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RESEARCH ARTICLE

Method Development and Validation of HPTLC Method for Simultaneous Estimation of Piperine, Bergapten, Plumbagin and Lupeol in *Chitrakadi Vati*

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Abstract

Background: *Chitrakadi Vati* is an *Ayurvedic* formulation containing several phytoconstituents. A reliable method for simultaneous quantification of its key components is essential for quality control and standardisation. **Aim:** To develop and validate a High-Performance Thin-Layer Chromatography (HPTLC) method for the simultaneous determination of four key phytoconstituents in *Chitrakadi Vati:* Piperine, Bergapten, Plumbagin and Lupeol. **Methods:** An HPTLC method was developed using silica gel $60F_{254}$ plates with a mobile phase of Petroleum ether: Ethyl acetate: Toluene: Formic acid (7:2:1:0.1 v/v/v/v). The method was validated for linearity, accuracy, precision, specificity, robustness, Limit of Detection (LOD) and Limit of Quantitation (LOQ). Robustness was further evaluated using a 2^3 factorial design, examining the effects of mobile phase volume, saturation time, and solvent front on retention factors. **Results:** The method demonstrated good linearity (R^2 = 0.9972-0.9989), precision (%RSD <2%), accuracy (97.68-102.80 % recovery) and specificity. LOD and LOQ ranged from 100.8-325 ng/band and 305.5-985 ng/band, respectively. The factorial design revealed that mobile phase volume and solvent front significantly impacted retention factors, while saturation time had minimal effect. **Conclusion:** The developed and validated HPTLC method proved effective for the simultaneous estimation of Piperine, Bergapten, Plumbagin and Lupeol in *Chitrakadi Vati*. The comprehensive validation, including the Design of Experiments for robustness evaluation, ensures the method's reliability for quality control applications.

Keywords: Bergapten, *Chitrakadi Vati*, HPTLC, Lupeol, Piperine, Plumbagin

1. Introduction

Renowned for its multiple health benefits, especially in boosting digestion and metabolism, *Chitrakadi Vati* is an *Ayurvedic* preparation with deep roots in traditional knowledge. Due to its therapeutic characteristics, this herbal blend enriched with a beneficial combination of strong components including *Chitraka, Pippali, Maricha* and *Sunthi* has been an integral part of *Ayurvedic* therapies for generations[1]. Research has demonstrated *Chitrakadi Vati's* efficacy in boosting hunger and supporting good digestion. It can be used as an appetiser by consuming before eating to help with weight loss, making it a useful supplement to holistic health regimens[2-4]. Regulation of *aama dosha* by *Chitrakadi Vati* enhances the secretion of the stomach's gastric acid. *Aama* contributes to the generation of endotoxins, which serve as the underlying cause of various autoimmune disorders including, Systemic Lupus Erythematosus (SLE), nephrotic syndrome, ankylosing spondylitis, scleroderma and rheumatoid arthritis. *Chitrakadi Vati* is beneficial in managing irritable bowel syndrome, loss of appetite, loose stools, abdominal bloating and the formation of gas. It aids in

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maintaining intestinal peristalsis and preventing the passage of undigested food along with loose stools[5].

The key ingredients in *Chitrakadi Vati* play pivotal roles in its efficacy. For instance, Piperine, found in *Pippali* and *Maricha* and Bergapten from *Chitraka* exhibit anti-inflammatory and antioxidant effects. The anti-inflammatory cardioprotective, anti-diabetic and anti-cancer characteristics of Lupeol, found in some components and antibacterial activity, antihyperglycemic, antiallergic antibacterial and anti-inflammatory qualities of Plumbagin from *Plumbago zeylanica* together enhance the therapeutic potential of *Chitrakadi Vati*[6-9].

Rigorous procedures are used in the preparation and standardisation of *Chitrakadi Vati* to guarantee its effectiveness and quality. Research has indicated that the medical benefits of this *Ayurvedic* composition, which include treatment of indigestion, bloating and appetite loss are associated with the existence of alkaloids and other biologically active chemicals. *Chitrakadi Vati's* standardised formulation has demonstrated efficacy in treating *Grahani,* enhancing digestion and managing a range of digestive diseases. These findings highlight the product's potential as a comprehensive *Ayurvedic* medicine treatment[10,11].

2. Materials and Methods

2.1 Standards and Reagents

Standards for Plumbagin, Bergapten, Lupeol and Piperine were purchased from Yucca Enterprises, Mumbai, Maharashtra, India. Analytical grade solvents and reagents, including petroleum ether, ethyl acetate, toluene and formic acid, were obtained from Suvidhinath Laboratories, Vadodara, Gujarat, India and Sisco Research Laboratories, Mumbai, Maharashtra, India. The *ayurvedic* formulation used was *Chitrakadi Vati*.

2.2 Instrumentation

The analytical setup included HPTLC CAMAG TLC Scanner 4 with Vision-CATS software version 3.0 for scanning and analysis. Samples were applied using the LINOMAT V applicator under a constant nitrogen gas pressure of 4-6 kg/cm^2. A Twin Trough Chamber (CAMAG), available in 10×10 cm and 20×10 cm dimensions, facilitated sample separation. Chromatographic separations were performed on 20×20 cm $60F_{254}$ TLC aluminium sheets pre-coated with silica gel, sourced from Merck, Germany. Measurements were conducted using a digital electronic balance (Shimadzu, India). Visualisation under UV light was done using a UV-1800 UV cabinet (Shimadzu, Japan). Sample preparation was aided by an ultrasonicator (Inco, Dubai). The Linomat syringe (CAMAG) with a 100µl capacity was used for sample application.

2.3 Preparation of Reference Standard Solutions

1mg of Plumbagin, Bergapten, Piperine and Lupeol standards each were measured and dissolved in 1ml methanol in separate Eppendorf tubes to prepare 1000 µg/ml solutions.

2.4 Preparation of Sample Solution

The sample solution was prepared by refluxing 100gm of the marketed *Chitrakadi Vati* powder in 200ml methanol for 30 minutes. The mixture was filtered and from the filtrate, 1mg of dried sample was dissolved in 1ml of methanol.

2.5 Chromatographic Conditions

For the simultaneous estimation of Plumbagin, Bergapten, Piperine and Lupeol, aluminium pre-coated Thin Layer Chromatography (TLC) plates with silica gel 60 F254 were used as the stationary phase. The mobile phase was optimised to petroleum ether acetate: toluene acid (7:2:1:0.1 v/v/v/v). The chamber was saturated with the mobile phase for 20 minutes at room temperature before the chromatographic run, which was 80mm in length. Detection wavelengths were selected based on spectrum studies: 263nm for Plumbagin, 254nm for Bergapten and 333nm for Piperine. For Lupeol, the plate was derivatised with Vanillin sulfuric acid reagent, heated in a hot air oven for 10 minutes and scanned at 594nm.

2.6. Quantification of Standards

The test sample was mixed with methanol and standards were applied onto a thin layer chromatography plate. Then, the plate was treated under determined conditions and scanned at different wavelengths: 258nm, 263nm and 333nm. After that, it was treated with vanillin sulfuric acid and scanned again at 594nm.

The areas of the peak were recorded and the amount of standard in the samples was found using the calibration point.

3. Method Validation

The evaluation of the method's validation parameters encompassed various critical aspects by the guidelines delineated in ICH Q2(R2)[12]. System suitability was ensured by analysing standard solutions of the target compounds under established chromatographic conditions, with assessments conducted for the retardation factor and peak area.

3.1 System Suitability

System suitability assessments were conducted by applying standard solutions of the four markers, spotted five times at a volume of 8.0 µl/spot, with a concentration of 2000 µg/ml. These were analysed under the established chromatographic conditions to evaluate both the retention factor and peak area.

3.2 Specificity

To assess specificity, the standard and test samples were carefully examined within the developed mobile phase to verify the presence of the target compounds among various other biomarkers. This confirmation was accomplished by comparing the Relative retention (R_f) values and UV spectra of the distinct bands corresponding to standard peaks.

3.3 Linearity

To establish linearity, a blend containing all four markers at 1000 µg/ml concentration was created. This mixture was then applied to a 10×10 cm TLC plate using spotting volumes varying from 8µl to 24µl. Subsequently, a calibration graph correlating concentration against peak area was plotted to derive a regression coefficient.

3.4 Accuracy

Accuracy assessment utilised the standard addition method, wherein the sample solution underwent spiking with predetermined quantities of Plumbagin, Bergapten, Piperine and Lupeol. Subsequently, the solution was reanalysed to compute both the mean percentage recovery and the percentage Relative Standard Deviation (% RSD). Concentration levels of 80%, 100% and 120% were employed three times to ensure robustness and reliability.

3.5 Precision

Precision studies encompassed assessments of repeatability, intra-day precision, and inter-day precision. Repeatability was ascertained by spot-analysing standard mixtures six times under consistent operating conditions but at varying time intervals to evaluate the consistency of results.

3.6 Intra-day and Inter-day

Intra-day precision was assessed by analysing standard solutions six times with intervals of 1 hour, while inter-day precision was conducted over three consecutive days. Both evaluations were carried out at concentrations of 1 mg/ml and with spot volumes ranging from 16µl to 24µl. These assessments aimed to evaluate the agreement between measurements over short-term and long-term timeframes.

3.7 Limit of Detection(LOD) and Limit of Quantitation (LOQ)

The Limit of Detection (LOD) and Limit of Quantitation (LOQ) for Plumbagin, Bergapten, Piperine, and Lupeol were determined by analysing standard solutions (1mg/ml) with varying spot volumes ranging from 8.0µl to 24.0µl. These values represent the minimum concentrations of the compounds that can be reliably detected and quantified. The LOD was calculated using the standard deviation and slope method.

3.8 Robustness Using the Design of the Experiment

In this study, a 2^3 factorial design was employed to assess the robustness of the system. The Design of Experiments (DoE) approach allows to systematically evaluate the impact of three critical factors on the system's performance.

Three key factors that could potentially influence the robustness of the system were identified. These were factor A (Volume of Petroleum ether), factor B (Saturation time) and factor C (Solvent front). By applying a 2^3 factorial design, the main effects and interactions of these factors were explored in a structured manner. This experimental design facilitated an efficient assessment of the system's

response to various combinations of the factor levels, providing valuable insights into the robustness of the system. The experimental domain of selected variables is given in Table 1.

Table 1. Variables and levels in 2^3 methods

Independent Variables	Low Level	High Level
A = Volume of petroleum ether	6.8	7.2
B = Saturation time	18	22
C = Solvent front	78	82
Responses	R1= R_f of Piperine R2= R_f of Bergapten R3= $R_f$of Lupeol R4= R_f of Plumbagin	

DoE was applied to assess the method's reliability by examining slight variations in the settings. The focus was on the R_f values of the drugs. The study utilised a design with two levels for three factors, known as a Three-Factor Two-level factorial design. Eight runs were conducted at high and low levels of the factors and the resulting responses were noted. The specific factors chosen for investigation are outlined in Table 2. To minimise any potential biases from unaccounted factors, all experiments were randomised.

Table 2. Design matrix of 2^3 factorial designs with all the runs

		Factor 1	Factor 2	Factor 3
Std.	Run	A: Volume of PE	B: Saturation Point	C: Solvent Front
		Ml	Min	Mm
5	1	6.8	18	82
3	2	6.8	22	78
2	3	7.2	18	78
1	4	6.8	18	78
4	5	7.2	22	78
7	6	6.8	22	82
8	7	7.2	22	82
6	8	7.2	18	82

4. Results

4.1 Optimisation of Mobile Phase

To establish an HPTLC method for the simultaneous analysis of Piperine, Bergapten, Plumbagin and Lupeol, various trials were performed according to the polarity of the mobile phase and different solvents and different solvent ratios were tried. After trials the mobile phase composition of Petroleum ether: ethylacetate: Toluene: Formic acid (7:2:1:0.1v/v/v/v) was selected as it showed good separation with the R_f values of 0.23 +/- 0.034,0.35 +/- 0.030,0.74+/- 0.041,0.58 +/- 0.041 for Piperine, Bergapten, Plumbagin and Lupeol

4.2 Selection of Detection Wavelength

Based on the spectrum studies it was found that the standards give the highest intensity at 333nm (Piperine), 258nm (Bergapten), 263nm (Plumbagin) and for the lupeol Vanilin sulphuric acid is used as a derivatisation reagent further dried and scanned at 594nm.

4.3 Method Validation

4.3.1 Linearity

All four standards showed a correlation coefficient of 0.9989 (Piperine), 0.9972 (Bergapten), 0.9986 (Plumbagin) and 0.9974 (Lupeol) at the range of 2000-6000ng/band. The overlay of Piperine at 333nm, the overlay of Bergapten at 258nm and the overlay of Plumbagin at 263nm are shown in Figures 1, 2 and 3 respectively and Figure 4 shows the overlay of Lupeol at 594nm and the summary of validation parameters is shown in Table 3.

4.3.2 Precision

The repeatability study was done by experimenting 6 times but at different times and average peak area and % RSD were calculated and shown in Table 4.

Intraday and Interday precision was done by repeating an experiment at 3 different hours (intraday) and 3 different days (Interday). Average peak area and % RSD were carried out and shown in Table 4.

4.3.3 Accuracy

For evaluation of recovery at three levels of concentrations, 80%, 100% and 120%, after spiking the standard the percentage recovery of Piperine was between 99.95-101.97, Bergapten was between 97.68-100.97, Plumbagin

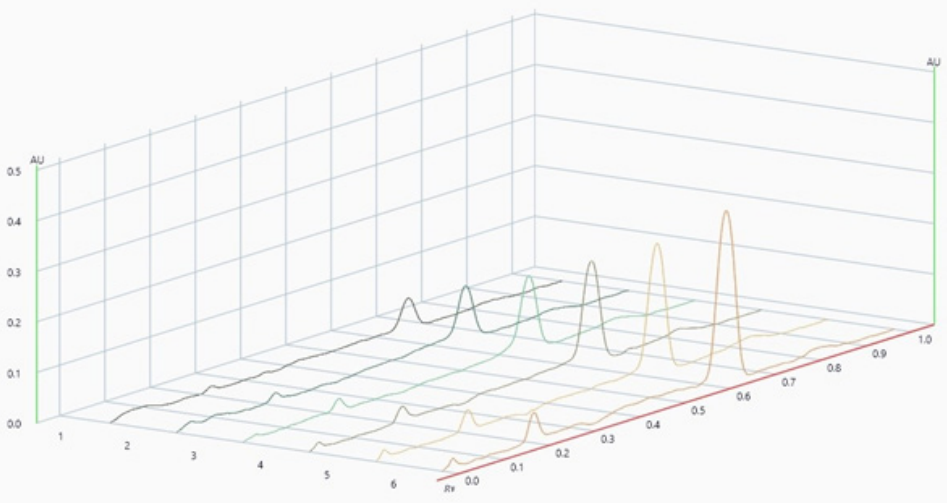

Figure 1. Overlay of Piperine.

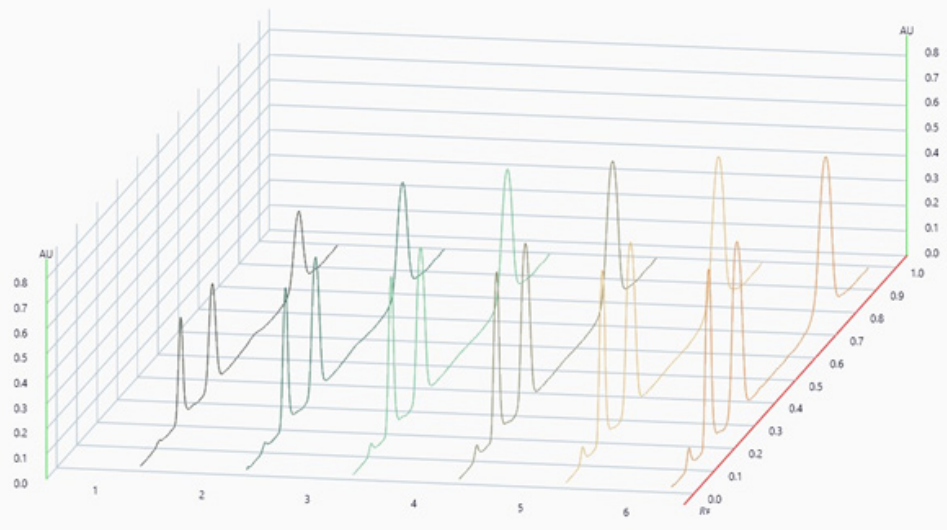

Figure 2. Overlay of Bergapten.

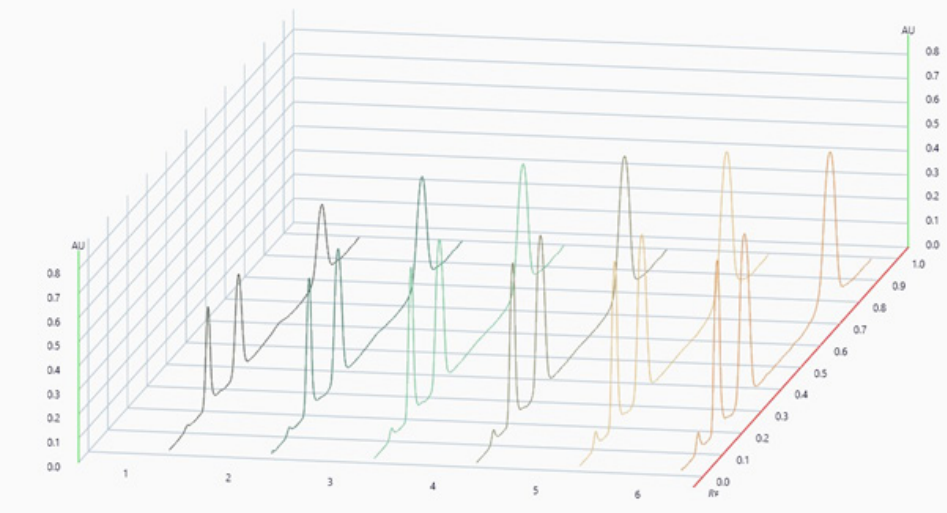

Figure 3. Overlay of Plumbagin.

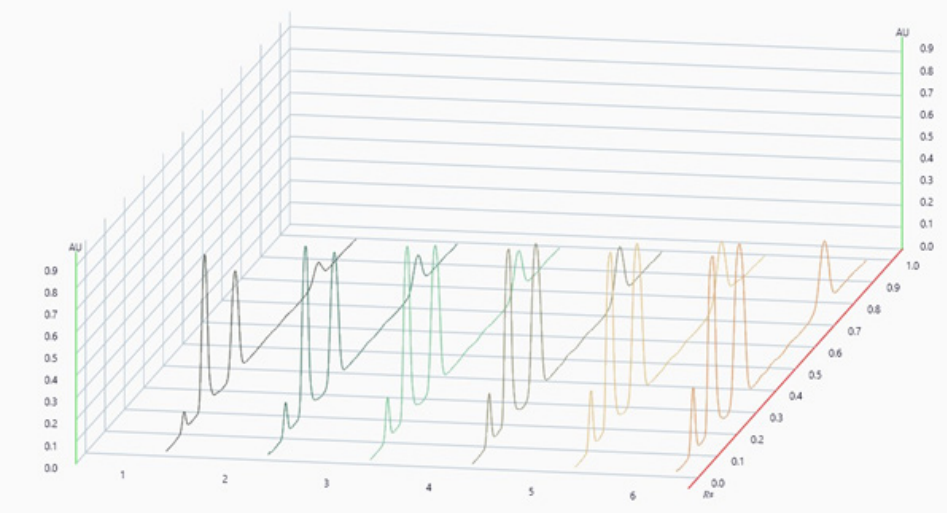

Figure 4. Overlay of Lupeol.

Table 3. Summary of validation parameter

Parameters	Piperine	Bergapten	Plumbagin	Lupeol
R_f value	0.23 +/- 0.034	0.35 +/- 0.030	0.74 +/- 0.041	0.58 +/- 0.041
Scanning wavelength (nm)	333	258	263	594
Linearity range (ng)	2000-6000	2000-6000	2000-6000	2000-6000
Regression equation	Y=1E-06x +0.0261	Y=3E-06x +0.0166	Y=5E-06x+0.0086	Y=2E-06x+0.0011
R^2	0.9986	0.9972	0.9989	0.9974
Intercept	0.0261	0.0166	0.0086	0.0011
Chamber saturation time (min)	20	20	20	20
Specificity	Specific	Specific	Specific	Specific
LOD (ng/band)	325 ng/band	229 ng/band	100.8 ng/band	165 ng/band
LOQ (ng/band)	985 ng/band	694 ng/band	305.5 ng/band	500 ng/band

Table 4. Intraday, interday and repeatability

Intraday			Interday			Repeatability
Mean Peak Area	**SD**	**% RSD**	**Mean Peak Area**	**SD**	**% RSD**	**% RSD**
Piperine						
0.03153	0.0002	0.87	0.03135	0.0003	1.16	1.45
Bergapten						
0.03049	0.0003	1.08	0.03076	0.0004	1.48	1.50
Plumbagin						
0.02653	0.0002	0.94	0.02670	0.0002	1.09	1.17
Lupeol						
0.00925	0.0001	0.92	0.009093	0.000139	1.52	0.92

was between 98.11-100.87 and Lupeol was between 99.56-101.81 as shown in Table 5.

4.3.4 Specificity

The peak purity of all four drugs in both the formulation and standard is assessed by comparing their spectra and shown in Table 6.

4.3.5 Robustness Study by Design of Experiment

The results of experiments were calculated using an expertly Design-Expert 13. The factorial model for the responsible variable R_f, about various drugs is displayed in Table 7.

The statistical analysis of the Design of Experiments (DoE) study was conducted to evaluate

Table 5. Recovery studies of lupeol, bergapten, plumbagin and piperine

Accuracy of Lupeol				
Spike%	**Concentration Present (ng)**	**Concentration Spiked (ng)**	**Concentration Recovered (ng)**	**% Recovery ± S.D**
80	2205	1764	3887	97.93±1.06
100	2205	2205	4390	99.54±1.66
120	2205	2646	4929	101.6±1.12
Accuracy of Bergapten				
Spike %	**Concentration Present (ng)**	**Concentration Spiked (ng)**	**Concentration Recovered (ng)**	**% Recovery ± S.D**
80	2315	2064	4192	100.6 ±1.36
100	2315	2580	4962	100.97 ±1.15
120	2315	2778	5179	101.68 ±1.58
Accuracy of Plumbagin				
Spike %	**Concentration Present (ng)**	**Concentration Spiked (ng)**	**Concentration Recovered (ng)**	**% Recovery ± S.D**
80	2007	1606	3545	98.11±0.91
100	2007	2007	4020	100.87±1.52
120	2007	2408	4377	99.13±1.22
Accuracy of Piperine				
Spike%	**Concentration Present (ng)**	**Concentration Spiked (ng)**	**Concentration Recovered (ng)**	**% Recovery ± S.D**
80	2530	2024	4534	99.56±1.07
100	2530	2530	5152	101.81±1.16
120	2530	3036	5722	102.8±1.82

Table 6. Peak purity of piperine, bergapten, lupeol and plumbagin

Formulation					**Piperine in Standard**	**Bergapten in Standard**	**Lupeol in Standard**	**Plumbagin in Standard**
Peak Purity	**Piperine**	**Bergapten**	**Lupeol**	**Plumbagin**				
r(s,m)	0.999574	0.999828	0.996934	0.998595	0.999280	0.999630	0.999668	0.999960
r(e,m)	0.996956	0.998667	0.981609	0.999175	0.995974	0.997807	0.993525	0.999652

the effects of various factors on the retention factor of four compounds: Piperine, Bergapten, Lupeol and Plumbagin. Table 8 presents several key statistical parameters for each response variable, including the Model P value, Polynomial equation, Coefficient of Variation percentage (C.V. %) and Adequate Precision ratio (Adeq Precision) is shown in Table 8.

The predicted models are presented as perturbation plots to understand the result better. For Piperine (Figure 5A), the perturbation plot shows its R_f is highly sensitive to the mobile phase volume, moderately affected by the solvent front, but minimally impacted by saturation time. The 3D plot (Figure 6A)reveals piperine's R_f increases with higher mobile phase volumes combined with higher solvent fronts. Bergapten's R_f exhibits moderate sensitivity to all three factors per the perturbation plot (Figure 5B). Its 3D surface (Figure 6B), indicates an increase in R_f at higher mobile phase volumes and solvent front. The lupeol perturbation plot (Figure 5C) suggests its R_f is strongly influenced by mobile phase volume, moderately by solvent front, but less by saturation time. The corresponding 3D plot (Figure 6C) points to higher R_f values at greater mobile phase volumes and solvent fronts. Plumbagin is fairly insensitive to saturation time, but its R_f is highly impacted by mobile phase volume and moderately by solvent front based on the steep

Table 7. 2^3 factorial designs for robustness and their responses

	Factor 1	Factor 2	Factor 3	Response 1	Response 2	Response 3	Response 4
Run	A: Volume of PE	B: Saturation Time	C: Solvent Front	Piperine	Bergapten	Lupeol	Plumbagin
	Ml	min	Mm				
1	6.8	18	82	0.225	0.32	0.579	0.702
2	6.8	22	78	0.223	0.325	0.586	0.718
3	7.2	18	78	0.25	0.349	0.59	0.72
4	6.8	18	78	0.24	0.338	0.581	0.718
5	7.2	22	78	0.25	0.355	0.598	0.739
6	6.8	22	82	0.232	0.332	0.572	0.72
7	7.2	22	82	0.265	0.38	0.636	0.802
8	7.2	18	82	0.248	0.358	0.631	0.779

Table 8. Summary of statistical analysis

Response	Piperine	Bergapten	Lupeol	Plumbagin
Model	2FI	2FI	2FI	2FI
P-value	0.0825	0.0963	0.0936	0.1004
Polynomial Equation	0.25575+0.01725A +0.00125B+0.008C +0.00175AB +0.012AC–0.002BC	0.352125+0.013625A +0.001375B+0.004625C +0.000875AB+0.008625 AC–0.002125BC	0.596625+0.017125A +0.001375B+0.007875C +0.001875AB+0.011875AC –0.001875BC	0.73725+0.02275A +0.0075B+0.0135C +0.003AB+0.017AC +0.00275BC
C.V. %	1.11	0.7028	0.5333	0.6714
Adeq Precision	23.4338	19.9781	20.7460	21.5980

perturbation slope (Figure 5D). The 3D surface (Figure 6D) shows that Plumbagin's increased R_f can be seen through a combination of higher mobile phase volumes and solvent fronts. Here in all standards saturation time has a very minimal effect at higher petroleum ether volumes.

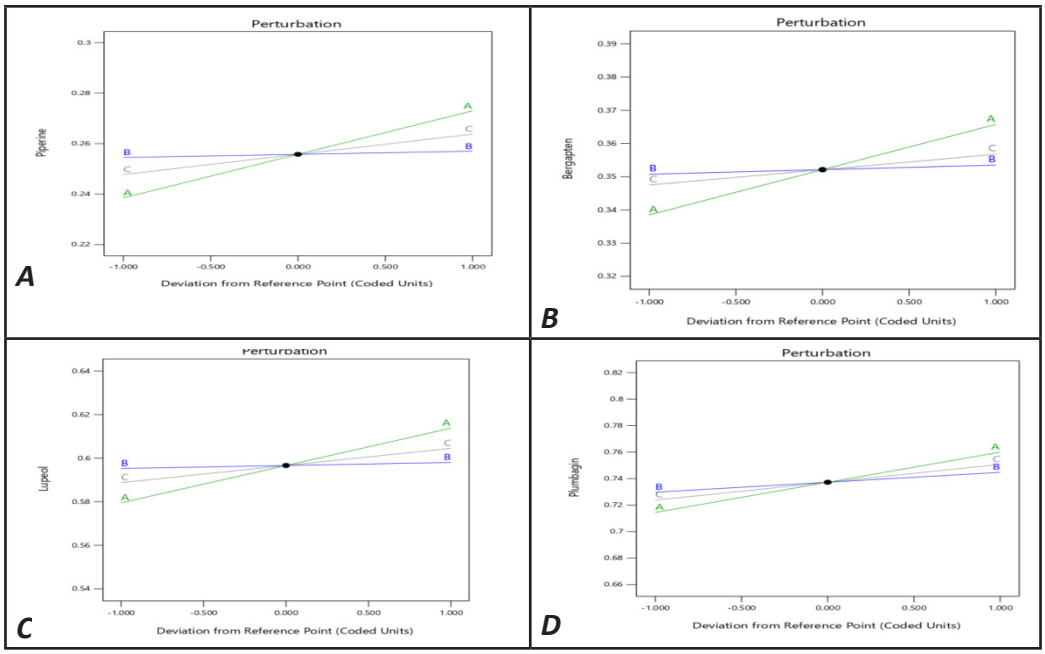

Figure 5. Individual effects of factors A, B and C on the R_f values of the four phytoconstituents. Perturbation graphs illustrate how each factor influences the R_f values of Piperine (Figure 5A), Bergapten (Figure 5B), Lupeol (Figure 5C) and Plumbagin (Figure 5D).

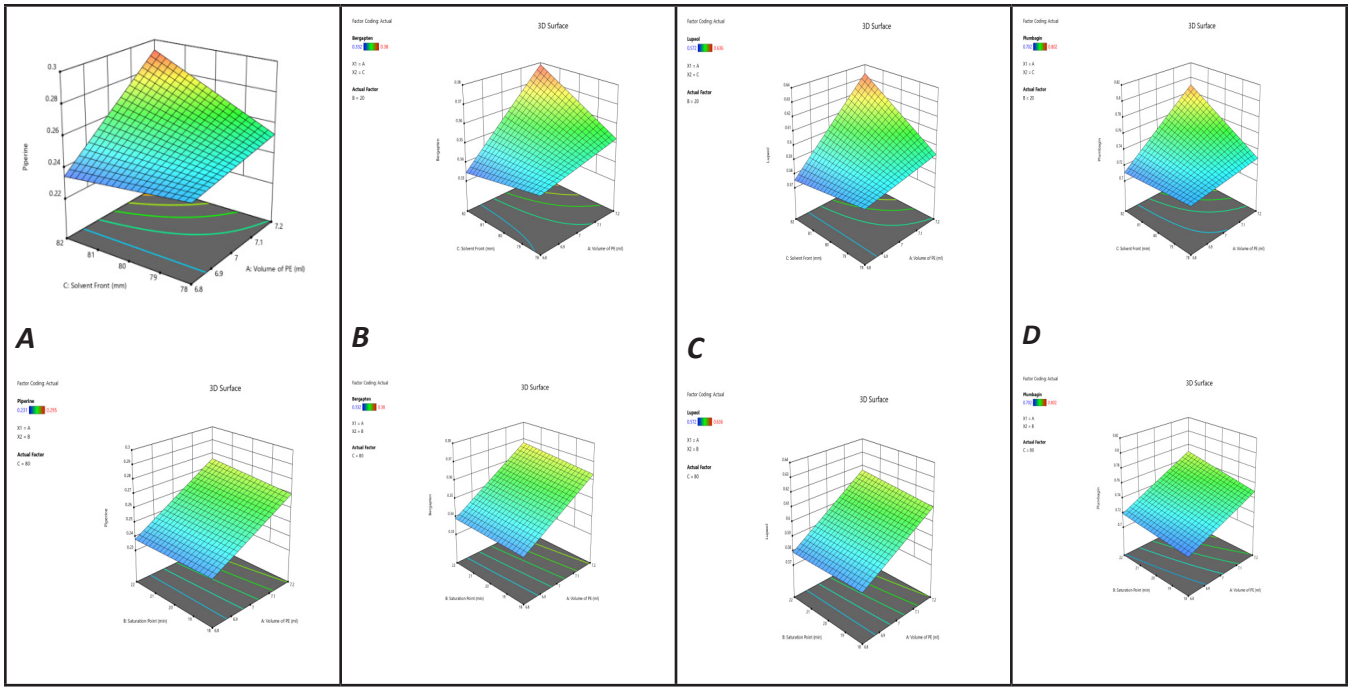

Figure 6. 3D graph showing the effect of factors (AC) and (AB) on (6A) the R_f value of Piperine (6B) the R_f value of Bergapten (6C) the R_f value of Lupeol (6D) the R_f value of Plumbagin.

5. Conclusion

The developed and validated HPTLC method demonstrated its efficacy in the simultaneous estimation of Piperine, Bergapten, Plumbagin and Lupeol in *Chitrakadi Vati*, a standardised *Ayurvedic* formulation. The method exhibited good linearity, precision, accuracy and robustness. The comprehensive validation approach, including the DoE for robustness evaluation, ensures the method's reliability.

6. References

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