Development and validation of UV Spectrophotometric method for estimation of β-carotene in *Cissus quadrangularis* stem extract

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Abstract **- A simple, precise and cost effective UV- visible spectrophotometric method for the estimation of β-carotene in *Cissus quadrangularis* stem extracts was developed and validated according to the ICH Q2 (R1) guideline. β-carotene is a carotenoid and a precursor of vitamin A, which is isolated from the stems of *Cissus quadrangularis* plant. Spiked β-carotene solution was scanned over UV-visible range for its wavelength of maximum absorbance. Various calibration standards of β-carotene were prepared. Calibration curve of concentration vs. absorbance was plotted. Various analytical method validation parameters viz. linearity, accuracy, precision, robustness, ruggedness, limit of detection and limit of quantitation were calculated. The maximum wavelength of β-carotene was found to be 459nm. The correlation coefficient over the concentration range of 5-25 µg/ml was found to be 0.9983.**

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 0.71 & 1.04 to 0.62 to 0.92 respectively. The total percent recovery of β-carotene was found to be 98.80 to 100.88%.

A simple, precise and cost effective UV- visible spectrophotometric method for the estimation of β-carotene in standardized extract of stems of *Cissus quadrangularis* was developed. The present validated UV- visible method can be efficiently used for the estimation of β-carotene in extracts of *Cissus quadrangularis* stems.

Index Terms **- UV- visible spectrophotometry, β-carotene, *Cissus quadrangularis*, validation.**

INTRODUCTION

β-carotene is a provitamin A carotenoid or a nutrient that body readily converts into vitamin A. β-carotene, isolated from the stems of *Cissus quadrangularis* which is commonly known as Asthisanharaka and Hadjod, belongs to family Vitaceae.

β-carotene is present in root, leaves and stems of *Cissus quadrangularis.* Stems are consisting of variety of chemicals with wide range of activities among all the chemicals within; β-carotene has gained the attention of researchers working in natural products due to its antioxidant property. It protects body from damaging molecules called free radicals. Its molecular weight 536.9 g/mol and chemical formula is $C_{40}H_{56}$. β-carotene prevents premature skin aging by acting as an antioxidant. It is soluble in organic solvent like ethanol, methanol and water. Abundant presence of β-carotene in several foods and plants including imparts prominent dietary supplement activity to extracts therefore standardized *Cissus quadrangularis* extracts are gaining commercial importance. Although, a precise UV- visible spectrophotometric method capable of estimating β-carotene in variety of dosage forms like powder and solutions is available in market., there is no single UV-visible spectrophotometric method available for estimation of β-carotene in extracts of *Cissus quadrangularis*. Therefore, considering the commercial importance and the needs of herbal industries, a simple yet precise and economical UV-visible spectrophotometric method capable of estimating β-carotene was developed and validated.

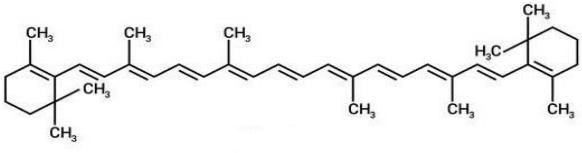


Fig 1: Chemical Structure of β-carotene

MATERIALS AND METHOD

Materials

The standard of β-carotene was purchased from Yucca Enterprises (Mumbai). Chloroform, Ethanol, Ethyl acetate, N-hexane and Methanol were of analytical grade were used for the proposed study.

Instruments

A double beam UV-visible spectrophotometer UV-2600, Shimadzu with spectra manager software were used for the analysis. Glass cells having 1 cm path length with 3 cm length were used for spectral measurement. Weighing balance with internal calibration mode was used for the accurate weighing purpose.

Preparation of standard stock solution

Accurately weighed 10 mg of β-carotene was transferred in to the calibrated volumetric flask and dissolved using 100 ml of chloroform to achieve a stock solution of 1000µg/ml (Stock-I). Stock-I solution was suitably diluted with chloroform to achieve solution of 100µg/ml (Stock-II).

Determination of wavelength of maximum absorbance (λmax)

Stock-II solution was scanned using full scan mode for the entire range of UV and visible i.e. 800 to 200nm with chloroform 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.

Preparation of calibration curve

Calibration curve was prepared by diluting the stock-II solution to achieve the five different calibration standards representing 5, 10, 15, 20, 25µg/ml strength. Absorbance of each calibration standard was measured at pre-identified λ_{max} 459nm using fixed wavelength measurement mode. The calibration curve representing concentration vs. absorbance was plotted. Above mentioned procedure was repeated pentaplicate so that reproducible results can be obtained.

Method Validation

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

Linearity and Range

Linearity of the proposed UV method was established using five 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 lower and upper 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 β-carotene were prepared in triplicate at level of 80%, 100% and 120% of its predefined concentration. To the predefined concentrations, different amounts of β-carotene were added (standard addition method) and the accuracy was calculated based on 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 three different β-carotene solutions of 5, 10 and 15µg/ml strength (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 intentionally adjusted to 23 and 28% and middle level quality control sample (5 µg/ml) of β-carotene was prepared using above mentioned co-solvent system separately. Samples (n=3) were analyzed at 459nm for β-carotene content. The results were calculated in terms of% RSD.

Ruggedness

Ruggedness study of the method was carried out by analyzing triplicate samples of β-carotene solution (5µg/ml) using two different analysts. Results were expressed 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 β-carotene in *Cissus quadrangularis* extracts

The *Cissus quadangulais* was shade dried at room temperature. Dried stems were grinded then screened through a sieve with mesh 80 to obtain uniform powder with particle size of 0.18mm. 20 gm of coarse powder was weighed and embedded in a thimble and put in soxhlet apparatus which was gradually filled with N-hexane, chloroform, Ethanol, Methanol and Water respectively in increasing order of polarity. The extraction experiment was carried out until solvent become colorless in siphon tube. The extracts were concentrated and after air drying, the respective extracts were weighed and percentage yield was calculated. Accurately weighed 10 mg of dry chloroform extract of *Cissus quadangulais* was transferred in to the calibrated volumetric flask and dissolved using 10 ml of chloroform to achieve a stock solution of 1000 µg/ml (Stock-I). Stock- II solution was suitably diluted with chloroform and analyzed for the β-carotene 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 β-carotene solution (5 µg/ml) was carried out using full scan mode of UV-Visible spectrophotometer (figure 2). Full scan was processed using UV software and the λ_{max} was identified with the help of software. The λ_{max} was found to be 459nm for β-carotene.

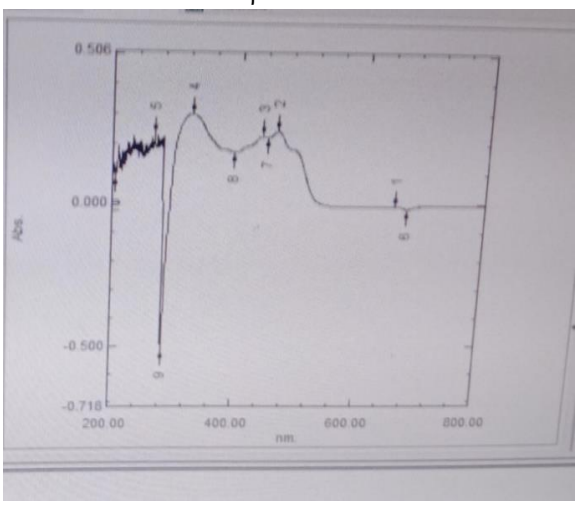


Fig 2: UV-visible spectra of β-carotene

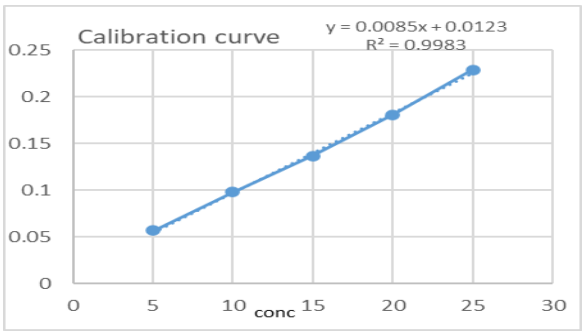
Preparation of calibration curve

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 β-carotene, calibration curve for β-carotene was developed using seven different calibration standards. The absorbance of different calibration standards at 459nm was recorded using fixed wavelength mode. Calibration

curve was repeated five times and the mean values ± standard deviation was reported as shown in Table 1.

Table 1: Calibration standard data for β-carotene

Sr. No.	Concentration (µg/ml)	Absorbance
1	5	0.057±0.0027
2	10	0.098±0.0042
3	15	0.137±0.0041
4	20	0.181±0.0055
5	25	0.229±0.0062



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 β-carotene covering a range of 5-25µg/ml was plotted. Details of concentrations and the respective mean absorbance values are shown in Table 1. Calibration curve when subjected to least square regression analysis yielded an equation.

y = 0.0085x+0.0123 with correlation coefficient 0.9983 as shown in Figure 3. 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 β-carotene, accuracy was established using recovery studies. At 80% standard addition, mean recovery of β-carotene was found to be 100.88% whereas at 100 and 120% standard addition, it was found to be 99.91 and 98.80% respectively.% RSD was found to be less than 2 for the β-carotene 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%.

Table 2: Accuracy data of UV method for β-carotene

Sr No.	Concentration (%)	Origin level (µg/ml)	Amount added (µg/ml)	% Recovery	Mean% Recovery	% RSD
1	80	10	8	99.81	100.88	0.47
2	80	10	8	100.8		
3	80	10	8	100.9		
4	100	10	10	100	99.91	0.22
5	100	10	10	99.6		
6	100	10	10	99.5		
7	120	10	12	98.8	98.80	1.14
8	120	10	12	97.4		
9	120	10	12	98.4		

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 10, 15and 20µg/ml levels β-carotene. The results in terms of mean absorbance values, standard deviation 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 0.712 and 1.04 whereas those of inter-day precision study were in between 0.621 and 0.923. Overall % RSD values of less than 2 demonstrated the precision of developed UV method.

Table 3: Intra-day precision data of UV method for β-carotene

Sr. No.	Concentration Range (µg/ml)	Absorbance (n=3)			Mean	SD	% RSD
		10.30am	12.30pm	2.30pm			

1	10	0.087	0.088	0.087	0.088	0.0006	0.712
2	15	0.095	0.094	0.095	0.094	0.0005	0.533
3	20	0.108	0.108	0.109	0.109	0.0011	1.04

Table 4: Inter-day precision data of UV method for β-carotene

Sr. No.	Concentration Range (µg/ml)	Absorbance (n=3)			Mean	SD	% RSD
		Day1	Day2	Day3			
1	10	0.093	0.094	0.095	0.094	0.0006	0.621
2	15	0.102	0.102	0.105	0.094	0.0005	0.593
3	20	0.112	0.112	0.111	0.112	0.0011	0.923

*Each value is an average of three determination.

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 change in absorbance frequency, temperature, 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 frequency did not affect the method performance. % RSD values were found to be in between 0.56 and 0.47 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 β-carotene

459nm				461nm		
Sr. No.	Concentration (µg/ml)	Absorbance	Statistical Analysis	Concentration (µg/ml)	Absorbance	Statistical Analysis
1	20	0.246	Mean=0.247 SD=0.0014 %RSD=0.56	20	0.358	Mean=0.357 SD=0.0017 %RSD=0.47
2	20	0.248		20	0.356	
3	20	0.247		20	0.358	

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, β-carotene solution was analyzed three times by two different Analysts. Sample analysis and data processing resulted into% RSD values between 1.11 and 1.50. 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 β-carotene

Analyst1				Analyst2		
Sr. No.	Concentration (µg/ml)	Absorbance	Statistical Analysis	Concentration (µg/ml)	Absorbance	Statistical Analysis
1	20	0.248	Mean=0.246 SD=0.0037 %RSD=1.50	20	0.253	Mean=0.251 SD=0.0028 %RSD=1.11
2	20	0.247		20	0.251	
3	20	0.245		20	0.249	

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 2.60 and 7.88 ng/ml respectively as shown in Table 7. Lower LOQ value indicated that proposed method would be suitable for analyzing the samples containing even small quantities of β-carotene.

Table 7: LOD & LOQ data for UV method for β-carotene

1	LOD	2.60 ng/ml
2	LOQ	7.88 ng/ml

Estimation of β-carotene in *Cissus quadrangularis* extracts

Developed UV method was successfully applied for estimation of β-carotene content in stem extracts by proposed UV method. β-carotene content in Soxhlet extracts of *Cissus quadrangularis* was found to be 1.27 ± 0.0058 mg/g feed.

CONCLUSION

A simple, accurate and precise UV-Visible spectrophotometric method for the estimation of β-carotene was developed and validated. The Proposed method was found to be robust and rugged in nature and was successfully used for the estimation of β-carotene.

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