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RESEARCH ARTICLE

Development and Greenness Assessment of Analytical Quality by Design Optimised Eco-friendly UV Spectrophotometric Methods for Analysis of Two Natural Antioxidants in Pure and Formulation

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Abstract

Utilizing analytical quality by design and green analytical chemistry principles, the present work introduces simple, robust, and environmentally benign UV methods. Two separate spectrophotometric methods were developed for the estimation of Silybin and Curcumin, where solvent, scan speed, and sampling interval are the estimated critical parameters. The detection was carried out at absorption maxima of 288nm for Silybin and 419nm for Curcumin with ethanol. To determine the critical method variables, a risk assessment was carried out using an Ishikawa diagram. Developed spectrophotometric methods were validated according to the International Council for Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use Q2 (R1) guidelines. The proposed methods showed good predictability and robustness. The new methodologies were found to be green according to the analytical greenness metric approach and software, the green analytical procedure index, and analytical eco-scale tools in comparison to the existing methods.

Keywords: Analytical Eco-scale, Analytical Greenness Metrics, Analytical Quality by Design, Curcumin, Green Analytical Procedure Index, Silybin

1. Introduction

Research on antioxidants has been a topic of enduring interest for decades. Antioxidants are stable molecules with many physiological benefits for humans. They limit oxygen consumption by free radicals by supplying an electron and shielding cells from free radical invasion by chain breaking, quenching chain initiating catalysts, and free radical scavenging mechanisms[1]. The use of antioxidants as dietary supplements aids in promoting good health. Several studies suggested their detailed mechanisms and addressed the potential benefits of both natural and synthetic antioxidants in medical, food, cosmetic, and therapeutic fields[2-4]. Their use in adjuvant treatment to reduce chemo and radiation therapies' adverse effects has gained significant prominence[5,6]. In the present study, three proven antioxidants were selected.

Milk thistle *(Silybum marianum)*, of the Asteraceae family, is an ancient polyphenolic plant flavonoid well known for its hepatoprotective, antioxidant, anti-inflammatory, neuroprotective, and antiviral properties. It consists of a major constituent known as

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silymarin, expressed as Silybin (SIL), which constitutes approximately 70-80 %. SIL is chemically (2R, 3R) - 3, 5, 7-trihydroxy-2- [(2R, 3R)-3-(4-hydroxy-3-methoxyphenyl)-2-(hydroxymethyl)-2,3-dihydro-1,4-benzodioxin-6-yl]-2,3-dihydrochromen-4-one. SIL exists as diastereomers silybin A and B (Figures 1a and 1b)[7-9]. A literature survey revealed that there have been several UV, HPLC, HPTLC, and LCMS methods reported[10].

Turmeric (*Curcuma longa)*, of the Zingiberaceae family, has been an Indian golden herb since ancient times. It contains Curcumin (CUR) as a major component along with other curcuminoids like demethoxycurcumin and bisdemethoxycurcumin. It has been a research topic of interest since ancient times to date because of its numerous benefits, like anticarcinogenic, antioxidant, anti-inflammatory properties etc[11]. CUR is chemically (1E, 6E) -1, 7-bis (4-hydroxy-3-methoxyphenyl) hepta-1, 6-diene-3, 5-Dione. It exists in enol and keto forms (Figures 1c and 1d)[12]. Total curcuminoids consist of not less than 90% in marketed dietary supplements, of which the majority is curcumin (77%) followed by demethoxycurcumin (19%) and bisdemethoxycurcumin (3%). Interestingly, the composition of the Curcuminoids varies based on the *Curcuma species*, cultivated land and region[13-15]. A literature survey revealed that there have been several UV, HPLC, HPTLC, and LCMS methods reported in the review article[16].

ICH Harmonised Tripartite guideline definition for Quality by design is a systematic approach to development that begins with predefined objectives and emphasizes product and process understanding and process control, based on sound science and quality risk management. When this concept is applied to analytical method development, it is termed Analytical Quality by Design (AQbD). By utilising the concept of AQbD, analysts can develop a robust method, minimize time and expense in studying the effect of numerous variables on the response at once using the Design of Experiments (DoE), and achieve regulatory flexibility by functioning inside the design space which is not possible in a conventional approach[17,18].

Green Analytical Chemistry (GAC) is a paradigm that has been very much focused on in recent decades as a result of the continuous efforts of the scientific community to advance in the development of processes and products that are more environmentally friendly. Several reviews describing the GAC principles and their application using alternative less hazardous solvents than toxic solvents have been published[19-21]. In short, the main aim of GAC is to avoid the use of toxic solvents and chemicals for a better and more sustainable future.

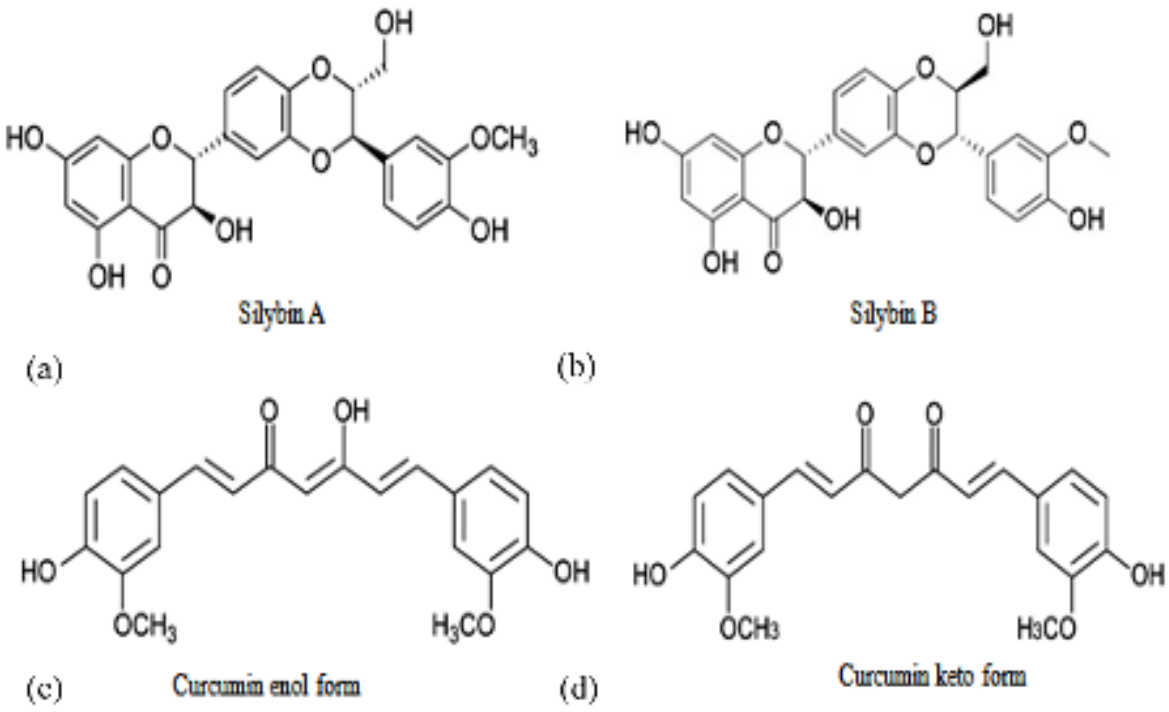


Figure 1. Chemical structures of (a) SIL A and (b) SIL B; (c) CUR enol form and (d) CUR keto form.

As mentioned earlier, the literature survey revealed that there have been several methods reported for the selected antioxidants, individually or in one or more combinations. Further toxic solvents like acetonitrile, methanol, etc., were utilized in all of these approaches. Very few methods for estimating silymarin and curcumin alone by UV were published, and ethanol (EtOH) in conjunction with hazardous solvents was reported[22,23]. In recent years, a few methods involving ethyl acetate, phosphate buffer, and tween 80[15,24,25] have been reported. Although these solvents are considered environmentally benign, their inability to biodegrade or be recycled remains a limitation, and the greenness of the methods was not assessed. Hence, the present goal of this work is to establish simple, precise, accurate, sensitive, inexpensive, eco-friendly UV methods using green solvents with the integration of the AQbD approach, aiming for a highly sustainable, easily adaptable routine analysis with the least possible wastage.

2. Materials and Methods

2.1 Materials

Pure SIL (>98%), and and CUR (>97%) reference standards were purchased from TCI Chemicals, Chennai, India. Silybon® tablets (140mg of silybin), Healthvit Curcumin capsules of 475mg (for UV studies) were procured from the local market. All the solvents used for analysis were of analytical grade, EtOH of premium grade (Hayman Group Ltd. East Ways Park Witham, UK), and Propylene Carbonate (PC) provided by Sisco Research Laboratories Pvt. Ltd.

2.2 Instrumentation, Apparatus and Software

Spectrophotometric scanning was carried out using a UV spectrophotometer (LAB INDIA UV 3092) double beam (Maharashtra, India) with 1.00cm quartz cells using UV–Win system software v5.2.0.1104. Digital Ultrasonic cleaner (LABMAN), Digital electronic balance (Shimadzu, Japan) and Whatman filter papers were used. DoE trailed version Design-Expert® 12 (Stat-Ease Inc., Minneapolis-USA) was used for AQbD design.

2.3 UV Method -1 Spectrophotometric Method for Estimation of SIL

2.3.1 Preparation of Standard and Working Solutions

Two separate standard solutions of SIL (100µg/mL) were prepared in EtOH and PC. Working standard solutions of SIL (10µg/mL) in EtOH and PC were obtained by appropriate dilutions. The working solutions were scanned individually over 200 to 800 nm against the respective diluents as blanks and further used for implementing the AQbD concept through DoE software.

2.4 UV Method -2 Spectrophotometric Method for Estimation of CUR

2.4.1 Preparation of Standard Solutions and Working Solutions

Two Stock solutions (100µg/mL) of CUR were prepared, dissolving 10mg each in two 100mL volumetric flasks with EtOH and PC as diluents. Working solutions of 5µg/mL CUR in EtOH and 5µg/mL CUR in PC were prepared freshly by dissolving 0.5mL to 10mL from the respective two stock solutions and were scanned individually over 200 to 800 nm against the respective diluents as blank and further used for implementing the AQbD concept through DoE software.

2.5 Design of Experiment for Both the UV Methods

Based on risk assessment by the Ishikawa diagram (Figure 2), factors like a solvent, scan speed, and sampling interval are the Critical Method Variables (CMVs) that directly impact both UV spectrophotometric methods' performance[26]. Hence, a suitable experimental design of a three-factor fractional factorial Central Composite Design (CCD) was implemented for the aforesaid variables for the study and optimisation.

3. Results and Discussion

Environmentally friendly analytical methods have gained significant attention from both pharma companies and academic researchers. Consequently, our main objective is to develop simple, accurate, and effective procedures employing non-hazardous solvents. Hence, the solvents utilised for the UV studies were EtOH, PC, and water, which are eco-friendly and categorized under green solvents[20,21].

3.1 Method Development for UV Studies

3.1.1 Selection of Wavelengths

SIL and CUR are indeed completely soluble in EtOH and PC solvents. Hence, both solvents were selected for the study from the perspective of sustainability to obtain UV spectrum in the range of 200-800 nm individually. The

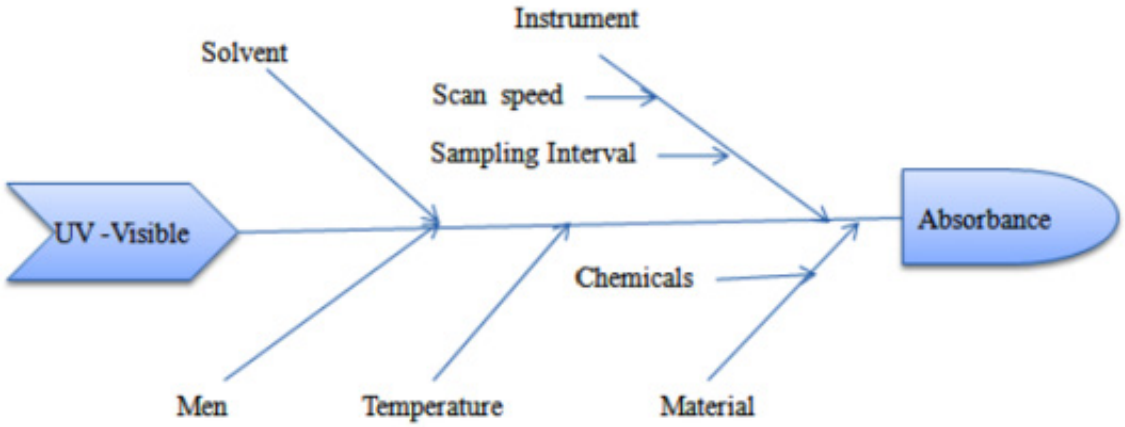


Figure 2. Ishikawa diagram of risk assessment for UV methods.

UV spectra of SIL in EtOH and PC (Figures 3a and 3b) exhibited maximum absorbance (λ_{max}) at 288nm, while the λ_{max} for CUR in EtOH and PC (Figures 3c and 3d) was observed at 419nm.

3.1.2 Central Composite Design for SIL

The AQbD methodology was started by identifying the CMVs that have a substantial impact on the Critical Analytical Attribute (CAA) i.e., response, and those were taken into consideration for optimization using the CCD for robustness. Solvent, scan speed, and sampling interval are recognised as CMVs that ultimately amend the absorbance (i.e., CAA) in both the UV methods. The selected factors and their coding are given in Table 1.

Twenty-six experiments for SIL were performed practically with minimal ten centre points according to face-centred CCD for an unbiased response, as shown in Table 2.

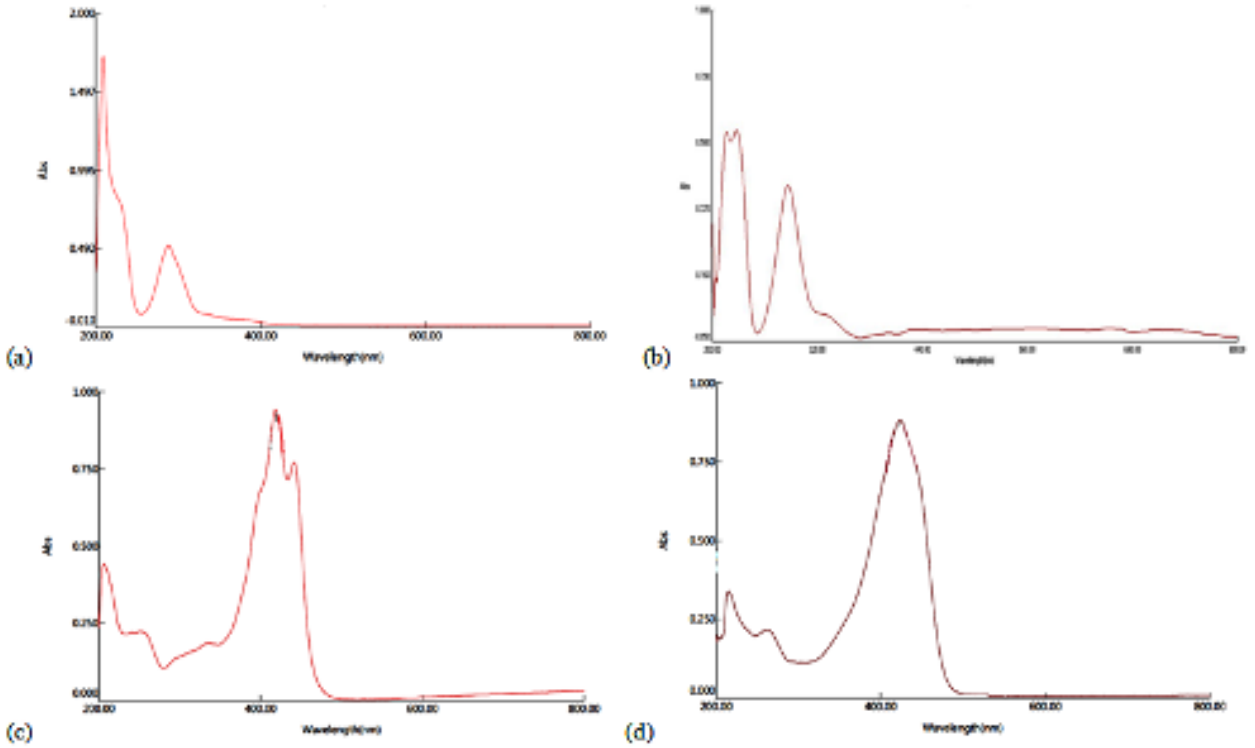


Figure 3. Absorbance maxima of (a) SIL in EtOH, (b) SIL in PC and Absorbance maxima of (c) CUR in EtOH, (d) CUR in PC.

Table 1. Factors and their coded values

Coded value	Scan speed	Sampling interval
-1 (Low)	Slow	0.5
0 (Medium)	Medium	1.0
1 (High)	Fast	2.0

Table 2. CCD experimental design for SIL

S. No.	Scan speed	Sampling interval	Solvent	Absorbance
1	1	0	EtOH	0.543
2	0	0	EtOH	0.527
3	0	0	PC	0.441
4	-1	1	EtOH	0.564
5	-1	-1	PC	0.422
6	-1	0	EtOH	0.559
7	1	1	EtOH	0.544
8	1	-1	PC	0.435
9	0	1	EtOH	0.567
10	-1	0	PC	0.431
11	-1	-1	EtOH	0.557
12	0	-1	PC	0.427
13	-1	1	PC	0.437
14	0	-1	EtOH	0.552
15	0	0	PC	0.422
16	0	0	PC	0.442
17	1	1	PC	0.424
18	0	1	PC	0.442
19	1	-1	EtOH	0.548
20	0	0	EtOH	0.532
21	0	0	EtOH	0.530
22	0	0	PC	0.441
23	0	0	EtOH	0.534
24	0	0	EtOH	0.536
25	0	0	PC	0.426
26	1	0	PC	0.445

Using DoE expert® trial version 12 software, the obtained data were analysed with an appropriate mathematical model. Model fitness was assessed as per ANOVA with a significant $p<0.05$. as shown in Table 3. Where the *F*-value is significant with a *p*-value <0.0001. Adjusted r^2 and predicted r^2 are in good agreement (<0.2). An adequate precision ratio of 21.489 indicates adequate signal, which indicates that the model can be used to navigate the design space. The equation in terms of coded factors is used to predict the response for the given values, which defines the relative impact of the factors. The final equation in coded form is, Absorbance = 0.49-2.583E-003 × A+3.083E-003 × B+0.056 × C-4.625E-003 × AB-4.917E-003 × AC-8.333E-005 × BC+3.181E-003 × A^2 × B^2 +5.681E-003. (1)

The residual versus predicted response plot is useful to examine any outliners of any observation outside the red line. There was no outlier for the model where the variables were within -3.7 to +3.7.

Perturbation, 2D contour plot, and 3D response surface plot of SIL in EtOH and Perturbation plots, 2D contour plot, and 3D response surface plots of SIL in PC explained the correlation between the variables and response, (Figure 4a-f). In the perturbation plot (Figure 4a) for solvent EtOH, there was a gradual decrease in the absorbance value as the scan speed increased, and a steady increase was observed with an increase in the sampling interval. 2D contour plot and 3D response plot (Figures 4b, 4c) also confirmed the same. Whereas in solvent PC (Figures 4d, 4e, and 4f) the absorbance is not much affected by scan speed but a rapid increase is observed with an increase in sampling interval.

3.1.3 Optimisation and Design Space for SIL

From the aforesaid analysis, the method was optimised by specifying the criteria in numerical form under the goal of the software to create the ideal conditions for the experiment. The default goals are in range for factors and none for response. The main objective was to get the maximum response within the coded values. Hence, the goal for absorbance was maximised, and projected solutions were observed.

Table 3. ANOVA summary of the model for SIL

Response	Drug	*F* value	*p*-value	r^2	Adjusted r^2	Predicted r^2	Adequate Precision	Std. dev.	C.V.%
Absorbance	SIL	87.60	<0.0001	0.9763	0.9652	0.9361	21.489	0.011	2.22

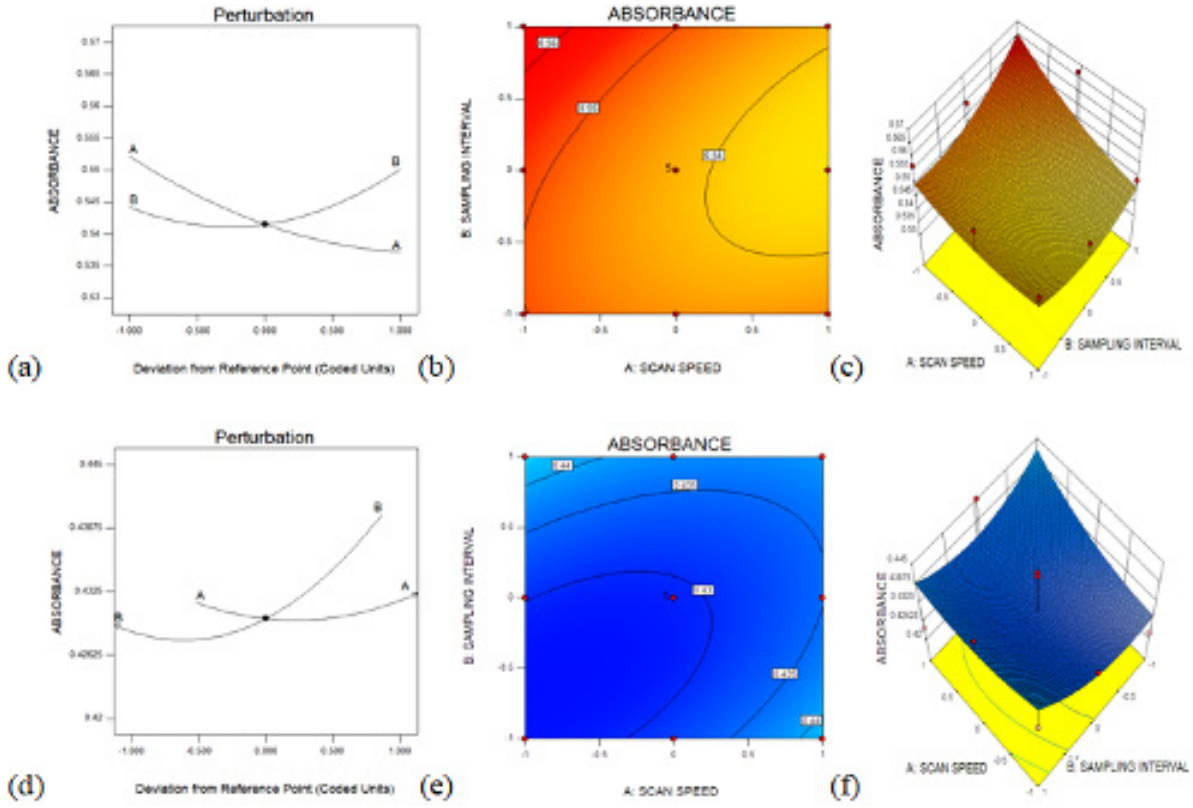


Figure 4. (a) Perturbation, (b) 2D contour plot, and (c) 3D response surface plot of SIL in EtOH and (d) Perturbation, (e) 2D contour plot and (f) 3D response surface plot of SIL in PC respectively.

From the optimised conditions, with the help of Derringer's desirability function, it was evident that at scan speed low, a sampling interval of 2.0, and solvent EtOH, the response, i.e., absorbance, is maximum with desirability of 0.990 (Figure 5a). The overlay plot (Figure 5b) showed the method operable region within which any slight change can be acceptable without having a significant effect on the response. Within the design space, the method was performed practically by selecting one of the predicted solutions suggested by the software, and the achieved results are in good agreement.

3.1.4 Final Optimal Conditions for SIL

From the 3D response surface plots, the preferable solvent for SIL was EtOH. Low scan speed and 2.0 sampling interval yielded the maximum response for SIL.

3.2 Central Composite Design for CUR

The selected factors and the coded values are represented in Table 1. Twenty-six experiments for CUR were performed practically with minimal ten centre points according to face-centred CCD for an unbiased response as shown in Table 4.

In the case of CUR, after performing the twenty-six runs as per the design matrix, thirteen each with EtOH and PC at different scan speeds and sampling intervals, the ANOVA summary was tabulated in Table 5, where the model fitness was significant, adjusted and predicted r^2 were in reasonable agreement (<0.2) and adequate precision greater than 4 (11.076) indicated adequate signal such that the model can be used to navigate the design space. The equation for coded factors is as follows:

$$\text{Absorbance} = 0.92 + 2.333E\text{-}003 \times A - 2.333E\text{-}003 \times B + 0.039 \times C - 2.875E\text{-}003 \times AB + 1.000E\text{-}003 \times AC - 4.833E\text{-}005 \times BC - 6.328E\text{-}003 \times A^2 + 0.012 \times B^2 \quad (2)$$

There were no outliers observed in the residual versus predicted plots, where all the observations are within +3.07 to -3.07, indicating they fitted well in the model.

It was observed from perturbation, 2D contour, and 3D response plots that with EtOH the absorbance increased with an increase in scan speed and a steep decrease with

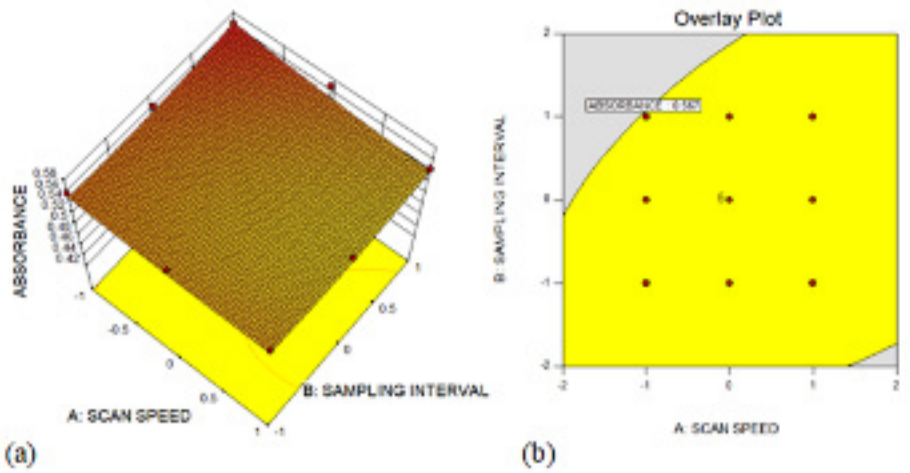


Figure 5. (a) Optimised 3D response plot and (b) Overlay plot of design space for SIL.

Table 4. CCD experimental design for CUR

S. No.	Scan speed	Sampling interval	Solvent	Absorbance
1	1	0	PC	0.877
2	0	0	EtOH	0.929
3	-1	0	EtOH	0.937
4	0	0	EtOH	0.944
5	0	0	PC	0.88
6	0	0	PC	0.876
7	-1	-1	EtOH	0.965
8	0	1	PC	0.88
9	-1	-1	PC	0.883
10	1	1	EtOH	0.953
11	-1	1	PC	0.891
12	0	0	EtOH	0.961
13	1	1	PC	0.902
14	0	0	PC	0.884
15	0	0	PC	0.906
16	1	0	EtOH	0.964
17	0	1	EtOH	0.965
18	0	0	EtOH	0.971
19	-1	0	PC	0.879
20	0	0	PC	0.885
21	-1	1	EtOH	0.974
22	1	-1	EtOH	0.979
23	0	-1	EtOH	0.991
24	0	-1	PC	0.893
25	0	0	EtOH	0.995
26	1	-1	PC	0.882

an increase in sampling interval (Figures 6a, 6b, and 6c). Whereas, with the solvent propylene carbonate, the absorbance was not significantly affected by scan speed but increased exponentially with an increase in sampling interval (Figures 6d, 6e, and 6f).

3.2.1 Optimisation and Design Space for CUR

Since the main objective is to attain maximum absorbance with the design, the goals were set accordingly. Keeping sampling interval within range, Scan speed and absorbance criteria were maximized and solutions were obtained by Derringer's desirability function. The optimized 3D response plot (Figure 7a) analysed that at maximum scan speed with a sampling interval of 0.5 and EtOH as a solvent, a higher desirability of 0.935 can be attained with maximum absorbance. The overlay plot (Figure 7b) has given the method operable region within which the optimized conditions were fixed and performed in practice where the results were in good agreement.

3.2.2 Final Optimal Conditions for CUR

From the 3D response surface plots, the preferable solvent for CUR was EtOH. The instrumental parameters of fast scan speed and 0.5 sampling interval yielded the maximum response.

4. Method Validation for the Developed Duo UV Methods

After development, the optimized spectroscopic methods were validated as per the ICH Q2 (R1) guidelines[27].

Table 5. ANOVA summary of the model for CUR

Response	*F* value	*p*-value	r^2	Adjusted r^2	Predicted r^2	Adequate Precision	Std. dev.	C.V.%
Absorbance	19.80	<0.0001	0.9031	0.8575	0.7989	11.076	0.016	1.73

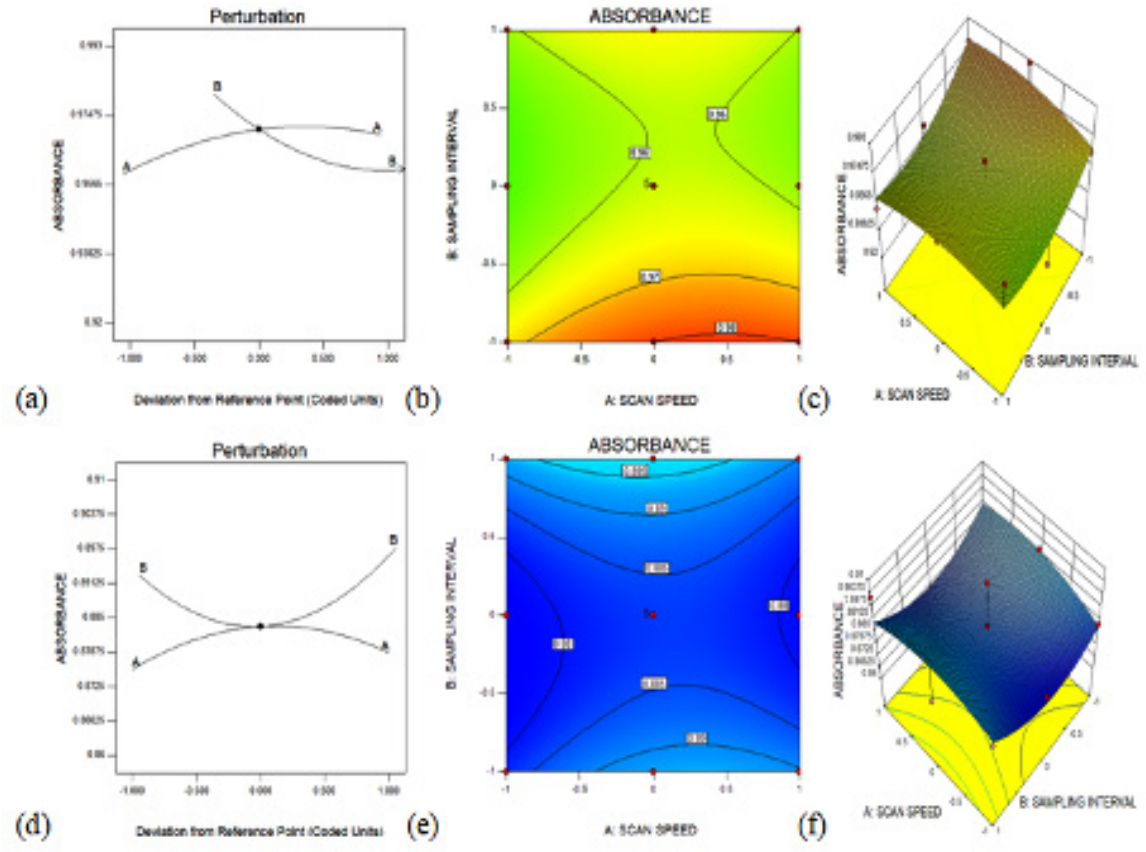


Figure 6. (a) Perturbation, (b) 2D contour plot and (c) 3D response surface plot for CUR in EtOH; and (d) Perturbation, (e) 2D contour plot and (f) 3D response surface plot for CUR in PC respectively.

4.1 Linearity

The linearity of the methods was established by preparing and analysing six different concentrations prepared from the respective standard stock solutions in the range of 7-13 µg/mL for SIL and 2.5-6.5 µg/mL for CUR. Calibration curves (Figures 8a and 8b) were plotted for each method as shown in the overlay spectra (Figures 8c and 8d) with concentration vs. absorbance. A linear response was observed, and regression analysis data are given in Table 6.

4.2 Limit of Detection (LOD) and Limit of Quantification (LOQ)

LOD and LOQ were calculated for both spectrophotometric methods to detect the lowest concentrations of SIL and CUR that can be detected and quantified, respectively, by the standard deviation approach, where the y-intercept (σ) and slope (S) of the calibration curve values are used in the following equations:

$$LOD = 3.3 \times \sigma \div S \quad (3)$$

$$LOQ = 10 \times \sigma \div S \quad (4)$$

LOD and LOQ values for SIL and CUR are listed in Table 6, and the values indicate the sensitivity of the proposed methods.

4.3 Precision

System precision, intra-day and inter-day precision were evaluated by analysing at 100% test concentration for both methods and expressed in terms of Relative Standard Deviation (RSD). The findings were less

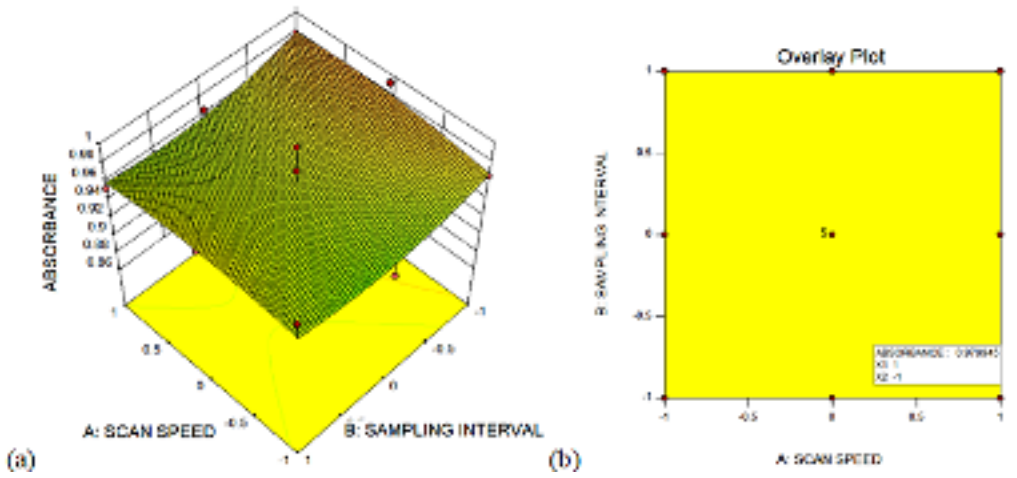


Figure 7. (a) Optimised 3D response plot and (b) Overlay plot of design space for CUR**.**

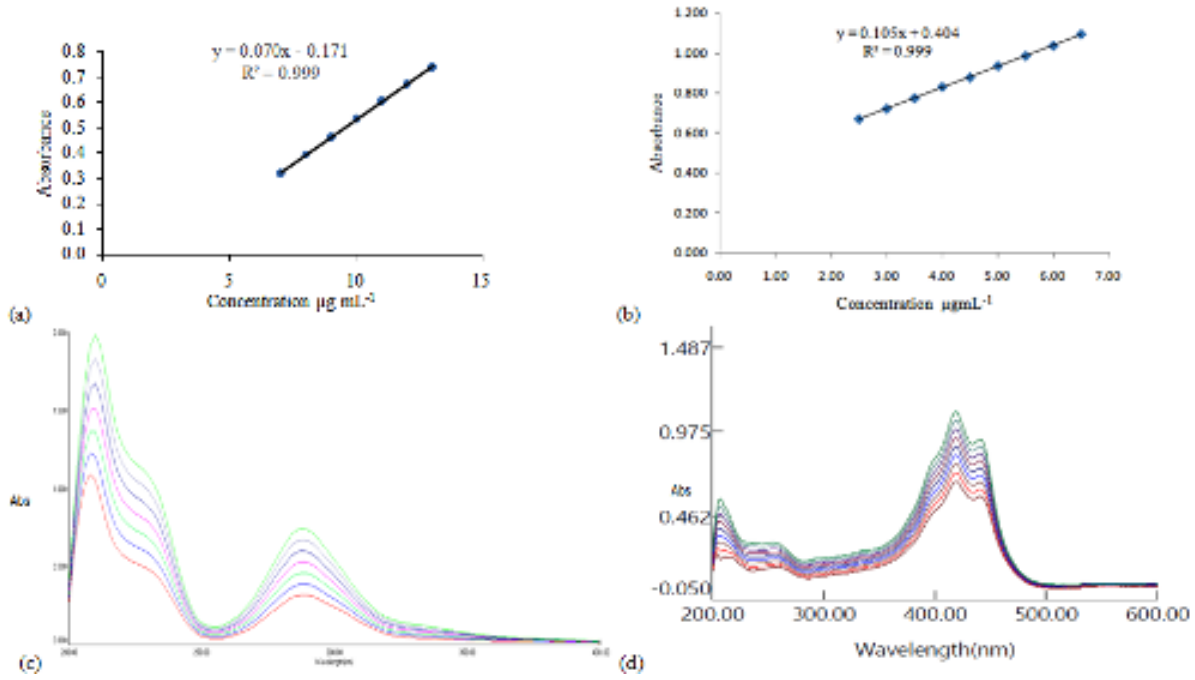


Figure 8. (a) Calibration curve of SIL, (b) Calibration curve of CUR, (c) Zero order overlay absorption spectrum of 10µg/mL SIL and (d) Zero order overlay absorption spectrum of 5µg/mL CUR.

than 1.5% as shown in Table 6 (SIL and CUR), showing that the precision was in good agreement for both methods.

4.4 Accuracy

The consistency of the developed method was confirmed by its accuracy. The percentage recovery for SIL (80%, 100%, and 120%) and CUR (50%, 80%, 100%, and 120%) was in the range of 98.54 - 99.23 % and 98.50-100.50 % respectively, by the standard addition method at all three levels. Samples were prepared in triplicate at each level, and the mean percentage recovery was calculated as indicated in Table 6.

4.5 Stability

The samples prepared for repeatability for both SIL and CUR were tested at room temperature 25±3 ºC for 0, 6, and 12hr (short term). The stability was measured by % assay value with that of freshly prepared standard solutions, and the results in terms of % RSD as shown

in Table 6 were found to be within the acceptance range (< 2%) indicating that samples are stable for 12hrs.

4.6 Assay for SIL Formulation

The applicability of the developed method was used to determine the SIL of commercially available tablets under optimized conditions. Ten tablets containing SIL were weighed and finely powdered in a mortar. A quantity of powder equivalent to 10mg of SIL was weighed accurately and dissolved in 100 mL of EtOH. The contents of the flask were sonicated for 15mins. to ensure complete dissolution. Suitable aliquots of the filtered liquid were used for appropriate dilutions (10µg/mL) for analysis. The results are summarized in Table 7.

Table 6. Validation parameters and results for the developed UV spectrophotometric methods

Parameter	SIL	CUR
Wavelength (nm)	288	419
Linearity (µg/mL)	7-13	2.5-6.5
Slope	0.0704	0.106
The standard deviation of the slope	0.0012	0.004
Confidence limit of the slope 95%	0.0704	0.1060
Intercept	0.1719	0.403
The standard deviation of Intercept	0.0088	0.0094
Confidence limit of the Intercept	0.1719	0.4025
Regression coefficient (r^2)	0.9998	0.999
LOD (µg/mL)	0.34	0.33
LOQ (µg/mL)	1.03	1.01
System precision (%RSD)	0.53	0.28
Confidence limit of system precision	0.002	0.002
Intraday precision (%RSD)	0.51	0.56
Confidence limit of Intra-day precision	0.4050	0.4409
Interday precision[c] (%RSD)	0.63	0.50
Confidence limit of Inter-day precision	0.3709	0.2204
Accuracy[d] (% w/w)	98.54–99.23	98.50–100.50
Confidence Limit of Accuracy	0.571	0.617
Solution Stability (%RSD)	0.38	1.32

[a] mean of six replicates, [b] mean of six determinations, [c] mean of 18 findings in three consecutive days, [d] mean of three findings at each level.

Table 7. Assay of SIL formulation

Tablet	Label claim (mg)	Amount found* (mg)	Recovery* (%)
Silybin	140	138.64	99.03

*Average of three determinations

4.7 Assay for CUR Formulation

10 mg of the commercial formulation of Healthvit curcumin capsule powder was dissolved in 100mL of EtOH and sonicated for 15mins to provide a stock solution for the evaluation of the established technique for CUR. The homogenised solution was filtered and used for appropriate dilutions to obtain a final test concentration of 5µg/mL and estimated the percentage of curcumin present by the absorbance ratio method and was found to be by the literature, as shown in Table 8.

Furthermore, the proposed method and reported methods for SIL and CUR are compared by taking advantage of the statistical *t*-test Table 9 and *F*- test Table 10. Both tests resulted in no significant variation for either drug.

5. Greenness Assessment of the Proposed Methods

Three green tools, namely Analytical Eco-Scale Assessment (ESA), Analytical GREEnness metric (AGREE), and Green Analytical Procedure Index (GAPI), were employed in assessing the greenness of the established spectrophotometric and chromatographic methods. Each tool is unique in its way when assessing the greenness profile.

ESA is a semi-quantitative approach where the computation of total Penalty Points (PP) is done based on the equation below, considering parameters like chemicals used and their quantities, waste generated, occupational hazards, energy consumed, etc. A method is considered ideal when its total value is > 75, acceptable when > 50, and inadequate if <50.

Analytical Eco scale = 100 – the sum of PP[28]

Table 8. Assay of CUR formulation

Capsule*	Amount tested (µg/mL)	Amount found (µg/mL)	Mean % recovery*
Healthvit	5	3.80	76.07

* Label claim turmeric rhizome extract 95%

Table 9. Student's *t*-Test: two-sample assuming equal variances

	SIL		CUR	
	Proposed	**Reported**	**Proposed**	**Reported**
	Variable 1	*Variable 2*	*Variable 1*	*Variable 2*
Mean	98.69524	98.57143	76.06357	76.01228
Variance	0.275986	0.510204	0.083008	0.056263
Observations	3	3	3	3
Pooled Variance	0.393095		0.069636	
Hypothesized Mean Difference	0		0	
Df	4		4	
t Stat	0.241853		0.238026	
P(T<=t) one-tail	0.410394		0.411778	
t Critical one-tail	2.131847		2.131847	
P(T<=t) two-tail	0.820788		0.823557	
t Critical two-tail	2.776445		2.776445	

Table 10. *F*-Test two-sample for variances

	SIL		CUR	
	Reported	**Proposed**	**Reported**	**Proposed**
	Variable 1	*Variable 2*	*Variable 1*	*Variable 2*
Mean	98.57	98.69	76.06	76.04
Variance	0.510	0.275	0.009	0.002
	Variable 1	*Variable 2*	*Variable 1*	*Variable 2*
Observations	3	3	3	3
Df	2	2	2	2
F	1.84		3.62	
P(F<=f) one-tail	0.35		0.21	
F Critical one-tail	19		19	

The eco scale score of the developed methods and reported methods are given in Table 11.

AGREE metrics is a software-based calculator representing all the 12 principles (a clock-like graph) of green analytical chemistry on a unified 0-1 scale shown as a pictogram with red, yellow, and green colours based on the method's performance. It is freely downloadable software, easy to operate, and aids fast analysis. A greener approach is indicated by a score that is closer to 1[29].

The AGREE metrics score of the developed methods and reported are given in Table 11.

GAPI is a new tool, more sophisticated than the ones previously discussed for determining the greenness of a method. From sample collection to final determination, the entire analytical method is represented as five pentagrams in different colours green (low), yellow (medium), and red (high) based on the impact on the environment[30].

GAPI for the proposed methods and reported methods are in Table 11.

6. Conclusion

The proposed work accomplished two spectrophotometric methods for the independent determination of SIL and CUR and one chromatographic method for the simultaneous estimation of SIL and CUR in their respective pharmaceutical dosage forms. Moreover, the proposed methods are advantageous owing to their greenness profile being eco-friendly and efficient in eliminating hazardous solvents. Also, the greenness of all the methods is assessed

Table 11. Comparison of greenness assessment between reported and proposed methods

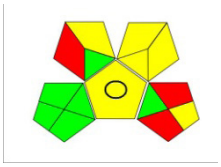
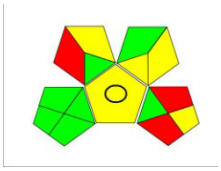
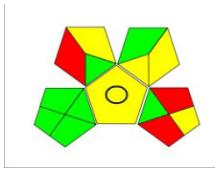
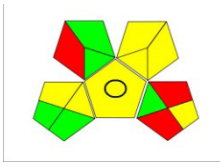
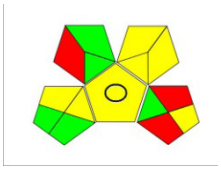
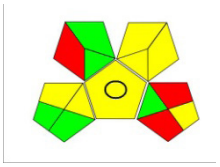
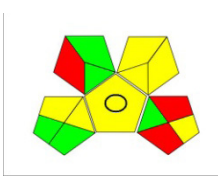
S. No.	Solvents used	Greenness assessment			Ref
		ESA	AGREE metrics	GAPI	
SIL UV					
1	Methanol Chloroform Sodium phosphate Waste	77			31
2	Methanol waste	88			32
3	Methanol Chloroform waste	81			33
4	Method 1: Methanol 3-Methyl Benzthiazolinone - 2 - Hydrozone (MBTH) & Cerric Ammonium Sulphate (CAS) H_2SO_4 Waste	78			34
	Method 2: $FeCl_3$, and 2,2-Bipyridine Waste	89			
5	Potassium permanganate, Methanol Chloroform waste	75			35
6	Method 1 Methanol $FeCl_3$ 1,10 phenanthroline waste	82			36

Table 11 *Continued*

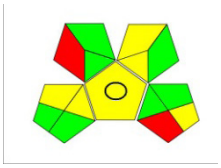
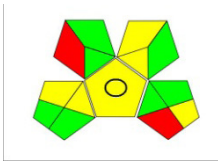
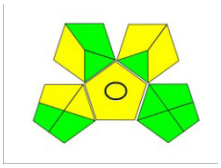
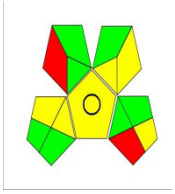
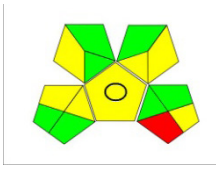
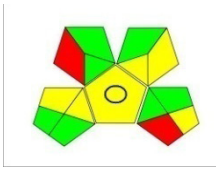
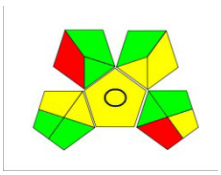
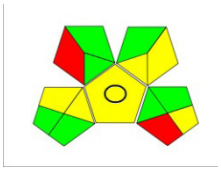
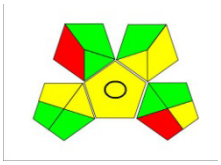
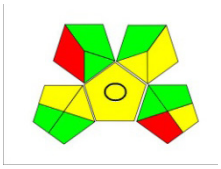
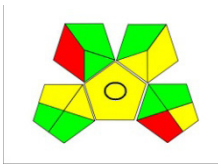
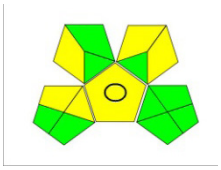
S. No.	Solvents used	Greenness assessment			Ref
		ESA	AGREE metrics	GAPI	
	Method 2 Methanol $FeCl_3$ Folin- Ciocalteu reagent (a mixture of phosphomolybdate and phosphotungstate) 1,10 phenanthroline Waste	64			
7	Ethanol Diazotised Sulphanilic acid Hcl Sodium carbonate Sodium nitrite waste	81			22
8	Ethanol Waste	93			Proposed method
CUR UV					
1	Methanol 6.4 pH phosphate buffer with 1.5% polysorbate 80	80			37
2	Tween 80 at 1 % waste	97			38
3	Methanol waste	88			39
4	Methanol waste	88			40

Table 11 *Continued*

S. No.	Solvents used	Greenness assessment			Ref
		ESA	AGREE metrics	GAPI	
5	Methanol waste	88			41
6	Ethyl acetate waste	93			15
7	Methanol waste	88			42
8	Methanol waste	88			43
9	Ethanol waste	93			Proposed method

using techniques like AGREE, ESA, and GAPI to indicate their highest greenness in comparison to the reported methods. All the developed methods were validated following ICH Q2(R1) guidelines for application in quality control laboratories for routine analysis.

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