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Development and Validation of UV-Visible Spectrophotometric Method for Estimation of Apigenin in Parsley Leaves Extract

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***Abstract*: A simple, precise and cost-effective UV- visible spectrophotometric method for the estimation of Apigenin according to the ICH Q2 (R1) guideline. The spiked Apigenin solution was scanned over UV-visible range for its wavelength of maximum absorbance. Various calibration standards of Apigenin were prepared and absorbance was recorded at wavelength of maximum absorbance. Calibration curve of concentration vs. absorbance was plotted and linearity and range was calculated. Various analytical method validation parameters viz. accuracy, precision, LOD, LOQ, robustness and ruggedness were calculated using QC standards. The maximum wavelength of Apigenin was found to be 268 nm. The correlation coefficient over the concentration range of 0.5-12 µg/mL was found to be 0.9997. Developed UV method was found to be precise during the intra-day and inter-day study and shows percent relative standard deviation in the range of 0.19 and 1.24 & 0.15 and 1.24respectively. The total percent recovery of Apigenin was found to be 99.94 and 100.10 %. Developed method was found to be robust and rugged for the intended use. Apigenin content of plant extract was successfully calculated using developed UV-Visible method. A simple, precise and cost-effective UV- visible spectrometry method for the estimation of Apigenin was developed. The said method was developed using economical percentage of organic phase in aqueous media as solvent. Said validated UV- visible method can be efficiently used for the estimation of Apigenin in bulk as well as in plant extract.**

***Keywords*: Apigenin, Validation, UV- visible spectrometry**

1. Introduction

Apigenin, is a natural product belonging to the flavone class. It is chemically known as 4′, 5, 7,-trihydroxyflavone is found in many plants. Apigenin (Fig. 1) is the aglycone of several naturally occurring glycosides [1], Apigenin is one of the predominant monomeric favonoids found in a daily diet [2]. Apigenin is synthesized in a number of plants as secondary metabolite. A variety of plants such as parsley, celery, onions, Oranges, chamomile, maize, rice, tea, wheat sprouts, some grasses etc., are known to synthesis Apigenin and its derivatives [3-4]. Flavones and some of their synthetic derivatives, have been shown four biological activities, including antioxidant, anti-inflammatory, antitumor, ant-genotoxic, antiallergic, neuroprotective, cardioprotective and antimicrobial [5-8]. Apigenin has large effects on various cancers. Interesting aspect of the effects of Apigenin on bacteria is its interactions with gut microbes [8-10]. Therefore, considering the therapeutic importance of the Apigenin and the need of simple yet precise and robust analytical methodology for the same, it was envisaged that development of UV-Visible spectrophotometric method for the determination of Apigenin in bulk and the formulation as well as in plant extract by using co-solvent system consisting economic percentage of organic solvent will be worth.

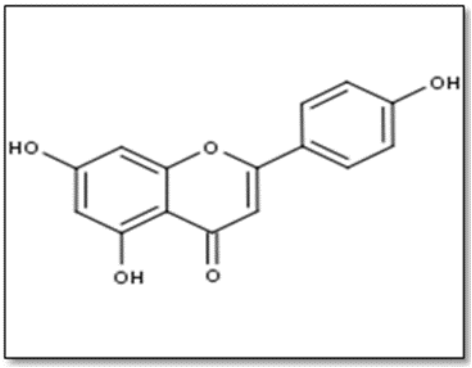

Fig. 1. Chemical structure of Apigenin

2. Materials and Methods

Apigenin (> 95 % by HPLC) was purchased from TCI Chemicals (India) Pvt. Ltd. All other chemicals of analytical grade were used for study.

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1) Instruments Used

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

2) Preparation of standard stock solution

Accurately weighed 5 mg of Apigenin 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). Stock- I solution was suitably diluted with co-solvent system of Methanol and water to achieve a solution of 100 µg/mL (Stock-II).

3) Determination of wavelength of maximum absorbance (λmax)

Stock-II solution was scanned using full scan mode with medium scanning speed for the entire range of UV and visible i.e. 800 to 200 nm with co-solvent system as a blank. After obtaining the spectrum, λmax was identified with the help of software. In order to achieve reproducible results, above method was repeated five times.

4) Preparation of calibration curve

Calibration curve was prepared by diluting the stock-II solution to achieve the seven different calibration standards representing 0.5, 1, 2, 4, 8, 10, 12 µg/mL strength. Absorbance of each calibration standard was measured at pre-identified λmax 268 nm using fixed wavelength measurement mode. The calibration curve representing concentration vs. absorbance was plotted using Excel program of Microsoft Office 2010. Above mentioned procedure was repeated five times so that reproducible results can be obtained.

5) Method Validation

Developed UV method for the estimation of Apigenin was validated as per the ICH guidelines [11-12]. Different parameters like linearity and range, accuracy, precision, robustness, ruggedness, limit of detection (LOD) and limit of quantitation (LOQ) were calculated using predefined calibration standards or quality control standards as described below.

6) Linearity and Range

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

7) Accuracy

The accuracy of the proposed UV method was evaluated using recovery studies after standard addition of analyte of interest. Three different solutions of Apigenin were prepared in triplicate at level of 80%, 100% and 120% of its predefined concentrations (0.6, 5 and 11 µg/mL). To the predefined concentrations, different amounts of Apigenin were added (standard addition method) and the accuracy was calculated on the basis of percent recovery. For calculating the percent recovery, following formula was used

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

Where,

SPS = Amount found in the spiked sample

S = Amount found in the sample

SP = Amount added to the sample

% RC = Percent recovery

8) Precision

The precision of the proposed UV method was established by performing intra- and inter-day UV analysis of quality control samples (0.6, 5 and 11 µg/mL). Apigenin solutions of 0.6, 5 and 11 µg/mL strength (n=5 for each concentration) were analyzed at morning, afternoon and evening time of three consecutive days. Deviation in the results was calculated in terms of % relative standard deviation (% RSD).

9) Robustness

Robustness of the developed UV method was established using different percentage of methanol in co-solvent system. Methanol percentage in co-solvent system was intentionally adjusted to 47 and 53 % and middle level quality control sample (5 µg/mL) of Apigenin was prepared using above mentioned co-solvent system separately. Samples (n=5) were analyzed at 268 nm for Apigenin content. The results were calculated in terms of % RSD.

10) Ruggedness

Ruggedness study of the method was carried out by analyzing triplicate samples of Apigenin solution (5 µg/mL) using two different instruments (V-530, Jasco and BA-UV-2600, Bioage). Results were expressed in terms of % RSD.

11) Limit of Detection (LOD)

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

$$LOD=3.3\times SD/S$$

Where, SD= Standard deviation of Y-intercepts

S= Slope

12) Limit of Quantitation (LOQ)

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

$$LOQ= 10\times SD/S$$

Where, SD= Standard deviation of Y-intercepts

S= Slope

13) Estimation of Apigenin from Parsley Leaves

Dried Parsely leaves were taken and powdered using twin blade mixer (Bajaj electrical ltd., Mumbai, India). To select uniform particle size, 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 a period of 10 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 Apigenin from Parsely leaves. Ten gm of powdered Parsely leaves was 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 1 h. After predefined extraction period,

solvent was distilled off under reduced pressure using rotary vaccum evaporator (Heidolph instruments GmbH & co. Germany) to obtain the dry extract. Accurately weighed 5 mg of dry extract of parsley leaves was transferred in to 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 co-solvent system and analyzed for the Apigenin content using proposed UV method.

3. Results and Discussion

A. Determination of wavelength of maximum absorbance

Identification of wavelength of maximum absorbance is prerequisite for quantitative UV analysis. Solution representing absorbance value less than 1 is generally considered to be suitable for the determination of wavelength of maximum absorbance. Considering the prerequisite and the suitability, determination of maximum wavelength for Apigenin solution (5 µg/mL) was carried out using full scan mode of UV-Visible spectrophotometer (Fig. 2). Full scan was processed using Jasco UV software and the λmax was identified with the help of software. The λmax was found to be 268 nm for Apigenin.

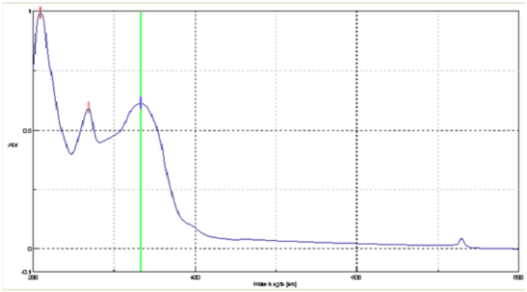

Fig. 2. UV-visible spectra of Apigenin

B. 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 a mathematical equation stating correlation between concentration and the response. As compare to graphical method, above stated method is widely accepted and reproducible in nature. Considering the utility of quantitative analysis of Apigenin, calibration curve for Apigenin was developed using seven different calibration standards. The absorbance of different calibration standards at 268 nm was recorded using fixed wavelength mode. Calibration curve was repeated three times and the mean values ± standard deviation was reported as shown in Table 1.

Table 1
Calibration standard data for Apigenin

S. no	Concentration (µg/mL)	Absorbance
1	0.5	0.0907±0.0043
2	1	0.1198±0.0021
3	2	0.2008±0.0064
4	4	0.3383±0.0018
5	10	0.6234±0.0047
6	12	0.7547±0.0058

C. Method validation

1) Linearity and Range

Linearity and range are the key parameters of 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, seven point calibration curve of Apigenin covering a range of 0.5-12 µg/mL was plotted. Details of concentrations and the respective mean absorbance values are depicted in Table 1. Calibration curve when subjected to least square regression analysis yielded an equation; y =0.0697x + 0.0572 with correlation coefficient 0.9997 as shown in Figure 3. From the linearity study, it was revealed that, developed UV method was linear over the concentration range of 0.5 to 12 µg/mL.

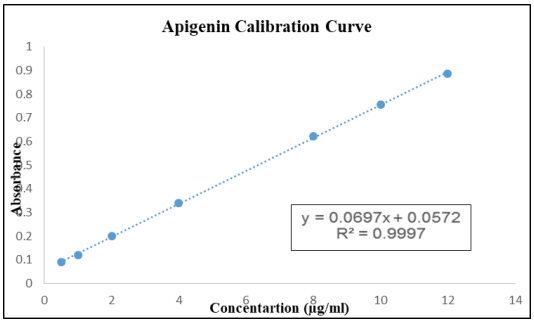

Fig. 3. Calibration curve for Apigenin

2) 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 case of UV method for Apigenin, accuracy was established using recovery studies. At 80 % standard addition, mean recovery of Apigenin was found to be 99.30% whereas at 100 and 120 % standard addition, it was found to be 99.94 and 100.10 % respectively. % RSD was found to be less than 2 for the Apigenin recovery studies as shown in Table 2. From the results of accuracy studies, it was observed that developed UV method is highly accurate as the percent recovery was in between 98 to 100% and the % RSD was well below 2%.

3) Precision

Precision is a measure of degree of scatter. It expresses the reproducibility of the measurements. It is expected that an analytical method should generate outcomes that are reproducible. Precise analytical method leads to accurate results. Considering the importance of reproducible yet accurate results, intra- and inter-day precision of developed UV method was established at 2, 12 and 24 µg/mL levels of Apigenin. 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 intra-day precision study were found to be in between 0.19 and 1.24 whereas those of inter-day precision study were in between 0.15 and 1.24. Overall, % RSD values of less than 2 demonstrated the precision of developed UV

method.

4) Robustness

proposed UV method was rugged as it showed % RSD values less than 2 as shown in Table 6.

Table 2
Accuracy data of UV method for Apigenin

S No.	Concentration (%)	Origin level (µg/mL)	Amount added (µg/mL)	% Recovery	Mean % Recovery	% RSD
1	80	0.6	0.48	99.58	99.30	0.1308
2	80	0.6	0.48	98.27		
3	80	0.6	0.48	100.14		
4	100	5	5	101.82	99.94	0.9974
5	100	5	5	98.51		
6	100	5	5	99.49		
7	120	11	13.2	101.08	100.10	0.1847
8	120	11	13.2	99.8		
9	120	11	13.2	99.44		

Table 3
Intra-day precision data of UV method for Apigenin

S No.	Conc. (µg/mL)	Morning			Afternoon			Evening		
		Mean	% Assay	%RSD	Mean	% Assay	%RSD	Mean	% Assay	%RSD
1	0.6	0.601	100.16	0.71	0.592	98.66	1.74	0.589	98.16	1.24
2	5	5.049	100.80	1.07	4.98	99.60	0.78	4.971	99.42	0.49
3	11	10.98	99.81	0.46	11.01	100.09	0.64	10.28	93.45	0.19

Table 4
Inter-day precision data of UV method for Apigenin

S No.	Conc. (µg/mL)	Day 1			Day 2			Day 3		
		Mean	% Assay	%RSD	Mean	% Assay	%RSD	Mean	% Assay	%RSD
1	0.6	0.589	98.16	1.02	0.592	98.66	1.24	0.598	99.66	0.987
2	5	4.987	99.60	0.78	5.014	100.28	1.12	4.971	99.42	0.158
3	11	10.99	99.90	0.48	11.04	100.36	0.97	10.97	99.72	0.849

Table 5
Robustness data of UV method for Apigenin

S.no	Concentration (µg/mL)	% Methanol	Conc. (µg/mL)	% RSD
1	5	48	4.98	0.4716
2	5	52	5.10	0.6938

Robustness of analytical method is the ability of a method to resist the change in its performance in spite of small, deliberate change in method parameters. It is an important parameter of analytical method as a small, un-intentional change in method parameters like solvent composition, buffer strength, pH etc. may occur during routine use and may hamper the performance of said method. It is expected that such change should not alter the performance of the analytical method. Therefore, robust analytical method is preferred. Robustness of proposed UV method was established by modifying the composition of co-solvent system. Change in methanol percentage (48 to 55 %) in co-solvent system did not affect the method performance. % RSD values were found to be in between 0.47 and 0.69 as shown in Table 5. % RSD values below 2 depicted that proposed UV method is robust in nature.

5) Ruggedness

Ruggedness of 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 impact of environmental/external factors. In order to establish the ruggedness of proposed UV method, Apigenin solution was analyzed using two different UV-Visible spectrophotometers of two different labs. Sample analysis and data processing resulted into % RSD values between 0.65 and 0.78. Results revealed that

Table 6
Ruggedness data of UV method for Apigenin

S.no.	Concentration (µg/mL)	Instrument	Conc. (µg/mL)	% RSD
1	5	Jasco	5.0118	0.7894
2	5	Bio-age	4.9978	0.6597

6) 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 proposed UV method was found to be 0.9639 and 2.92µg/mL respectively as shown in Table 7. Lower LOQ value indicated that proposed method would be sensitive enough to quantify the Apigenin content of samples at its lower level.

Table 7
LOD & LOQ data for UV method for Apigenin

1	LOD	0.9639 µg/mL
2	LOQ	2.92 µg/mL

D. Estimation of Apigenin content in marketed formulation

Developed UV method was successfully applied for estimation of Apigenin content in dried parsley leaves extract. By proposed UV method, Apigenin content in Soxhlet extract of dried parsley leaves was found to be 41.49 ± 0.51 mg/g feed.

4. Conclusion

A simple, accurate and precise UV-Visible spectrophotometric method for the estimation of Apigenin in dried parsley leaves extract was developed and validated. Proposed method was found to be robust and rugged in nature and was successfully used for the estimation of Apigenin present in dried parsley leaves extract.

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