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RESEARCH ARTICLE

Application of Quality Risk Assessment and Design of Experiment in Optimizing Chromatographic Method for Estimation of Total Sarsasapogenin from the Roots of *Asparagus racemosus* Wild

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Abstract

The roots of the plant *Asparagus racemosus* (Wild) (family: Liliaceae) are used alone as a lactogenic and general tonic or incorporated as a major ingredient in many herbal and *Ayurveda* formulations. Shatavarin glycosides containing sarsasapogenin as aglycone were found bioactive, and present in predominantly higher amount. The experiments were planned to develop an analytical method for estimation of total sarsasapogenin from the extract using HPTLC, through a systematic approach after identifying the potential failure modes, termed as critical process parameters to achieve the analytical target profile. The associated risks were mitigated by studying the combined effect of the critical process parameters on area, R_f, and tailing factor corresponding to the sarsasapogenin peak, assigned as critical method attributes. The optimization studies were carried out through Design of Experiment approach and process parameters were finalized to achieve analytical target profile. The developed method could resolve the peak of sarsasapogenin from the hydrolyzed methanolic extract prepared from the root powder using silica gel-backed pre-coated aluminum plates. The optimized mobile phase was Hexane:Ethyl acetate:Formic acid (7.0:1.9:0.35, v/v/v); the plates were subjected to post-chromatographic derivatization using 5% v/v hydrochloric acid solution, and heating of the plats for 3 min at 110°C. The chromatogram was recorded by scanning the plates in TLC scanner using fluorescence mode at 366 nm. The developed analytical method was found to yield linear response in the range of 40-280 ng/spot. The limit of quantification was determined to be 35.88 ng/spot. The method was found selective, accurate, and robust. The amount of sarsasapogenin was found to be determined using the developed method and found to be 2.31% w/w on dried weight basis from the dried root powder. This is the first report of a systematic approach in estimating sarsasapogenin from *Asparagus* sp.

Keywords: *Asparagus racemosus*, AQbD, HPTLC, Quality by Design, Quality Risk Management, Sarsasapogenin

1. Introduction

Asparagus racemosus (Wild) (family: Liliaceae) (*A. racemosus*); is traditionally known as *Shatavari*[1]; recognized as the '*Rasayana*' drug in *Ayurveda*. *Rasayana* drugs are believed to promote overall health by improving cellular vitality, immunity as well as resistance[1-4]. The roots of *A. racemosus* are used due to their promising

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effect as lactogenic and general tonic[5]. *A. racemosus* was found to possess galactagogue, antispasmodic, stomach tonic, aphrodisiac, antidiarrhoeal, laxative, antidysentiric, anti-tubercular, hypoglycaemic, antioxidant, antifungal, anticoagulant, antiulcer, nutritive, mucilaginous, carminative, hepatoprotective, and immune-stimulant activity[1-4,6-11]. The plant was reported to contain Vitamin A, B_1, B_2, C, E, and folic acid. Moreover, the plant was reported to contain essential oils, asparagine, arginine, tyrosine, flavonoids (kaempferol, quercetin, and rutin), resin, and tannin[12,13]. The major bioactive constituents of genus *Asparagus* are a group of steroidal saponins[14]. Several steroidal saponins such as shatavarin-I to shatavarin-V have been isolated from the genus *Asparagus*. Shatavarin glycosides are classified on the basis of the type of aglycon moiety, which was found either to be spirostenol (shatavarin-IV, shatavarin-V, asparanin B) or furostanol (shataravin I, curriloside G, asparoside A). Aglycone moiety in spirotenol type shatavari glycoside was found to be sarsasapogenin which was linked to sugar molecules through a hemiacetal linkage[15]. There have been analytical methods reported for the estimation of shatavarin-IV from variety of matrices using HPTLC (High Performance Thin Layer Chromatography) and HPLC (High Performance Liquid Chromatography)[16]. Moreover, a few analytical methods have been reported for the estimation of sarsasapogenin from the extracts prepared from *A. racemosus*. Being a chemical devoid of chromophoric group and extended conjugation, shatavarin glycosides had poor absorptivity in UV region, and reported to be estimated either using HPLC coupled with evaporative light scattering detector, mass detector[17-19], or using HPTLC with post chromatographic derivatization approach. As a part of glycoside, sarsasapogenin could be present in a free state as well as in binding with glycone part. An analytical method capable of estimating sarsasapogenin from hydrolyzed fraction would provide information about total sarsasapogenin present in the plant extract, which further would be equivalent to the total amount of bioactive spirostanol type of glycosides present in the plant extract. Few analytical techniques have been described for estimating sarsasapogenin using HPTLC, but those techniques lack sensitivity and the procedure adopted for sample preparation from the plant material appeared to be evolved empirically. A sensitive method was envisaged to be developed using HPTLC by adopting Analytical Quality by Design (AQbD) approach for the estimation of total sarsasapogenin through a rationalized process of sample preparation, risk analysis, and optimization of chromatographic parameters to mitigate the identified potential risks[20]. The method was to be validated for linearity, selectivity, sensitivity, precision, accuracy, and robustness as per the ICH guidelines.

2 Materials and Methods

2.1 Chemicals and Reagents

The plants were collected from the medicinal plant garden in the month of May. The plant was identified as *Asparagus racemosus* (Wild) (family: Liliaceae) by the taxonomist at J and J College of Science, Nadiad, Gujarat, India. Analytical grade acetone, methanol, hydrochloric acid (37% v/v), ethyl acetate, hexane, chloroform, sulphuric acid, formic acid, and anhydrous sodium sulphate were used. These chemicals and solvents were purchased from Loba Chemie, Mumbai, India. Sarsasapogenin was procured from Sigma-Aldrich (more than 90% pure) and used without further purification.

2.2 Instruments and Software

HPTLC system (CAMAG,) consisting of Linomat V semi-automatic spotting device, twin trough glass chamber (20 × 10 cm), TLC scanner IV, ultraviolet (UV) cabinet with dual-wavelength UV lamps, dipping chamber, TLC plate heater, syringe (100 µL capacity, Hamilton) and controlling software (WinCATs) was used for chromatographic studies. Rotary Vacuum Evaporator (Heidolph), and electronic analytical balance (Shimadzu AUX-220) were used for studies. The data analysis was carried out using Design Expert software (Trial Version).

2.3 Preparation of Plant Extract

Accurately weighed *A. racemosus* root powder (100 g) was sonicated with acetone (300 ml, 50˚C, 1 h). The extract was discarded and the plant material was dried at room temperature. The defatted plant material was further extracted using methanol (300 ml, 50˚C for 2 hrs) in a Soxhlet apparatus, and the filtrate was evaporated using a rotary vacuum evaporator (45˚C). The residues obtained (~40g) were stored in a glass desiccator. Accurately weighed methanolic extract (3g) was mixed with

methanolic hydrochloric acid. The content was refluxed with acidic solution for set duration and temperature. The solution was further concentrated under reduced pressure to get a brownish gummy residue. These residues were partitioned between water and chloroform (50 ml (50% v/v), three times), and chloroform layer was collected and pooled. The pooled fractions were concentrated in a rotary vacuum evaporator (40˚C) to get a brownish mass (~1.2 g).

2.4 Optimizing Hydrolytic Conditions

The hydrolysis process had three critical failure modes. The effect of the identified failure modes on amount of sarsasapogenin was further studied through Box-Behnken design. The experimental design was evolved using Design Expert software, through the set levels of the failure modes (Table 1). The experiments were performed after following the experimental conditions and amount of sarsasapogenin was determined for each run. Two-way ANOVA (Analysis of Variance) was used to ensure the model's significance, and the correlation coefficient value was used to conclude the curve fitting and mathematical formula. The perturbation chart and contour plots were used to study the relationship between failure modes and amount of sarsasapogenin.

2.5 Preparation of Standard Solution for Estimation of Sarsasapogenin from Hydrolyzed Extract of *A. racemosus* Wild

Sarsasapogenin (10 mg) was accurately weighed and dissolved in methanol at room temperature with the help of a bath sonicator, yielded a reference standard solution (1000 μg/ml). The reference standard solution was diluted using methanol to prepare working standard solution (50 μg/ml). Various aliquots from working standard solution were transferred to different volumetric flasks and diluted using methanol to prepare a series of dilutions containing sarsasapogenin; covering the range of 40 ng/spot to 280 ng/spot.

2.6 Application of AQbD Approach for Optimizing Chromatographic Conditions

2.6.1 Evolving Analytical Target Profile (ATP) and Identifying the Failure Mode Using Preliminary Screening

R_f (Retardation Factor) value (0.4-0.6), peak area (≥2000), and Tailing Factor (TF) (0.8-1.2) for the peak corresponding to sarsasapogenin were the ATP evolved for HPTLC-based method to be optimized for estimation of sarsasapogenin from hydrolyzed extract. Failure Mode and Critical Effect Analysis Approaches (FMCEA) served as tools to identify potential failure modes, which might be encountered while achieving ATP. The process was initiated with recognition of potential failure modes in order to develop an analytical method. Through prior chromatographic knowledge and experimental trials, various failure modes were identified and depicted in Ishikawa diagram.

2.6.2 Quality Risk Assessment Using Risk Priority Number Ranking (RPN) and Filtering Method

Area, R_f and TF were considered to be Critical Method Attributes (CMA). The occurrence and severity of all identified potential risk parameters were studied on CMA. Scores have been assigned to Occurrence (O), Severity (S), and Detectability (D) of failure mode for carrying out a risk assessment. Scale was decided as per the possibility

Table 1. Experimental design employed for optimizing hydrolytic conditions

Sr. No.	Failure Modes	Levels	
		-1	+1
1	Concentration of Acid (% v/v) [Factor A]	10	20
2	Time of Reflux (h) [Factor B]	3.0	7.0
3	Temperature of Reflux (°C) [Factor C]	65.00	75.00

Table 2. Experimental design employed for screening failure modes

Sr. No.	Failure Modes	Levels	
		Maximum	Minimum
1	Volume of Hexane in the mobile phase (Factor: A) (ml)	6.0	8.0
2	Volume of Ethyl Acetate in the mobile phase (Factor: B) (ml)	1.5	2.3
3	Volume of Formic Acid in the mobile phase (Factor: C)(ml)	0.2	0.4
4	Heating Time after dipping the plate (Factor: D) (min)	3	7
5	Heating Temperature for derivatization (Factor: E) (°C)	100	120
6	Concentration of Acid in derivatization reagent (Factor: F) (% v/v)	3	7
7	Dipping Time for the plate (Factor: G) (s)	3	7
8	Saturation Time for the chamber (Factor: H) (min)	12	18
9	Volume of Mobile Phase (Factor: I)(ml)	8	12
10	Run Distance (Factor: J) (cm)	6	8
11	Time Between Heating and Scanning (Factor: K) (min)	8	12

of O, S, and D, very low (02), low (04), medium (06), high (08) and very high (10) for scoring of failure mode. Value for S, O and D were assigned to a potential risk parameter and RPN for a particular failure mode was calculated using the formula RPN = S X O X D.

2.6.3 Screening of Failure Mode to Apply Critical Effect Analysis

Based on prior chromatographic knowledge and experimental runs; more than forty likely failure modes were identified and further, it was found that there were eleven identified failure modes were crucial for the development of HPTLC method. Taguchi design was adopted to analyse the critical effect of the failure modes on CMA (Table 2). There were twelve experimental runs suggested to be performed in the laboratory. The mean value of area, R_f and TF were obtained to determine the statistical significance of the model. The factors found critical in the screening experiments were selected to be evaluated thoroughly for optimizing the chromatographic conditions.

2.6.4 Optimization of Critical Factors Using DoE

The failure modes affecting the CMA in statistically significant manner during the screening experiment were considered for Box-Behnken Design. Three different levels, low (-1), mean (0) and high (+1) were selected on the basis of prior knowledge for each failure mode (Table 3). The optimization of the analytical method was achieved after studying the combined effect of the factors on the CMA through a systematic approach. Forty-six suggested experimental runs with five central points were executed. These runs were performed in the laboratory and the responses corresponding to CMA was recorded

Table 3. Summary of the level of CPP studied for effect on CMA for optimization using DoE

Sr. No.	Selected CPP	Levels	
		Minimum	Maximum
1	Volume of Hexane in the mobile phase (Factor: A) (ml)	6.0	8.0
2	Volume of Ethyl Acetate in the mobile phase (Factor: B) (ml)	1.50	2.50
3	Volume of Formic Acid in the mobile phase (Factor: C) (ml)	0.2	0.4
4	Run Distance (Factor: C) (cm)	60.0	80.0
5	Heating Time for plate after derivatization (Factor: D) (min)	3.0	7.0

at the end of each analysis. The data analysis was carried out, which included the determination of statistical significance, curve fitting details, perturbation plots, and contour plots. The curves showing the combined effect of the selected failure modes on CMA were generated through mathematical equations obtained from the data analysis. The studies suggested few failure modes could affect CMA in statistically significant manner, which constituted Critical Process Parameters (CPP).

2.6.5 Control Strategy and Design Space Navigation for Failure Mode Risk Mitigation

Method Operable Design Ranges (MODR) were generated for optimization of CPP and achieving ATP. Failure modes were studied for their combined effects on CMA through perturbation plots and contour plots. The risk associated with individual CPP was controlled by optimizing the failure mode to achieve ATP. The validation of the model was carried out by randomly selecting three runs from the suggested experimental runs and the experiments were performed in the laboratory by keeping independent variables at the selected value level. Value corresponding to Area, R_f, and TF was recorded after each run. The mathematical model yielded the value for Area, R_f, and Tailing Factor too. The values obtained from experimental data and mathematical models were compared to confirm the validity of the model in predicting the results.

2.6.6 Control Strategy and Optimized Chromatographic Condition

The control strategy was evolved from suggested MODR for the selection of the ranges of CPP. The preferred conditions for the development of HPTLC based analytical method for the estimation of total sarsasapogenin content from the hydrolyzed extract was evolved using control strategy and studies of contour plots. Silica gel G_{60} F_{254} coated on Aluminum sheet was used as stationary phase for chromatographic separation. Spotting was carried out with LINOMAT-V semiautomatic spotter with a constant flow of nitrogen. Instrument was controlled by WinCATs ver. 1.4.7 software. Spotting of samples was done in the form of bands having size 8 × 0.45 mm with the help of Camag 100 µl application syringe (Hamilton, Switzerland) on precoated silica plates. The rate of application was set constant as 150 nl/sec. The distance between two bands was set as 15 mm, 10 mm away from the edges of plate and 10 mm from the bottom of the plate. Hexane: Ethyl acetate: Formic acid (7.0:1.9:0.35, v/v/v) was used as optimized mobile phase and the plate was developed in twin trough chamber (20 cm × 10 cm) previously saturated for 15 min at room temperature. Migration distance was kept as 70 mm from the application position. The plate was derivatized using 5% v/v hydrochloric acid solution, dipping time was kept 5 s and heating time was 3 min at 110˚C. Time between plate heating and scanning was kept 10 min. Camag TLC scanner IV operated by WinCATs ver. 1.4.7 software was used for scanning the plate in fluorescence mode at 366 nm. The slit dimension was set as 6 × 0.45 mm and the scanning speed was kept at 20 mm/sec. Mercury lamp was used as a source of radiation which took almost 4 min to warm up. Evaluation was performed using linear regression of peak areas.

2.7 Method Validation

The developed HPTLC method for estimation of total sarsasapogenin was validated to evolve selectivity, linearity, precision, sensitivity, accuracy, and robustness as per ICH Q2 (R1) guideline.

2.7.1 Selectivity

The selectivity of the analytical method was established by measuring correlation coefficient for the spectra recorded at different positions (starting, middle, and end of the peak) in the visible region for the spot corresponding to sarsasapogenin in the track of sample solution determined using WinCATs software. Value of correlation coefficient, more than 0.950 was set as acceptance criteria.

2.7.2 Linearity

Equal volume of each solution from set of serial dilutions was spotted on the TLC plate covering the range of 40 ng/spot to 280 ng/spot sarsasapogenin, and the area corresponding to each spot was integrated using WinCATs software and plotted against the concentration after developing the plate following the optimized chromatographic conditions. Average value of y-intercept, regression coefficient (r^2), and the slope of regression line was determined and reported along with standard deviation.

2.7.3 Sensitivity

The sensitivity of the developed analytical method was determined and expressed in terms of limit of detection (LoD) and limit of quantification (LoQ). Visual inspection approach was adopted to determine the LoD and LoQ for

sarsasapogenin while performing linearity studies. The values of LoD and LoQ obtained by determining S/N ratio in chromatogram was then confirmed mathematically, using standard deviation of response and slope of intercept.

2.7.4 Precision

The precision of the developed analytical method was determined by performing intra- day, inter-day precision and repeatability. In precision three selected concentrations from the linearity studies were spotted on TLC plate and the plates were developed following the optimized chromatographic conditions to investigate the precisions. The studies were performed six times in a day for inter-day precision studies, daily once for six days for intra-day precision studies and repeatability studies were carried out by applying the sample solution for multiple times on a plate.

2.7.5 Accuracy

The standard addition method was used to conduct accuracy studies. In a pre-analyzed sample solution, a known amount of sarsasapogenin was spiked at three distinct levels (80, 100 and 120%). The amount of sarsasapogenin was determined by analysing the spiked sample solutions. The mean value of percentage recovery was determined.

2.7.6 Robustness

Robustness of the developed method was ensured by incorporating the deliberate changes in the optimized chromatographic conditions within certain ranges. Volume of hexane, volume of ethyl acetate, volume of formic acid, migration distance, heating time, and heating temperature were selected as variables to be studied for their action on area and R_f corresponding to sarsasapogenin peak, to establish the robustness. One factor at a time approach was used for the studies. Peak area and R_f corresponding to sarsasapogenin was measured for each run and the data are compared with the control run using one-way ANOVA.

2.7.7 Estimation of Sarsasapogenin from Sample Solution

The sample solutions were prepared from plant material following the procedure optimized for the preparation of sample solution. The developed analytical method was used to analyze total sarsasapogenin from the dried root powder of *A. racemosus* using external standardization approach.

3. Results and Discussion

3.1 Process Optimization for Hydrolysis of Extract

Sarsapongenin (Figure 1) is a spirostanol type of steroidal compound which constitutes the aglycone part of saponin glycosides obtained from the roots of *A. racemosus*. Conversion of glycosidic sarsasapogenin to aglycone form, and preparing an enriched fraction containing sapogenins would minimize the interference, and this was achieved by hydrolyzing the methanolic extract using hydrochloric acid. The hydrolyzed sapogenins were collected in chloroform through

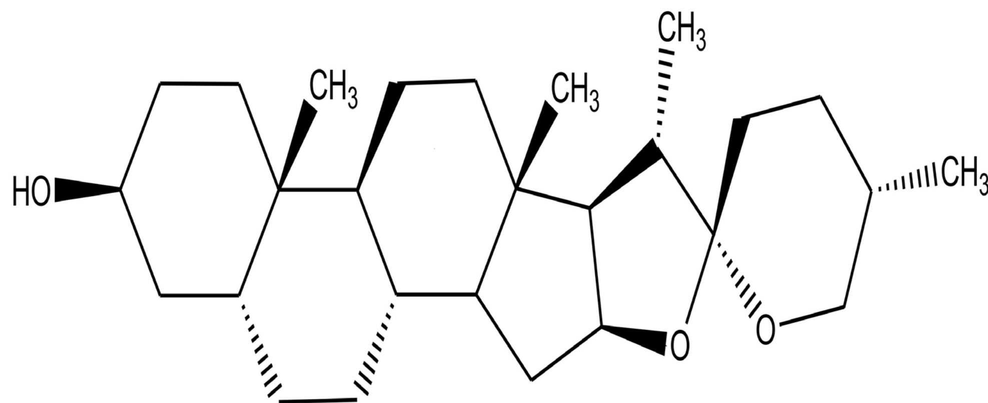


Figure 1. Chemical structure of sarsasapogenin.

Table 4. Design metrics for studies performed to optimize extraction process

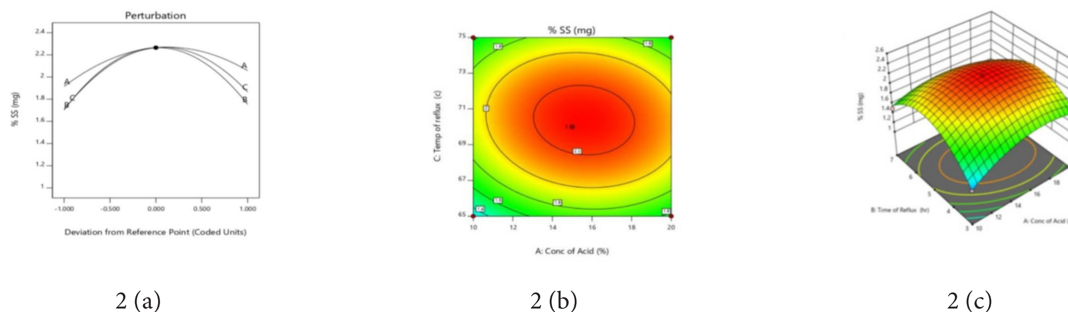
Sr. No.	Concentration of Acid (% v/v)	Time of Reflux (h)	Temperature of Reflux (˚C)	Percentage Sarsasapogenin (Avg mg % w/w)
1	20	5	65	1.5
2	15	3	75	1.4
3	20	5	75	1.6
4	15	5	70	2.28
5	10	5	65	1.3
6	10	3	70	1.2
7	15	5	70	2.25
8	20	7	70	1.4
9	15	7	75	1.2
10	10	7	70	1.5
11	15	3	65	1
12	15	7	65	1.4
13	10	5	75	1.63
14	20	3	70	1.7

(n = 3) 3 g Methanolic extract refluxed with 30 ml methanol and 30 ml aq. Hydrochloric acid

partition. The process was optimized by studying the combined effect of identified failure modes on the yield of sarsasapogenin by adopting DoE (Design of Experiments) approach. Box-Behnken design was selected considering the ability of the design to reduce the number of experiments with minimal effect on the amount of information generated. Duration and temperature of refluxing, as well as concentration of acid, were identified as failure modes to be studied, while amount of sarsasapogenin in the sample solution was a dependent variable (Table 4). The model was found statically significant ($p = 0.0014$), and the identified failure mode, concentration of acid [Factor: A], had a significant impact on the amount of sarsasapogenin estimated from the sample solution (Table 5). The difference between adjusted R^2 and predicted R^2 was 0.13, with a good precision value of 21.97. PRESS value was found to be 0.29. The statistical analysis revealed that the adopted model had lack of fit value statistically non-significant which made the model appropriate for adopting to construct and navigate MODR. The effect of selected failure modes on dependent variables was graphically represented through perturbation and contour plots (Figures 2 a, b and c). The perturbation graph suggested that heating temperature had the maximum effect, while heating time and concentration of acid had an almost similar effect on the amount of sarsasapogenin obtained after hydrolyzing the methanol

Table 5. Statistical analysis of data for extraction process

	F-value	p-value	Statistical Significance
Model statistics	8.00	0.0169	Significant
Concentration of acid (Factor: A)	9.41	0.0279	Significant
Temperature of Reflux (Factor: B)	0.0383	0.8525	Non-significant
Time of Reflux (Factor: C)	0.0369	0.8553	Non-significant



2 (a) 2 (b) 2 (c)

Figure 2. Optimization of hydrolyzed fraction using DoE and presented in the form of Perturbation Graph, Contour Plot and 3D Graph, **(a)**. Perturbation Graph showing interaction between concentration of acid, time of reflux and temperature of reflux on amount of sarsasapogenin estimated, **(b)**. Contour plot showing interaction between temperature set for reflux and concentration of acid, **(c)**. 3D surface showing interaction between concentration of acid and temperature on % Sarsasapogenin.

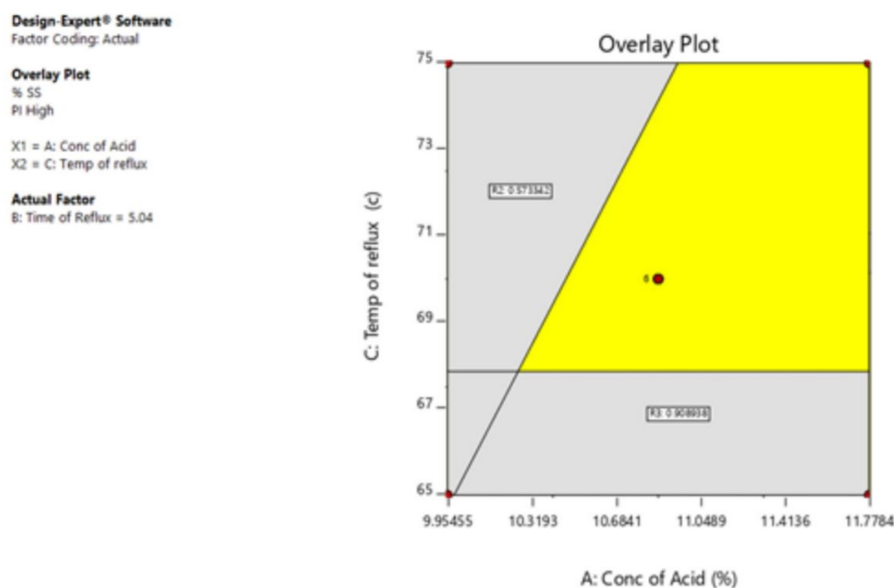


Figure 3. Overlay plot showing MODR for preparing the sample solution from the plant material.

Table 6. Assessing the fitness of the adopted model in optimizing hydrolytic conditions

Sr. No	Value of Failure mode			Difference Between Predicted and Experimentally Obtained Value for Dependent Variables (%)
	Concentration of acid (%v/v)	Time of reflux (h)	Temp. of reflux (˚C)	% Difference
1	15	3	75	1.37
2	10	5	75	9.72
3	20	5	65	6.22

(n = 3) Methanolic extract (3g) refluxed using methanol and aqueous Hydrochloric acid (30 ml)

extract. The results suggested that when the concentration of acid and temperature of reflux were 15% v/v and 70˚C, respectively, maximum amount of sarsasapogenin was determined with the set reflux time of five hours. Overlay graph was plotted using the highest anticipated value of the sarsasapogenin, which yielded MODR (Figure 3). The MODR was successfully validation (Table 6) indicating that the MODR could be navigated in order to control the failure modes to get optimal content of sarsasapogenin. Based on experiments and graphical analysis of the data, the optimal condition for the hydrolysis was chosen (3g methanol extract hydrolyzed for 5 hrs at 70˚C using 100 ml 15% v/v hydrochloric acid), and partitioned with chloroform.

3.2 DoE Approach for Chromatographic Method Optimization

FMEA is a quality management tool to identify, prioritize and manage the risks associated with the processes. Failure modes describe the way in which a process may fail to perform as intended and critical effect analysis refers to studying the consequences or effects of those failure modes on the outcome. Failure modes for the proposed analytical process were identified by prior chromatographic knowledge and preliminary experimental studies. The process yielded forty identified failure modes, categorized in 6 M and appropriately placed in Fishbone diagram (Figure 4). Failure mode risk assessment was carried out by assigning risk priority number and criticality score to individual failure modes. Preliminary experimentation and past knowledge of HPTLC method development has constituted the base for RPN to evolve failure mode risk assessment. The method risk parameters having RPN score greater than 70 were considered as priority method risk parameters to be controlled for the development of the chromatographic method (Figure 5). The studies showed that mobile phase composition (volume of hexane and ethyl acetate in the mobile phase), volume of acidic modifier (0.03 to 0.05 ml), saturation time (12 to 18 mins), volume of mobile phase (8-12 ml), concentration of acid in the mobile phase (3-7 % v/v), plate dipping time (3-7 s), plate heating time (3-7 mins) had RPN score greater than 70 in the category of method and material. Migration distance (60 to 80 mm), heating temperature (100-120), time between heating and scanning (8-12 mins) were also critical method risk parameters, with RPN scores of more than 70; for the machine.

Eleven likely critical risk factors identified through the risk assessment process were further investigated for their main effect on CMA using Taguchi Screening Design (Table 7). The analysis of the main effect of eleven method risk parameters on CMA showed that the model was significant (Table 8), and there was only less than 5% chance that the F-value could occur largely due to noise and depicted using pareto chart (Figure 6). It is evident from the studies that amount of ethyl acetate and formic acid in the mobile phase, heating time of the plate after derivatization, and run distance

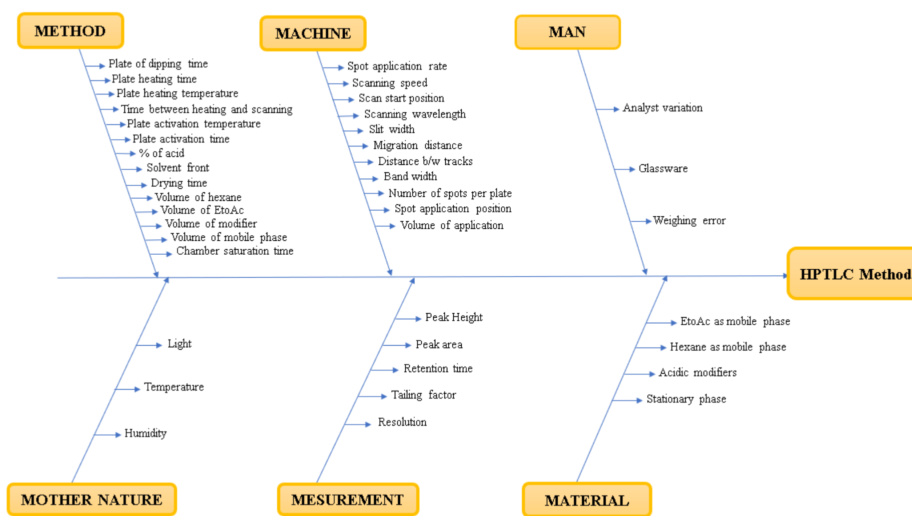


Figure 4. Fishbone diagram showing possible failure modes.

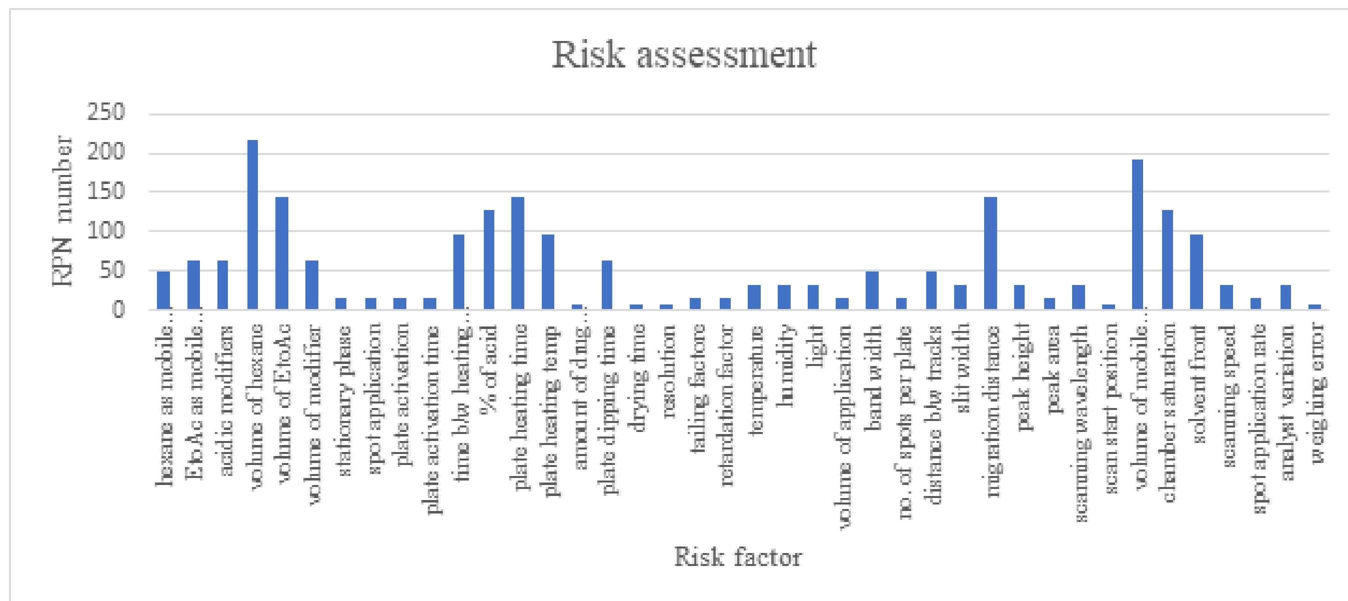


Figure 5. Graphical presentation of RPN determined as part of QRM approach.

Table 7. Design metrics for Taguchi Screening Design

Factor 1	Factor 2	Factor 3	Factor 4	Factor 5	Factor 6	Factor 7	Factor 8	Factor 9	Factor 10	Factor 11	Response 1	Response 2	Response 3
Hexane	Ethyl Acetate	Formic Acid	Heating Time	Heating Temp	Conc. of Acid	Dipping Time	Saturation Time	Volume of Mobile Phase	Run Distance	Time Between Heating and Scanning	Area	R_f	Tailing Factor
mL	mL	mL	min	°C	%v/v	s	min	mL	cm	min			
8	2.3	0.2	3	120	3	7	12	12	8	8	2357.16	0.45	1.25
6	1.5	0.2	3	100	7	7	18	12	8	12	1555.43	0.48	1.25
8	2.3	0.2	7	100	7	3	12	8	8	12	2538	0.46	1
6	2.3	0.4	7	100	7	7	12	12	6	8	1601.2	0.59	1
6	2.3	0.2	7	120	3	7	18	8	6	12	1975	0.57	0.834
8	1.5	0.4	7	100	3	7	18	8	8	8	1788.53	0.34	0.75
8	1.5	0.4	3	120	7	7	12	8	6	12	1031.74	0.49	1
6	2.3	0.4	3	120	7	3	18	8	8	8	1316.36	0.74	1
8	1.5	0.2	7	120	7	3	18	12	6	8	1574.4	0.34	1.25
8	2.3	0.4	3	100	3	3	18	12	6	12	1440.2	0.5	0.834
6	1.5	0.4	7	120	3	3	12	12	8	12	1638.7	0.63	1
6	1.5	0.2	3	100	3	3	12	8	6	8	1452.9	0.53	1.14

(n = 3) using sample solution. Sample Conc. 5µg/spot

significantly affected ($p < 0.05$) the area corresponding to sarsasapogenin peak. R_f was found significantly ($p < 0.05$) affected by amount of hexane, ethyl acetate and formic acid in the mobile phase, while formic acid was the only factor found affecting TF in significant manner ($p < 0.05$). The studies also showed that, amount of formic acid in mobile phase might reduce the area and TF of the peak, while it might increase R_f of sarsasapogenin peak.

Table 8. Statistical analysis of Taguchi Design for identifying critical failure modes

CMA	Failure Modes	F-value	p-value	Statistical Significane
Area	Model	9.25	0.0063	significant
	B-Ethyl Acetate	8.81	0.0208	
	C-Formic Acid	12.81	0.0090	
	D-Heating Time	7.10	0.0323	
	K-Run Distance	8.28	0.0238	
R_f	Model	11.08	0.0032	significant
	A-Hexane	22.15	0.0015	
	B-Ethyl Acetate	6.01	0.0398	
	C-Formic Acid	5.09	0.0541	
TF	Model	5.21	0.0315	significant
	C-Formic Acid	6.64	0.0299	
	J-Volume of Mobile Phase	3.74	0.0839	

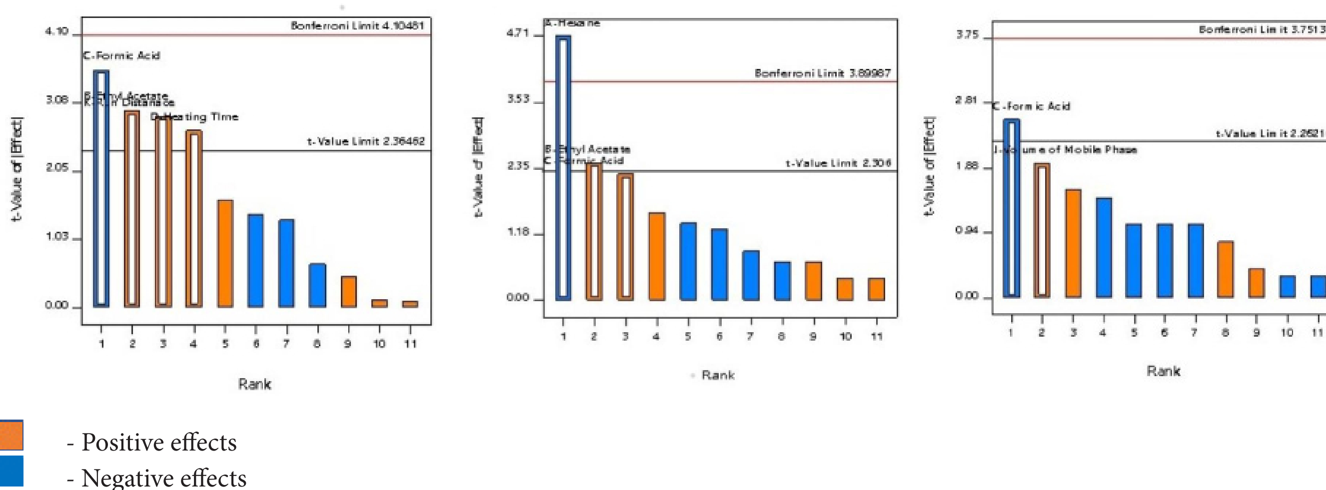


Figure 6. Pareto chart of optimization of chromatographic parameters. **(a).** Pareto chart showing the significance of the effect of failure modes on Area. **(b).** Pareto chart showing the significance of the effect of failure modes on R_f. **(c).** Pareto chart showing the significance of the effect of failure modes on TF. Factor Coding: A = Hexane, B = Ethyl Acetate, C = Formic Acid, D = Heating Time, E = Heating Temperature, F = Concentration of Acid, G = Dipping Time, H = Saturation Time, I = Volume of Mobile Phase, J = Run Distance, K = Time Between Heating and Scanning.

Screening studies suggested the potential failure modes to be studied for their combined effects on the CMA to optimize the chromatographic conditions. These failure modes were considered as CPP. The cause effect of CPP on CMA was studied using BBD (Table 9), which confirmed that the model was significant, and identified CPP having statistically significant effect on CMA (Table 10). The difference between Predicted R^2 and Adjusted R^2 value, PRESS value, and Adeque Precision were used to identify the best fit equation for respective CMA. The results suggested that the linear model could be adopted for area, R_f, and TF, and there was only a 5% possibility that the model's F-value might be largely due to noise. The curve fitting revealed that predicted R^2 value for

the CMA was in reasonable agreement of adjusted R^2 value. The value of Adequate precision was more than four, suggested that the model could be used to navigate the MODR. The regression analysis was cross-validated using the PRESS which measures the disparity between the data and an estimated model. The lower PRESS value confirmed the validity of the regression analysis (Table 11). The results revealed that the effect of formic acid and plate heating time had statistically significant effect on area of the peak corresponding to sarsasapogenin. R_f was significantly affected by the volume of hexane, ethyl acetate, and formic acid, while the volume of formic acid in the mobile phase and run distance had a substantial impact on TF. Equations 1, 2 and 3 represent the derived equations for Area and R_f and TF.

Area	$2238.60 - 119.38 * A - 18.84 * B - 179.16 * C - 96.68 * D + 245.01 * E$
R_f	$0.54 - 0.08 * A + 0.09 * B + 0.04 * C + 0.02 * D + 0.01 * E$
Tailing factor	$0.93 + 0.04 * A + 0.01 * B + 0.05 * C + 0.08 * D - 0.02 * E$

Where, A = Hexane B = Ethyl Acetate C = Formic Acid D = Run Distance and E = Heating Time

The perturbation graph plotted using the equation suggested that between the selected factors, heating time as well as run distance were the factors had the significant impact on the area of the peak, and amongst these two factors heating time had the major impact on the area. The extensive analysis revealed that incorporating changes to the mobile phase ratio and migration distance had no effect on the peak area of sarsasapogenin (Figure 7a). The contour plot illustrates the impact of heating duration and migration distance on the area of the peak corresponding to sarsasapogenin, when the volumes of hexane, ethyl acetate, and formic acid were held constant. The plot indicated that in order to get a peak area value greater than 1500 for sarsasapogenin, the heating period should be greater than 3 mins, and the migration distance should be between 65 and 75 mm (Figure 7b). Perturbation graph for R_f showed that the volume of hexane and ethyl acetate in the mobile phase had a noticeable impact on the R_f value (Figure 8a). The results also suggested that heating time and migration distance had negligible effect on the R_f of the sarsasapogenin peak. When the volume of formic acid, and run distance were held constant, the contour plot demonstrated the impact of hexane and ethyl acetate on the R_f of the peak corresponding to sarsasapogenin. The plat revealed that in order to achieve a value of R_f between 0.4 and 0.6, the volume of hexane and ethyl acetate should be about 6.5 ml and 7.5 ml (Figure 8b). Volume of formic acid had a significant impact on the tailing factor of the peak, where it was highly affected by the run distance and volume of formic acid in mobile phase (Figure 9a). The contour plot showed that in order to get a tailing factor value between 0.8 and 1.2, formic acid volume should be greater than 0.3 ml, and a migration distance should be kept between 65 and 75 mm (Figure 9b).

ATP would be achieved by optimizing the CPP affecting CMA in significant manner. The chromatographic parameters were thus optimized by navigating the design space and controlling the CPP. The software suggested number of solutions with a combination of CPP to achieve ATP value, depicted as overlain graph (Figure 10). The yellow-colored area in the overlain graph covered all suggested solutions meeting the ATP. Three points were picked from the suggested solutions for validation and risk review of the mathematical model, and compared with the results obtained after performing the experiments to ensure the prediction power. The results showed that variation was lesser than ten percentages (Table 12). This indicated that the model had a high prediction power for the suggested MODR, and MODR could be navigated optimizing CPP which could yield a robust HPTLC method for estimation of sarsasapogenin.

Data gathered during the method development and verification stages were used as part of control strategy. The data was used to correlate method attributes and the ability of the method to meet the ATP criteria. The control strategy included fixing of the parameters influencing the study results. Though control strategy might not be different from the traditional approach, but it was established using comprehensive datasets and ensured a link between the purpose of the method and the performance of the method. One point was selected from the MODR as the control strategy to optimize the chromatographic parameters in the method developed for the estimation of total sarsasapogenin. The chromatogram with optimized chromatographic conditions (Table 13) are recorded, which showed well resolved and Guassian peak corresponding to sarsasapogenin (Figure 11).

Table 9. Design matrix for studies performed to optimize chromatographic parameters

Sr. No	Hexane	Ethyl Acetate	Formic Acid	Run Distance	Heating Time	R1: Area	R2: R_f	R3: Tailing Factor
	mL	mL	mL	mm	min			
1	7	1.9	0.2	60	5	2296	0.465	0.75
2	7	2.3	0.3	70	3	2048.65	0.575	0.92
3	7	1.9	0.3	70	5	1802.75	0.585	0.857
4	7	1.9	0.3	80	7	2393.25	0.54	0.83
5	6	1.9	0.3	70	3	1917.65	0.65	1
6	7	1.5	0.3	80	5	2050.75	0.43	0.84
7	7	1.9	0.3	80	3	2218.85	0.535	1.125
8	7	1.9	0.2	80	5	2634.1	0.545	0.9
9	8	1.9	0.3	70	3	2071.1	0.49	0.9
10	8	1.5	0.3	70	5	2068.9	0.37	1
11	7	1.9	0.3	70	5	2017.4	0.53	1
12	7	2.3	0.3	70	7	2288.6	0.61	0.8
13	7	1.9	0.3	70	5	2320.95	0.57	1
14	7	1.9	0.3	70	5	2182.7	0.59	1
15	8	1.9	0.3	80	5	1556.9	0.55	1.3
16	6	1.9	0.2	70	5	2811.15	0.525	0.875
17	7	1.9	0.2	70	7	2493.8	0.47	0.87
18	7	1.5	0.3	60	5	2771.6	0.41	0.75
19	7	1.9	0.3	70	5	2438.45	0.58	0.87
20	6	1.9	0.3	80	5	1728.55	0.68	1.08
21	8	1.9	0.3	70	7	2278.3	0.49	0.87
22	8	2.3	0.3	70	5	2159.85	0.5	1
23	7	1.9	0.3	70	5	2550.85	0.5	0.94
24	8	1.9	0.2	70	5	2280.6	0.42	1
25	7	1.9	0.2	70	3	1947.1	0.44	0.91
26	7	2.3	0.3	80	5	2405.2	0.61	0.875
27	7	1.5	0.4	70	5	2171.4	0.43	0.87
28	7	1.9	0.4	70	3	2048.9	0.595	0.8
29	7	1.5	0.2	70	5	2628.5	0.36	0.71
30	7	1.9	0.3	60	7	2800.5	0.485	0.75
31	7	1.9	0.3	60	3	2213.75	0.44	0.9
32	6	1.9	0.4	70	5	2605.25	0.54	1
33	6	1.9	0.3	70	7	2775.6	0.695	0.8
34	7	2.3	0.4	70	5	2113.95	0.59	1.125
35	6	1.5	0.3	70	5	2308.75	0.57	1
36	6	1.9	0.3	60	5	1972.3	0.705	0.875

Table 9 to be continued...

Sr. No	Hexane	Ethyl Acetate	Formic Acid	Run Distance	Heating Time	R1: Area	R2: R_f	R3: Tailing Factor
37	7	1.9	0.4	80	5	1733.1	0.595	1
38	7	1.9	0.4	60	5	1726.3	0.56	0.8
39	7	2.3	0.3	60	5	2383.1	0.61	0.87
40	6	2.3	0.3	70	5	2314.4	0.74	0.8
41	7	1.5	0.3	70	7	2774.75	0.48	1
42	8	1.9	0.4	70	5	2003.8	0.565	1
43	7	1.5	0.3	70	3	1740.7	0.52	1
44	7	1.9	0.4	70	7	2322	0.6	1.16
45	8	1.9	0.3	60	5	2104.1	0.485	1
46	7	2.3	0.2	70	5	2500.05	0.595	1

(n = 3) using sample solution. Sample Conc. 5 µg/spot, saturation time 15 min, conc. of acid 5%v/v H_2SO_4, dipping time 5 sec, heating temp. 110˚C, time between heating and scanning 10 min.

Table 10. Statist ical analysis for assessing the effect of CPP on CMA

CMA	CPP	F-value	p-value	Statistical Significance
Area	Model Statistics	5.46	0.0006	Significant
	Amount of Hexane	3.35	0.0746	
	Amount of Ethyl Acetate	0.0835	0.7741	
	Amount of Formic acid	7.55	0.0090	
	Run Distance	2.20	0.1461	
	Heating Time	14.11	0.0005	
	Lack of Fit (F = 0.87, p = 0.646)			Non-significant
R_f	Model Statistics	18.30	<0.0001	significant
	Amount of Hexane	38.09	<0.0001	
	Amount of Ethyl Acetate	39.65	<0.0001	
	Amount of Formic acid	10.72	0.0022	
	Run Distance	2.64	0.1122	
	Heating Time	0.3902	0.5357	
	Lack of Fit (F = 2.05, p = 0.217)			Non-significant
TF	Model Statistics	2.98	0.0222	significant
	Amount of Hexane	2.18	0.1479	
	Amount of Ethyl Acetate	0.2573	0.6148	
	Amount of Formic Acid	2.91	0.0957	
	Run Distance	8.37	0.0061	
	Heating Time	1.20	0.2800	
	Lack of Fit (F = 2.85, p = 0.120)			Non-significant

Table 11. Selection of experimental model to optimize chromatographic parameters

Sr. No	CMA	p-value for Model	R2	Adjusted R2	Predicted R2	Adequate Precision	PRESS	Suggested Model
1	Area	0.0006	0.4056	0.3312	0.2112	9.0038	3.612X 10^6	Linear
2	R_f	<0.0001	0.6958	0.6578	0.5910	17.2624	0.1346	Linear
3	TF	0.0222	0.2716	0.1806	0.0118	6.3680	0.6381	Linear

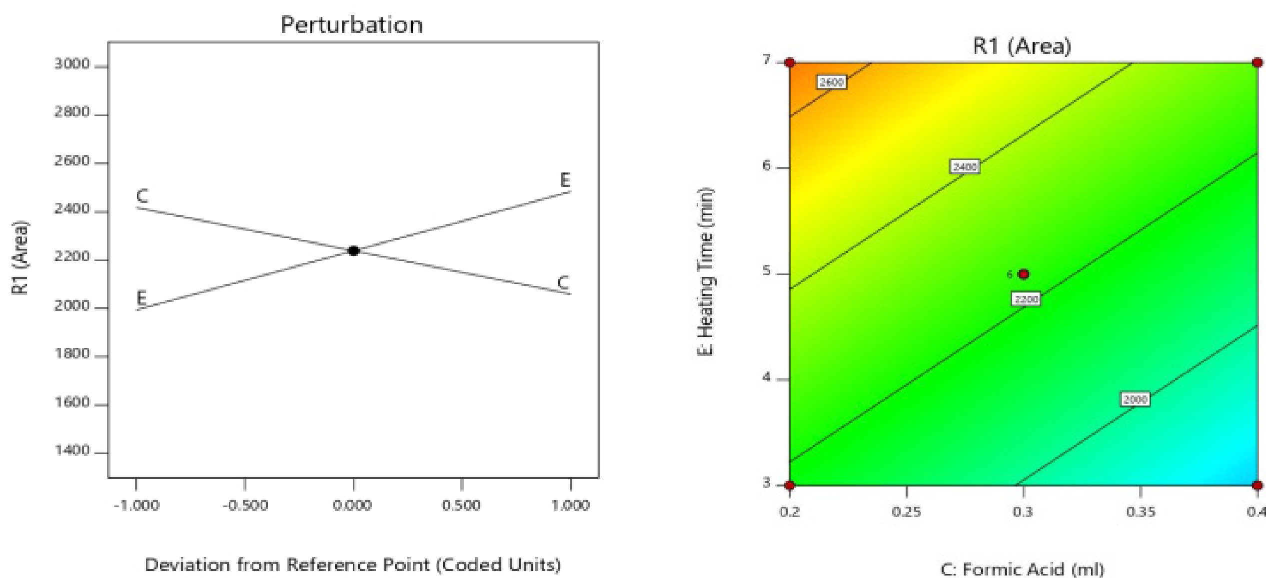


Figure 7. Perturbation chart and contour plot for area. **(a).** Perturbation chart showing deviation from a reference point on the area for the selected factors. **(b).** Contour plot for optimizing CPP for area. Factor Coding: A = Hexane, B = Ethyl Acetate, C = Formic Acid, D = Run Distance, E = Heating Time.

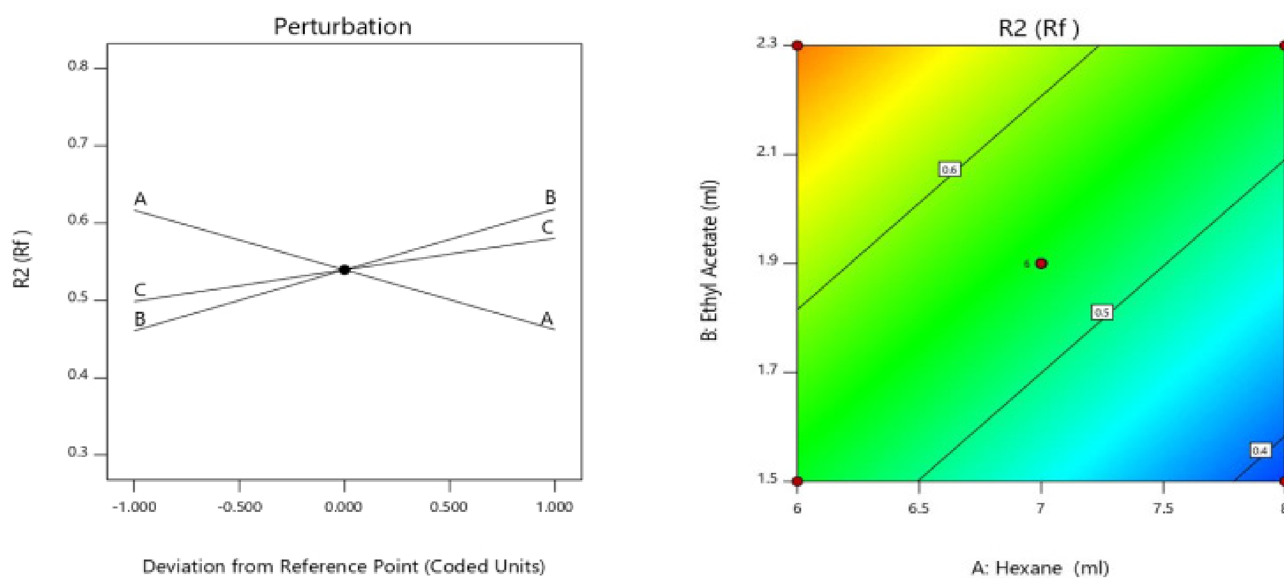


Figure 8. Perturbation chart and contour plot for R_f. **(a).** Perturbation chart showing deviation from a reference point on R_f for the selected factors. **(b).** Contour plot for Optimizing CPP for R_f. Factor Coding: A = Hexane, B = Ethyl Acetate, C = Formic Acid, D = Run Distance, E = Heating Time.

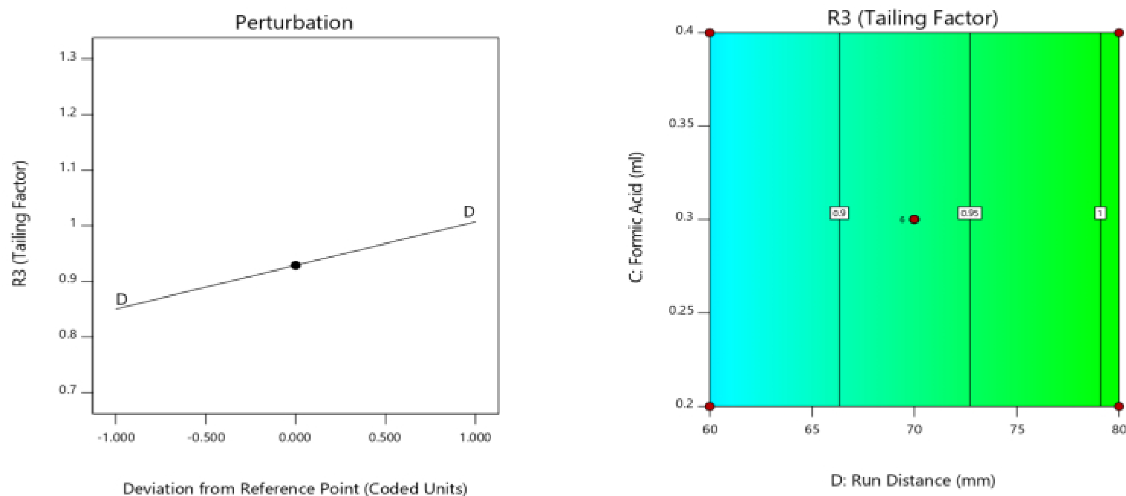


Figure 9. Perturbation chart and contour plot for TF. **(a).** Perturbation chart showing deviation from a reference point on TF for the selected factors. **(b).** Contour plot for optimizing CPP for TF. Factor Coding: A = Hexane, B = Ethyl Acetate, C = Formic Acid, D = Run Distance, E = Heating Time.

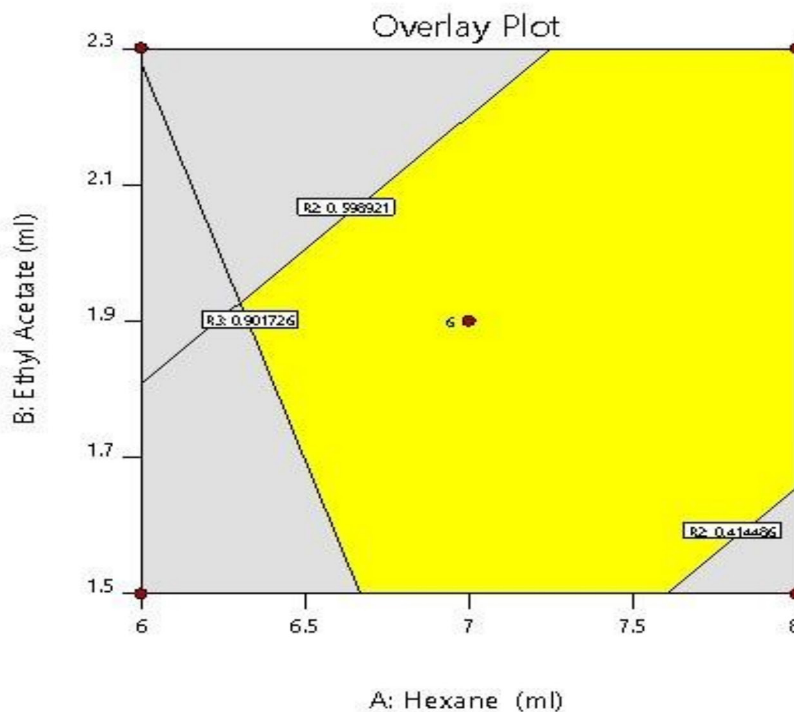


Figure 10. Method Operable Design Region (MODR) for the HPTLC method.

Table 12. Validation of experimental design

Sr. No	Value of CPP					Difference Between Predicted and Experimentally Obtained Value for CMA (%)		
	Hexane	Ethyl Acetate	Formic acid	Run distance	Heating time	Area	R_f	TF
1	8.00	1.90	0.40	70.00	5.00	-6.49	2.58	1.48
2	7.00	1.50	0.30	70.00	3.00	-3.26	-3.09	2.65
3	7.00	1.90	0.20	60.00	5.00	2.30	1.67	-5.72

Table 13. Optimized chromatographic condition

Sr. No.	Parameters	Description
1	Stationary phase	Silica gel G_{60} F_{254} coated on Aluminum sheet
2	Mobile phase	Hexane: ethyl acetate: formic acid (7:1.9:0.35, v/v/v)
3	Saturation time	15 min
4	Migration Distance	70 mm
5	Concentration of acid	5 % v/v sulphuric acid
6	Heating time	3 min
7	Heating Temperature	110 °C
8	Band length	8 mm
9	Scanning wavelength	366 nm (mercury lamp)
10	Time between heating and scanning	10 min
11	Slit size	(6.00 X 0.45) mm
12	Scan Speed	20mm/s

3.3 Analytical Method Validation

The HPTLC based analytical method was developed and optimized for estimation of sarsasapogenin was subjected to validation studies following ICH Q2 (R1) Guideline. The value of selected parameters to evolve system suitability were summarized (Table 14). The selectivity of the analytical method is the ability to measure an analyte accurately in the presence of interference. Selectivity of the method was determined by scanning the spot of sarsasapogenin in standard as well as sample track. The regression analysis of the spectra recorded at three different positions of the spots confirmed that the coefficient value was found 0.999, suggested that the peak was spectrally pure. The appearance of the spot at the similar R_f value in track of standard as well as of sample solution and value of regression coefficient for spectra recorded for spot corresponding to sarsasapogenin in sample track approaching to one established the selectivity of the developed method. The linearity of developed HPTLC method was established for sarsasapogenin using spots ranging from 40-280 ng/spot. Concentration range and area of the respective peak were noted and subjected to the least square linear regression analysis. The value of regression coefficient approaching one suggested the linearity of the response. The value of LoD and LoQ for sarsasapogenin was found to be 11.82 ng/ml and 35.88 ng/ml respectively. The results showed that the developed HPTLC method was sensitive to detect and estimate sarsasapogenin from the sample solution. A low standard deviation of the developed analytical method revealed that the developed analytical method was precise. Repeatability studies were performed by repetitively measuring the area corresponding to sarsasapogenin from sample solution. A low value of percentage standard deviation suggested that, the system was in order and could respond evenly for all the instances. Accuracy of the method was established

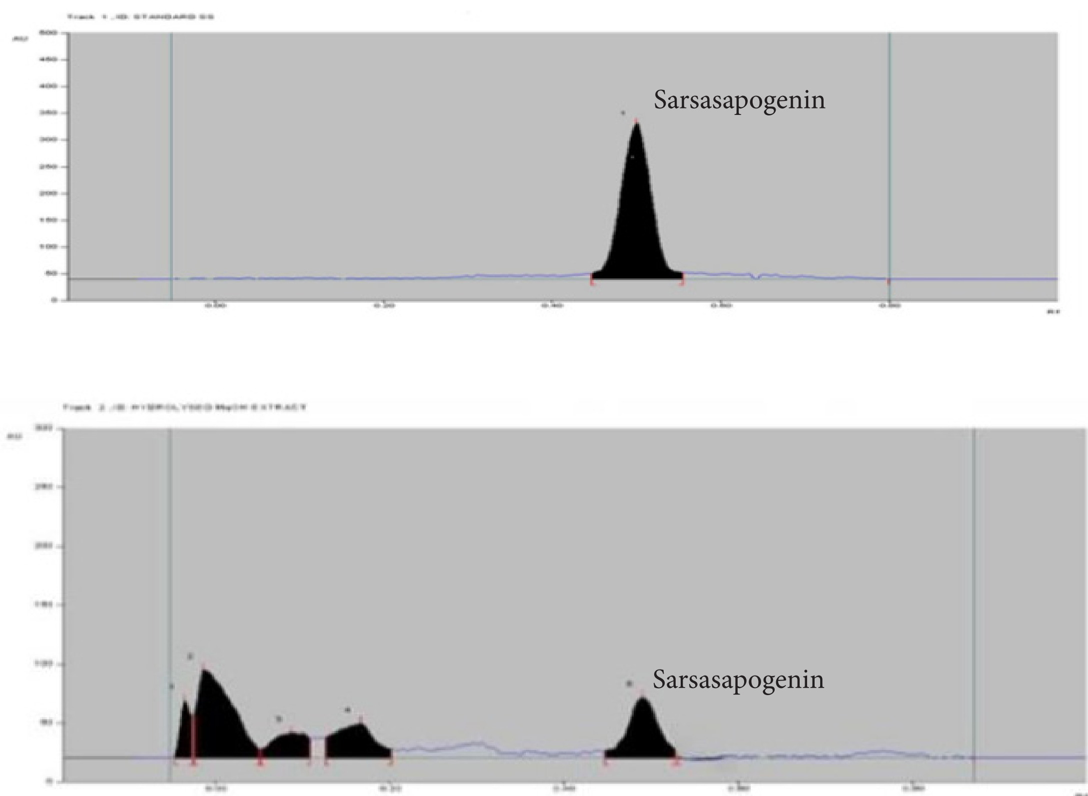


Figure 11. Chromatogram recorded following optimized chromatographic conditions showing the peak of standard sarsasapogenin. **(a).** Chromatogram of standard sarsasapogenin (0.5µg/spot), **(b).** Chromatogram of sample solution (5µg/spot).

Table 14. Summary of validation parameter

Sr. No	Parameters	Results
1	Selectivity	r^2 = 0.995 (standard) r^2 = 0.992 (sample)
2	Linearity (ng/spot)	40 - 280 ng/spot
3	Regression coefficient	0.998
4	y- Intercept Mean ± SD	904.55 ± 12.93
5	LoD (ng/spot)	11.82 ng/spot
6	LoQ (ng/spot)	35.88 ng/spot
7	Intraday Precision(%RSD)	0.57 - 0.67%
8	Interday Precision(%RSD)	0.76 - 1.07%
9	Repeatability	0.67 %
10	Accuracy (%Recovery)	99.35 - 101.20%
11	Robustness [(F_{exp}< F_{crit}) p > 0.05, null hypothesis was to be accepted]	Robust

by performing recovery studies by adopting standard addition method. The average recovery value was found in the range of 99-101%, indicating that the developed analytical method was capable of measuring the amount of sarsasapogenin from the sample solution to very close to the actual amount. Robustness of the developed analytical method was ensured by altering the selected critical analytical parameters within certain limits. The parameters were carefully chosen on the basis of the effect of a variable observed on the area and R_f corresponding to sarsasapogenin while developing the analytical method. It should be noted here that the studies were performed by altering only one of the selected factors at a time. Statistical analysis of the data sets using ANOVA showed $F_{exp} < F_{crit}$ (Null hypothesis to be accepted). The studies showed that when the data sets were subjected to one-way ANOVA, it was revealed that $F_{exp} < F_{crit}$. It suggested that mean value of the groups was not significantly different to each other. The results of the studies, thus, indicated that the data sets comprising the area and R_f corresponding to peak of sarsasapogenin obtained after altering the selected critical chromatographic parameters were not statistically different to each other. The results revealed that the developed method was robust within the tested variations in optimized chromatographic conditions.

3.4 Estimation from Hydrolyzed Extract and Marketed Formulation

The developed analytical method was used to analyze total sarsasapogenin from the dried root powder of *A. racemosus*. Optimized experimental conditions were used to prepare hydrolyzed fraction from the extract. The sample solution was then subjected to analysis following optimized chromatographic conditions and the area of the peak corresponding to sarsasapogenin was integrated. The value of peak area was used to determine the concentration of sarsasapogenin from the sample solution using external standardization method. The amount of total sarsasapogenin was determined to be 2.31% w/w from the root powder; on dried weight basis.

4. Conclusion

An analytical approach was evolved for estimation of total sarsasapogenin from the roots of *A. racemosus*. A sensitive analytical method was developed using HPTLC as part of quality control strategy after adopting AQbD approach. The method was developed after identifying the possible failure modes and characterizing the combined effect of critical process parameters on critical method attributes through design of experiments. The developed method was validated for the purpose, and validated analytical method was employed for estimation of total sarsasapogenin from the dried root powder of *A. racemosus*. The developed method, being evolved through a systematic approach, could be adopted as part of routine quality control strategy. This is probably the first analytical studies based on AQbD approach for estimation of sarsasapogenin from *Asparagus* spp.

5. Acknowledgement

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