharashtra, India.

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ABSTRACT

A novel, simple, precise, accurate, and cost effective UV- visible spectrophotometric method for the estimation of daidzein in soy dry extracts according to the ICH Q2 (R1) guideline. Spiked daidzein solution was scanned over the UV-visible range for its wavelength of maximum absorbance. Various calibration standards of daidzein ranging from 0.5 to 8 µg/ml were prepared. The calibration curve of concentration vs. absorbance was plotted. Various analytical method validation parameters viz. linearity, range, accuracy, precision, LOD, LOQ, and robustness, etc. were calculated. The wavelength of maximum absorbance of daidzein was found to be 248 nm. The correlation coefficient at the calibration range of 0.5-8 µg/ml was found to be 0.999. The validation of the developed UV method was carried out by conducting linearity, accuracy, precision, robustness, ruggedness, limit of detection, and limit of quantitation studies. The developed UV method was found to be precise for the intra-day and inter-day study and shows percent relative standard deviation in the range of 0.54 to 1.52 & 0.67 to 1.69 respectively. The total percent recovery of daidzein was found to be 99.57 to 100.38 %. A simple, precise, and cost-effective UV-visible spectrometry method for the estimation of daidzein in a standardized dry extract of Soybean was developed. The said method was developed using a solvent containing an economical percentage of organic phase in aqueous media. Said validated UV- visible method can be efficiently used for the estimation of daidzein in soy dry extracts.

KEYWORDS: UV- visible spectrometry, Daidzein, Validation.

INTRODUCTION

Daidzein (4', 7-dihydroxyisoflavone) whose chemical structure is shown in Figure 1 is a naturally occurring isoflavonic phytoestrogen belonging to the non-steroidal estrogens.^[1-2] Daidzin is present predominantly in the form of glucosides in various plants, including red clover (*Trifolium pratense*), alfalfa (*Medicago sativa*), soybean (*Glycine max*), and some legumes (Leguminosae).^[3-5] Among them, soybean and soybean products are considered as the most abundant source of daidzein. In humans, recent research has shown the viability of using daidzein in medicine for menopausal relief, osteoporosis, blood cholesterol, and lowering the risk of some hormone-related cancers, and heart disease.^[6-8] Considering the therapeutic importance of daidzein, some of the analytical methods were reported from all over the globe but till today, there is no single UV visible spectrophotometric method available for the estimation of daidzein in soy dry extracts using cost-effective, non-reactive, and biocompatible solvents.^[9-10] Therefore, considering the commercial importance and the needs of herbal industries, a simple yet precise and economical

UV-visible spectrophotometric method capable of estimating daidzein was developed and validated.

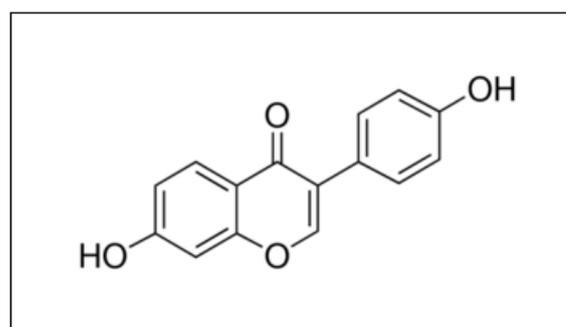


Figure 1: Chemical structure of Daidzein.

MATERIALS AND METHOD

Materials

Daidzein (purity 98% by HPLC) was purchased from TCI Chemicals (India) Pvt. Ltd. All the other chemicals of analytical grade were used for the proposed study.

Instruments Used

A double beam UV-visible spectrometer (UV-530, Jasco) with spectra manager software was used for the analysis. Quartz cells having a 3 cm length with 1 cm path length were used for spectral measurement. Weighing balance (Essae, Vibra HT) with internal calibration mode was used for the accurate weighing purpose.

Preparation of standard stock solution

Accurately weighed 5 mg of daidzein was transferred into the calibrated volumetric flask and dissolved in 5 ml mixture of Methanol and Water (50:50 v/v) to achieve a stock solution of 1000 µg/ml (Stock-I). Further diluting Stock- I solution with a co-solvent system of Methanol and water to get the desired concentration of 10 µg/ml (Stock II).

Determination of wavelength of maximum absorbance (λ_{\max})

Stock-II solution was scanned using full scan mode for the entire range of UV and visible i.e. 800 to 200 nm with a co-solvent system of Methanol and Water as a blank. After obtaining the spectrum, λ_{\max} was identified with the help of software. To achieve reproducible results, the above method was repeated five times.

Preparation of calibration curve

The calibration curve was prepared by diluting the stock-II solution to achieve the six different calibration standards representing 0.5, 1, 2, 4, 6, 8 µg/ml strength. The absorbance of each calibration standard was measured at pre-identified λ_{\max} ; 248 nm using fixed wavelength measurement mode. The calibration curve representing concentration vs. absorbance was plotted. Above mentioned procedure was repeated five times so that reproducible results can be obtained.

Method Validation

The developed UV method for the estimation of daidzein was validated as per the ICH guideline.^[11-12] Different validation parameters like linearity, accuracy, precision, robustness, ruggedness, limit of detection (LOD), and limit of quantitation (LOQ) for the proposed method were evaluated.

Linearity and Range

The linearity of the proposed UV method was established using six different calibration standards. Based on the analysis of calibration standards, calibration curves in terms of absorbance vs. concentration plots were developed and subjected to linear least square regression analysis. The correlation coefficient value was considered to be an important factor for establishing the linearity of the proposed method. The interval between the upper and lower concentration limit with acceptable linearity was reported to be the range of the proposed UV method.

Accuracy

The accuracy of the proposed UV method was evaluated using recovery studies after the standard addition of analyte of interest i.e. daidzein. Three different solutions of daidzein were prepared in triplicate at a level of 80%, 100% and 120% of its predefined concentration. To the predefined concentrations, different amounts of daidzein were added (standard addition method) and the accuracy was calculated based on percent recovery. For calculating the percent recovery following formula was used.

$$\% \text{ RC} = (\text{SPS}-\text{S}/\text{SP}) \times 100$$

Where,

SPS = Amount found in the spiked sample

S = Amount found in the sample

SP = Amount added to the sample

% RC = Percent recovery

Precision

The precision of the proposed UV method was established by performing Intra- and inter-day UV analysis of predefined samples. The study was performed at three concentration levels. The intra-day precision study was carried out by preparing fifteen different daidzein solutions of 0.5, 4, and 8 µg/ml strength (5 solutions of each concentration) and analyzing the same in the morning, afternoon, and evening time of the same day. Deviation in the results was calculated in terms of % relative standard deviation (% RSD). Similarly, the inter-day precision study was carried out by analyzing the above-mentioned solutions at three consecutive days.

Robustness

The robustness of the developed UV method was established using a different percentage of methanol in the co-solvent system. The methanol percentage in the co-solvent system was intentionally adjusted to 45 and 54 % and a middle-level quality control sample (4 µg/ml) of daidzein was prepared using the above mentioned co-solvent system separately. Samples (n=5) were analyzed at 248 nm for daidzein content. The results were calculated in terms of % RSD.

Ruggedness

Ruggedness study of the method was carried out by analyzing triplicate samples of daidzein solution (4 µg/ml) using two different instruments (V-530, Jasco and BA-UV-2600, Bioage). Results were expressed in terms of % RSD.

Limit of Detection (LOD)

The LOD of the developed UV method was calculated by using the following formula

$$\text{LOD} = 3.3 \times \text{SD}/\text{S}$$

Where, SD= Standard deviation of Y-intercepts

S= Slope

Limit of Quantitation (LOQ)

The LOQ of the developed UV method was calculated by using the following formula

$$\text{LOQ} = 10 \times \text{SD} / \text{S}$$

Where, SD= Standard deviation of Y-intercepts

S= Slope

Estimation of daidzein in soy dry extracts

Soybean (*Glycine max (L.)* seeds were dried at 50°C using a Microtray drier (S.B. Panchal and company, Mumbai, India) and powdered using a twin-blade mixer (Bajaj electrical ltd., Mumbai, India). To select uniform particle size, the powder was sifted in a sieve shaker (CIP Machineries, Ahmedabad, India) with sieves of different sizes (12, 24, 45, 85 and 120 mesh, Swastika electric and scientific works, Ambala, India) for 15 min. Powder passed through 120 mesh sieve was collected and used for further extraction.

Soxhlet assisted extraction (SAE) technique was used for the extraction of Soybean. Fifty g of powdered Soybean seeds were placed in a thimble (Borosil, Mumbai, India) which was inserted into a Soxhlet apparatus. The material was exhaustively extracted with 95% ethanol. SAE was performed for 8 h. After the predefined extraction period, the solvent was distilled off under reduced pressure using a rotary vacuum evaporator

(Heidolph Instruments GmbH & co. Germany) to obtain the dry extract.

Accurately weighed 5 mg of dry extract of Soybean seeds was transferred into the calibrated volumetric flask and dissolved using 5 ml of ethanol to achieve a stock solution of 1000 µg/ml (Stock-III). Stock- III solution was suitably diluted with a co-solvent system and analyzed for the daidzein content using the proposed UV method.

RESULTS AND DISCUSSION

Determination of wavelength of maximum absorbance

The identification of the wavelength of maximum absorbance is a prerequisite for quantitative UV analysis. Solution representing absorbance value less than 1 is generally considered to be suitable for the determination of the wavelength of maximum absorbance. Considering the prerequisite and the suitability, the determination of maximum wavelength for the daidzein solution was carried out using a full scan mode of UV-Visible spectrophotometer (figure 2). A full scan was processed using UV software and the λ_{max} was identified with the help of software. It was found to be 248 nm for daidzein.

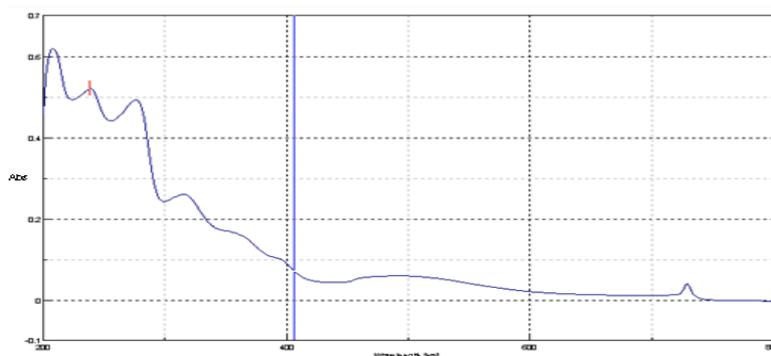


Figure 2: UV-visible spectra of daidzein.

Preparation of calibration curve

Quantification of unknown samples by UV-Visible spectrophotometer or any other instrumental method of analysis needs a reproducible calibration curve and an equation stating the correlation between concentration and the response. As compared to the graphical method, the above-stated method is widely accepted and reproducible. Considering the utility of the quantitative analysis of daidzein, the calibration curve for daidzein was developed using six different calibration standards. The absorbance of different calibration standards at 284 nm was recorded using a fixed wavelength mode of UV-Visible spectrophotometer. The calibration curve was repeated five times and the mean values \pm deviation was reported as shown in Table 1.

Table 1: Calibration standard data for daidzein.

Concentration (µg/ml)	Absorbance
0.5	0.0521 \pm 0.0015
1	0.1255 \pm 0.0027
2	0.2017 \pm 0.0029
4	0.4161 \pm 0.0037
6	0.6177 \pm 0.0012
8	0.8947 \pm 0.0035

Method validation

Linearity and Range

Linearity and range are the key parameters of an analytical method that demonstrates the limit within which the intended method is to be used for its optimum performance. Considering the prime importance of linearity and the range, a six-point calibration curve of daidzein covering a range of 0.5-8 µg/ml was plotted.

Details of concentrations and the respective mean absorbance values are depicted in Table 1. The calibration curve when subjected to least square regression analysis yielded an equation; $y =$

$0.1117x+0.044$ with a correlation coefficient 0.999 as shown in Figure 3. From the linearity study, it was revealed that the developed UV method was linear in the pre-defined concentration range of calibration standards.

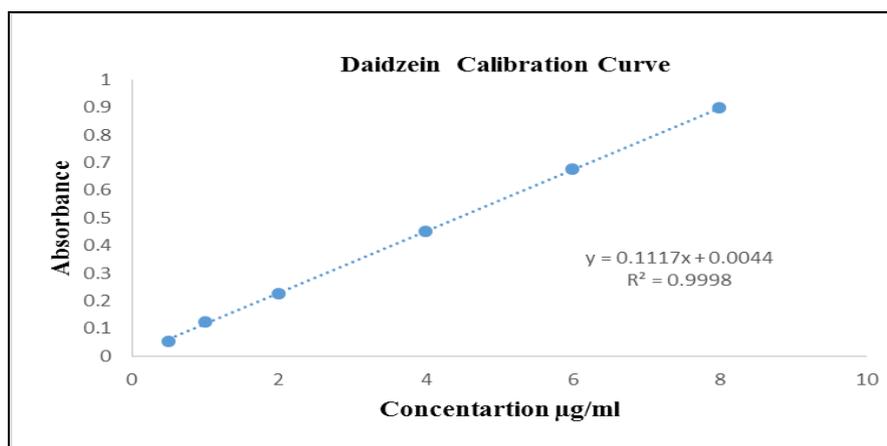


Figure 3: Calibration curve for daidzein.

Accuracy

Accuracy is a measure of the closeness of the experimental value to the actual amount of the substance in the matrix. Accuracy is to be established over the entire calibration range of the analytical method so that at any point of determination, results obtained would be reliable. In the case of the UV method for daidzein, accuracy was established using recovery studies. At 80 % standard addition, the mean recovery of daidzein was

found to be 99.57% whereas, at 100 and 120 % standard addition, it was found to be 99.78 and 100.38% respectively. % RSD was found to be less than 2 for the daidzein recovery studies as shown in Table 2. From the results of accuracy studies, it was observed that the developed UV method is highly accurate as the percent recovery was between 99.57 to 100.38%, and the % RSD was well below 2%.

Table 2: Accuracy data of the UV method for daidzein.

S No.	Concentration (%)	Origin level(µg/ml)	Amount added(µg/ml)	% Recovery	Mean % Recovery	% RSD
1	80	1	0.8	99.48	99.57	0.2223
2	80	1	0.8	99.41		
3	80	1	0.8	99.83		
4	100	4	4	99.82	99.78	0.3929
5	100	4	4	11015		
6	100	4	4	99.37		
7	120	8	9.6	101.14	100.38	0.7908
8	120	8	9.6	99.56		
9	120	8	9.6	100.43		

Precision

Precision is a measure of the degree of scatter. It expresses the reproducibility of the measurements. It is expected that an analytical method should generate reproducible outcomes. A precise analytical method leads to accurate results. Considering the importance of reproducible yet accurate results, intra- and inter-day precision of the developed UV method was established at 1, 4, and 8 µg/ml levels of daidzein. The results in terms of mean absorbance values, percent assay, and % RSD for the intra- and inter-day precision study are demonstrated in Table 3 and Table 4 respectively. % RSD values of the intra-day precision study were found to be between 0.54 and 1.52 whereas those of inter-day precision study was in between 0.67 and 1.69. Overall, %

RSD values of less than 2 showed the precision of the developed UV method.

Table 3: Intra-day precision data of UV method for daidzein.

S. NO	Concentration Range (µg/ml)	Morning			Afternoon			Evening		
		Mean	% Assay	% RSD	Mean	% Assay	% RSD	Mean	% Assay	% RSD
1	1	0.9917	99.17	1.04	1.0278	102.78	0.66	1.0218	102.18	1.44
2	4	3.9528	98.82	1.06	3.9955	99.88	1.35	3.9808	99.92	1.52
3	8	7.9939	99.92	0.66	7.9811	99.76	0.54	8.0252	100.31	0.80

Table 4: Inter-day precision data of UV method for daidzein.

S. NO	Concentration Range (µg/ml)	Day 1			Day 2			Day 3		
		Mean	% Assay	% RSD	Mean	% Assay	% RSD	Mean	% Assay	% RSD
1	1	1.01	101.38	1.05	1.01	101.65	1.69	1.01	101.47	1.50
2	4	3.97	99.40	1.31	3.99	99.92	0.90	3.99	99.94	0.86
3	8	8.00	100.00	0.67	7.97	99.66	1.60	8.01	100.17	0.72

Robustness

The robustness of the analytical method is the ability of a method to resist the change in its performance in spite of a small, deliberate change in method parameters. It is an important parameter of the analytical method as a small, unintentional change in method parameters like solvent composition, buffer strength, pH, etc. may occur during routine use and may hamper the performance of the said method. It is expected that such a change should not alter

the performance of the analytical method. Therefore, a robust analytical method is preferred. The robustness of the proposed UV method was established by modifying the composition of the co-solvent system. The change in methanol percentage (45 to 55%) in the co-solvent system did not affect the method performance. % RSD values were found to be between 0.106 and 1.31 as shown in Table 5. % RSD values below 2 depicted that the proposed UV method is robust in nature.

Table 5: Robustness data of the UV method for daidzein.

S. NO	Concentration (µg/ml)	% Methanol	Amount found	% RSD
1	4	55	0.4351	1.31
2	4	45	0.4423	1.06

Ruggedness

The ruggedness of the analytical method is the ability of a method to resist the change in its performance in spite of influential environmental factors like temperature. Rugged analytical methods are preferred as these methods are free from the impact of environmental/external factors. In order to establish the

ruggedness of the proposed UV method, the daidzein solution was analyzed using two different UV-Visible spectrophotometers of two different labs. Sample analysis and data processing resulted in % RSD values between 0.76 and 1.34. Results revealed that the proposed UV method was rugged as it showed % RSD values less than 2 as shown in Table 6.

Table 6: Ruggedness data of the UV method for daidzein.

S. No.	Concentration (µg/ml)	Instrument	Amount found	% RSD
1	4	Jasco	0.4484	0.76
2	4	Bio-age	0.4226	1.34

Limit of Quantitation (LOQ) and Limit of Detection (LOD)

LOQ represents the lowermost concentration that can be analyzed with acceptable accuracy and precision. Generally, LOQ is the first calibration standard. LOD and LOQ of the proposed UV method were found to be 3.21 and 9.72 µg/ml respectively as shown in Table 7. Lower LOQ value indicated that the proposed method would be suitable for analyzing the samples containing even small quantities of daidzein.

Table 7: LOD & LOQ data for UV method for daidzein.

LOD	3.21 µg/ml
LOQ	9.72 µg/ml

Estimation of daidzein in soy dry extracts

The developed UV method was successfully applied for the estimation of daidzein content in soy dry extracts. By the proposed UV method, daidzein content in Soxhlet extracts of Soybean (*Glycine max (L.)*) was found to be 2.5 ± 0.41 mg/g feed.

CONCLUSION

A simple, accurate, and precise UV-Visible spectrophotometric method for the estimation of daidzein in soy dry extracts was developed and validated. The proposed method was found to be robust and rugged in nature and was successfully used for the estimation of daidzein present in soy dry extracts.

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