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Analytical Quality by Design Driven Development and Validation of an UV-Visible Spectrophotometric Method for Quantification of Xanthohumol in Bulk and Solid Lipid Nanoparticles

Short Title in English: AQbd Driven Method Development and Validation

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ABSTRACT
INTRODUCTION: Xanthohumol (XH) is a prenylated chalcone available naturally and having diverse pharmacological activities. It has some limitations in the physiological environment like biotransformation, less GIT absorption. To overcome the limitations, we prepared nanoformulations (solid lipid nanoparticles) of XH. Therefore, an analytical method is required for the estimation of XH in the bulk nanoformulations so, we developed and validated QbD based UV-spectrophotometric method as per ICH Q2 (R1) guidelines.
METHODS: The new AQbd based UV-Visible spectrophotometric technique is developed and validated for estimation of xanthohumol in bulk and solid lipid nanoparticles as per ICH guidelines Q2 (R1). The critical method variables (CMVs) are selected based on risk assessment studies. Optimization of method variables was performed by using CCD model.
RESULTS: Multi-regression ANOVA analysis showed R2 value 0.8698 which is nearer to 1 that indicates the model was best fitted. The optimized method by CCD is validated for its linearity, precision, accuracy, repeatability, LOD and LOQ and specificity. All the validated parameters were found to be within the acceptable limits (%RSD<2). The method was linear between 2-12µg/ml concentration with R2 value 0.9981. Method was accurate with % recovery 99.3%-100.1%. The LOD and LOQ was found to be 0.77 and 2.36 µg/mL respectively. The precision investigation was also confirmed the method was precise with %RSD<2.
DISCUSSION AND CONCLUSION: The developed and validated method was applied to estimate the XH in bulk and solid lipid nanoparticles. The developed method was specific to XH which was confined by the specificity study.
Keywords: AQbd, solid lipid nanoparticles, Validation, UV-Visible spectrophotometric method

INTRODUCTION

Xanthohumol (XH) is a natural prenylated chalcone obtained from hops. It possesses potential pharmacological applications and used against inflammation[1,2], cancer[3,4], diabetes[5], melanoma[6], hyperlipidemia[7], invasion[8], angiogenesis[9], and obesity[7,10]. Due to these excellent therapeutic activities of XH, there is an immediate need of developing simple, cost effective, rapid, sensitive and accurate method to quantify XH in several matrices[4]. As per our knowledge there is no such UV-visible spectrophotometric method available to quantifying XH in bulk and in lipid based nanocarriers. Therefore, in present research analytical quality by design (AQbd) have been utilized to develop novel simple and cost effective method for estimation of XH in bulk and in lipid based nanocarriers. The structure of XH was represented in **Figure 1**.
Since last decade AQbd approach is attaining great importance in method development and validation of various analytical methods. It is generally termed as a systematic approach for developing methods that starts with the already defined objectives and also impart special focus on understanding the process, product and process control along with quality risk management[11]. Analytical science is regarded an important element in the development of pharmaceutical products and thus coincides with the product life cycle. AQbd approach requires less time and also decrease the number of trials of experimentation as compared to conventional type of

developing the analytical method[12,13]. It mainly employs the concept of design of experiments (DOE) and quality risk management (QRM) to discover the likely risks as well as interactions associated amongst the method variables, therefore saving a significant amount of time effort and money. The DOE is an integral part of AQbd because it provides best possible method performance. It also permits to construct the statistically significant model that allows different factors and their interactive impact on responses to be estimated. Therefore, adopting principles of AQbd will provide a significant benefit in terms of complete understanding the performance of the method. The application of AQbd in the development of UV-visible spectrophotometric method has utmost priority than other conventional methods[14]. Initially, before proceeding to method development we have to select the analytical target profile for the defined objectives of the study needs to be selected. Following that, risk assessment studies were used to identify the critical method variables (CMVs) and critical analytical attribute (CAA). CMVs impacting the performance of the method were optimized by Central composite design (CCD) to ensure the quality within the stated targets [15,16]. The current research work is mainly based on the application of AQbd principles to decrease the variability occur during measurement of XH to find the best solution. Therefore, simple, robust, economic, UV-visible spectrophotometric method has been developed and validated as per International Conference of Harmonization (ICH) guidelines Q2 (R1)[17,18].

EXPERIMENTAL

Reagents and chemicals

XanthoFlav (XH) was gifted by Simon H. Steiner, Hopfen, GmbH, Mainburg, Germany. Methanol of UV grade was purchased from Loba chemicals, Mumbai, India. The solid lipid Compritol E was gifted by Gattefosse Pvt Ltd (Mumbai,India). Lipoid E 80SN was gifted by Lipoid GmbH Germany. Pluronic F 68 was purchased from Loba chemicals India. Sephadex -G-25 was purchased from GE healthcare, Hyderabad, India. All other chemicals used were of analytical grade.

Instrumentation

UV-visible spectrophotometer (1800), Shimadzu with a set of 1cm quartz cuvettes was used for the photometric analysis of the sample. Design expert version 11, Stat-ease, Minneapolis, USA software is used for optimization. A 0.1mg sensitive analytical digital balance was used for weighing all the components (Shimadzu).

Analytical method development and optimization

Defining analytical target profile (ATP) and critical analytical attribute (CAA)

The ATP was established for the systematic development of XH estimation by outlining all of the required quality features of the analytical method using the principles of the AQbd strategy. Method objective was defined based on the assessment of the literature and profile of the analyte. The motivation for selecting the UV-visible spectrophotometric approach was due to its simplicity and speed of analysis when compared to more advanced analytical methods. To satisfy ATP, XH absorbance is selected as CAA [15,19].

Establishment of cause-effect relationship and risk management

Generation of CAA was made by studying the relationship between variables of the method and this CAA was used for analysis of control-noise -experiment (C-N-X). Ishikawa fish bone representation is used for depicting the correlation between CAA and method variables **(Figure 2)**. The C-N-X strategy utilized the risk assessment matrix to determine the crucial quality variables which are risky. Further, rankings were assigned to the recognized risky variables and the overall score was used to determine the CMVs **(Table 1)**. During analysis of C-N-X, variables such as detecting wavelength, sampling interval, scanning speed, sample integrity, solvent variation were investigated. Furthermore, sampling interval and scanning speed were discovered as CMVs and treated with appropriate experimental design (CCD) for investigative evaluation and optimization [15,16,20].

Determination of absorption maxima (λ_{max}) for XH analysis

Absorption maxima of XH was determined by scanning the solution of 10µg/mL from 800-200 nm by taking methanol as blank. Absorption maxima spectrum of XH was represented in **Figure 3**. The λ_{max} of XH was identified as 369 nm and is used for further analysis [14].

Optimization of the method by CCD

Identification of optimum conditions of the method and to assure robustness CCD was used. Based on the risk assessment studies, the optimization of the selected CMVs were done by performing 13 experiments with five center points by using CCD. The response variable (absorbance) of XH was evaluated by CCD and is measured at 369 nm using 10 µg/mL standard solution. Design expert version 11, Stat Ease, Inc, Minneapolis, USA, software is used to best fit the obtained data in to suitable mathematical model [15]. Polynomial equations were created as per ANOVA for significant model terms with p values less than 0.05. Fit summary of the model for the selected CAA suggested quadratic model as best fit. Correlation coefficient and lack of fit were used to evaluate the suitability of the model. The correlation between the CAA and CMVs were investigated by using contour plots and response surface plots. Furthermore, the numerical and graphical optimization is used to improve the method conditions by software.

The design space created by the DoE technique was used to define method control strategies, within which minor changes in method performance were tolerated and considered resilient.

Selection of solvent

The solvent for the analysis was selected based on the solubility studies of XH in various solvents, such as DMSO, methanol, chloroform and water. XH has shown highest solubility in methanol and DMSO. Methanol has been selected as a solvent for spectrophotometric method development because DMSO produces toxicity and it has stability issues.

Preparation of stock solutions

Primary stock solution was prepared by dissolving accurately weighed 100mg of XH in 100mL of methanol which gives the solution of concentration 1mg/mL or 1000µg/mL.

The secondary stock solution was prepared from primary stock solution by taking 10 mL of primary standard and volume is made with methanol up to 100 mL, which gives a solution of concentration 100µg/mL. By using secondary stock further dilutions were made for analysis.

Analytical method validation

UV-visible spectrophotometer (Shimadzu, UV-6000) operated with spectral bandwidth of 1nm was used for analytical method development and validation. Validation parameters include, precision, linearity and range, accuracy, repeatability, specificity, limit of detection (LOD) and limit of quantification (LOQ), robustness were evaluated as per ICH guidelines Q2 (R1) [18,21,22].

Linearity

Six samples of diverse concentrations (2-12µg/mL) were prepared from secondary standard and used for executing the linearity parameter of XH in methanol. It was executed for three days in triplicate (n=9). Linearity cure were plotted by using the obtained data. Correlation coefficient equation and regression equation was determined by using same data [14,23].

Limit of detection (LOD) and limit of quantification (LOQ)

The determination of LOD and LOQ for the method was performed by standard deviation (σ) and slope of the standard curve [18]. LOD and LOQ was given by the following equations:

LOD= 3.3*σ/S and LOQ= 10*σ/S

Where, σ is standard deviation and S is slope of the standard curve

Precision

UV method precision was determined in terms of variations in intraday and interday (Intermediate day) precision. The levels of precision were examined for three diverse known concentrations (4µg/mL, 6µg/mL and 8µg/mL) of XH prepared from the secondary stock solution for intraday precision of XH three concentrations were determined by taking absorbance of the samples in triplicates, three times in a day. Interday precision was executed by measuring the absorbance for the selected samples for three days in triplicate. Using linearity curve %RSD was calculated for the samples [18,24].

Repeatability

Determination of repeatability of the UV method was performed by measuring the absorbance of the XH solution in methanol for six times at 4µg/mL concentration [18].

Accuracy

The accuracy of the UV method was estimated by standard addition method. In this method standard stock solution of known amount was added to the test solution (6µg/mL, prepared from secondary stock solution) of XH at various levels like 80%, 100%, 125%. The absorbance for the prepared solutions were determined and the concentration was calculated again in triplicate by using linearity curve [15,18].

Specificity

Specificity test was performed by using blank solid lipid nanoparticles (SLNs) (only excipients). Blank solid lipid nanoparticles were prepared and known amount of XH was added to the dispersion. The resulting dispersion was mixed vigorously. 1mL of sample was taken and subjected to nanoparticle lysis. XH extraction was made by using methanol up to 5mL. The sample was analyzed by the developed method after filtering through 0.22µm filter [15,18].

Analysis of in-house prepared SLNs of XH

Preparation of solid lipid nanoparticles

The XH loaded SLNs were prepared by using homogenization-ultrasonication method. It mainly includes two steps, firstly preparation of lipophilic phase and later preparation of aqueous phase. The lipophilic phase is prepared by melting solid lipid (Compritol E ATO) 10 °C above its melting point. XH and lipophilic surfactant (lipoid E 80SN) was added to the molten lipid and stirred well. Aqueous phase was prepared by dissolving Pluronic F-68 in water. Both aqueous phase and lipid phase was heated to the same temperature i.e., above the melting point of the lipid. Then at hot condition aqueous phase was added to the lipid phase drop wise with continuous stirring. The mixture was subjected to high shear homogenizer for 30min at 6000rpm followed by

probe sonication at 40% amplitude, 35 pulse rate for 10min. The resultant dispersion was cooled to room temperature for solidification and precipitation of SLNs [25].

Analysis of solid lipid nanoparticles

The prepared solid lipid nanoparticles were analyzed by the developed method and validated method for determining the percentage entrapment efficiency and percentage drug loading.

Percentage Entrapment efficiency (%EE) and percentage drug loading (%DL)

The %EE of the XH-SLN was determined by separating the entrapped and unentrapped XH using Sephadex G-25 chromatography. The drug present in XH-SLN was extracted after lysis of lipid particles by mixing with methanol followed by filtration through 0.22µm filter. Then both entrapped and unentrapped XH content was determined in triplicate by using above developed and validated UV method. Concentration of the XH was calculated by using the calibration curve [21,26].

%EE and %DL was calculated by using following equation:

$$\%EE=\frac{Amount\ of\ drug\ used\ for\ formulation-amount\ of\ unentrapped\ drug}{Total\ amount\ of\ drug\ in\ formulation}\ X\ 100 \qquad Eq\,(1)$$

$$\%DL=\frac{Amount\ of\ entrapped\ drug\ in\ solid\ lipid\ nanoparticles}{Total\ weight\ of\ solid\ lipid\ nanopartcles}\ X\ 100 \qquad Eq\,(2)$$

RESULTS

Analytical method development and optimization

The solubility of XH was found to be freely soluble in methanol and DMSO, based on the toxicity, stability parameter methanol is selected as the solvent for UV-visible spectrophotometric method development and validation. The developed and validated method was used for the characterization (%EE and %DL) of prepared SLNs. The absorption maxima spectrum was determined by using methanol and was found to be 369 nm (Figure 3). To obtain CMVs for developing final spectrophotometric conditions, an AQbd approach was used. The C-N- X approach was used to identify CMVs using the Ishikawa fish-bone diagram and C-E risk assessment matrix. The risk levels of CMVs were identified based on the literature[27] and ranked according to their severity. The parameters with higher severity was selected and optimized by using CCD. Total scores for various method variables were calculated and prioritized for DoE investigation. The influence of CMVs on CAA was assessed by CCD. The UV-visible spectrophotometer is used to conduct 13 randomized trials in order to get impartial response with not more than five center points (**Table 2Aand 2B**) and the overlay spectrum of all the responses are depicted in **Figure 4 (A-B)**. CCD has given optimized spectrophotometric conditions with scanning speed -0.147 nm/sec and sampling interval as -0.259 nm with a desirability nearer to 1 i.e. 0.903. All the important parameters evaluated are found to be present within the specified limits. In addition, numerical and graphical optimization was performed to identify the best option within the given design space. Furthermore, the impact of the variables such as scanning speed and sampling interval on response (absorbance) is evaluated. The responses from all the trials were fitted in various kinetic models (linear, 2FI, quadratic, cubic) as it showed best fit to quadratic model. Investigation of ANOVA for quadratic model showed the p value equal to 0.0053 with R^2 value 0.8698 indicating the model is significant (**Table3**). The effect of CMVs on CAA was studied by using response, contour and 3D plots (**Figure 5)**. The graphical optimization was performed by superimposing the contour of critical response with contour plots using design expert software that lead to overlay plot with two regions (yellow and gray). The overlay plot of the optimized method was represented in **Figure 6A**. The design space with yellow shade indicates the area with possible response values whereas gray area indicates the design space with the responses that does not meet the criteria. The optimum conditions were selected based on overlay plot and desirability criteria.

Effect of scanning speed and sampling interval on absorbance

The impact of scanning speed and sampling interval on absorbance was studied from 3D plots, 2D contour plots and polynomial equation (3) **(Figure 5)**. For interpretation and optimization purposes, a 3D surface plot and 2-D contour plot were used. According to the contours produced under optimal circumstances, the investigation should proceed with specified center values for both CMVs. In all three 3-D response surfaces for the response, a similar pattern was seen for both CMVs. At low levels of sample interval, a curvilinear rise in response was seen with a progressive increase in scanning speed. Similarly, with low scanning speeds and increasing sample intervals, a small increase in responsiveness was seen. However, at low levels of both CMVs, a minimal reaction was seen. The actual vs predicted plot illustrated that the data obtained from the experiments were lies within the specified limits **Figure 6B**. The different colored points in the figure represents the higher R^2 values imply that the model can explain the majority of the variation. The blue, green and red points represents the lower, middle and higher values respectively. Model appropriateness was suggested by satisfactory p-values in ANOVA and low projected residual sum of squares (PRESS) values **(Table 3)**. The polynomial equation of quadratic model is clearly showing that there is a significant positive impact of scanning speed and sampling interval individually on absorbance. However, there is negative effect of scanning speed and sampling interval combinedly on absorbance. The effects of interaction of CMVs, individually and in combination with CAA as well as desirability of the method due to these interactions were illustrated in **Figure 7**.

The polynomial equation for the model is as follows

Absorbance=+1.27+0.0170*A+0.0386*B-0.0020*AB+0.0592*A^2+0.0750*B^2 Eq (3)
Where, A is scanning speed and B is Sampling interval

Analytical method validation

Linearity

The obtained linearity chart of XH **(Figure 8)** was analyzed by its correlation coefficient. The data obtained was represented in **Table 4**. XH linearity range in methanol was 2- 12 µg/mL with a R^2 >0.9981.

LOD and LOQ

The sensitivity of the method was assessed by estimating LOD and LOQ. LOD and LOQ for the developed UV method of XH in methanol was found to be 0.77 and 2.36 µg/mL respectively.

Precision and Repeatability

Determination of precision was performed under prescribed conditions by measuring the absorbance multiple times with the homogenous sample. The results for both interday and intraday were shown in **Table 5A and Table 5B**. The results of the interday and intraday precision illustrated that the developed method was stable and precise as the %RSD values are <2. Repeatability study was performed by taking absorbance of the XH (4µg/mL) for six times and the percentage drug recovered was calculated by comparing it with the standard graph. The % RSD of drug recovered was found to be less than 2.

Accuracy

Accuracy study was performed by % recovery method. The results are summarized in **Table 6**. The % recovery was found to be 99.3%-100.1%. The % RSD values were found to be within the acceptable limits (%RSD <2). Therefore, it is concluded that the developed method was accurate.

Specificity

The specificity of the developed method was evaluated by using blank SLNs. The % drug recovery from the blank SLNs mixed with known amount of XH was found to be 99.75± 0.23% which indicated the developed method was specific towards XH. There is no interference with the excipients used in the development of solid lipid nanoparticles. **Figure 3 (A-C)** represents the individual spectrums which clearly showing that there is no peak at the absorption maxima of XH. Therefore, there is no interaction between the excipients used in SLNs with XH.

Analysis of in-house prepared SLNs

The prepared nanoparticles were analyzed for the content of drug encapsulated in the SLNs by using the Eq^n (1) and (2). The results of % EE and %DL is summarized in the **Table 7**. The %RSD<2 is suggesting that the method is efficient for estimation of XH in nanoformulations without any interference.

DISCUSSION

Many UV-spectrophotometric methods have been developed for various drugs for their estimation in bulk and in pharmaceutical formulations. In recent years, researchers have also developed UV-spectrophotometric methods for estimation of drugs in nanoformulations and also demonstrated AQbd approach during their developmental process [15,18]. The present work has been designed based on the past developed methods for estimation of XH in nanoformulations such as SLNs. Till date there is no UV-visible spectrophotometric method has been reported for XH by using AQbd approach. All the results of validation parameters are lies within in the acceptable limits. The method's specificity and selectivity were accomplished due to the absence of interference from widely used excipients (Compritol E ATO, lipoid E80SN, Pluronic F-68) in the SLNs. The recovery and accuracy of XH from SLNs were found to be 99.3%-100.1% at wavelength 369 nm. The LOD and LOQ values are found to be 0.77 and 2.36 µg/mL respectively. The results of precision studies were found to be %RSD<2 which is an acceptable value. Therefore, the developed method was reliable and robust method, interference free which can be used for the estimation of XH in bulk and also in nanoformulations.

CONCLUSION

A new robust, simple, cost-effective UV-visible spectrophotometric method has been developed by utilizing AQbd approach for the estimation of XH in bulk and nanoformulations. The quality of the analytical method was assured by using the AQbd process. The findings point to the method's originality, simplicity, accuracy, and precision. Statistical analyses of the technique validation results support the established methods' suitability for application in quality control laboratories. The developed method is more efficient to estimate the XH in nanoformulations without interfering with the excipients used in the formulation. Therefore, this developed method can find its applicability in pharmaceutical industries to estimate XH effectively.

AUTHOR CONTRIBUTIONS

V.H.; Data curation, experiments, method development, validation and writing original draft, D.T.; conceptualization; interpretation, overall supervision, review and editing, RK; validation, conceptualization, supervision, review and editing, assistance in experiments, and formal analysis, P.G.; review and editing, S.M.; assistance in experiments, and formal analysis, S.K.S.; review and editing, M.G.; review and editing; All authors have approved the final version of the manuscript.

LIST OF ABBREVATION

XH- Xanthohumol, AQbd – analytical quality by design, DOE- design of experiments, QRM- quality risk management, CMVs- critical method variables, CAA- critical analytical attributes, CCD- central composite design, ICH- international conference of harmonization, ATP- analytical target profile, DMSO- dimethyl sulfoxide, LOD- limit of detection, LOQ- limit of quantification, SLNs- solid lipid nanoparticles, %EE- percentage entrapment efficiency, %DL- percentage drug loading, ANOVA- analysis of variance, 3D- three dimensional, 2D- two dimensional, PRESS- projected residual sum of squares, %RSD- percentage relative standard deviation.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable

HUMAN AND ANIMAL RIGHTS

No animals/humans are used for conducting this research

CONSENT FOR PUBLICATION

Not applicable

AVAILABILITY OF DATA AND ANIMALS

The authors confirm that the data supporting the findings of this study are available within the article

FUNDING

Not applicable

CONFLICTS OF INTEREST

Authors declare no conflicts of interest

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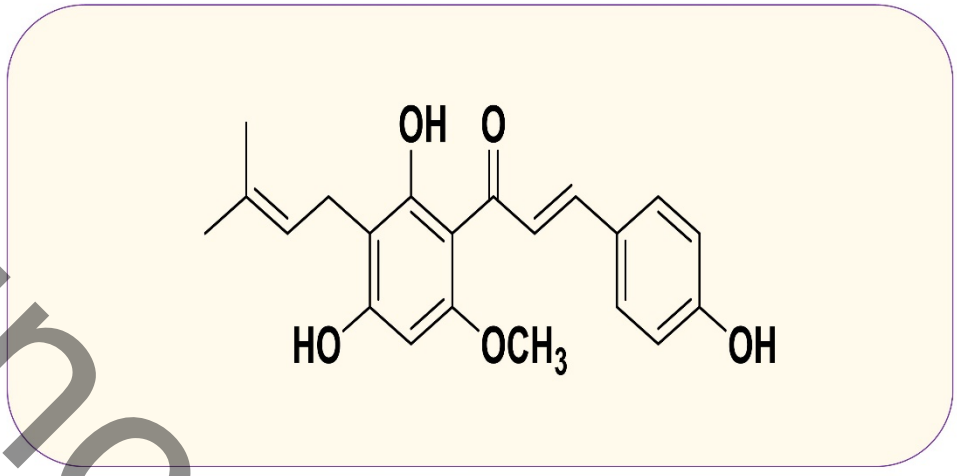

Figure 1. Structure of XH

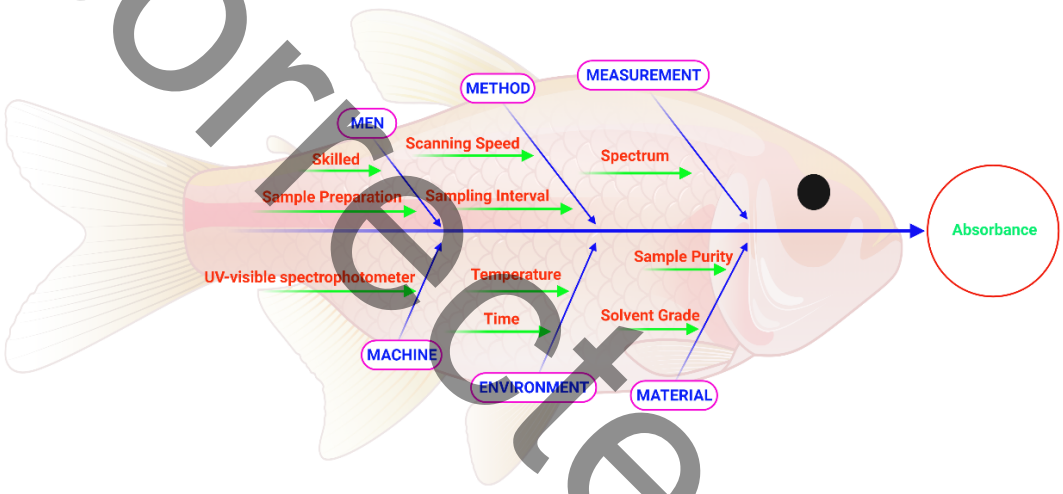

Figure 2. Depicting diagram of Ishikawa fish bone that illustrates the cause-effect correlation between method variables and analytical attributes

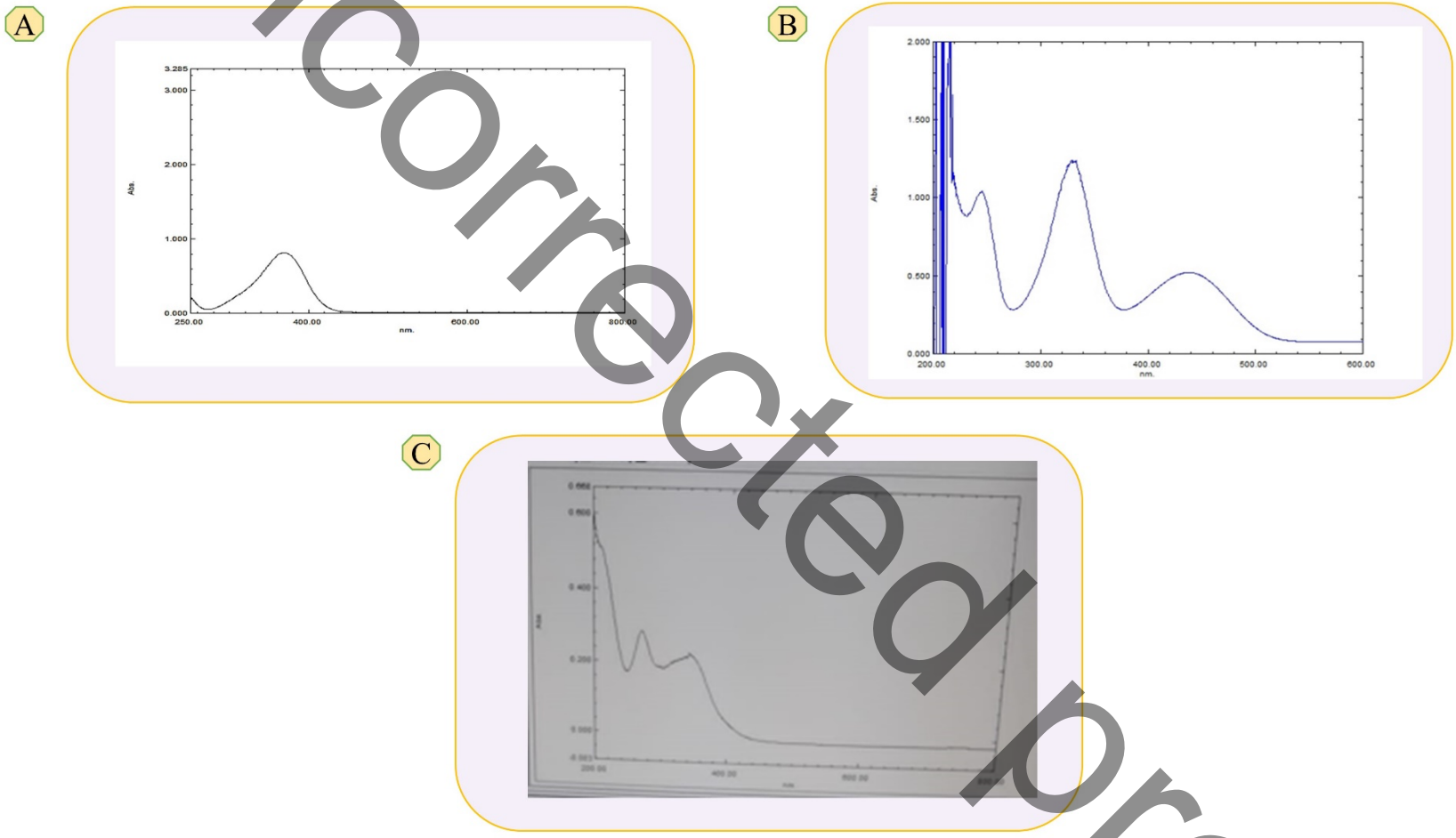

Figure 3. Absorption maxima spectrum of A) Bulk Xanthohumol B) XH-SLNs C) Blank SLNs (Excipients without drug)

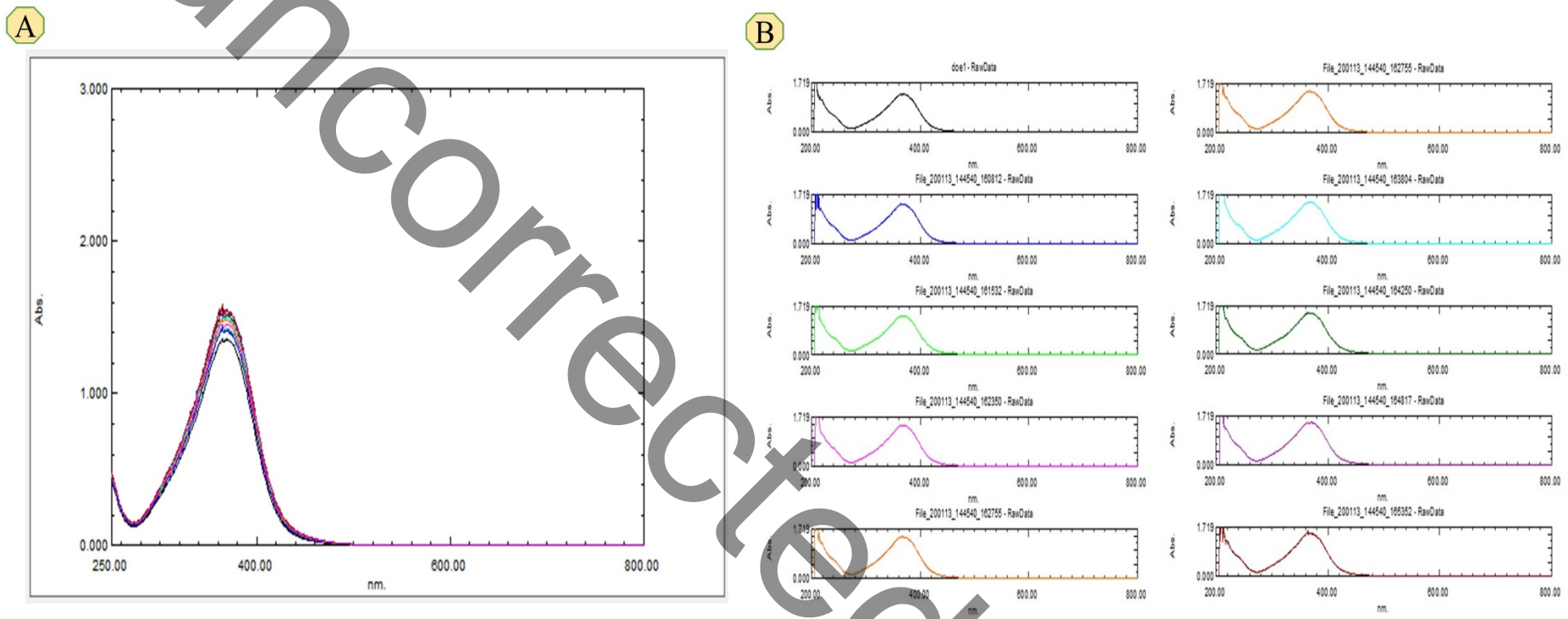

Figure 4. A) Overlay spectrums of all the experiments given by design B) Individual spectrums of experiments given by design

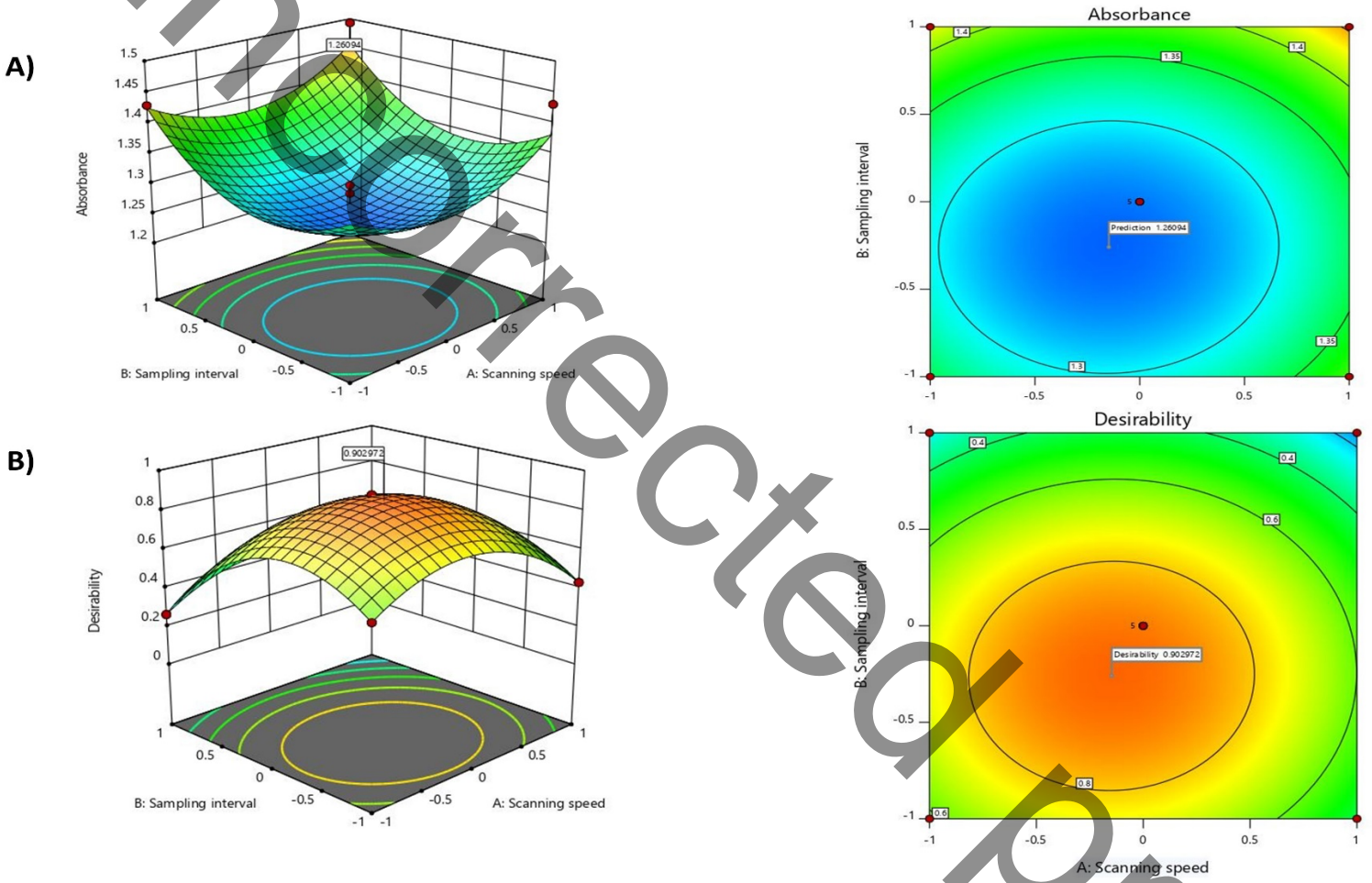

Figure 5. Depicting the 3D and contour plots; A) illustrating the effects of scanning speed and sampling intervals on absorbance. B) Showing the desirability of the optimized method.

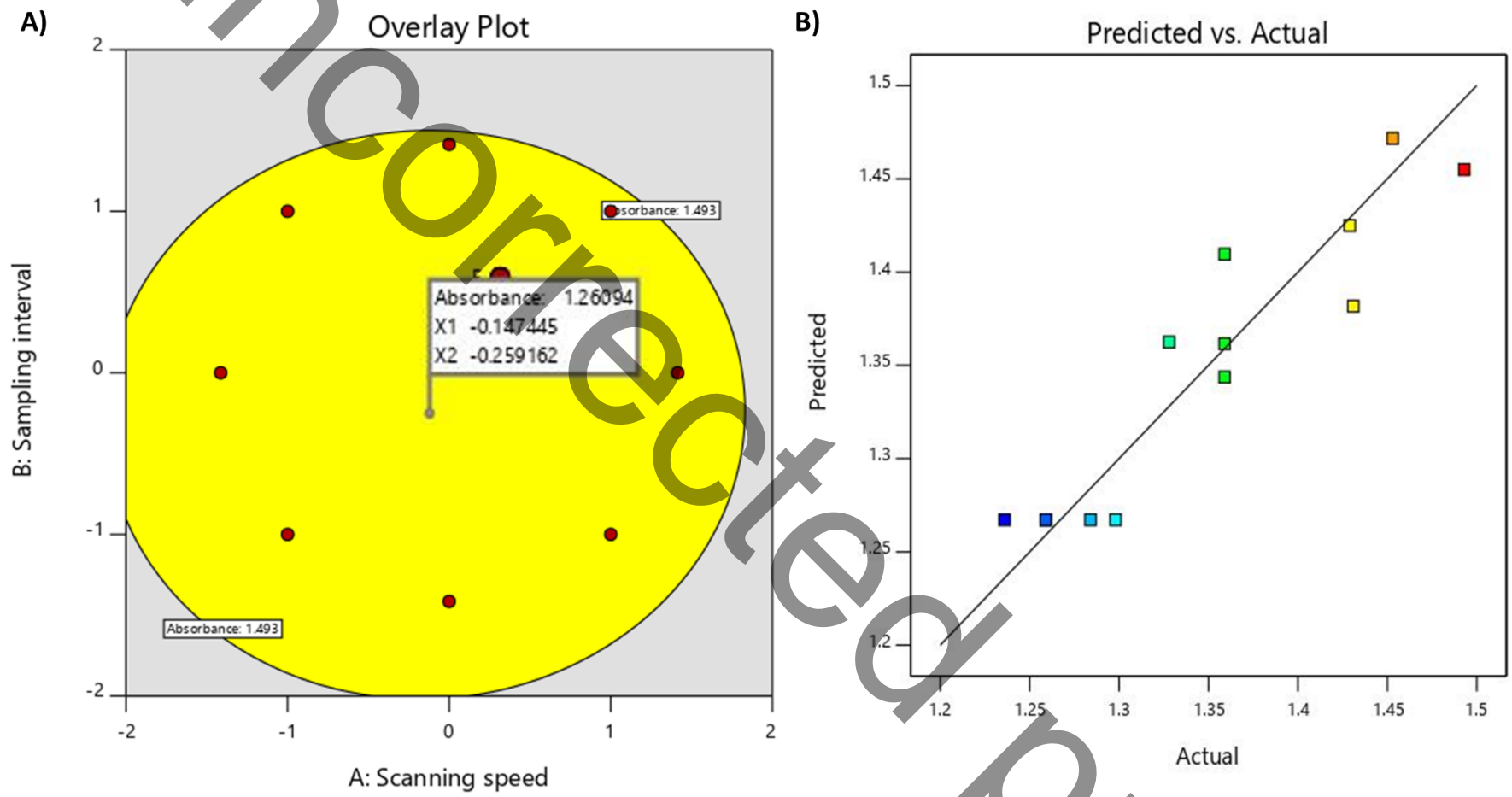

Figure 6. A) Representing the overlay plot of the optimized method B) Illustrating the correlation between predicted and actual values

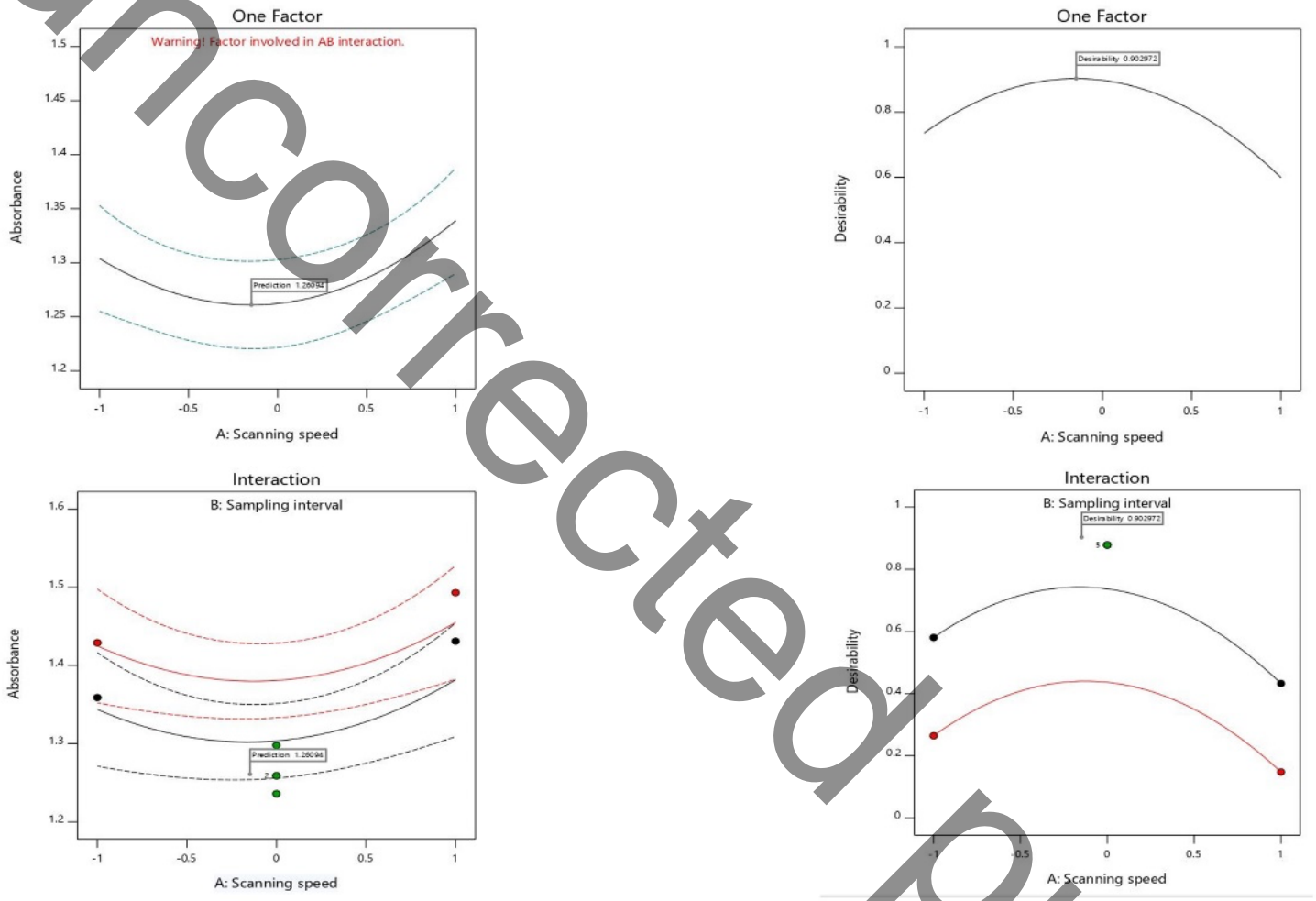

Figure 7. 2D representation of combined and individual effects and desirability of the optimized method

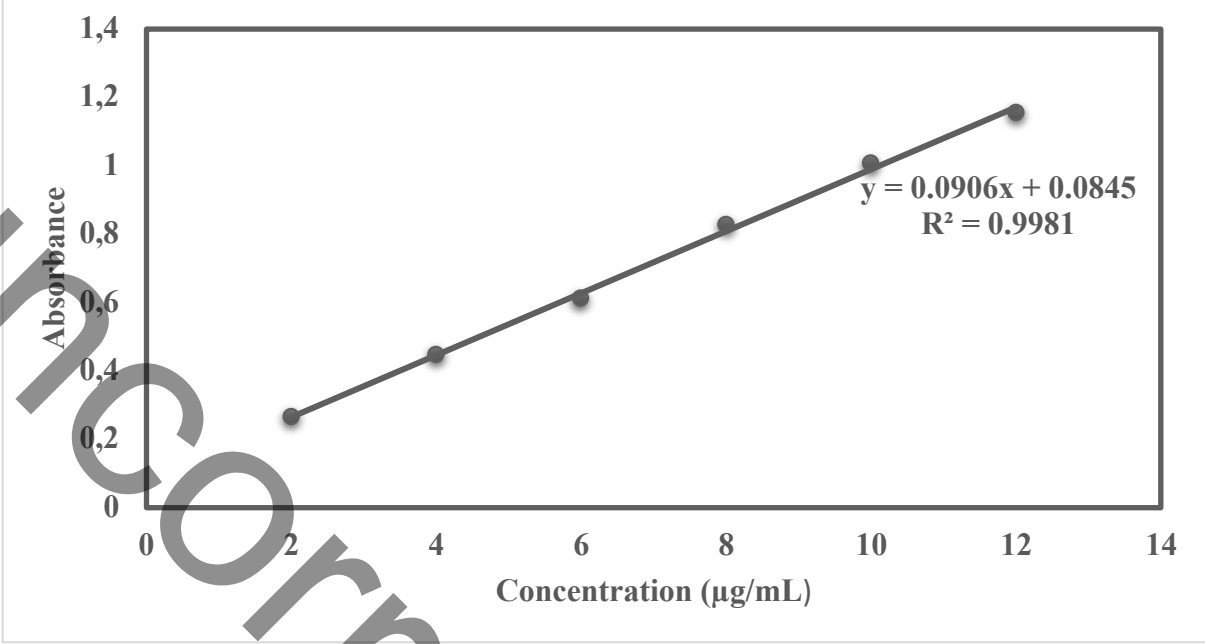

Figure 8. Depicting linearity chart of Xanthohumol in methanol

Table 1: C-N-X based risk assessment of CMVs for method development

CMVs	Level of risk on absorbance	C/N/X	Strategy used
Sampling speed	1	X	DOE
Sampling interval	1	X	DOE
Solvent	0	C	Controlled
wavelength	-1	C	369nm
Purity of sample	-1	N	Quality
Preparation of sample	-1	C	Controlled
Equilibration of detector	-1	C	Controlled

Level: 1- high, 0- medium, -1- low; **C/N/X:** Control/ Noise/Experimental

Table2A: Design table representing the range of variables used for optimizing the method

Run No	A	B
1*	0	0
2	+1	0
3	+1	+1
4	-1	+1
5*	0	0
6	-1	-1
7*	0	0
8	0	+1
9	0	-1
10	-1	0
11	+1	-1
12*	0	0
13*	0	0

*Represents center points of the model; A: scanning speed; B: sampling interval

Table2B: Representing the decoding of DOE codes

Level	A: Scanning speed	B: Sampling interval
-1 (Low)	Slow	0.5nm
0 (Medium)	Medium	1.0nm
+1 (High)	Fast	2.0nm

Table 3: Representing the Quadratic ANOVA model, Lack of Fit for scanning speed and sampling interval on absorbance

Source	Sum of squares	df	Mean squares	F-value	p-value
Model	0.0706	5	0.0141	9.35	0.0053
Scanning speed (A)	0.0023	1	0.00023	1.53	0.2559
Sampling interval (B)	0.0119	1	0.0119	7.89	0.0262
AB	0.0000	1	0.0000	0.0106	0.9209
A^2	0.0244	1	0.0244	16.15	0.0051
B^2	0.0391	1	0.0391	25.88	0.0014
Residual	0.0106	7	0.0015		
Lack of fit	0.0082	3	0.0027		
Pure error	0.0023	4	0.0006		
Cor Total	0.0812	12			
Sequential Model sum of squares					
Means vs Total	23.68	1	23.68		
Linear vs Mean	0.0142	2	0.0071	1.06	0.3816
2FI vs linear	0.0000	1	0.0000	0.0022	0.9640
Quadratic vs 2FI	0.0564	2	0.0282	18.67	0.0016
Cubic vs Quadratic	0.0026	2	0.0013	0.7998	0.4996
Residual	0.0080	5	0.0016		
Total	23.77	13	1.83		
Lack of Fits Tests					
Linear	0.0646	6	0.0108	18.42	0.0070
2FI	0.0646	5	0.0129	22.10	0.0052
Quadratic	0.0082	3	0.0027	4.69	0.0847
Cubic	0.0057	1	0.0057	9.70	0.0357
Pure Error	0.0023	4	0.0006		

Table 3 (continue): Representing the data of model summary, Fit summary and Fit statistics.

Model summary Statistics					
	Standard Deviation	**R^2**	**Adjusted R^2**	**Predicted R^2**	**PRESS**
Linear	0.0818	0.1752	0.0103	-0.3568	0.1102

2FI	0.0863	0.1754	-0.0994	-1.4285	0.1972
Quadratic	0.0389	0.8698	0.7768	0.2340	0.0622
Cubic	0.0400	0.9014	0.7633	-3.5146	0.3666
Fit Summary	**Sequential p-value**	**Lack of Fit p-value**	**Adjusted R^2**	**Predicted R^2**	
Linear	0.3816	0.0070	0.0103	-0.3568	
2FI	0.9640	0.0052	-0.0994	-1.4285	
Quadratic	0.0016	0.0847	0.7768	0.2340	
Cubic	0.4996	0.0357	0.7633	-35146	
Fit statistics					
Standard deviation	0.0389		**R^2**	0.8698	
Mean	1.35		**Adjusted R^2**	0.7768	
C.V%	2.88		**Predicted R^2**	0.2340	
			Adeq precision	7.7458	

Table 4: Depicting the linearity parameters of xanthohumol

Concentration (µg/ml)	Day1 (Abs)			Day2(Abs)			Day3(Abs)			Avg
	Trial I	Trail II	Trail III	Trial I	Trail II	Trail III	Trial I	Trail II	Trail III	
2	0.299	0.286	0.292	0.235	0.285	0.268	0.208	0.253	0.271	0.266
4	0.485	0.480	0.487	0.436	0.454	0.475	0.397	0.402	0.399	0.446
6	0.602	0.610	0.608	0.621	0.610	0.622	0.592	0.618	0.626	0.612
8	0.826	0.811	0.825	0.852	0.848	0.844	0.805	0.810	0.815	0.826
10	1.012	1.001	1.010	0.999	1.005	1.012	1.007	1.001	1.020	1.007
12	1.312	1.302	1.310	1.216	1.285	1.295	1.193	1.267	1.222	1.155

Table 5A: Interday precision data of xanthohumol

Concentration(µg/ml)	Absorbance			Mean±SD	%RSD	% Average potency
	Morning	Afternoon	Evening			
4	0.450 0.449 0.448	0.447 0.453 0.455	0.453 0.456 0.454	0.451±0.003335	0.743	102.2
6	0.693 0.692 0.692	0.692 0.693 0.696	0.692 0.692 0.690	0.692±0.001389	0.200	113.0
8	0.897 0.902 0.904	0.898 0.901 0.895	0.898 0.900 0899	0.899±0.002925	0.325	108.8

Table 5B: Depicting intraday precision data of Xanthohumol

Concentration(µg/ml)	Absorbance			Mean±SD	%RSD	% Average potency
	Day 1	Day 2	Day 3			
4	0.463 0.464 0.466	0.468 0.468 0.466	0.467 0.469 0.468	0.466±0.002066	0.443	104.4
6	0.684 0.684 0.686	0.686 0.687 0.685	0.687 0.683 0.685	0.685±0.001488	0.217	111.9
8	0.924 0.925 0.922	0.926 0.922 0.925	0.923 0.926 0.925	0.924±0.001642	0.177	111.8

Table 6: Depicting accuracy data of xanthohumol

Solvent used	Amount of standard added (%)	Percentage recovery (%)	% RSD
Methanol	**80**	**99.3±0.00215**	**0.785**
	100	**100.1±0.00452**	**0.321**
	125	**99.54±0.00256**	**0.685**

Table 7: Results of percentage of XH entrapped and Percentage drug loading in solid lipid nanoparticles

Formulation	% of XH entrapped ±SD	% of XH unentrapped ±SD	% total amount of XH recovered ±SD	% RSD	% DL
F1	76.64 ± 0.64	22.43 ±0.22	99.07± 0.46	0.469	11.6
F2	63.2±0.35	34.91±0.165	98.11±0.19	0.195	8.34
F3	72.2±0.65	25.93±0.15	98.13±0.75	0.764	12.8