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Research Article

Development and Validation of an Economical UV-Visible Spectrophotometric Method for the Estimation of Naringenin in Marketed Formulation

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ABSTRACT

A simple yet economical UV-Visible Spectrophotometric method was developed and validated for the quantification of Naringenin in a marketed formulation. UV analysis was performed in a methanol–water (25:75 v/v) co-solvent system, with absorbance measured at the wavelength of maximum absorbance (λmax) of 290 nm. The method was validated according to ICH Q2 (R1) guidelines. The said method was found to be linear over 1 to 17 µg/mL, with a coefficient of determination (r^2) greater than 0.999. The intra-day accuracy of the proposed method was found to be in the range of -0.57 to +0.61%, while the inter-day accuracy was found to be in the range of -1.31 to +1.99%. Precision of the proposed UV-Visible Spectrophotometric method was found to be satisfactory with % RSD of 0.79 to 1.73 (intra-day) and 0.28 to 1.12 (inter-day). Deliberate variation of the solvent composition (methanol: water 30:70 and 20:80 v/v) resulted into % RSD values below 0.6%, indicating the robustness of the method. The LOD and LOQ were found to be 0.352 and 1.067 µg/mL, respectively. The validated method was successfully applied for the estimation of Naringenin in a marketed syrup formulation (label claim: 1.2 mg/5 mL), and the percent assay was found to be 96.69 ± 1.69% (n=3) of the label claim, which is in close agreement with the labelled amount. These findings indicate that the developed analytical method is accurate, precise, and suitable for the routine quality control analysis of Naringenin in pharmaceutical formulations.

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INTRODUCTION:

Naringenin (4′,5,7-trihydroxyflavanone; molecular formula C15H12O5; molar mass 272.25 g/mol) is a flavanone-type flavonoid that occurs abundantly in citrus fruits such as grapefruit, sweet orange and pomelo, and is also reported in tomatoes and several aromatic herbs [1, 2]. In the plant, Naringenin is found mostly in its glycosidic form, chiefly as naringin (Naringenin-7-O-neohesperidoside), the compound responsible for the bitter taste of grapefruit. The free aglycone is liberated from these glycosides by enzymatic or acid hydrolysis [3, 4].

A wide range of biological activities has been attributed to Naringenin. Experimental and clinical reports depict the antioxidant, anti-inflammatory, lipid-lowering, antidiabetic, cardioprotective and anticancer effects of Naringenin [1, 5, 6, 7, 8]. Because of such properties, Naringenin and Naringenin-rich citrus extracts are increasingly incorporated into nutraceutical and herbal products, and interest in their pharmaceutical development continues to grow [2, 9].

Despite the various beneficial pharmacological actions of Naringenin, there are many challenges associated with Naringenin. It is poorly soluble in water, shows limited oral bioavailability and undergoes extensive conjugation after absorption [9, 10, 11]. Complexation with cyclodextrins and encapsulation in nanocarriers have therefore been explored to improve its delivery [12, 13]. Each of these developments relies on dependable analytical procedures to quantify Naringenin in raw materials, finished formulations and plant extracts, both for standardisation and for routine quality control.

A newly developed analytical procedure must be demonstrated to be fit for its intended purpose before it is used for routine work. The International Council for Harmonisation (ICH) provides the accepted framework for demonstration through its guidelines on the validation of analytical procedures [17, 18, 19]. The aim of the present work was to develop a simple, precise and economical UV-Visible Spectrophotometric method for Naringenin using a co-solvent system with a low proportion of organic solvent, to validate it for linearity, range, accuracy, precision, robustness, LOD and LOQ as per ICH Q2 (R1), and to apply it to the estimation of Naringenin in marketed formulation.

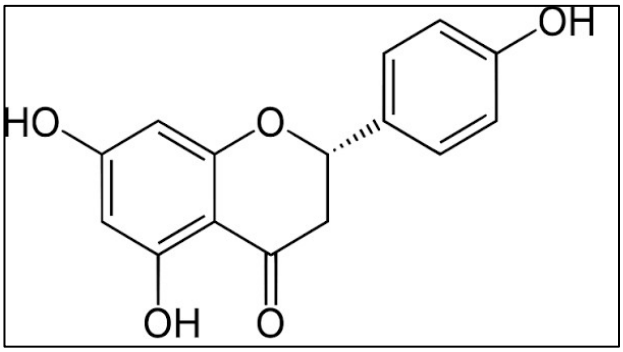


Figure 1: Chemical Structure of Naringenin

MATERIALS AND METHODS:

2.1 Instrumentation:

A pre-calibrated double-beam UV-Visible Spectrophotometer (Model V-530, Jasco) with Spectra Manager Software was utilized in developing the UV-Visible Spectrophotometric method. Quartz cuvettes having height 3 cm and optical path 1 cm were used for the analysis. The analytical balance (Essae Vibra HT) was used for the weighing purpose which featured internal calibration system. An ultrasonic bath (PCI Analytics, India;6.5 L capacity) was used for complete solubilization.

MATERIALS:

Naringenin was purchased from TCI Chemicals (India) Pvt. Ltd., Chennai. Methanol was purchased from Merck. HPLC-grade (Type 1) water was obtained from water purification system (Lablink Xtrapure). For method development all chemicals used were of at least analytical grade.

2.3 Preparation of Standard and Stock Solutions:

Accurately weighed 100 mg of Naringenin was transferred into a 100 ml volumetric flask and dissolved in 100 ml methanol to prepare the mother stock (1000 µg/mL). Further dilutions were prepared using co-solvent system comprising of methanol and water in a 25:75 (v/v) ratio to achieve working (stock I) solution having strength 100 µg/mL and was suitably diluted to achieve stock II solution of strength 10 µg/mL.

2.4 Determination of Wavelength of maximum absorbance (λmax):

The maximum absorbance wavelength (λmax) of Naringenin was determined using UV-Visible Spectrophotometer. The UV-Visible Spectrophotometer was set in the spectrum mode and the baseline correction was performed by setting the instrument to autozero using the co-solvent as a blank. A working stock II solution of 10 µg/mL was scanned over the wavelength of 200 to 800 nm. The scanning was three times repeated using freshly prepared stock II solutions to achieve reproducible results.

2.5 Preparation of Calibration Curve:

Freshly prepared stock I solution of Naringenin was diluted with co-solvent to achieve five calibration standards viz., CAL-STD-1 (1 µg/mL), CAL-STD-2 (5 µg/mL), CAL-STD-3 (9 µg/mL), CAL-STD-4 (13 µg/mL), CAL-STD-5 (17 µg/mL) respectively. The UV-Visible Spectrophotometer was set in fixed wavelength measurement mode, and the absorbance of each CAL-STD was measured at the pre-identified absorbance maxima of 290 nm. Three times the procedure was repeated to ensure the reproducibility of the results. The results were expressed in terms of Mean ± SD.

2.6 Method Validation:

The developed UV-Visible Spectrophotometric method for Naringenin was validated using ICH Q2 (R1) Guidelines. The proposed method was assessed for its linearity, accuracy, precision, robustness, limit of detection (LOD), limit of quantification (LOQ).

2.6.1 Linearity and Range:

The linearity of the proposed UV-Visible Spectrophotometric method for Naringenin was evaluated using pre-established five calibration standards, as mentioned above. By using calibration curves of absorbance vs. concentration the linear least-squares regression analysis was carried out. The linearity of the method was confirmed based on the correlation coefficient (r^2) value. The range of the UV-Visible Spectrophotometric method was stated to be in between upper and lower concentration limits with satisfactory linearity.

2.6.2 Accuracy:

The accuracy of the UV-Visible Spectrophotometric method for Naringenin was evaluated using three quality control standards (QC-STD), such as QC-STD-1 (2 µg/mL), QC-STD-2 (8 µg/mL), QC-STD-3 (16 µg/mL) respectively, were prepared in triplicate and used for

development of the said method. The QC-STD were analysed for its Naringenin content using proposed UV-Visible Spectrophotometric methods in three different times in a day. The intra-day and inter-day accuracy of the proposed method was established in terms of % difference obtained by using following formula,

$$\%\ Difference = \frac{Mean\ measured\ concentration - Nominal\ concentration}{Nominal\ concentration} \times 100$$

2.6.3 Precision:

The QC-STDs (2 µg/mL, 8 µg/mL, 16 µg/mL) were used to determine precision of the said method in terms of % Relative Standard Deviation (RSD). The intra-day and inter-day precision was established by analysing the QC-STDs at three different time intervals in a day and the same process was repeated on three consecutive days. The precision of the method was established in terms of % RSD, which was calculated by using following formula,

2.6.4 Robustness:

$$\%\ RSD = \frac{Standard\ Deviation(SD)}{Mean} \times 100$$

The robustness of the proposed UV-Visible Spectrophotometric method for Naringenin was assessed by introducing a deliberate change of two different co-solvent mixtures (methanol: water in ratio of 30:70 v/v and 20:80 v/v) to check whether such variation affected results. The three QC-STDs were analysed with different solvent ratios and absorbance values were used to calculate the mean standard deviation (SD), and percent relative standard deviation (%RSD). The robustness of the method was confirmed by deliberate minor variations in analytical procedure, with all calculated% RSD values remaining within the acceptable limit of ≤ 2%.

2.6.5 Limit of Detection (LOD):

The LOD of the developed UV-Visible Spectrophotometric method was calculated using the following formula,

$$LOD = \frac{3.3 \times SD}{S}$$

Where, SD = Standard Deviation of Y-intercepts

S = Slope of the calibration curve

2.6.6 Limit of Quantification (LOQ):

The LOQ of the developed UV-Visible Spectrophotometric method was calculated using the following formula,

$$LOQ = \frac{10 \times SD}{S}$$

Where, SD = Standard Deviation of Y-intercepts

S = Slope of the calibration curve

2.7 Estimation of Naringenin in Marketed Formulation:

The marketed Naringenin syrup was labelled to contain 1.2 mg of Naringenin per 5 mL (0.24 mg/mL), which corresponds to 48 mg in the 200 mL pack. A volume of syrup equivalent to 10 mg of Naringenin (41.7 mL) was accurately measured, transferred to a 100 mL volumetric flask, and dissolved in about 50 mL of methanol. The mixture was ultrasonicated for 15 min to ensure complete extraction, and the volume was made up to 100 mL with methanol to give a solution of 0.1 mg/mL (100 µg/mL). The obtained solution was filtered through a 0.22 µm (25 mm) syringe filter to remove insoluble excipients. A 650 µL of filtered solution mixed with 4350 µL of the co-solvent system so as to achieve the solution of 13 µg/mL strength (theoretical concentration). Said solution was subjected to analysis by using proposed UV-Visible Spectrophotometric method. The estimation was carried out in triplicate. The Naringenin content was calculated from the regression equation of the calibration curve and reported as a percent assay.

RESULTS AND DISCUSSION

In pharmaceutical analysis, chromatography and the spectroscopy are the widely used techniques for the qualitative as well as quantitative analysis. In chromatography, the HPLC and GC are the preferred tools for the quantitative analysis but both the tools are costly and involves comparatively longer duration of analysis. High-performance liquid chromatography (HPLC) is the technique most frequently used to determine Naringenin and related citrus flavonoids [4, 14]. However, chromatographic analysis requires expensive instrumentation, large volumes of high-purity solvents, and trained operators, which may limit its utilization in all

quality-control or academic laboratories. UV-Visible Spectrophotometry offers a practical alternative as it is inexpensive, widely available and easy to validate.

The ultraviolet spectra of flavonoids display two principal absorption regions, Band I (usually above 300 nm), associated with the cinnamoyl system of the B-ring, and Band II (usually 240–295 nm), associated with the benzoyl system of the A-ring. In flavanones such as Naringenin, the C-ring is saturated at the 2,3-position, so conjugation between the B-ring and the carbonyl group is interrupted; as a result, Band II is intense whereas Band I appears only as a weak shoulder [15, 16]. This characteristic spectrum makes direct spectrophotometric quantification feasible. Overall, when exposed to UV-Visible light, the chemical structure of the Naringenin results into good UV absorption which makes it easy to develop the UV-Visible Spectrophotometric method for the Naringenin.

3.1 Determination of the wavelength of maximum absorbance (λ max):

To achieve accurate quantitative estimation of Naringenin by UV-Visible Spectrophotometry, it is essential to identify the λmax as it directly influences assay sensitivity & improves reliability of results. A solution containing Naringenin (10 µg/mL) was repeatedly scanned over the wavelength range of 200 to 800 nm using the spectrum measurement mode. The recorded spectrum was analysed using the instrument's software, the maximum absorption peak was observed at

290 nm, which was subsequently selected as the analytical wavelength for all further measurements.

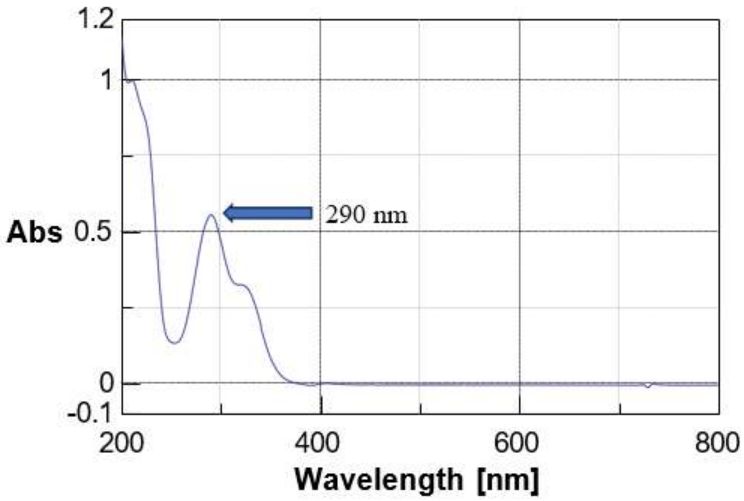


Figure 2: UV-Visible Spectrum of Naringenin

3.2 Development of a calibration curve:

Before development of calibration curve, the co-solvent system was optimized for the preparation of Naringenin solutions. In order to make the method economical as well as sensitive, the percentage of methanol in the co-solvent system was adjusted to 25% which resulted into greener UV-Visible spectrophotometric method development. The optimised co-solvent system comprising 25% methanol and 75 % water was utilized further for all the solution preparations.

For the quantitative estimation of unknown samples using the UV-Visible Spectrophotometric method, a mathematical relationship between concentration and absorbance was established through the calibration curve. A calibration curve was constructed using five standard solutions at concentrations of 1, 5, 9, 13 and 17 µg/mL, designated as CAL-STD-1, CAL-STD-2, CAL-STD-3, CAL-STD-4, and CAL-STD-5 respectively. The absorbance of each standard solution was measured in triplicate at 290 nm using the fixed-wavelength mode of the UV-Visible Spectrophotometer. The mean absorbance and corresponding standard deviation were calculated for each concentration and the calibration curve was plotted for the same.

Table 1: Calibration Standard Data for Naringenin

Naringenin Concentration (µg/mL)	Absorbance (Mean ± S.D)
1	0.0533 ± 0.0006
5	0.2627 ± 0.0003
9	0.4859 ± 0.0071
13	0.6935 ± 0.0071
17	0.9332 ± 0.0130

(n=3)

3.3 Method Validation:

Once an analytical method has been developed, it must be validated to confirm that it is reliable and suitable for its intended purpose. Method validation ensures the quality, reproducibility, and dependability of the results obtained. In both academic and industrial settings, this process commonly follows the ICH Q2 (R1) guideline, which is the internationally accepted standard for the validation of analytical methods. Each validation parameter is assessed against a specific acceptance criterion. A method is regarded as reliable and scientifically acceptable

only when all parameters meet their respective criteria. In this study, the following parameters were evaluated to validate the developed method for Naringenin.

3.3.1 Linearity and Range:

The linearity of the developed method was evaluated using five calibration standards of Naringenin over a concentration range of 1 to 17 µg/mL. Regression analysis of the calibration data showed a strong linear relationship between absorbance and concentration. The mean absorbance values for the calibration standards are presented in Table 1. All three calibration curves demonstrated excellent linearity, with coefficients of determination (r^2) greater than 0.999. The minimal variability observed in the slopes and intercepts indicates that the linear response is suitable for the intended application, as shown in Figure 3 (A–C). Based on this assessment, the developed UV-Visible Spectrophotometric method was found to be linear over the stated concentration range.

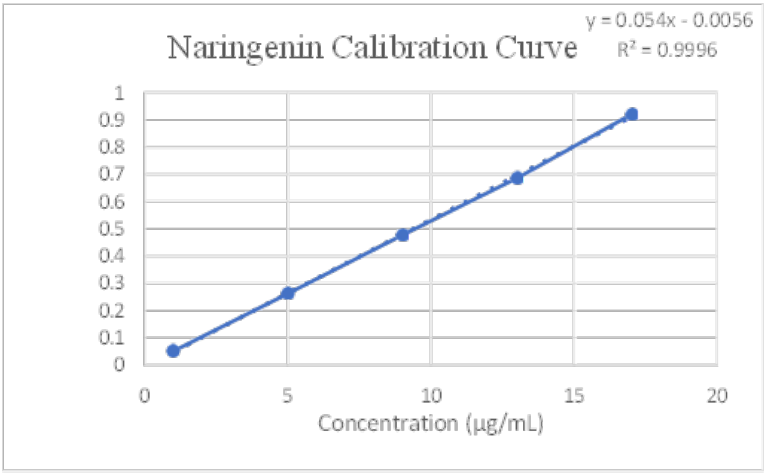


Figure 3: (A): Calibration Curve of Naringenin (Replicate 1)

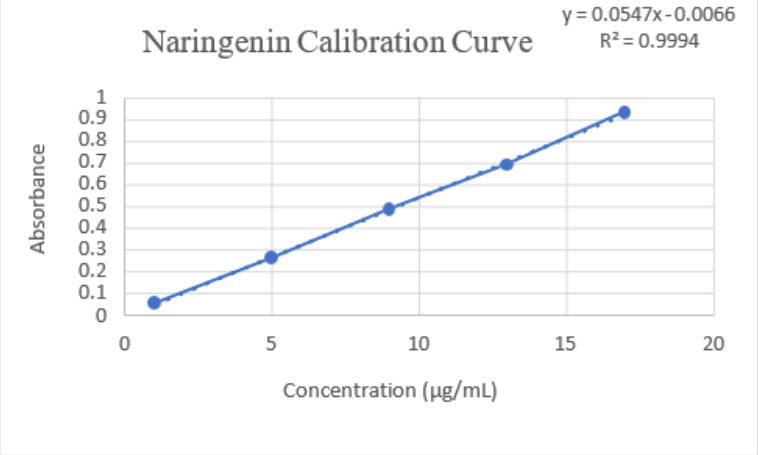


Figure 3: (B): Calibration Curve of Naringenin (Replicate 2)

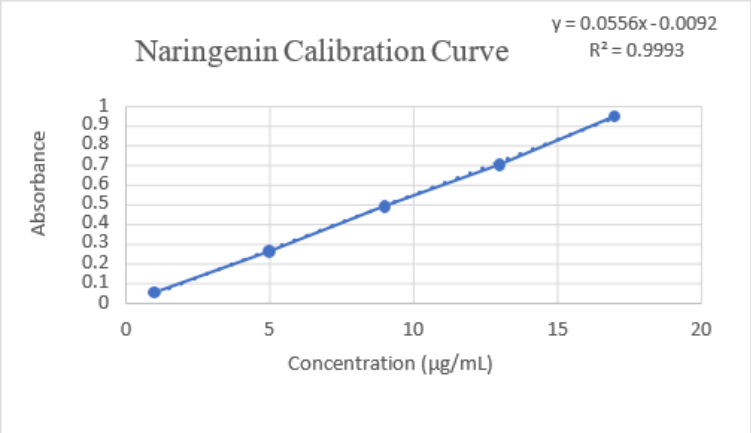


Figure 3: (C): Calibration Curve of Naringenin (Replicate 3)

3.3.2 Accuracy:

Accuracy refers to how closely the experimental value agrees with the nominal (true) reference value. To obtain reliable results, accuracy must be maintained across the entire calibration range of the method. The intra-day and inter-day % difference values are presented in Table No. 2 and 3 respectively. The intra-day accuracy of the proposed method was found to be in the range of -0.35 to +0.61% while the inter-day accuracy was found to be in the range of -1.31 to +1.99%. These values fall within the acceptable limits (≤ 2), indicating that the proposed UV-Visible Spectrophotometric method for Naringenin was accurate.

Table 2: Intra-day Accuracy Data of the UV-Visible Method for Naringenin

Naringenin Concentration Level	Nominal Concentration (µg/mL)	Mean Measured Concentration (µg/mL)	% Difference
LQC	2	2.0006	+0.0308
	2	2.0122	+0.6115
	2	2.0033	+0.166
MQC	8	7.9714	-0.3573
	8	8.01389	+0.1737
	8	8.01497	+0.1871
HQC	16	15.9926	-0.0462
	16	16.0457	+0.2856
	16	16.0063	+0.0393

Table 3: Inter-day Accuracy Data of the UV-Visible Method for Naringenin

Naringenin Concentration Level	Nominal Concentration (µg/mL)	Mean Measured Concentration (µg/mL)	% Difference
LQC	2	1.9737	-1.3135
	2	2.0398	+1.9934
	2	2.0025	+0.1280
MQC	8	8.0535	+0.6696
	8	8.0343	+0.4296
	8	7.9123	-1.0958
HQC	16	16.2720	+1.7003
	16	15.9798	-0.1261
	16	15.7927	-1.2953

3.3.3 Precision:

Precision describes how closely repeated measurements agree with one another, and it reflects the consistency of the results obtained. A reliable analytical method should produce reproducible outcomes. For the developed UV-Visible Spectroscopic method, intra-day and inter-day precision were evaluated at three concentration levels: 2, 8, and 16 µg/mL. The corresponding % RSD values are presented in Table 4 and 5. The intra-day precision of the proposed method was found to be in the range of 0.79 to 1.75 whereas the inter-day precision was found to be in the range of 0.2876 to 1.1261. The intra-day and inter-day % RSD values within acceptable limits (≤ 2) indicates that the proposed UV-Visible Spectrophotometric method is precise and suitable for the routine quantification of Naringenin.

Table 4: Intra-day Precision Data of the UV-Visible Method for Naringenin

	Morning			Afternoon			Evening		
Naringenin Conc. Range (µg/mL)	Mean	SD	% RSD	Mean	SD	% RSD	Mean	SD	% RSD
2	2.0006	0.0351	1.7576	2.0122	0.0348	1.7326	2.0033	0.0269	1.3458
8	7.9714	0.1029	1.2913	8.0138	0.0638	0.7962	8.0149	0.0804	1.0034
16	15.9926	0.1875	1.1724	16.0456	0.2476	1.5436	16.0063	0.2458	1.5356

Table 5: Inter-day Precision Data of the UV- Visible Method for Naringenin

	Day 1			Day 2			Day 3		
Naringenin Conc. Range (µg/mL)	Mean	SD	% RSD	Mean	SD	% RSD	Mean	SD	% RSD
2	1.9737	0.0139	0.7049	2.0398	0.0092	0.4549	2.0025	0.0225	1.1261
8	8.0535	0.0595	0.7399	8.0343	0.0521	0.6486	7.9123	0.0552	0.6984
16	16.2720	0.1453	0.8934	15.9798	0.0459	0.2876	15.7927	0.0639	0.4050

3.3.4 Robustness:

The robustness of the proposed method for Naringenin was evaluated by making deliberate changes in concentration of co-solvent system (methanol: water in ratio of 30:70 v/v and 20:80 v/v). All calculated % RSD values within the acceptable limit of ≤ 2 as shown in table 6. The results demonstrated that the proposed analytical method of Naringenin is robust and remains unaffected under deliberate variations.

Table 6: Robustness Data of the UV-Visible Method for Naringenin

Naringenin Concentration (µg/mL)	Methanol: Water (v/v)	Absorbance (Mean ± S.D.)	%RSD
2	30:70	0.1018 ± 0.0004	0.4428
8		0.3991 ± 0.0004	0.1012
16		0.7925 ± 0.0003	0.0378
2	20:80	0.1019 ± 0.0005	0.5403
8		0.3996 ± 0.0006	0.1562
16		0.7881 ± 0.0029	0.3783

3.3.5 Limit of Quantification (LOQ) and Limit of Detection (LOD):

The limit of quantification (LOQ) represents the lowest concentration of an analyte that can be measured with acceptable precision & accuracy. In the present study, the proposed UV-Visible Spectrophotometric method showed an LOD 0.352 µg/mL & LOQ of 1.067 µg/mL as summarized in Table 7. The lower values of LOD and LOQ demonstrates that the proposed UV-Visible Spectrophotometric method for the estimation of Naringenin is sensitive.

Table 7: LOD and LOQ Data of the UV-Visible Method for Naringenin

Parameter	Values
LOD	0.352 µg/mL
LOQ	1.067 µg/mL

3.4 Estimation of Naringenin in the marketed formulation:

The proposed UV-Visible Spectrophotometric method was developed and validated for routine analysis. Before it is adopted for routine use, its suitability for analysing real samples must be established. In order to do so, the quantification of Naringenin in a commercially available syrup formulation was performed using the proposed UV-Visible Spectrophotometric method. The Naringenin content was calculated from the regression equation of the calibration curve, and the % assay was determined as a percentage of the label claim. The % assay values obtained from the triplicate determinations are presented in Table 8 as shown below. The results were in close agreement with the labelled amount, indicating that the method estimates the Naringenin

content of the formulation accurately and precisely. These findings showed that the method is suitable for routine quality control analysis of Naringenin in pharmaceutical formulations.

Table 8: Naringenin Content in the Marketed Formulation

Sr. No	Formulation	Brand Name	Label claim of Naringenin	% Assay of Naringenin (Mean ± S.D.)
1	Syrup	Zincovit CL	1.2 mg/5 ml	96.69 ± 1.69

(n=3)

CONCLUSION:

A simple, rapid, precise, accurate and economical UV-Visible Spectrophotometric method has been developed and validated for the quantitative estimation of Naringenin as per ICH Q2 (R1). The method was linear over the range of 1 to 17 µg/mL, and its accuracy, precision, robustness, LOD and LOQ were found to be within the acceptable limits. The use of a methanol: water co-solvent with a low proportion of organic solvent makes the method inexpensive and environmentally considerate. The method was successfully applied for the determination of Naringenin in marketed formulation and can be adopted for routine quality-control analysis of Naringenin in formulations.

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