IJBPAS, June, 2025, 14(6): 3608-3619

ISSN: 2277–4998

International Journal of Biology, Pharmacy and Allied Sciences (IJBPAS)

'A Bridge Between Laboratory and Reader'

www.ijbpas.com

GREEN ANALYTICAL PRINCIPLES INTEGRATED ANALYTICAL QUALITY BY DESIGN METHOD FOR SIMULTANEOUS ESTIMATION OF PTEROSTILBENE AND RESVERATROL

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Received 2nd Sept. 2024; Revised 19th Dec. 2024; Accepted 1st May 2025; Available online 1st June 2025

https://doi.org/10.31032/IJBPAS/2025/14.6.9694

ABSTRACT

The QbD methodology has been used to establish a simple, rapid, precise, sensitive, and accurate Reverse Phase High Performance Liquid Chromatography (RP-HPLC) approach for the quantitative measurement of Resveratrol and Pterostilbene in pharmaceutical product. With the use of Design Expert Software, a central composite design was chosen in order to achieve the desired optimum conditions. Ideal chromatographic conditions were obtained on the Waters Alliance-e2695 by using Inertsil ODS 3V (250x 4.6mm, 5µm) column and the mobile phase containing Acetonitrile: 0.1%, Formic Acid in the ratio of 18.9:81.1% v/v at a flow rate of 1.202 ml/min. Pterostilbene and Resveratrol were detected at 273nm using a photodiode array detector with a 10- minutes run time. Pterostilbene and Resveratrol were found to have retention durations of 2.968 and 4.659, respectively. The resolution of 7.57 was obtained. Studies on forced degradation were carried out. All parameters were confirmed to be within acceptable ranges after the suggested approach was validated in accordance with ICH criteria. AGREE software was employed to assess the greenness of the proposed method. The approach developed for the quantitative analysis of Pterostilbene and Resveratrol was found to be simple, eco- friendly, precise, accurate, and robust.

Keywords: Pterostilbene, Resveratrol, RP-HPLC, QbD, AGREE

INTRODUCTION:

Pterostilbene, a stilbenoid, has anti-inflammatory, pro-apoptotic, antioxidant, antineoplastic, and cytoprotective properties. It scavenges reactive oxygen species, boosts antioxidant enzyme production. It inhibits or prevents activation of carcinogenic signaling pathways, and causes tumor cells to undergo apoptosis directly **[1]**. Resveratrol is a stilbene derivative, produced by plants using stilbene synthase. It has two isomers: trans-(E) and cis-(Z). When heated or exposed to UV light, trans-resveratrol can isomerize into the cis-form. Red grapes contain high amounts of plant polyphenol Resveratrol, which has been suggested to treat hyperlipidemia, prevent fatty liver, diabetes, atherosclerosis, and aging **[2]**.

The US FDA promotes risk-based strategies and QbD concepts in development, manufacturing, and regulation of pharmaceutical products. The design space, critical material attributes (CMAs), critical product points (CPPs), and optimal conditions are identified through the application of the DoE tool in process development or formulation **[11]**. Green Analytical Chemistry (GAC) mainly focuses on 12 principles to assess the analytical method focusing on environmental and human safety **[16]**. Pterostilbene and Resveratrol in combination is used as dietary supplement, acts as neuroprotective. According to literature till date, there was no method development for Pterostilbene and Resveratrol in combined capsule dosage form. UV spectroscopic and RP-HPLC methods for the development of Pterostilbene and resveratrol individually in dosage forms or in plant extracts has been reported **[3-10]**. Resveratrol was developed simultaneously with other drugs **[12-13]**. So, there is a need for the development of Pterostilbene and Resveratrol simultaneously in combination form for routine analysis by employing QbD approach. Hence, the present study was developed and validated by QbD for Pterostilbene and Resveratrol in combined capsule dosage form.

MATERIALS AND METHODS:

Instrumentation

The HPLC system with PDA detector of the Alliance model (Waters e 2695-Empower software 2.0 versions). Design expert software with 13.0 version was used for method optimization. AGREE software (V. 0.5 beta version) was employed to assess the method greenness. Every chemical and reagent that acquired was of HPLC quality. Acetonitrile and methanol were procured from Rankem laboratories.

Standard Solution Preparation

Weighed separately, Pterostilbene (6 mg) and Resveratrol (60 mg) were put to a 10 ml volumetric flask along with diluent. After the components were dissolved by sonication, the mixture was reconstituted using the same solvent to reach the desired volume. Pipetting a milliliter of the stock solutions into a 10-mililiter flask, the milliliter was diluted to the proper concentration using diluent (600ppm of Resveratrol and 60ppm of Pterostilbene) to the appropriate level.

Sample Solution Preparation

A 10 mL volumetric flask containing78 mg of weighed Pterostilbene and Resveratrol sample was subjected to dilution with diluent and sonication for duration of 30 minutes. After that, surplus volume was eliminated by centrifuging the leftover solvent for thirty minutes. An injection filter with a 0.4- micro opening was then used to filter the stock solution.

Diluent- Methanol

QbD based Method Development

Design expert software (version 13) was employed by RSM. Central Composite Design (CCD) was used to optimize chromatographic conditions. Two factors i.e (A) Flow rate, (B) Mobile phase ratio and four responses such as retention time of peak 1 (R1), resolution (R2), plate count of peak 1 (R3), retention time of peak 2 (R4) has been chosen. High and low coded levels were identified. 13 experimental runs were generated. The developed method was optimized by Response Surface Methodology (RSM).

Method Validation

The suggested method was validated in accordance with ICH recommendations.

Degradation Studies

The degradation studies have been performed to selected sample, the stress conditions that are performed includes acidic, alkaline, hydrolysis, reduction, oxidation, thermal, and photolytic.

Greenness Assessment

The developed HPLC method's greenness was assessed using the Analytical Greenness Calculator (V. 0.5 beta version). The 12 GAC principles served as the basis for evaluating greenness. These principles' evaluations were categorized on a 0–1 scale. A pictogram has been used to represent the outcome. The center of the pictogram represents the total score. A scale of 1 denotes green, a value of 0.6 denotes yellow, and a scale of 0 denotes red.

RESULTS AND DISCUSSION:

QbD

Based on preliminary studies, factors and responses were chosen in Design Expert

software as shown in **Table 1**. 13 experimental runs were shown by incorporating low and high coded values of factors into the software. Polynomial equations and ANOVA were applied to validate the responses. Because the quadratic model had the greatest least square regression coefficients for each of the four responses, the design expert program chose it above the other models (R1, R2, R3, and R4). The answers R1, R2, R3, and R4 have p-values less than 0.2, according to an ANOVA. The model is therefore important. Lack of fit had no measurable impact. The 13 experimental runs given by QbD software was shown in **Table 2**.

Plot perturbation (as shown in **Figure- 4i, 4ii, 4iii, 4iv**) reveals that the data was completely confined within the model's line of best fit, with just negligible deviations. Critical factors have a large impact on chosen responses, as indicated by the total influence of all critical variables, as shown in contour and 3D responses surface plots **(Figure 4)**. The response values are represented by the colors blue, red, and green, respectively, denoting the lower, higher, and intermediate values.

Optimized Chromatographic Conditions

Based upon the ideal conditions **(Table 3)**, Pterostilbene and Resveratrol were discovered to have retention times of 2.968 and 4.659 respectively. In **Figure 1**, the optimized chromatogram was demonstrated.

Method Validation

In accordance with ICH recommendations, validation parameters were used to validate the developed method. **Table 5** presents a summary of all the validated parameter data.

Specificity:

The specificity was analyzed by identifying interfering peaks in chromatograms and there were no interfering peaks observed in placebo and blank.

System Suitability:

In suitability resolution parameters, plate count, peak area, tailing factor, and retention time were all determined. **Table 4** presents the tabulated results of the parameters of system suitability.

Linearity:

Six different concentrations of solutions (15-90µg/ml of Pterostilbene and 150-900µg/ml of Resveratrol) were prepared. Standard calibration curve was constructed and regression coefficient was calculated. Calibration curves of Resveratrol and Pterostilbene were depicted in **Figures 2 & 3**.

Table 1: Factors

Factor	Name	Units	Type	SubType	Minimum	Maximum	Coded Low	Coded High	Mean	Std. Dev
A	Flow rate	ml/min	Numeric	Continuous	0.8964	1.60	-1 ↔ 1.00	+1 ↔ 1.50	1.25	0.2041
B	Mobile phase ratio	%	Numeric	Continuous	5.86	34.14	-1 ↔ 10.00	+1 ↔ 30.00	20.00	8.16

Table 2: 13 Experimental runs given by QbD

Std	Run	Factor 1 A: Flow rate (ml/min)	Factor 2 B: Organic Phase ratio (%)	Response 1	Response 2	Response 3	Response 4
13	1	1,25	20	4.506	7.56	3336	2.846
5	2	0.8964466094067 3	20	6.367	8.38	5670	4.249
3	3	1	30	4.939	7.09	4438	3.370
8	4	1.25	34.142135623731	3.795	5.60	3061	2.666
1	5	1	10	6.342	10.49	4938	3.710
12	6	1.25	20	4.311	7.72	3774	2.644
4	7	1.5	30	3.320	5.36	2502	2.283
7	8	1.25	5.857864376269	5.419	9.97	3691	3.065
2	9	1.5	10	3.819	6.97	2613	2.397
6	10	1.6035533905933	20	3.196	5.39	2229	2.148
11	11	1,25	20	4.471	7.40	3439	2837
10	12	1.25	20	4.468	7.43	3253	2.832
9	13	1.25	20	4.463	7.42	3273	2.831

Table 3: Ideal chromatographic conditions

Parameters	Observation
Instrument used	Waters HPLC with an auto sampler and PDA detector.
Injection volume	10µl
Mobile Phase	Acetonitrile (ACN): 0.1% Formic acid (18.9:81.1)
Column	Inertsil ODS 3V (250x4.6 mm, 5 µ)
Detection Wave Length	273nm
Flow Rate	1.202 mL/min
Runtime	10min
Temperature	Ambient (25°C)
Mode of separation	Isocratic

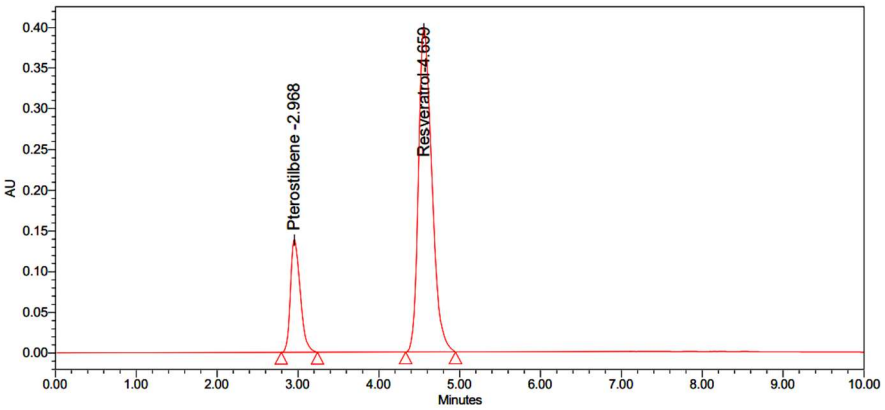


Figure 1: Optimized chromatogram

Table 4: Parameters of System Suitability

S.no	Parameter	Pterostilbene	Resveratrol
1	Retention time	2.968	4.659
2	Resolution	----	7.57
3	Plate count	3526	5858
4	Tailing factor	1.07	1.12
5	%RSD	0.13	0.22

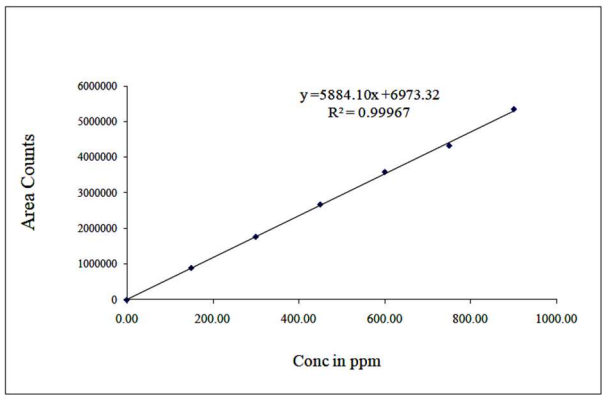


Figure 2: Calibration curve of Resveratrol

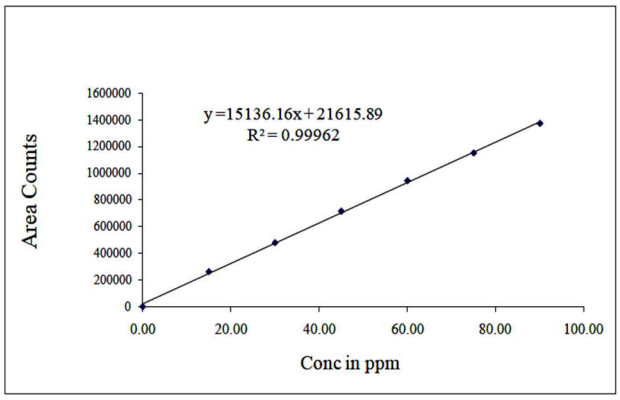


Figure 3: Calibration Curve of Pterostilbene

Precision:

Method precision, system precision, and intermediate precision were analyzed by injecting six replicate sets. % RSD was found to be less than 2%.

Accuracy:

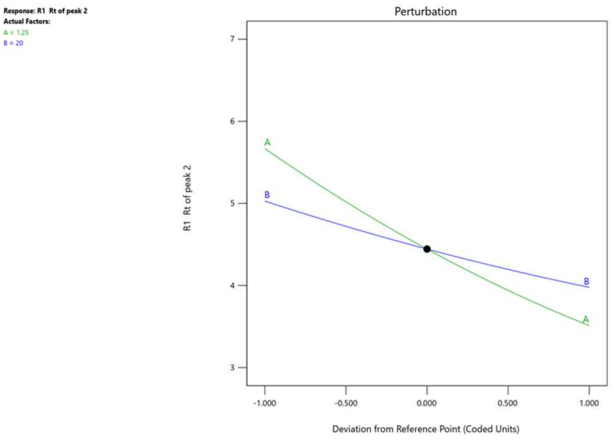
Three levels of Accuracy samples were made using a standard addition technique. For every accuracy level, three injections were administered, and the mean percentage recovery for Pterostilbene and Resveratrol was found to be 100.3% and 99.8% respectively

Robustness:

Robustness is analyzed by making deliberate changes to actual parameters such as organic phase ratio and flow rate. The % RSD was calculated and found to be less than 2%

LOD and LOQ:

These parameters are calculated based upon sloping technique. The method's sensitivity is demonstrated by the decreased LOD and LOQ values for both drugs **(Table 5)**.



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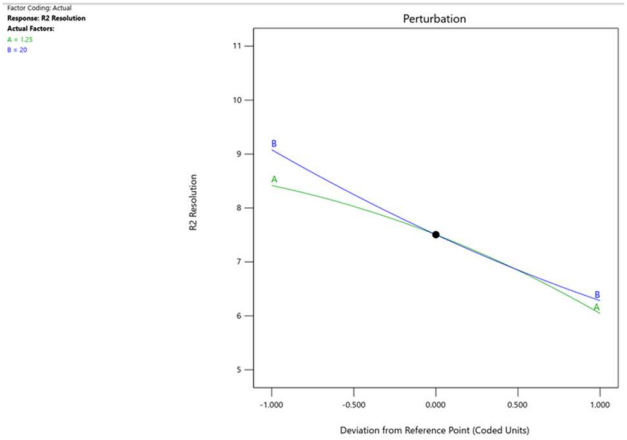

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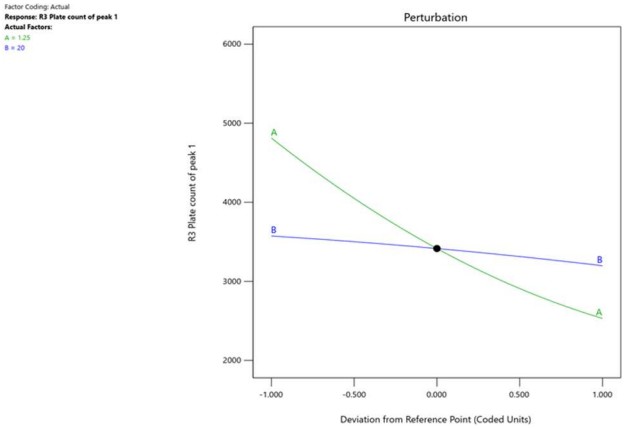

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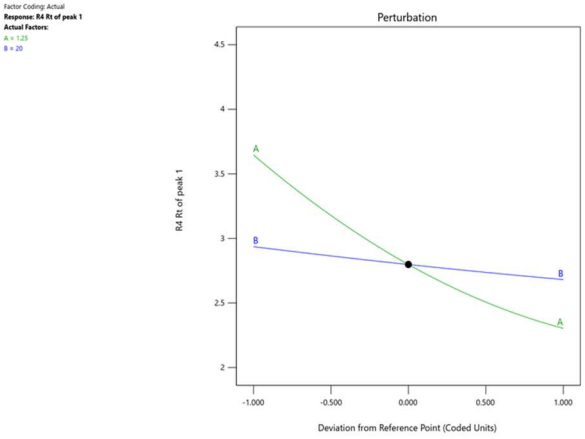

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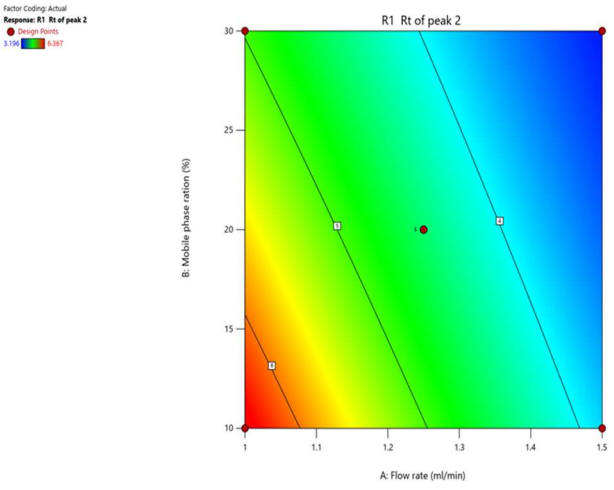

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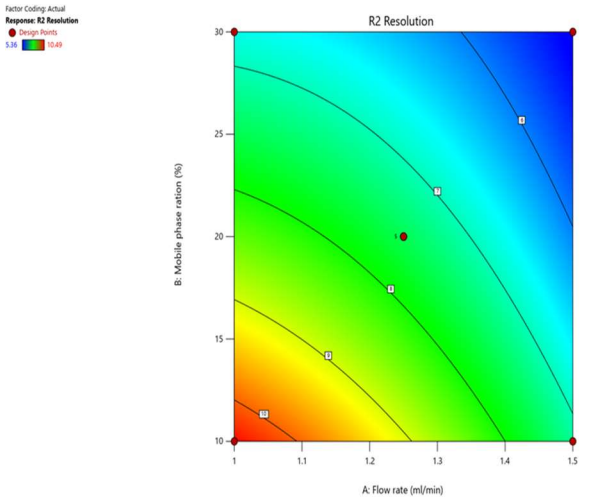

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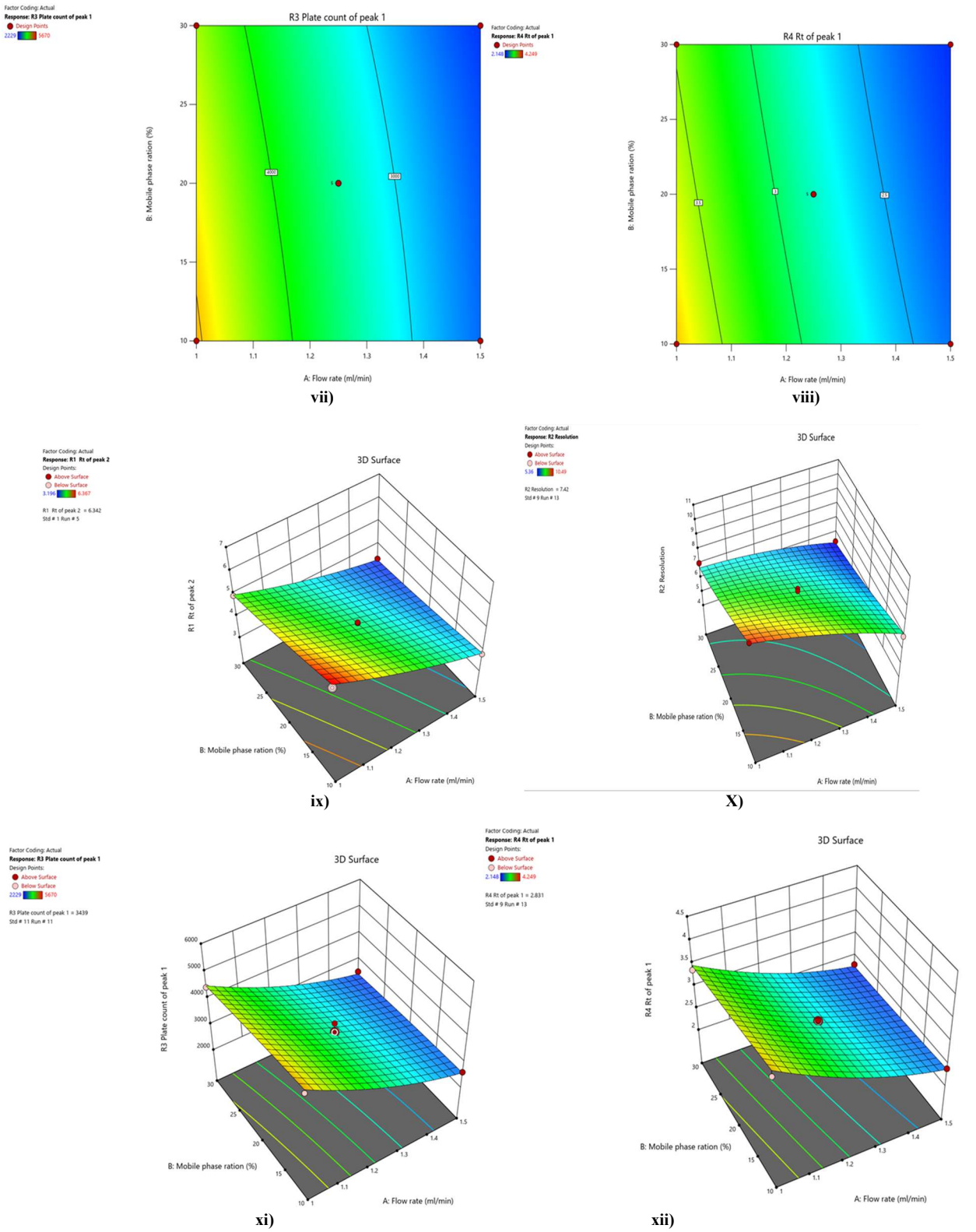

Figure 4: i) Perturbation plot for R1 responses, ii) Perturbation plot for R2 responses, iii) Perturbation plot for R3 responses, iv) Perturbation plot for R4 responses, v) Contour plot for R1, vi) Contour plot for R2, vii) Contour plot for R3, viii) Contour plot for R4, ix) 3D Surface plot for R1, x) 3D Surface plot for R2, xi) 3D Surface plot for R3, and xii) 3D Surface plot for R4

Table 5: Summary of Results

Parameters	Pterostilbene	Resveratrol
Specificity	Specific	Specific
System Suitability (% RSD)	0.13	0.22
Regression Coefficient	0.99962	0.99967
Mean % Recovery	100.3%	99.8%
System Precision (% RSD)	0.13	0.22
Intermediate Precision (% RSD)	0.24	0.82
Less Flow (% RSD)	0.21	0.75
More Flow (% RSD)	0.40	0.45
Less Organic Phase (% RSD)	0.38	0.21
More Organic Phase (% RSD)	0.25	0.70
LOQ (µg/ml)	0.180	0.180
LOD (µg/ml)	0.054	0.054

Assay of Pharmaceutical Dosage Form

Assay was performed for the selected capsule dosage form. The amount of sample obtained for Pterostilbene and Resveratrol were 6.00 and 60.12 µg/ml. The % assay for Pterostilbene and Resveratrol were found to be 100% and 100.2% respectively.

Forced Degradation Studies

A range of stress conditions, including thermal, photolytic, oxidative, hydrolysis, reduction, acidic, and alkaline, are applied to sample solutions in stability indicating studies. From the results, it states that the maximum degradation takes place in oxidative stress for both drugs (Pterostilbene-13.2%, Resveratrol-15.3%). Minimum degradation takes place in photolytic condition 0.7% for Pterostilbene and in reductive stress 1.1% for Resveratrol. Although, the percentage degradation not exceeded more than 20% in any of the stressed conditions.

Greenness Assessment

The overall greenness score is 0.71 and represents green color which indicates high score. A high score is indicated by the color green, a medium score by the color yellow, and a poor score by the color red. The middle of the pictogram represents overall score of the greenness of analytical method. So, based on this it is conformed that the developed method is eco-friendly. The greenness score was pictured in **Figure 5**.

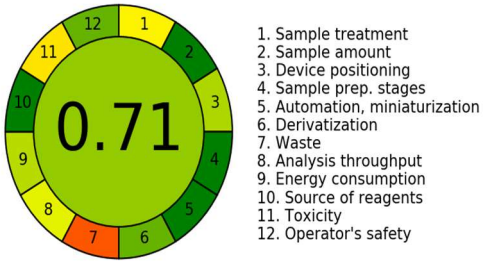

Figure-5: Analytical Greenness

CONCLUSION:

The GAC integrated QbD technique for the simultaneous estimation of Pterostilbene and Resveratrol using RP-HPLC was successfully developed and validated. The method's optimization is done using the QbD methodology. As for forced degradation studies minimum degradation takes place in photolytic condition. According to the results, every validation parameter was discovered to be within reasonable bounds. The proposed method was found to be eco-friendly, specific, sensitive, linear, accurate, precise, and robust. The present study is suitable for routine analysis for the simultaneous estimation of pharmaceutical dosage form.

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