RESEARCH ARTICLE

Quality by Design Approach in Quantitative Determination of the Red Dye from Extract of *Onosma Echioides* (Boraginaceae) by UV-visible Spectrophotometer

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Received: 11th February 2021; Revised: 9th June, 2021; Accepted: 13th July, 2021; Available Online: 25th September, 2021

ABSTRACT

Implementation of the Quality by Design (QbD) approach to analytical method development for quantifying red dye from the extract of *Onosma echioides* was performed. The soxhlet extraction technique was prepared by the dried root bark of *O. echioides* extract with petroleum ether (60–80°C). A column chromatographic technique used a mobile phase toluene: formic acid (99:1) toluate the major coloring compound from the petroleum ether extract. The extract was standardized using an isolated compound by High-Performance Thin Layer Chromatography (HPTLC). To develop the UV-visible spectrophotometric method, the two-level full factorial model with three variable factors was designed. QbD approach facilitated to calculate the spectrophotometric risk factor of the variable. The QbD approach proved that the selected spectrophotometric condition of each factor was lying in the middle of the design space and has wide boundaries and space. The middle values of the range were selected for method validation. The calibration curve showed a good linear relationship over the concentration range of 10 to 70 ppm. The LOQ and LOD were 0.95 ppm and 2.86 ppm, which indicates adequate method sensitivity. The mean RSD of interday precision, intraday precision and robustness was less than 2, indicating that the method is precise and robust. The accuracy of the method was found to be 105.65%. Thus, an accurate method was developed for the quantification of red dye from extract of *O. echioides* by using QbD approach. The proposed spectrophotometric method along with QbD approach, can be used as an alternative tool in the drug quality control laboratories for the quantitative determination of red dye in *O. echioides* in the extract.

Keywords: 1,4-naphthoquinones, *Onosma echioides,* Quality by Design, UV-visible spectrophotometric, Validation.

International Journal of Pharmaceutical Quality Assurance (2021); DOI: 10.25258/ijpqa.12.3.25

How to cite this article: Pananchery JF, Jadhav ML, Gadgoli CH, Jain AS. Quality by Design Approach in Quantitative Determination of the Red Dye From Extract of *Onosma Echioides* (Boraginaceae) by UV-visible Spectrophotometer. International Journal of Pharmaceutical Quality Assurance. 2021;12(3):292-298.

Source of support: This project was funded under Minor Research Project, Mumbai University.

Conflict of interest: None

INTRODUCTION

Quality by Design (QbD) has lately been widening its domain at a winged pace getting across diverse terrains. The recent literature reports indicate a spurt in the applications of QbD principles in modern herbal drug industries. QbD approach, verily, banks upon the combined use of risk assessment and Design of Experiments (DoE) for efficient and cost-effective extraction of bioactives from diverse plant materials. Such approaches not only provide benefits of efficient extraction process but also help in unearthing and understanding the scientific minutiae among the critical material attributes (CMAs), critical process parameters (CPPs) and critical quality attributes (CQAs) to control the potential sources of variation.[1,2]

Plants from the family *Boraginaceae* are popularly known for the presence of naphthaquinone derivatives such as alkyl derivative of 1,4-naphthaquinone called as alkannin. The isomeric form of alkannin is shikonin.[3,4] These compounds are found to be located in the external layers of the roots.[4]

Many plants of the genus *Onosma* family *Boraginaceae* are present in nature. The root bark of *Boraginaceae* yields a liposoluble red dye.[4] 1,4-naphthoquinone dimer is the major coloring component in the petroleum ether (60–80°C)

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extract of *O. echioides*.[5] 1,4 naphthoquinones are known for their wound-healing,[4,5] anti-inflammatory,[6] and antitumour[7] property and are even used in colored cosmetic formulations like hair oil, lipstick, rouge, eye-shadow.[8] The red dye is used in the dyeing industry to color cotton[9] and wool[10] fabrics. Research work on the colorization of vanaspati[11] with the red dye is available. For the standardization of the dye, a simple, robust spectrophotometric method is required for reducing batch to batch variation, as the natural products are subjected to variations in chemical constituents, which can further lead to variation in color strength and medicinal activity.

The literature search revealed that the methods available for estimation of red dye in plants of the Boraginaceae family include UV[12] and HPLC,[13,14] however, none of these methods are developed and validated using QbD approach. The present study was undertaken to develop and validate a simple rapid UV-visible spectrophotometric method for quantifying red dye from *O. echiodes* root bark extract using QbD approach, which can be utilized for routine analysis.

MATERIALS AND METHODS

Procurement of Raw Material and Authentication

The dried root of *O. echioides* was procured from the local market, Mumbai and was exomorphically authenticated at Agharkar Research Institute, Pune. The voucher specimen is deposited in the Institute for reference. Solvents were procured from Research Lab, Mumbai. (Specimen no. PYC # 261211)

Preparation of Extract

Dried root bark of *O. echioides* was powdered in a mixer and the powdered crude drug (100 g) was extracted with petroleum ether (60-80^{o}C) using Soxhlet Extraction apparatus for 18-20 hours. The extract was concentrated in a rotary flash evaporator until syrupy mass was obtained and the syrupy mass was then heated in a vacuum oven until dryness.

Isolation of Marker Compound

A column chromatographic technique was utilized for the isolation of major coloring compounds from the pet—ether extract of root bark of *O. echioides*. Silica gel for coumn chromatography (60-120#) was used as stationary phase was packed in a column (450 mmx20mm), and the constituents were eluted isocratically by using mobile phase toluene: formic acid (99:1)[12] with flow rate of 1 mL/min. A fixed volume (5 mL) of eluent was collected in different tubes, and TLC analysis of each eluent was carried out. All the eluents indicating a single spot with Rf 0.43 were pooled together and dried under vacuum to isolate the major coloring component, which is the marker compound.

Spectroscopic Studies on the Isolated Color Compound

The isolated color compound was recrystallized from toluene and checked for purity by performing TLC using mobile phase viz., toluene: formic acid (99:1).[15] The structure of isolated color compound[5] is shown in Figure 1 with molecular weight 684, and the empirical formula is $C_{40}H_{44}O_{10.}$

Standardization of the Color Extract

UV-Visible Spectroscopic Analysis

The isolated color compound was scanned from 200 to 800 nm to determine λ_{max} determination. The isolated major color compound was dissolved in methanol, and UV spectrum was recorded using UV visible spectrophotometer.

Quantification of the Isolated Color Compound

Quantification of the isolated color compound in pet. ether extract of dried root bark of *O. echioides* was carried out by high-performance thin Layer Chromatography (HPTLC).

The solution of the pet. ether extract was prepared by dissolving 200 mg of the solvent-free extract in 100 mL pet ether (60–80^{o}C) to get a concentration of 2000 ppm, and 5 µL of the same was then loaded on silica gel GF 254 TLC plate. The isolated marker compound (10 mg) was dissolved in 10 mL pet ether (60–80^{o}C) to get the concentration of 1000 ppm, and this was utilized as a standard solution for loading on HPTLC plates. The Silica gel GF 254 HPTLC plates were loaded with 2, 4, 6, 8, and 10 µL. The plates were developed as per the conditions mentioned above in Table 1.

The standard curve was prepared by plotting the area on the Y-axis and the concentration of isolated compounds on the X-axis. The content of isolated compounds in the pet. The ether extract was determined by extrapolating from the standard curve. The extract, thus standardized for the content of the isolated compound, was utilized for further studies.

Preparation of Sample

The standardized dried pet. ether extract was subjected to column chromatography**.** Silica gel for chromatography (60-120 #) was used as a stationary phase. The stationary phase was packed in a column (450 mm X 20 mm) and the constituents were eluted isocratically by using mobile phase toluene: formic acid (99:1) with a flow rate of 1ml/min. A fixed volume (5 mL) of eluent was collected in different test tubes, and TLC analysis

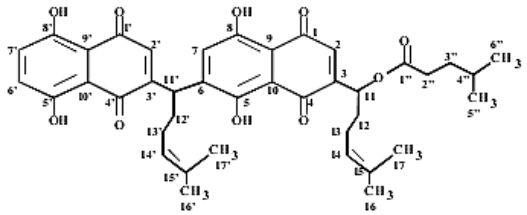


Figure 1: Structure of isolated compound

Table 1: Chromatographic conditions

Application mode	*Camag Linomat 5, Hamilton Syringe*
Development mode	Ascending technique.
Mobile Phase	Toluene:Formic acid (99 : 1)
Chamber saturation	20 minutes
Development distance	80 mm
Scanner	Camag Scanner 3
Detection	At 520 nm

of each eluent was carried out. All three coloring components were collected, pooled, dried, and used as color extract for further analysis.

Method Development

Selection of Solvent

For the analytical method development, the drug should be completely soluble in the solvent used and should give constant results. To serve this purpose, various solvents like methanol, ethanol, toluene, chloroform were tried. From these, methanol and ethanol were used as variable parameters for the QbD approach.

Preparation of Stock Standard and Working Solution

A stock solution was prepared by weighing 50 mg of color extract in 50 mL volumetric flask and dissolved in methanol to obtain a 1000 µg/mL concentration.

Working solution (100 µg/mL) was prepared by diluting 1-mL of stock solution to 10 mL. It was used for the initial spectral scan in spectrophotometric method. Further dilutions for linearity were prepared from the stock solution by the allegation method.

Selection of Wavelength

The working standard was scanned, and the spectrum was recorded as shown in Figure 2. According to the spectral scan, the wavelength was selected as 516 nm.

Implementation of QbD Approach

The experimental design and statistical analysis of data were performed by Design-Expert 10.0 software, Full Version (Stat Ease Stat-Ease, Inc., Minneapolis, MN, USA).

Design 1

Initially, the two-level factorial design with four factors was selected (Table 2). The factors included change in solvent, wavelength, spectral bandwidth, and response time. The first two factors were manually changed, while the latter two were instrumental variables. The total number of experiments was 16, and the response was recorded as absorbance. This model was not suitable as the factor "Change in solvent" was showing a significant effect. Hence another model was designed excluding this factor.

Design 2

The two-level full factorial model with three variable factors was finalized (Table 3). The factors included change in wavelength, change in spectral bandwidth, and response time. The number of experiments was eight, and the response was recorded as absorbance. The dilution of concentration 20 ppm was used for all experimental trials, and it was found that the model can be executed for the validation parameters. Thus, the study was extended, and the response was recorded with three validation parameters: linearity, precision, and accuracy. The experimental design and response recorded are as shown in Table 4. The responses of this model were analyzed by ANOVA test with the help of software. Also, the pareto charts and desirability plots were observed for this study.

Design 3

In accordance with the interpretation from design 2, it was decided to narrow the range of factor A. Thus, slight changes were made in the narrow range of 514 to 518 nm was selected for change in wavelength factor (Table 3), and responses were recorded as shown in Table 5. Correspondingly, the software analyzed the responses in the form of ANOVA test, pareto charts, and desirability plots.

After the completion of the design of experiments, the validation of the analytical method was performed within the obtained design space.

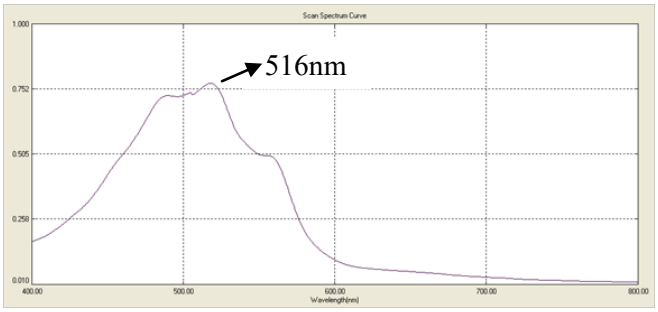


Figure 2: UV spectrum of the dye in methanol

Validation of Spectrophotometric Method

For the method validation, the factors with highest desirability value were selected from the desirability plots. The validation was performed according to ICH Q2 (R1) guidelines.

Linearity Studies

The dye obeyed beer's law in the concentration range of 10-70 µg/mL at high- and low-level wavelength at 512 nm and 520 nm using methanol. The absorbances were plotted against the corresponding concentrations to obtain the calibration graphs.

Precision

Precision studies were performed by using standard solutions containing three concentrations that are 20, 30, 40 µg/mL.

Table 2: 2^4 Full Factorial Design (Design 1)

		Levels	
UV Spectrophotometer Variables		*Low*	*High*
A	Change in Solvent	Ethanol	Methanol
B	Change in Wavelength (nm)	512	520
C	Change in Response time (sec)	0.2	1
D	Change in Spectral bandwidth (nm)	1	2

Table 3: 2^4 Full Factorial Design (Design 2 and 3)

UV Spectrophotometer Variables		*Levels*			
		Design 2		*Design 3*	
	Low	*Low*	*High*	*Low*	*High*
A	Change in Wavelength (nm)	512	520	514	518
B	Change in Response time (sec)	0.2	1	0.2	1
C	Change in Spectral bandwidth (nm)	1	2	1	2

Table 4: Responses of Design 2

Std.	*Run*	*Factor A: Change in wavelength nm*	*Factor B: Response Time sec*	*Factor C: Spectral Bandwidth nm*	*Response 1 Linearity R-square*	*Response 2 Precision RSD (n=6)*	*Response3 Accuracy % (n=3)*
7	1	512	1	2	0.998	1.4587	103.95
6	2	520	0.2	2	0.997	1.8102	106.81
4	3	520	1	1	0.998	1.6118	105.64
1	4	512	0.2	1	0.998	1.2959	104.01
5	5	512	0.2	2	0.997	1.4017	104.32
3	6	512	1	1	0.998	1.4126	103.34
8	7	520	1	2	0.998	1.9501	106.81
2	8	520	0.2	1	0.998	1.5702	106.60

Table 5: Responses of Design 3

Std.	*Run*	*Factor A: Change in wavelength nm*	*Factor B: Response Time sec*	*Factor C: Spectral Bandwidth nm*	*Response 1 Linearity R-square*	*Response 2 Precision RSD (n=6)*	*Response3 Accuracy % (n=3)*
7	1	514	1	2	0.999	0.021442	105.1971
8	2	518	1	2	0.999	0.021574	106.6114
3	3	514	1	1	0.998	0.02336	104.2484
2	4	518	0.2	1	0.998	0.020074	106.7289
4	5	518	1	1	0.998	0.113869	106.0208
1	6	514	0.2	1	0.998	0.022542	105.1945
5	7	514	0.2	2	0.998	0.024996	105.8236
6	8	518	0.2	2	0.998	0.024967	107.2672

Intra-day Precision

The precision of the methods in terms of repeatability was determined by analyzing three concentrations per three replicates of the dye. Depending on absorbances obtained for each concentration, standard deviation, and percentage relative standard deviation was calculated.

Inter-day Precision

Intermediate precision was assessed by analyzing the colour solutions on three consecutive days over a period of three days. The same parameters were calculated for inter-day precision.

Accuracy

Accuracy was determined by using the standard addition method at three different levels. The solution of dye having a concentration of 30 µg/mL was used for the study. 80%, 100%, and 120% of standard color solutions were spiked to the above solutions.

Specificity

For determination of interference of other excipients, 30 µg/mL of PEG 400 was added to 30 µg/mL standard colour solution. Interference of excipient was determined by finding percentage concentration of the standard drug in each dilution.

Sensitivity

The limit of detection (LOD) and limit of quantification (LOQ) were calculated using equations 1 and 2.

$$LOD = \frac{3.3 \times \sigma}{S} \qquad LOQ = \frac{10 \times \sigma}{S}$$

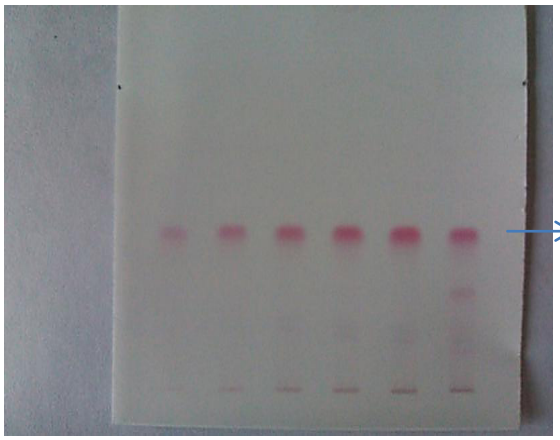
Where σ is the standard deviation of intercept, S is the slope of the calibration curve.

RESULTS AND DISCUSSION

Petroleum ether extract of dried root bark of *O. echioides* was prepared using soxhlet extraction apparatus. Isolation of major coloring compound from the petroleum ether extract was performed by a column chromatographic technique using a mobile phase toluene: formic acid (99:1) with a flow rate of 1 mL/min (Figure 3). The isolated color compound was recrystallized from toluene and standardized by UV-visible spectroscopic analysis and HPTLC analysis. The concentration of major coloring compounds in extract was 32.6% w/w.

QbD Approach for Spectrophotometric Method Development

An experimental design is an experimental setup to simultaneously evaluate several factors at a given number of levels in a predefined number of experiments. Several types of experimental designs (Two levels full factorial, two-level fractional factorial, Placket-Burman, mixed-level designs) are available, and these designs allow the simultaneous examination of qualitative, quantitative, and mixture related factors. A two-level full factorial design was selected for the



OEI: Isolated compound 2 µL
OEII: Isolated compound 4 µL
OEIII: Isolated compound 6 µL
OEIV: Isolated compound 8 µL
OEV: Isolated compound 10µL
E: Isolated compound 5µL

Figure 3: Quantification of Isolated major coloring compound in pet. ether extract of dried root bark of *O. echioides*

present study to determine the main effects and all interactions between the factors.

The results obtained from the eight experiments of both designs were analyzed individually through Design-Expert software. The responses recorded were in terms of linearity, precision, and accuracy. For linearity, the R-squared values of all calibration graphs were measured. Similarly, relative standard deviation and percentage recovery were calculated for precision and accuracy, and all these data were fitted into the summary window of the experimental design sheet in the software.

ANOVA Test

The data was processed by selecting the model as "Two Level Factorial" and then the ANOVA test was applied to all sets of experiments.

In design 2, for the first two responses, all probability values (p-value) were more than 0.05, indicating that there were NO significant model terms. While for the third response, the first factor that is a change in wavelength was showing a considerable effect and its p-value was 0.0410. In design 3, for the first two responses, all probability values (p-value) were more than 0.05 indicating that there were NO significant model terms. While for a third response, the first factor that is change in wavelength was showing the significant effect and its p-value was 0.0276.

Pareto Charts

For Design 2 (Figure 4), the pareto chart for linearity demonstrated that factor A and B was having positive effect while factor C had a negative effect as indicated by blue color and all the factors were within limit of t value. The second pareto chart for precision demonstrated that factor A and C were not within the t-value limit and showed significant effects, but all factors showed a positive effect. The third pareto chart for accuracy demonstrated that that factor A and C were not within the limit of t value and hence showing significant

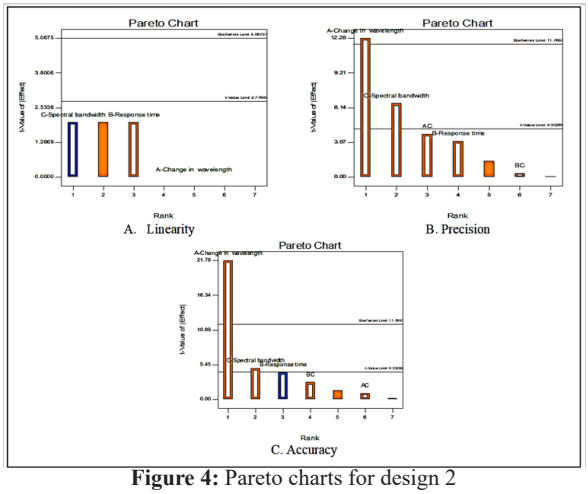


Figure 4: Pareto charts for design 2

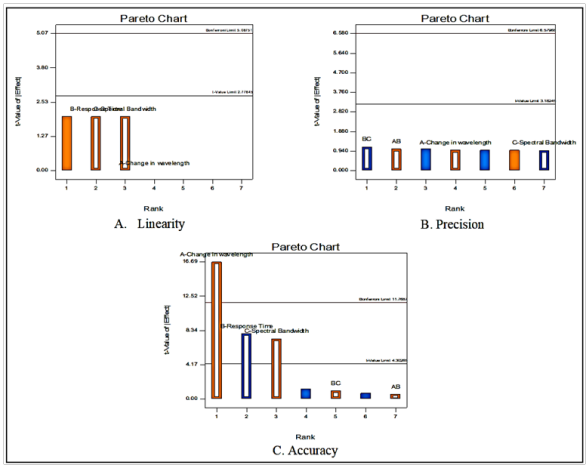


Figure 5: Pareto charts for design 3

effects while factor B has a negative effect and rest all showed a positive effect.

For Design 3 (Figure 5), the pareto chart for linearity demonstrated that all factors were having positive effect as indicated by the red color and all the factors were within the limit of t-value. The second pareto chart for precision demonstrated that factors A and C were showing negative effects as indicated by blue color and the remaining factors showed a positive impact. The third pareto chart for accuracy demonstrated that factors A, B, and C were not within the t-value limit, showing significant effects, while factor B had a negative effect.

Desirability Plots

To evaluate the desired range of spectrophotometric condition of these variables, desirability plots were established. These assured the desired acceptable result obtained in the range of the spectrophotometric condition of three variables in the desired space. The yellow to a green region in the design space graph indicated that the responses were in an acceptable range. The green to blue region showed that the responses were below the desired level.

The desirability plot for the combined effect of factors A and B for design 2 (Figure 6.a) demonstrated that the design

space for the present method could vary in the complete range of both factors. Also, the results will be more accurate in the central region, having constant spectral bandwidth of 1.5 with a response time between range 0.5 to 0.7 and change in wavelength between 514 to 516 with a desirability 0.77.

The desirability plot for the combined effect of factors A and C design 2 (Figure 6.b) demonstrated that the design space for the present method could vary in the complete range of both factors. Also, the results will be more accurate in the central region, having a constant response time of 0.6 with spectral bandwidth between range 1.5 to 1.8 and change in wavelength between 514 to 518 with desirability 0.77.

The desirability plot for the combined effect of factors B and C design 2 (Figure 6.c) demonstrated that the design space for the present method could not vary in the complete range of both factors. The results will be more accurate in the region with a constant wavelength 515.5 with response time between 0.4 to 0.6 and spectral bandwidth between 1.5 to 1.8 with desirability 0.77. The design space cannot be varied in the response time of range 0.6–1 relative to the spectral bandwidth of range 1.5 to 1.

The desirability plot for the combined effect of factors A and B for design 3 (Figure 7a) demonstrated that the design space for the present method could vary in the complete range of both factors. Also, the results will be more accurate in the central region, having constant spectral bandwidth of 1.5 with a response time between range 0.5 to 0.8 and change in wavelength between 515.5 to 516.5 with desirability 0.771817.

The desirability plot for the combined effect of factors A and C for design 3 (Figure 7.b) demonstrated that the design space for the present method could vary in the complete range of both factors. Also, the results will be more accurate in the central region, having a constant response time of 0.6 with spectral bandwidth between range 1.3 to 1.6 and change in wavelength between 515.5 to 516.5 with desirability 0.771817.

The desirability plot for the combined effect of factors B and C for design 3 (Figure 7.c) demonstrated that the design space for the present method could vary in the complete range of both factors. The results will be more accurate in the region with constant wavelength 516 with response time between 0.6 to 0.65 and spectral bandwidth between 1.4 to 1.5 with desirability 0.771817.

From the above design plot, it facilitated to calculation of the spectrophotometric risk factor of the variable. It has also confirmed the design space in between multiple interactions of the variable in the acceptable responses. The above design proved that the selected spectrophotometric condition on each variable was lying in the middle of the design space and had wide boundaries and space. The middle values of the range were selected for method validation.

Validation of UV Spectrophotometric Method

Linearity

The linear regression data for the calibration curves showed good linear relationship over the concentration range 10–70 ppm. Linear regression equation was found to be Y = 0.014 X+ 0.005 (r^2 = 0.998).

Precision

The precision of the developed spectrophotometric method was expressed in terms of % relative standard deviation (% RSD). The study was extended for Intra-day and Inter-day precision. The results depicted revealed high precision of the method is presented in Table 6.

Accuracy

The proposed method when used for extraction and subsequent estimation of the drug from the extract after standard addition with 80%, 100% and 120 % of additional drug; afforded recovery in the acceptable range as listed in Table 7.

Specificity

The identity of the colour extracts in the excipients was confirmed by comparing the absorbance with those of their respective standard and reported as percent relative standard deviation (%RSD) as listed in Table 8.

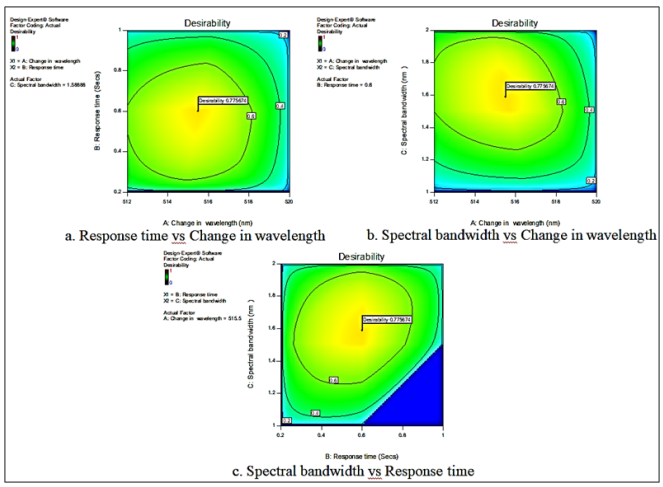


Figure 6: Desirability graphs on CQA for Design 2

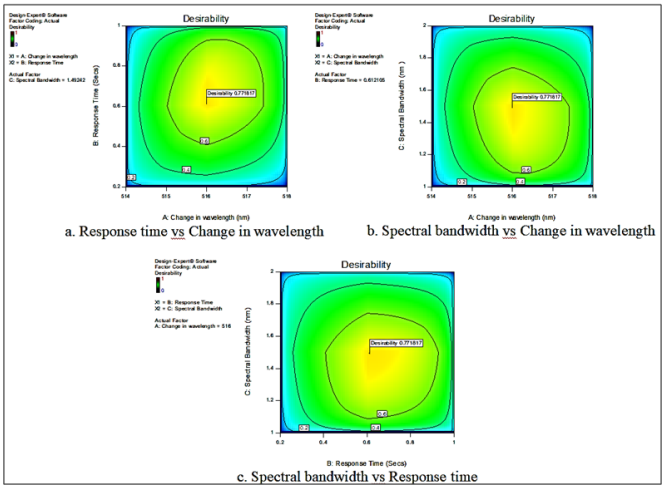


Figure 7: Counter desirability graphs on CQA for Design 3

Table 6: Precision data

		Inter day (RSD)		
Concentration	*Intra day (RSD)*	*Day 1*	*Day 2*	*Day 3*
20	1.57	1.92	1.98	1.96
30	1.63	1.38	1.43	1.44
40	1.63	1.43	1.49	1.49
Mean	1.60	1.58	1.63	1.63
		1.61		

Table 7: Accuracy data

	Concentration (ppm)		*Percent*	
Level	*Standard*	*Bulk*	*Recovery*	*Mean*
80 %	24	30	106.17	105.65
100 %	30	30	104.68	
120 %	36	30	106.09	

Table 8: Specificity data

Name of Excipient	*Concentration of colour (ppm)*	*Concentration of Excipient (ppm)*	*Absorbance*	*Standard Deviation*	*RSD*
PEG 400	30	30	0.451	0.0035	0.7851
	30	30	0.444		
	30	30	0.447		

LOD and LOQ

Detection limit and quantification limit were calculated by the method as described earlier. The LOQ and LOD were 0.95 ppm and 2.86 ppm. This indicates the adequate sensitivity of the method.

CONCLUSION

The proposed spectrophotometric method can be concluded as accurate, precise, specific, and economical. Implementation of the QbD approach resulted in more robust methods with less time consumption, consistency, reliability, and quality data. The proposed spectrophotometric methods and QbD approach can be used as an alternative tool in the drug quality control laboratories for quantitative determination of red dye in *O. echioides*.

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