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# DEVELOPMENT AND VALIDATION OF UV-SPECTROPHOTOMETRIC METHOD FOR ESTIMATION OF TANGERETIN IN *ORANGE PEEL* POWDER

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## ABSTRACT

**Aim:** To develop and validate a simple, precise and cost effective UV-visible spectrophotometric method for the estimation of tangeretin in *Orange Peel* Powder extracts. All the parameters of the analysis were chosen according to ICH Q2 (R1) guideline. **Methods:** Tangeretin solution was scanned over UV-visible range for its wavelength of maximum absorbance. Various calibration standards of tangeretin were prepared. Calibration curve of concentration vs. absorbance was plotted. Various analytical method validation parameters were calculated. **Results:** The maximum wavelength of tangeretin was found to be 327 nm. The correlation coefficient at concentration range of 0.5-10 µg/ml was found to be 0.9999. The validation study of the developed UV method was carried out by conducting linearity, accuracy, precision, robustness, ruggedness, limit of detection and limit of quantitation studies. Developed UV method was found to be precise for the intra and inter day studies and showed standard deviation in the range of 0.12 to 1.56 & 0.35 to 1.71 respectively. The total percent recovery of tangeretin was found to be 98.29 to 100.02%. **Conclusion:** A simple, precise and cost-effective UV- visible spectrometry method was developed for the estimation of tangeretin in standardized extract of Powder of *Orange Peel*. The said method was developed using solvent containing economical percentage of organic phase in aqueous media. Said validated UV- visible method can be efficiently used for the estimation of tangeretin in extracts of *Orange Peel* Powder.

**KEYWORDS:** UV- Visible Spectrometric Method, Tangeretin, *Orange Peel*, Validation.

## INTRODUCTION

*Orange peel* commonly known as *Citrus vulgaris, Citrus bigaradia, Citrus aurantium amara, Biga-rade orange, Bitter orange, Seville orange*. The orange peel is the fresh or dried outer part of the pericarp of *Citrus aurantium* Linn, belonging to family Rutaceae.[1-2] *Orange Peel* powder consists of variety of chemicals with wide range of activities. Among all the chemicals in Orange Peel Powder, tangeretin (Fig.1) has gained the attention of researchers working in natural products .[3-5] Tangeretin is a O-Polymethoxylated flavone that is found in Orange peels with chemical formula $C_{20}H_{20}O_7$. The biological activity of tangeretin is readily absorbed in tissues and that it has many beneficial properties such lowering of cholesterol, anti-tumor activity, and neuroprotective action and anti-cancer activity.[6-8] It induces apoptosis in leukemia cells without being toxic to normal cells. Tangeretin stops the growth of cancer cell in the G1 phase.[9-10] It is soluble in organic solvent like methanol and ethyl acetate, Insoluble in water. Till date, there are reports no economical UV-visible spectrophotometric method for estimation of tangeretin in extracts of *Orange Peel* Powder. The said method was developed using solvent containing economical percentage of organic phase in aqueous media. Even, a precise economical UV- visible spectrophotometric method capable of estimating tangeretin in variety of dosage forms like powder and solutions and standardized extract is also unavailable. Therefore, considering the commercial importance and the needs of herbal industries, a simple yet precise and economical UV-visible spectrophotometric method capable of estimating tangeretin was developed and validated.

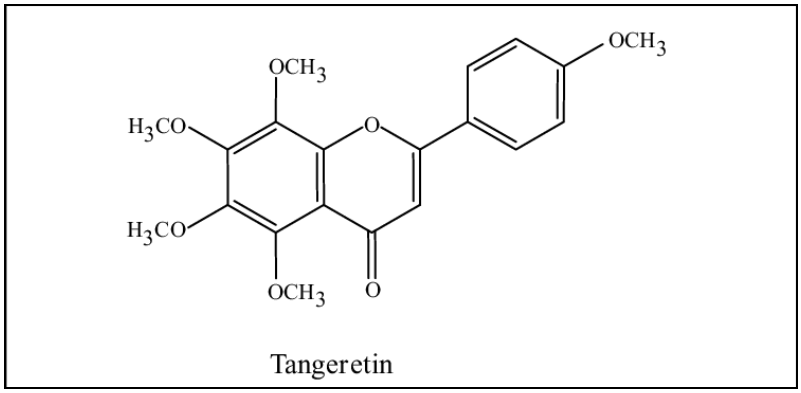


**Fig. 1: Chemical structure of Tangeretin.**

## MATERIALS AND METHOD

### Materials

Tangeretin was purchased from TCI Chemicals (India) Pvt. Ltd, Chennai. Methanol was purchase from Merck. All the chemicals of analytical grade were used for the proposed study.

**Instruments Used**

A double beam UV-visible spectrometer (UV-530, Jasco) connected to a computer loaded 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 (Essae, Vibra HT) with internal calibration mode was used for the accurate weighing purpose.

**Preparation of standard stock solution**

Accurately weighed 1 mg of tangeretin was transferred in to the calibrated volumetric flask and dissolved using 1 ml co-solvent system consisting of methanol and water (25:75 v/v) to achieve a stock solution of 1000 µg/ml (Stock-I). Stock- I solution was suitably diluted with co-solvent system to achieve solution of 100 µg/ml (Stock-II).

**Determination of wavelength of maximum absorbance ($\lambda_{max}$)**

Stock-II solution was scanned using full scan mode for the entire range of UV and visible i.e. 200 to 800 nm with co-solvent system as a blank. After obtaining the spectrum, $\lambda_{max}$ was identified with the help of software the spectrum is shown in (figure.2). In order to achieve reproducible results, above method was repeated five times.

**Preparation of calibration curve**

Calibration curve was prepared by diluting the stock-I solution to achieve the seven different calibration standards representing 0.5, 1, 3, 5, 7, 9 & 10 µg/ml strength. Absorbance of each calibration standard was measured at pre-identified $\lambda_{max}$; 327nm 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**

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

**Linearity and Range**

Linearity of the proposed UV method was established using seven different calibration standards. Based on 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.

**Accuracy**

The accuracy of the proposed UV method was evaluated using recovery studies after standard addition of analyte of interest. Three different solutions of tangeretin were prepared in triplicate at the level of 80%, 100% and 120% of its predefined concentration. To the predefined concentrations, different amounts of tangeretin 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) × 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. Intra-day precision study was carried out by preparing nine different solutions of 0.5, 5and 10 µg/ml strength of tangeretin (3 solutions of each concentration) and analyzing the same at morning, afternoon and evening time of same day. Deviation in the results was calculated in terms of % relative standard deviation (% RSD). Similarly, inter-day precision study was carried out by analyzing the above-mentioned solutions at three consecutive days.

**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 kept at 20 and 50% and tangeretin was dissolved in said co-solvent system separately. Triplicate samples were analyzed at 327 nm for absorbance. Levels of tangeretin in each sample were estimated using respective calibration curve. The results were calculated in terms of % RSD.

**Ruggedness**

Ruggedness study of the method was carried out by analyzing triplicate samples of tangeretin solution (4 µg/ml) on two different Instruments (V-530, Jasco and BA-UV-2600, Bioage) and absorbance were noted in terms of % RSD.

**Limit of Detection (LOD)**

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

LOD=3.3×SD/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 following formula

LOQ= 10×SD/S

Where, SD= Standard deviation of Y-intercepts

S= Slope

**Estimation of tangeretin in *Orange Peel* Powder extracts**

*Orange Peel* powder were dried at 50$^{o}$C using a Micro tray drier (S.B. Paschal and company, Mumbai, India) 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 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.10gm of powdered *Orange Peel* Powder was placed in a thimble (Borosil, Mumbai, India) which was inserted into a Soxhlet apparatus. The material was exhaustively extracted with methanol. SAE was performed for 2 h. After predefined extraction period, solvent was distilled off under reduced pressure using rotary vacuum evaporator (Heidolph instruments GmbH & co. Germany) to obtain the dry extract. Accurately weighed 1 mg of dry extract of *Orange Peel* Powder was transferred in to the calibrated volumetric flask and dissolved using 1 ml of methanol 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 tangeretin content using proposed UV method.

## RESULTS AND DISCUSSION

### 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 tangeretin solution was carried out using full scan mode of UV-Visible spectrophotometer. Full scan was processed using UV software and the $\lambda_{max}$ was identified with the help of software. It was found to be 327 nm for tangeretin (Fig.2).

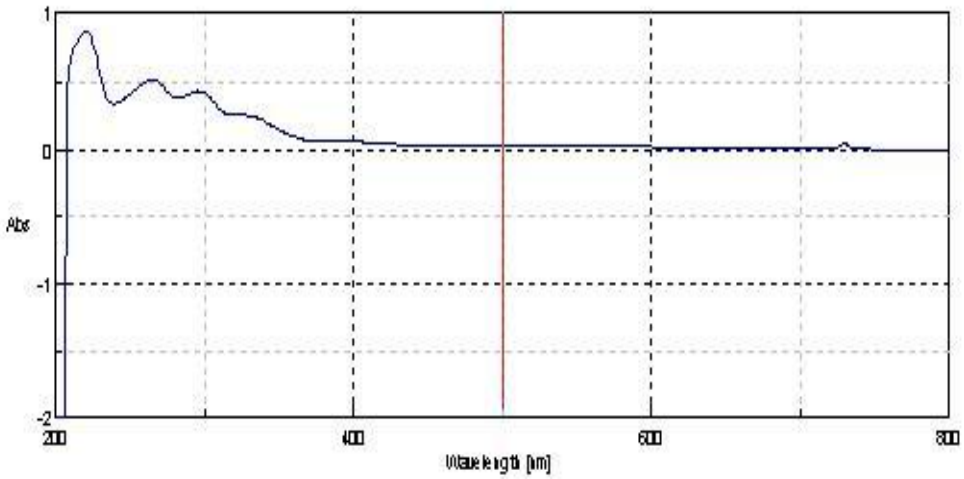


**Fig. 2: UV-visible spectra of Tangeretin.**

### 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 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 tangeretin, calibration curve for tangeretin was developed using seven different calibration standards. The absorbance of different calibration standards at 327 nm was recorded using fixed wavelength mode of UV-Visible spectrophotometer. Calibration curve was repeated five times and the mean values ± deviation was reported as shown in Table 1.

**Table 1: Calibration standard data for Tangeretin.**

| Concentration (µg/ml) | Absorbance |
|---|---|
| 0.5 | 0.048±0.0011 |
| 1 | 0.0946±0.0014 |
| 3 | 0.2804±0.0018 |
| 5 | 0.4847±0.0015 |
| 7 | 0.6771±0.0008 |
| 9 | 0.8776±0.0010 |
| 10 | 0.9668±0.0009 |

## Method validation

### 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 tangeretin covering a range of 0.5-10 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 = 0.0097x+0.004 with correlation coefficient 0.999 as shown in Figure 3.

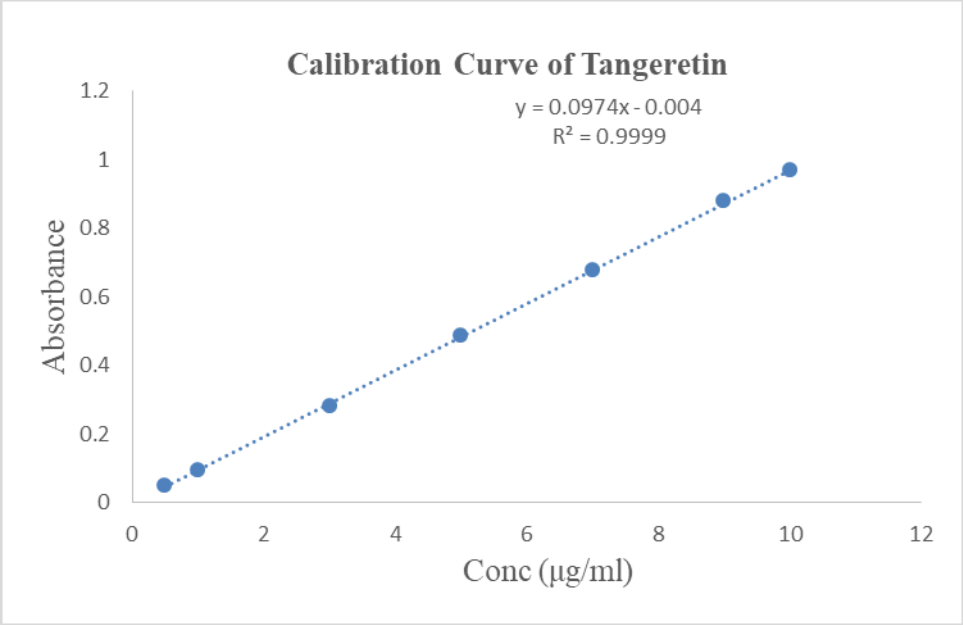


**Fig. 3: Calibration curve for Tangeretin.**

From the linearity study, it was revealed that, developed UV method was linear in the pre-defined concentration range of calibration standards.

**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 tangeretin, accuracy was established using recovery studies. At 80 % standard addition, mean recovery of tangeretin was found to be 98.44% whereas at 100 and 120 % standard addition, it was found to be 100.02 and 99.29% respectively. % RSD was found to be less than 2 for the tangeretin recovery studies as shown in Table 2.

**Table 2: Accuracy data of UV method for Tangeretin.**

| Concentration (%) | Origin level (µg/ml) | Amount added (µg/ml) | % Recovery | Mean % Recovery | % RSD |
|---|---|---|---|---|---|
| 80 | 1 | 0.8 | 100.11 | 98.44 | 1.77 |
| 80 | 1 | 0.8 | 98.65 | | |
| 80 | 2 | 0.8 | 96.58 | | |
| 100 | 5.5 | 5.5 | 100.66 | 100.02 | 0.6955 |
| 100 | 5.5 | 5.5 | 100.12 | | |
| 100 | 5.5 | 5.5 | 99.28 | | |
| 120 | 9.5 | 11.4 | 99.36 | 99.29 | 0.5344 |
| 120 | 9.5 | 11.4 | 98.72 | | |
| 120 | 9.5 | 11.4 | 99.78 | | |

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 102% and the % RSD was well below 2%.

**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 0.6, 4 and 9.5 µg/ml levels of tangeretin. 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.

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

| | Morning | | | Afternoon | | | Evening | | |
|---|---|---|---|---|---|---|---|---|---|
| Concentration Range (µg/ml) | Mean | % Assay | % RSD | Mean | % Assay | % RSD | Mean | % Assay | % RSD |
| 1 | 0.9824 | 98.24 | 1.56 | 0.9946 | 99.46 | 1.37 | 0.9973 | 99.73 | 1.46 |
| 5.5 | 5.50 | 100.10 | 0.81 | 5.48 | 99.79 | 1.51 | 5.47 | 99.62 | 1.33 |
| 9.5 | 9.34 | 98.35 | 0.12 | 9.36 | 98.60 | 0.64 | 9.35 | 98.43 | 0.29 |

**Table 4: Inter-day precision data of UV method for Tangeretin**

| | Day 1 | | | Day 2 | | | Day 3 | | |
|---|---|---|---|---|---|---|---|---|---|
| Concentration Range (µg/ml) | Mean | % Assay | % RSD | Mean | % Assay | % RSD | Mean | % Assay | % RSD |
| 1 | 0.9914 | 99.14 | 1.46 | 0.9874 | 98.74 | 1.71 | 0.9809 | 98.09 | 1.35 |
| 5.5 | 5.49 | 99.84 | 1.22 | 5.49 | 99.90 | 1.20 | 5.50 | 100.1 | 1.36 |
| 9.5 | 9.35 | 98.46 | 0.35 | 9.36 | 98.54 | 0.58 | 9.34 | 98.32 | 0.72 |

% RSD values of intra-day precision study were found to be in between 0.12 and 1.56 whereas those of inter-day precision study were in between 0.35 and 1.71. Overall, % RSD values of less than 2 showed the precision of developed UV method.

**Robustness**

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; 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 ethanol percentage (20 to 30 %) in co-solvent system did not affect the method performance. % RSD values were found to be in between 0.87 and 1.43 as shown in Table 5. % RSD values below 2 showed that proposed UV method is robust in nature.

**Table 5: Robustness data of UV method for Tangeretin.**

| Concentration (µg/ml) | % MeOH | Absorbance | % RSD |
|---|---|---|---|
| 4 | 20 | 0.3701 | 0.8783 |
| 4 | 25 | 0.3648 | 1.4336 |
| 4 | 30 | 0.3754 | 1.2440 |

**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, tangeretin solution was analyzed using two different UV-Visible spectrophotometers of two different labs. Sample analysis and data processing resulted into % RSD values between 1.35 and 1.41. Results revealed that proposed UV method was rugged as it showed % RSD values less than 2 as shown in Table 6.

**Table 6: Ruggedness data of UV method for Tangeretin.**

| Concentration (µg/ml) | Instruments | Absorbance | % RSD |
|---|---|---|---|
| 4 | Jasco | 0.3749 | 1.4140 |
| 4 | Bioage | 0.3731 | 1.3572 |

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

LOQ represents the lowermost concentration that can be analyzed with acceptable accuracy and precision. LOD and LOQ of proposed UV method was found to be 0.77 and 2.33 µg/ml respectively as shown in Table 7.

**Table 7: LOD & LOQ data for UV method for Tangeretin.**

| | |
|---|---|
| LOD | 0.77 µg/ml |
| LOQ | 2.33 µg/ml |

Lower LOQ value indicated that proposed method would be suitable for analyzing the samples containing even small quantities of tangeretin.

**Estimation of Tangeretin in *Orange Peel* powder extracts**

Developed UV method was successfully applied for estimation of tangeretin content in *Orange Peel powder* extracts. By proposed UV method, tangeretin content in Soxhlet extracts of *Orange Peel* Powder was found to be 0.020 ± 0.41 g/100g Powder.

**CONCLUSION**

A simple, accurate and precise UV-Visible spectrophotometric method for the estimation of tangeretin in *Orange Peel powder* extracts *was* developed and validated. Proposed method was found to be robust and rugged in nature and was successfully used for the estimation of tangeretin present in *Orange Peel powder* extracts.

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