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DEVELOPMENT AND VALIDATION OF HPLC METHOD FOR SIMULTANEOUS DETERMINATION OF MOISTURIZERS, PRESERVATIVES AND ANTI-OXIDANTS IN COSMETIC FORMULATIONS BY DESIGN OF EXPERIMENTS.

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ABSTRACT

Excipients are substances other than the active drug or prodrug that has been appropriately evaluated for safety. The present study aims at to develop and validate single RP HPLC method using QbD aspects to determine Ceramide PC 104, Cholesterol, Linoleic acid, Squalene, Sorbic acid, Phenoxyethanol and α-Tocopherolacetate simultaneously. The method is based on separation employing HPLC with mobile phase (A) 0.1% ortho phosphoric acid - methanol (70:30 % v/v) and (B) methanol- acetonitrile (80:20 % v/v) eluted in gradient mode. The analytes were separated on aWaters symmetry C18 column 250 x 4.6 mm 5μ at a flow rate of 1.3mL/min and detected at two wavelengths,210nm and 264nm respectively. Seven compounds used in moisturizing creams are well separated using a single HPLC method, the method is found accurate, precise, specific, linear and robust. It can be applied for quality control release of a cream formulation containing Ceramide PC 104, Cholesterol, Linoleic acid, Squalene, Sorbic acid, Phenoxyethanol and α-Tocopherol acetate.

Key words: Moisturizes, Ceramide, Preservatives, HPLC, DOE and Cream.

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INTRODUCTION

There is a vast array of moisturizers available in the market today and consumer demand for these products is growing. These products range from value brands that provide basic moisturization to luxury therapeutics with claims of anti-aging benefits. A recent study found that moisturizers are the third most commonly recommended OTC topical skin products (13.4%) behind hydrocortisone (27.6%) and anti-infectives (23.4%)[1].These preparations are used to restore the barrier function of the epidermis, and increase the water-content of the epidermis. Traditionally, moisturization was believed to inhibit transepidermal water loss (TEWL) by occlusion. Water originates in the deeper epidermal layers and moves upward to hydrate cells in the stratum corneum (SC), eventually being lost to evaporation[2].The moisturizing treatment involves repairing the skin barrier, increasing water content, reducing TEWL, restoring the lipid barrier's ability to attract, hold and redistribute water. The ideal characteristics would be an effective moisturizer, an emollient, an aid in restoring the lipid barrier, cosmeticallyelegant and acceptable.Loden suggests that skin care products not only form an inert, epicutaneous layer, but they also penetrate, influence the structure and function of the skin[3].Shantikumaret. al.[4,5] have showed the applicability of design of experiments (DOE) to chromatographic methods, even the importance of simultaneous estimation was also elucidated for various pharmaceuticals[6]. The history of DOE dates back to 1920 when it was originally proposed by Ronald A. Fischer, a British scientist, in order to maximize the knowledge gained from experimental data. The approach proposed by him overcomes the limitations of traditional approaches by considering all variables simultaneously and obtaining the most relevant data

with minimal effort[4]. Thus in the present study DOE approach was used during method development so as

to ensure valid and supportable conclusions. Ceramide (Fig.-1) is chemically N, N'-(2-hydroxypropane-1, 3-diyl) bis (N-(2-hydroxyethyl) palmitamide) which constitutes 50 % of human SC lipids. They are considered to play a critical role in the barrier properties of SC. Linoleic acid (Fig.-1) is chemically (9Z, 12Z)-octadeca-9, 12-dienoic acid. It helps to reduce the water loss through the skin epidermis, further improving its conditions[7]. Cholesterol (Fig. 1) is chemically (8S,9S,10R,13R,14S,17R)-10,13-dimethyl-17-((R)-6-methylheptan-2-yl)-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-3-ol which is used in cosmetics which maintain skins normal function. It is also used as a stabilizer, emollient and water-binding agent. Squalene (Fig.-1) is a hydrocarbon and a triterpene used in cosmetics and more recently as an immunologic adjuvant in vaccines. Preservatives such as phenoxy ethanol, sorbic acid (Fig.-1) were widely used to kill or inhibit the growth of microorganisms. Antioxidants such as tocopheryl acetate (Fig.-1) are included in preparations to inhibit oxidation by reacting with free radicals blocking the chain reactions.
In literature individual analytical methods are available for determination of quality of Ceramides (CE), preservatives, anti-oxidants in cosmetic products. These ingredients are used in cosmetic cream formulations and hence there is need for a common analytical method to apply to various dosage forms[8,9].Therefore an attempt was made to develop a single method using single mobile phase to determine the quality of these components simultaneously.

EXPERIMENTAL

Instrumentation

Agilent 1100 series HPLC system equipped with Quaternary pump with an online degasser, auto sampler, thermostatted column compartment and variable wavelength detector. Waters alliance HPLC system equipped with 2695 separations module and 2489 UV-VIS detector or 2998 Photodiode array detector. Waters Empower2 software is used for data acquisition, data processing.

Materials and Reagents

CE PC 104is procured from Macrocare Tech. Ltd., Cheongwon Chung Buk, South Korea; LA is procured from ACME synthetic chemicals, Goregaon (W) Mumbai, India; CH is procured from CRODA Inc. USA; SQ is procured from Sarvotham care Ltd, Hyderabad, India;PE, SA; Ortho phosphoric acid(OPA) AR grade is procured from Merck,India. Methanol (MeOH), acetonitrile (ACN) HPLC grade were purchasedfrom Merck, India; Tetrahydrofuran (THF) from Merck, India; High pure water is from Mill-Q water purification system from Millipore.

Chromatographic conditions

The HPLC analysis was performed using Water symmetry C-18 column (250 mm × 4.6 mm, 5 μm). The temperature of column compartment was maintained at 40 °C. A gradient elution at a flow rate of 1.3 mL min-1 was employed and detection was carried out at 210 and 260 nm. Eluent A was 0.1%OPA - MeOH (70:30 v/v) and eluent B was MeOH - ACN (80:20 v/v). Run time of 60 min with a gradient elution: 0.0 – 8.0 min (15 % B), 8.0 – 12 min (15 – 77 % B), 8.0 – 23 (77 - 87% B), 23 – 26 (87 - 97 % B), 26.0 – 50 (97 % B), 50 – 55 (97 - 15 % B) and 55 – 60 (15 % B) was used.The mixture of methanol and acetonitrile (80:20 v/v) was used as a diluent.

Preparation of standard and sample solution

Stock solution: Stock solutions (SS) were prepared in THF at a concentration of a) SS of moisturizers (SSM) 2mg/ml of CH, 2mg/ml of LA, 4mg/ml of SQ and 2mg/ml of CE, b) SS of preservatives (SSP)

0.2mg/ml of PE and 0.5mg/ml of SA,c) SS of anti-oxidant (SSA) 0.5mg/ml of TA.Standard solution or system suitability solution is prepared by mixing5ml ofSSM, 4ml of SSP and 1ml of SSA, diluted to 50 ml with mixture of Diluent.

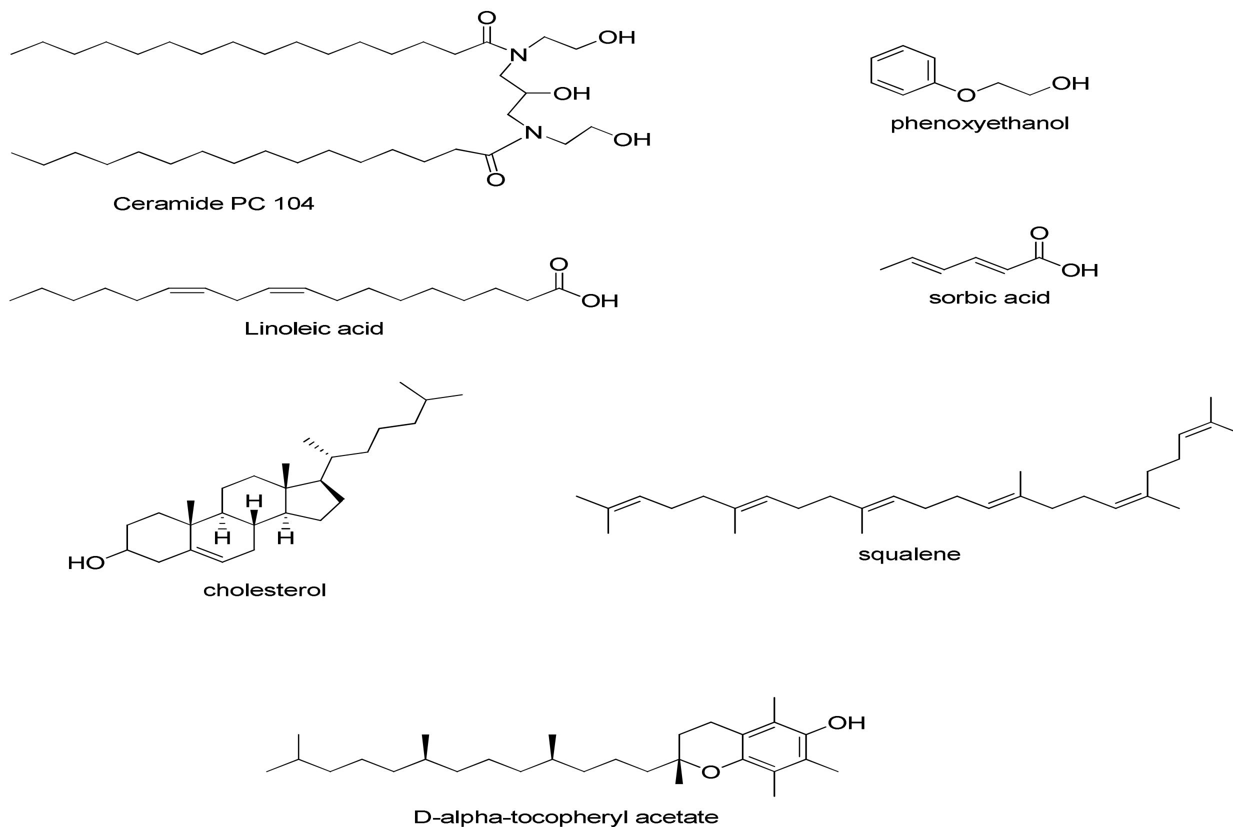

Fig.-1: Structures of all the components separated in the present study.

Sample solution : Sample preparation of pure compounds: About 20mg of CE, CH, LA, 40mg of SQ, 4mg of PE, 2mg of SA, 1mg of TA were weighed into 100ml volumetric flask to this 20ml of THF was added and contents are dissolving by means of sonication,and the final volume is made up to 100 ml with diluent.

Formulation sample preparation: Cream formulationcontaining1-3% of SQ, 0.5-2% of CE ,0.5-2%of CH, 0.5-2%of LA, 0.1-0.3%of PE, 0.05-0.15%of SA and0.025-0.1% of TA are dispersed in 20ml of THFby means of mechanical shaking or sonication. To the above dispersion 30 ml of diluent is added and sonicated to completely extract the ingredients into solution, diluted to 100ml with diluents and mixed will. The extract is centrifuged and the clear solution is injected for chromatography.

RESULTS AND DISCUSSION

Method development

A systematic approach is followed during method development.

Column selection

The selected analytes are highly non-polarity and have poor aqueous solubility.Stationary phase for separating these compounds need to be optimally carbon loaded which shall retain non polar compounds, length of the column is also important for resolving the closely eluting peaks. When low carbon loaded columns are tried PE and SA are eluted in void and CE, TA, CH peaks are not resolved. Narrow pore size

columns generated higher pressure on repeated injections due to presence of waxy materials in cream placebo. Higher injection volumes on less than 5μ particle size stationary phase resulted in peaks splitting, whereas low injection volumes resulted in low responses. Hence longer column (250mm) with carbon loading of 19, pore size of 100A, particle size of 5μ is selected and Water symmetry C_{18} 250 x4.6 mm, 5μ gave best results.

Wave length selection

Experiments demonstrated that the selected compounds have different UV absorption maxima (λ_{max}). λ_{max} of PE is 221nm, LA is 204nm, CE is 207nm, TA is 206nm, CH is 205nm, SQ is 207nm and SA is 264nm. Hence 210nm is selected for quantifying PE, LA, CE, TA, CH and SQ. 264nm is selected for quantifying SA (Fig. 6).

Diluents

Solubility of analytesis crucial for selection of diluents[10]. Majority of selected compounds are soluble in non-aqueous solvents and moderately polar organic solvents like THF and ACN. The sample extraction was carried out using two solvents. Analytes are dissolved in THF and diluted with a solvent mixture of ACN - MeOH (15:85 v/v).

Sample preparation

Extraction of components from the sample matrix (cream) need optimization of parameters like sonication time (in diluent) (table-1), weight of the sample(table-1(b)), dilution volumes (THF and diluent), diluents composition (diluent) (table-1), filtration of final aliquot, also the injection volume, needed optimization.

Using these parameters the method was optimized and the details of the final optimized method were shown above. The chromatogram showing neat separation of all components was depicted in Fig.-6. While table-2 shows the chromatographic peak characteristics.

Method Validation

The developed method is validated for accuracy, precision, specificity, limit of detection, limit of qualification, linearity and robustness, according to the ICH guidelines[11].

Accuracy and precision

Accuracy is performed at three levels to the test concentration (50%, 100% and 150%), by spiking pure compound solution to placebo. Recovery is found to be within the limits of 95% to 110% (Table-3). Precision of the test method is demonstrated on pure compounds and also by analyzing the six preparations made by spiking pure compound on placebo at the test concentration level. The % RSD of six preparations with respect to each component is less than 2% (Table-3).

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

LOD and LOQ are established to all seven compounds based on standard deviation of response and slope. The correlation of the linear curve is not less than 0.999 (Table-3).

Table-1: Development Parameters.

Parameter	% Assay						
Sonication time (min)	PE	SA	LA	CE	TA	CH	SQ
10	102.45	125	110.7	87.35	88.8	105.85	102.85
15	103.6	125.05	111.3	86.95	89.55	104.1	102.95
20	103.7	125.45	113.2	85.6	89.6	105.35	103.2

30		103.85	126.1	112.45	89.25	90.4	106.45	104.1
Weight variation								
1 gram in 100ml		104.3	135.1	107.5	94	97	108.3	107
2 grams in 100ml		103.4	134.3	111.4	95.5	97.9	104.4	103.4
3 grams in 100ml		102	133.1	109.6	92.7	94.8	103.2	101
5 grams in 100ml		98.6	132.8	107.3	88.3	93.5	102.7	100.5
10 grams in 100ml		93.4	132.1	104.2	83.6	92.2	101.1	98.6
Filtration / Centrifuging								
PTFE 0.45µ		103.9	133.8	108.7	91.9	94.4	106.6	104.6
PVDF 0.45µ		103.8	133.7	108.5	91.6	90.8	105.1	105
Hydrophilic PVDF 0.22µ		103.8	133.8	108.5	92.3	90.7	103.1	104.8
Nylon		104	133.8	109.5	92	91.9	105.5	105
Centrifuge		104.3	134.4	109.2	92.2	94.9	105.6	105.1
Diluents composition (MeOH : Water)								
80:20		97.2	130	102.9	84.0	67.3	91.8	55.0
Diluents composition (MeOH : ACN : Water)								
80:10:10		101.4	132.6	110.2	94.3	95.3	107.3	101.2
Diluents composition (MeOH : ACN)								
75:25	Mean(3)	97.7	130.3	110.0	91.2	96.9	103.4	103.4
80:20	Mean(3)	102.7	133.5	108.9	91.9	95.4	104.7	103.9
85:15	Mean(3)	102.8	133.2	109.3	93.3	96.2	104.7	104.7
90:10	Mean(3)	102.5	132.8	110.7	92.0	102.5	104.3	104.0
Injection volume (µl)								
40		†	†	✓	✓	✓	✓	✓
30		*	*	✓	✓	✓	✓	✓
20		✓	✓	✓	✓	✓	✓	✓
10		✓	✓	✓	•	•	•	✓

✓ : Peak shape is satisfactory,*: distorted peak, †: peak split, •: low response.

Table-2: Chromatographic peak characteristics.

Peak	PE	SA	LA	CE	TA	CH	SQ
R.T (min)	6.6	8.1	22.4	36.7	38	39.4	49
% RSD of peak area. (5)	0.5	0.5	0.5	1	1.3	1.4	0.3
Resolution	1	3.2	32.8	27.5	2.5	4.5	9.9
Plate count	3729	4331	42279	98669	91729	64244	56574
Tailing	1	1	1.1	1.1	1	1	1

Specificity

Specificity of test method is demonstrated by performing stress study/ degradation on pure moisturizers, the degradants are well separated from peaks of interest. LA degraded under exposure to light, CE

degraded more in acid over base and light, CH degraded more under exposure to light over thermal, peroxide & base while SQ is found to be degraded less than 2% in all stress conditions. Also degradation if done on formulation with acid at different concentration of 0.1N, 0.5N and 2N at 60°C for 30 minutes to have adequate level of degradation, similarly with base of 0.1N, 0.5N and 2N at 60°C for 30 minutes. Oxidative stress is done by 3% and 10% hydrogen peroxide (H2O2), thermal stress by maintaining 60°C for 24 hours. Photolytic degradation by exposing to 1.2 million Lux hours and 200 kilo watts per m^2 (Table-3).

Linearity

Linear range of the test method is established from LoQ to 200% of the test concentration, the correlation is not less than 0.9997 and bias at 100% is less than +/- 2 (Table-3).

Robustness

The conventional approach for optimizing analytical methods in laboratory is by one-factor-at-a-time, where each experimental parameter is optimized separately and independently of other parameter is possible by using Design of experiments (DoE)[12] which is "Quality by design (QbD) approach". In contrast, factorial designs involve simultaneous optimization of all factors at once. Factorial designs offer a simple, efficient, and statistically valid method for optimizing analytical methods. The robustness is evaluated for flow rate, temperature of column, polar solvent composition in mobile phase B. These three factors are assigned as variables and the resolution between the closest peaks are fixed as responses in a design expert software 8.0.7.1 for full factorial 2^3 design. The experimental domain was defined and a zero-level (center), in which all variables are fixed at their mean value (Table-4), was included in order to minimize the risk of missing non-linear relationships.

A minimum obtained value of individual resolution (R_s) values of 2.3 was used as a selection criterion. Proposed eleven experiments were carried out and R_s values of all consecutive peak pairs were calculated. The resolution R1 between CE & TA and resolution R2 between TA & CH values of obtained peak pairs are tabulated. In the full factorial 2^3 experimental design, a linear mathematical model of the measured response is often applied for the evaluation of the influence of investigated factors.

An often used linear model is: $y = b_0 + b_1x_1 + b_2x_2 + b_3x_3 + b_{12}x_1x_2 + b_{13}x_1x_3 + b_{23}x_2x_3 + b_{123}x_1x_2x_3$ Where y represents the estimated response, b_0 is the average experimental response, coefficients b_1, b_2, and b_3 are the estimated effects of the factors considered. The extent to which these terms affect the performance of the method is called the main effect. The coefficients b_{12}, b_{13}, b_{23} and b_{123} are called interaction terms.

Table-3: Summary of results of validation parameters.

Accuracy (Recovery)	Label claim (% w/w)	PE (0.2)	SA (0.1)	LA (1)	CE (1)	TA (0.05)	CH(1)	SQ (2)
50%	Mean(3)	102.4	100.6	107.4	104.1	104.7	101.6	104.3
100%	Mean(6)	105.1	102.4	107	104.5	102.8	103.4	103.8
150%	Mean(3)	102.9	99.7	104.5	102.3	100	101.7	99.9
Precision	Prep-1	105.8	102.7	106.8	105.4	101	105.3	105.3
	Prep-2	105.7	102.8	107	104.4	103.3	103.8	103.9
	Prep-3	103.5	101.4	107.9	104.9	102.6	103.1	103.7
	Prep-4	105.3	102.5	106.6	103.1	103	101.8	103.1
	Prep-5	105.3	102.3	106.8	104.6	104.6	103.3	103.2
	Prep-6	105	102.4	106.8	104.3	102.3	102.9	103.4
	mean	105.1	102.4	107	104.5	102.8	103.4	103.8

	% RSD	0.8	0.5	0.4	0.7	1.2	1.1	0.8
Stress Study of formulation (% Degraded)	A-2 N	2.5	3.7	66.8	36	9.7	2	12.4
	A-0.5 N	3.9	ND	18.3	24	0.8	3.8	2.5
	A-0.1 N	5.6	1.3	23.3	18	1.1	8.7	3.6
	B-2 N	3	0.6	1.6	100	100	4.9	4.3
	B-0.5 N	4.1	ND	0.3	60.5	100	7.7	1.3
	B-0.1 N	3.9	0.4	12.1	ND	41.1	9.2	0.3
	O-3%	4.5	0.2	5.5	ND	0.4	4.7	5.7
	O-10%	5.8	ND	4.1	ND	1.1	4.3	4.6
	Thermal	3.9	5.7	4.9	5.6	ND	3.3	0.2
	Photolytic	ND	51.5	ND	0.7	ND	5.2	4.1
Stress study of Pure compound (% Degraded)	A	NA	NA	ND	65.68	NA	ND	ND
	B	NA	NA	ND	20.98	NA	3.87	0.34
	O	NA	NA	ND	ND	NA	4.11	0.44
	Thermal	NA	NA	ND	0.51	NA	4.46	0.24
	Photolytic	NA	NA	7.53	4.47	NA	14.12	1.13
Limits of detection & quantification (µg/ml)	LOD	0.125	0.031	0.525	0.234	0.099	1.618	0.409
	LOQ	0.38	0.094	1.591	0.708	0.3	4.903	1.238
	Correlation	0.9999	0.9999	0.9999	0.9999	1	0.9991	0.9999
Linearity	Slope	35686.71	184251.2	5784.954	13527.17	50332.12	3507.021	42464.5
	Intercept	3835.94	14542.97	15410.89	-4237.07	1535.98	-7314.47	517980.8
	Correlation	0.99998	0.99997	0.9999	1	0.99972	0.99993	0.99965
	Bias at 100%	0.27	0.38	1.29	-0.16	0.3	-1.05	2.86

N - Normality, NA- Not analyzed, ND-No degradation, A- Acid, B-Base, O- Peroxide, Prep- Preparation.

Table-4: DoE proposed experiments and respective results.

Exp no.	Flow (ml/min)	Temperature (°C)	ACN Composition (%)	R1	R2
1	1.5	35	25	1.1	4.5
2	1.5	45	25	2.9	1.2
3	1.1	35	25	1.6	5.5
4	1.3	40	20	3	2.8
5	1.2	45	15	4.8	1.3
6	1.1	35	15	3.2	4.8
7	1.5	35	15	2.7	4
8	1.3	40	20	3	2.7
9	1.3	40	20	3	2.8
10	1.5	45	15	4.7	1
11	1.1	45	25	3.6	1.9

In this way, the factorial design provides information about the importance of interaction between the factors. The number of coefficients is equal to the number of experiments (in experiment 8). The zero-level experiment was not included in the calculation of coefficients. Also, b_0 is the intercept of the linear

model, b_1, b_2 and b_3 are the main effects, b_{12}, b_{13} and b_{23} are two factor interactions and b_{123} are a three-factor interaction.

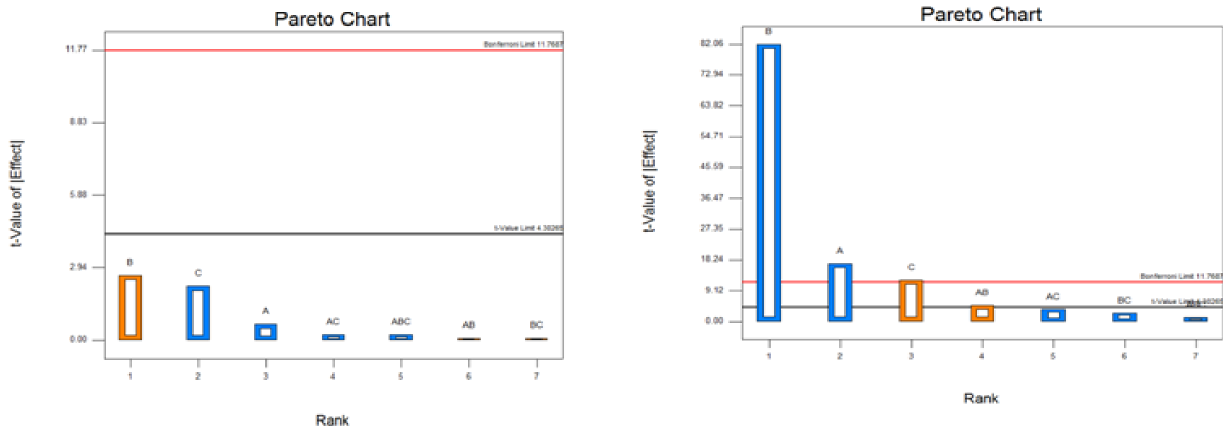

Fig.-2: (a) Pareto of R1: t-value of respective variables, (b) Pareto of R2: t-value of respective variables. A- Flow (ml/min), B-Temperature (°C), C- ACN composition (%).

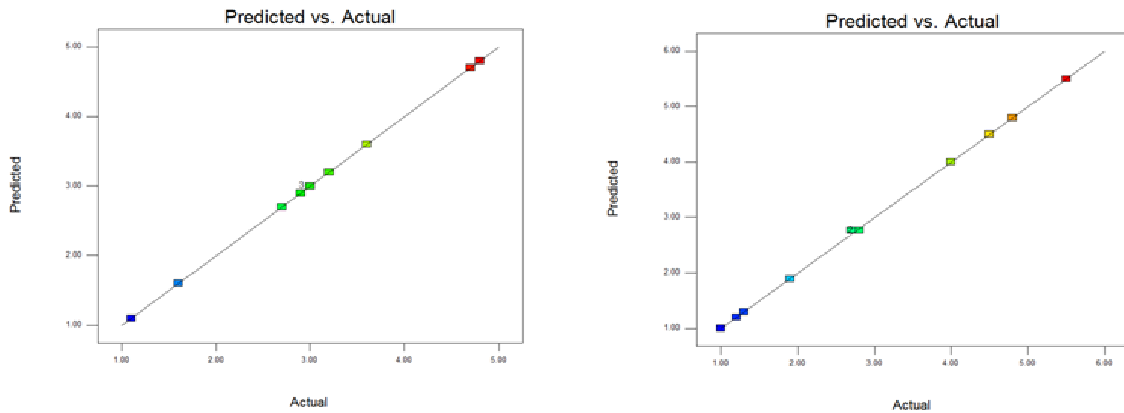

Fig.-3: Actual versus Predicted plots for resolution R1 and R2 respectively.

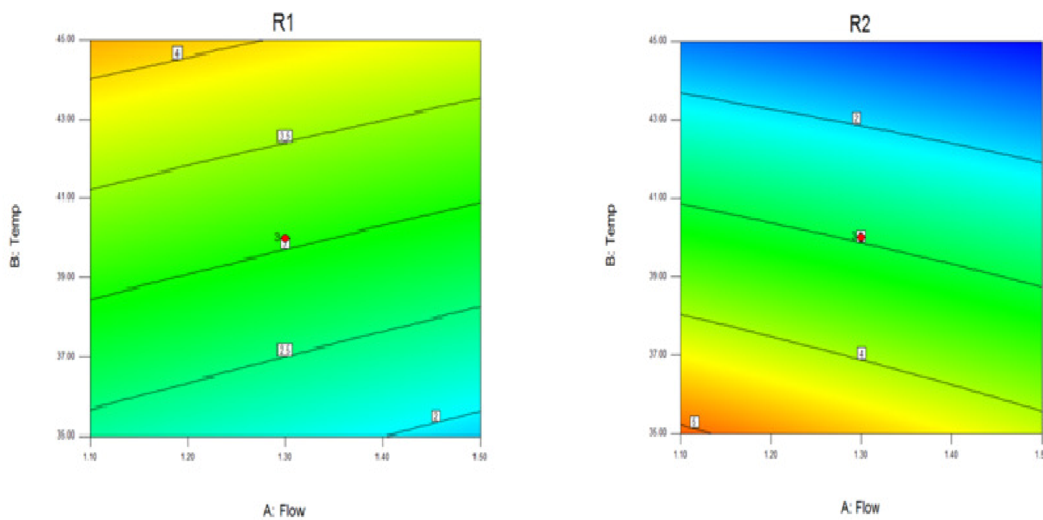

Fig.-4: Contour diagram for R1 and R2

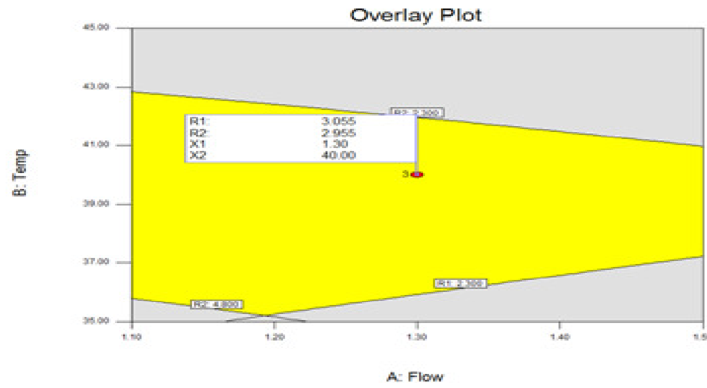

Fig.-5: Overlay plot of R1 and R2 showing the design point.

PE SA LA CE TA CH SO

210 nm

Phenoxy Ethanol - 6.697
Sorbic Acid - 8.137
Linoleic Acid - 22.482
Ceramide PC-104 - 36.708
Alpha tocopherol - 38.050
Chlosterol - 39.469
Squalene - 49.093

260 nm

Blank at 210 nm

Minutes

Fig.-6: Absorption maxima (λ_{max}) spectrum and chromatograms of Samples at 210 nm, 260nm, blank at 210 nm.

The ANOVA resulted in:

a. Pareto chart for Resolution (R1) between CE and TA shows positive effect with Temperature and negative effect with ACN composition. For Resolution (R2) between TA and CH positive effect is with ACN composition and negative effect is with Temperature (Fig.-2).
b. Predicted vs actual points are liner for both R1 and R2 (Fig.-3).
c. Contour diagram is constructed which presents the R1 and R2 values as a function of two variables while the third is kept constant at zero-level (center) (Fig.-4).
d. Three dimensional view of design point (Fig.-5).
e. The overlay plot of resultant maximum values for R1 and R2. This plot defines edge of failures of the resolutions R1 and R2 for the variation in flow and temperature. Also the design point is away from the edges.

CONCLUSION

A validated HPLC method for simultaneous analysis of CE PC 104, CH, LA, SQ, SA, PE and TA in cream formulations discussed. It is successfully applied to cosmetic formulation. Robustness testing using DOE approach helps in reducing the efforts and getting to a valid conclusion with minimum number of runs. The present study can be adopted by various departments like quality control etc. for qualitative and quantitative determinations of commercially manufactured creams quality control release and stability testing.

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