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COMBINED MIXTURE DESIGN-DOE AND ION PAIR REAGENT: A SYNERGISTIC MODEL FOR THE SEPARATION OF MULTIPLE AMINO ACIDS IN RP-HPLC

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Design of Experiments (DoE), RP-HPLC, OFAT, Ion Pair Regent

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ABSTRACT: The aim of the present study is to apply the Design of Experiments (DoE) to develop an assay method for the separation of amino acids by RP-HPLC. The method used in this study is the DoE Combined-Randomized method. Design of Experiments allows interpreting the results with better outcomes and enhanced understanding process. Ion pair reagent-Octane 1 Sulphonic acid sodium salt monohydrate of 0.01M at pH 2.2 is used along with Methanol and Tetra-hydro Furan. The column used is INETSIL C_8, with 5µ Particle size and 4.6×250 mm column dimensions. The Solvent composition and ion pair concentration is evaluated as variables in the DoE. The wavelength for all amino acids is 205 nm. Conclusively DoE is an efficient tool for the separation of amino acids. Method development was established, and the design is validated. The proposed method has adequate reproducibility and accuracy for the estimation of the amino acids in the routine analysis.

INTRODUCTION: Amino acids are a group of organic compounds. They contain two functional groups, such as amino and carboxyl [12]. The carboxyl (COOH) is acidic, while the amino group (NH_2) is basic. There are a total of 22 amino acids that are classified into essential which cannot be synthesized by the body and are to be supplied through the diet they are phenylalanine, valine, threonine, tryptophan, methionine, leucine, isoleucine, lysine, and histidine, conditionally essential where this synthesis is conditionally limited arginine, cysteine, glycine, glutamine, proline, and tyrosine, non-essential these can be synthesized in the body itself alanine, aspartic acid, asparagine, glutamic acid, serine, selenocysteine, pyrrolysine. The lack of chromophores [17] in most of the amino acids makes the HPLC method development as the most challenging task. Most of the derivatization techniques in RP-HPLC methods are reported until now. Separation of the amino acids in the reverse phase without the derivatization technique is the present work's main objective. For this normal C_8 Column is used. This separation is aided by the help of the Design of Experiment Software[4,] and the developed method is executed, and the design validation is performed.

Available literature references mainly focus on the determination of amino acids in RP-HPLC[1], Determination of amino acids in HPLC [2, 5, 6, 9, 13, 14,

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[15, 17], Separation of amino acids by HPLC [5], By Precolumn derivatization in HPLC [7], Determination of amino acids without derivatization in HPLC [10], Un derivatized amino acid determination [11], Quantitative analysis of amino acids in RP-HPLC [13], Determination of amino acids in food substances by HPLC [17, 18]. Based on the literature review and some OFAT approaches, some factors and variables are selected for the study, and they are implemented in the Design Expert Software and the separation thus aided by the few numbers of runs/ experiments.

MATERIALS AND METHODS:

Chemicals and Reagents: L-Cystic Acid Monohydrate, L-Cysteine, Glycine, L-Alanine, L-Tyrosine, L-Histidine, L-Asparagine, L-Aspartic Acid are procured from Sigma Aldrich, L-Proline, L-Glutamine from Avra Synthesis, L-Phenylalanine, L-Glutamic Acid from Spectro chem, L-Valine from Fluka, DL-Methionine from TCI, L-Serine from Merck and L-Threonine from Chem Impex. HPLC grade Acetonitrile (ACN) JT Baker, HPLC grade Methanol, Tetra Hydro Furan (THF), Orthophosphoric acid, and Octane-1 Sulphonic acid sodium salt monohydrate was procured from Merck Life sciences Pvt Ltd. Water was from Milli-Q.

Apparatus and Equipment: High-Performance Liquid Chromatography was carried with Waters HPLC, Make Alliance, Model E2695 with Empower-3 software isocratic elution capability, a Spectrophotometric PDA detector HPLC 2998, and an autosampler with temperature control.

INERTSIL C_8, (5µ, 4.6×250 mm particle size, GL-Sciences) was utilized in this study. Other equipment used was analytical Balance (Model-225D-101N, Sartorius), Micro Balance (BM-20, AND Company Limited), Ultrasonic cleaner (3200 EP S3, SOLTEC), and pH was observed by using pH/Ion analyzer (LP139SA, Polmon, Bangalore, India). All glassware was used was made of Borosil. Design-Expert 10 software was used during DoE studies so as to generate experimental designs and to analyze the obtained responses.

One Factor at a Time (OFAT) Approach: Different experiments with the OFAT approach by varying different method conditions like mobile phase composition, pH of the mobile phase were conducted. In initial trials with water and Acetonitrile (ACN) the Amino acids do not retain in the column, so an anionic ion pair reagent is used, and there is no much resolution between the peaks, so methanol was taken as another mobile phase to increase the resolution. Peak shapes are not good in the above combination, so THF is added to the methanol but did not get any suitable method that has a resolution for all the analytes.

Method Procedure: Octane-1 Sulphonic acid sodium salt monohydrate pH 2.2 (Concentrations as per DoE) as Mobile phase A, Methanol as Mobile phase B, THF as Mobile phase C. Amino acids preparation- L-Cysteine, L-Cystic Acid Monohydrate, Asparagine, Proline, Glutamic Acid, Serine, Threonine, Phenyl Alanine, Aspartic Acid, Glutamine, Glycine, Alanine, Valine, Methionine, Tyrosine, Histidine each of 50 mg is weighed in a 50 ml volumetric flask and makeup to 50 ml with diluent. The mixture is sonicated to dissolve.

Application of DoE during the Method Development: The design of experiments considers multiple factors to experiment in a single experiment, and all the factors were varied in each of the sets of experiments as per predetermined statistical modeling. A simple combined-randomized design was optimized to develop the method for the separation of Amino acids with each high and low levels of each selected factor or variable. Four different factors or variables are selected to determine the lack of fit or curvature of the design. A total of 28 runs of different combinations are given by the DoE.

These amino acids were subjected to the different combinations of the mobile phase composition, ion-pair concentrations, and the trials are executed using HPLC.

Design Evaluation for Adequacy and Statistical Significance: To evaluate the effectiveness of the experimental design, Combined-Randomized design was assessed through a statistical measure of power, lack of fit, and pure error. These three statistical parameters determine the adequacy of the design model created. An additional graphical evaluation was performed through a fractional design space (FDS) graph. Statistical evaluation

tools like Power lack of fit, pure error, and VIF value are evaluated to ensure the adequacy of the design. As the design was proven to be adequate, all the experimental runs were executed, and the results of each response were evaluated for statistical significance by ANOVA tool. ANOVA includes Model F–value, adjusted R-square, predicted R-square, and adequate precision as statistical measures.

Design Prediction and Validation: As the design model was proven to be statistically significant, further results were evaluated for the effects of variables on responses with the help of Trace plots, Contour plots, mix process plots, and 3D Mix process plots to understand which variable is having a significant effect on the responses. The next step of DoE involves the prediction of solutions as per the desired outcome and validating the suggested solutions against experimental data. DoE design was aided with the desired separation values of solutions that were predicted. Out of the suggested solutions, two solutions were selected and evaluated with numerical optimization and overlay graph to understand the method operable design region (MODR) of experimental design.

RESULTS AND DISCUSSION: Based on the initial OFAT approaches, the constraints or variables are selected. From the initial OFAT trails, mobile phase composition has an effect over the resolution between the peaks [19-20], and ion pair concentration has an effect over the resolution. So, the Ion pair concentration, mobile phase composition (Ion pair Buffer: Methanol: THF).

TABLE 1: DOE DESIGN SUMMARY

Study Type	Combined
Subtype	Randomized
Design type	I-optimal
Design bodel	Quadratic x Quadratic
Runs	28
Blocks	No blocks

ANOVA Evaluation: ANOVA indicates the statistical significance of the model. The adjusted R square and predicted R square values should be in reasonable agreement (difference less than 0.2), and adequate precision shall be more than 4. This indicated that the model is capable of predicting solutions from the available experimental run data and that there is a good correlation between study variables and observed responses.

TABLE 2: ANOVA EVALUATION

S. no.	R1	R2	R3	R4	R5
R-Squared	0.9566	0.9615	1.0000	0.4826	0.9814
Adjusted R-Square	0.9311	0.9453	0.9998	0.4180	0.9686
Predicted R-Square	0.8218	0.9051	0.9464	0.3401	0.9419
Adequate Precision	25.708	26.341	343.331	10.116	31.049
Difference between Predicted R-Squared and Adjusted R-Squared	-0.1093	-0.0402	-0.0534	-0.0779	-0.0267

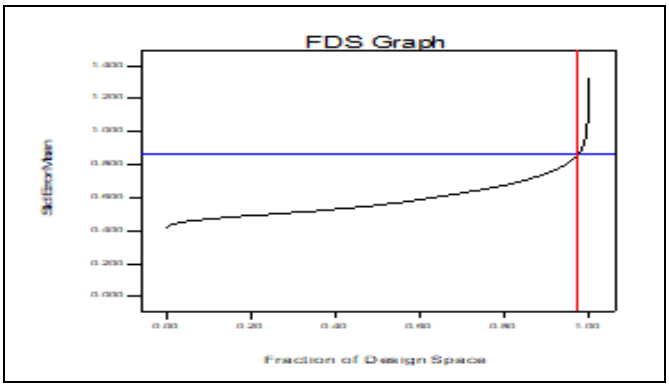

FIG. 1: FRACTION OF DESIGN SPACE

Fraction of Design Space Evaluation: FDS discuss the design space that is being predicted by design. The design space should be less than or equal to a specified value. Design space is the "Ratio of obtained value to the total value." The ideal FDS score is 80% or 0.8 or above and 100% for the Quality by Design work. In the present Design, the obtained FDS score from the graph is found to be 0.97, which is in the range to accept the design. So, the design can be used further to obtain the best results.

Factor/Factors and Variable/Variables Selection: The factors and Variables are selected based on the initial One factor at a time trails, and they are included in the Design.

Here, the mobile phase Composition is selected as a Variable, and the Ion-Pair Reagent concentration is chosen as a factor

TABLE 3: EXPERIMENTAL SETUP BY DoE

Run	A	B	C	D	R1	R2	R3	R4	R5
1	%	%	%	g	-4.41	-1.45	-7.67	-0.12	16.811
2	94.2	3	2.76	3	-2.26	-1.43	-4.28	1.2	12.459
3	94.6	3	2.38	2.04	-0.85	0.05	-1.94	1.29	7.705
4	92.9	3	4.07	1	-2.26	-1.43	-4.28	1.2	12.459
5	94.6	3	2.38	2.04	-1.04	-0.34	-2.17	0.56	8.57
6	90.0	6.20	3.70	2	-0.8	-0.2	-2.32	0.59	7.907
7	87.5	7.50	4.93	2.47	-1.06	-0.18	-2.46	0.06	10.558
8	85	10	5	3	-3.25	-1.69	-6.93	1.35	16.991
9	91.4	6.50	2	3	-0.53	0.08	-1.83	1.29	7.645
10	90.2	6.64	3.11	1	-0.36	0.24	-1.68	0.64	6.822
11	87.5	7.45	5	1.35	-1.04	-0.35	-2.32	1.72	9.198
12	88.7	9.27	2	1.61	-1.22	-0.44	-2.65	7.42	10.3557
13	92.4	5.24	2.27	1.5	-0.44	0.24	-1.65	0.89	6.445
14	85.5	10	4.46	1	-0.31	0.19	-1.4	0.91	6.701
15	89.4	5.51	5	1	-1.71	-0.52	-3.61	-0.12	12.781
16	89.1	7.19	3.65	3	-1.93	-0.84	-5.03	1.17	14.755
17	87.5	10	2.44	3	-0.63	0.08	-1.97	2.07	7.777
18	88	10	2	1.09	-1.09	-0.35	-2.87	3.21	9.91
19	95	3	2	1.05	-1.04	-0.34	-2.17	0.56	8.57
20	90.0	6.20	3.70	2	-0.58	0.09	-1.89	1.43	7.643
21	90.2	6.64	3.11	1	-1.04	-0.34	-2.17	0.56	8.57
22	90.0	6.20	3.70	2	-0.16	-0.31	-2.36	0.74	8.426
23	87.0	10	2.95	2.01	-0.62	-0.12	-1.52	0.01	7.046
24	85.3	9.67	5	1.98	-1.29	-0.41	-2.75	-0.7	12.154
25	90.0	4.93	5	3	-0.88	-0.3	-1.99	0.54	8.427
26	91.6	3.32	5	2	-0.98	-0.35	-2.41	0.23	8.775
27	92.3	3	4.61	2.53	-1.37	-0.64	-2.92	1.24	10.455
28	90.7	7.23	2	2.01	-1.71	-0.52	-3.61	-0.12	12.781

A, Buffer (Octane-1 Sulphonic acid sodium salt monohydrate). B, Methanol. C, Tetrahydrofuran. D, Ion Pair reagent concentration. R1, Resolution b/w L Cysteine & Glutamic acid. R2, Resolution b/w L-Cysteine & Alanine. R3, Resolution b/w L Cysteine & Aspartic acid. R4, Resolution b/w Cystine & Valine. R5, RT of L-Cysteine.

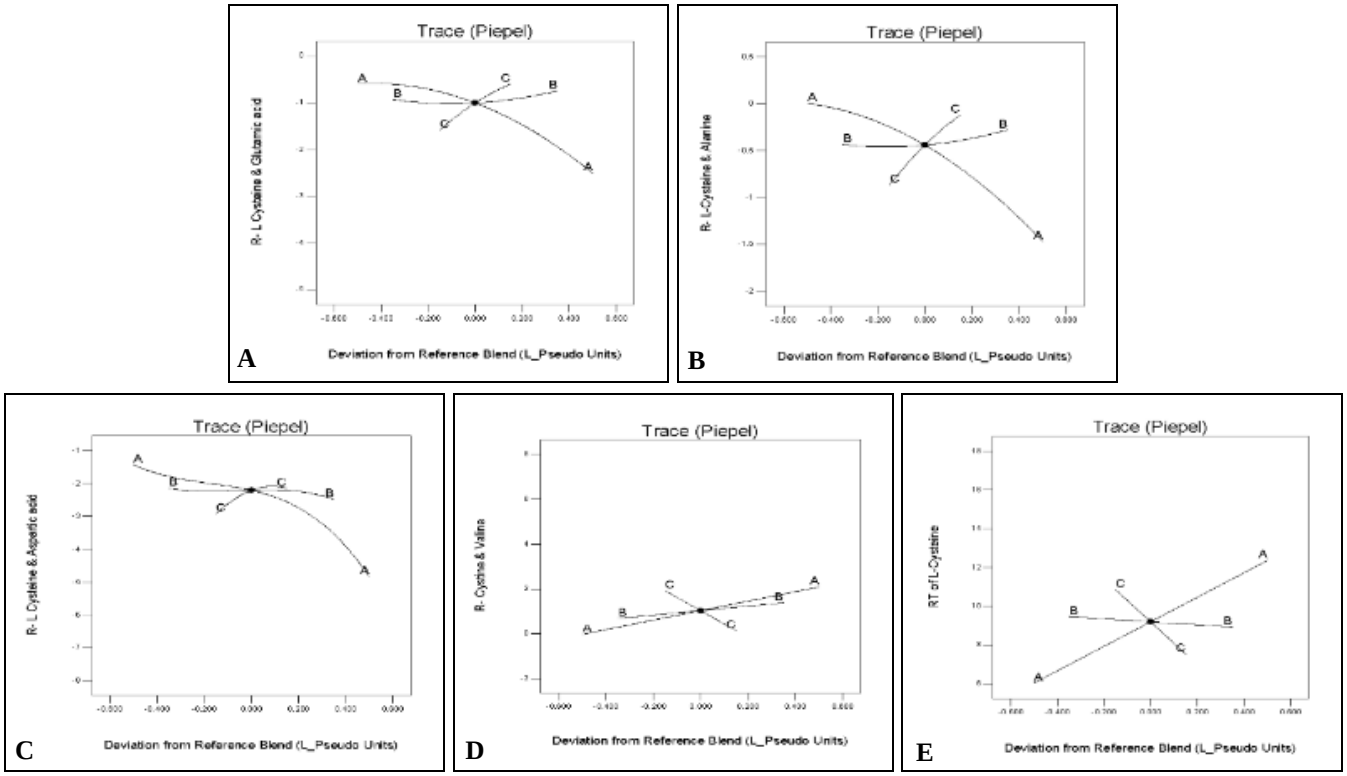

FIG. 2: TRACE PLOTS R-1 TO R-5. (A), Resolution b/w L Cysteine & Glutamic acid. (B), Resolution b/w L-Cysteine & Alanine. (C), Resolution b/w L Cysteine & Aspartic acid. (D), Resolution b/w Cystine & Valine. (E), RT of L-Cysteine

Model-Graphs Evaluation: After ANOVA Evaluation, the design is further subjected to the Graphical evaluation.

Graphical Evaluation: Graphical evaluation is done by evaluating the perturbation plot- "This helps in comparing the all factors at a single point in a design." Contour-Plot - "This is a 2-dimensional plot (2D) after responses that which are plotted with the combination of factor numeric/Mixture component", 3D Surface Plots- This project the contour plot. And Model graphs.

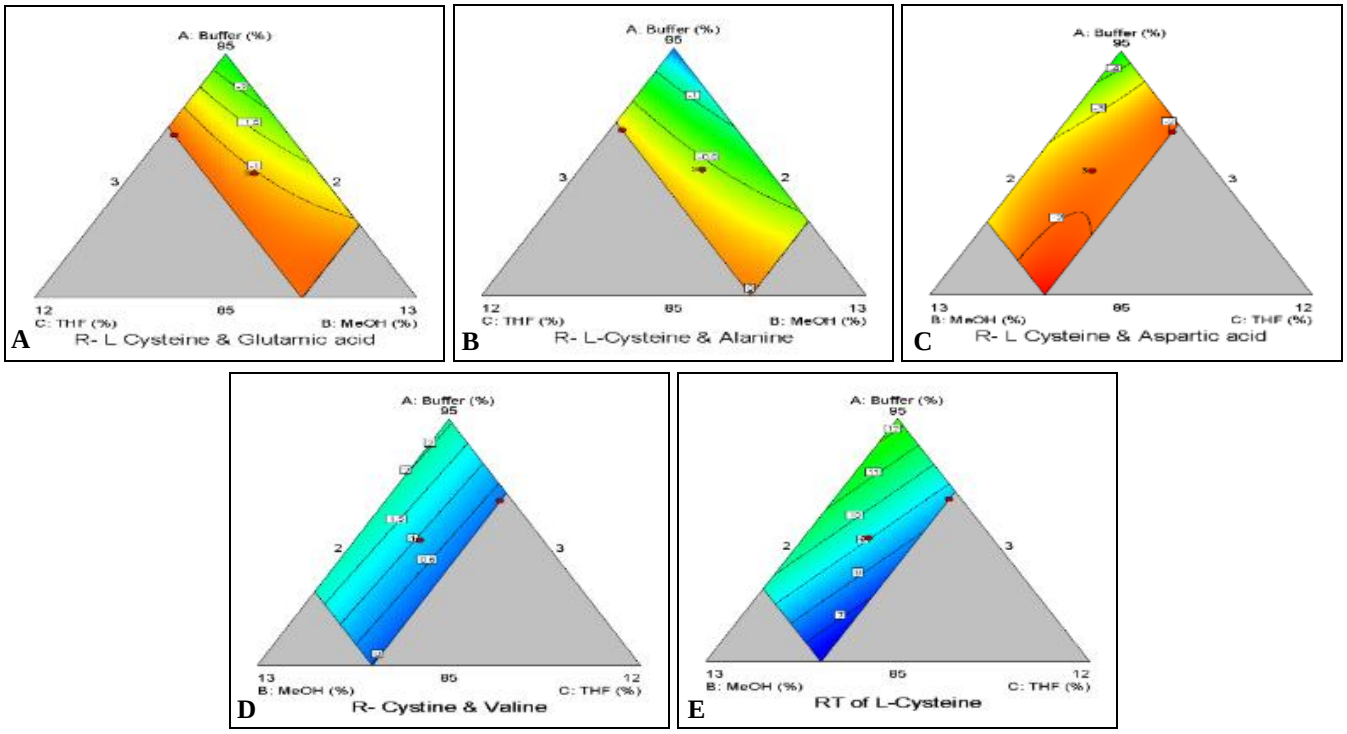

FIG. 3: CONTOUR PLOTS R-1 TO R-5. (A), Resolution b/w L Cysteine & Glutamic acid. (B), Resolution b/w L-Cysteine & Alanine. (C), Resolution b/w L Cysteine & Aspartic acid. (D), Resolution b/w Cystine & Valine. (E), RT of L-Cysteine

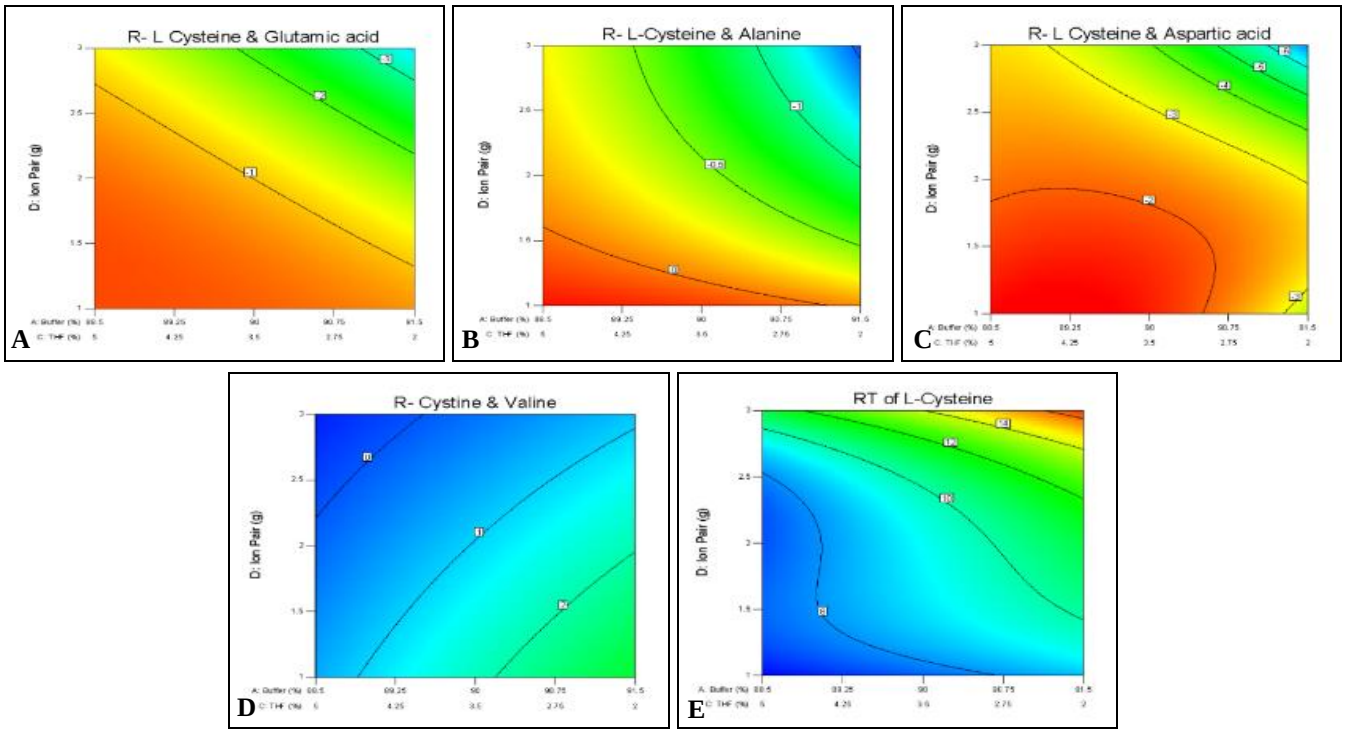

FIG. 4: MIX PROCESS PLOTS R-1 TO R-5. (A), Resolution b/w L Cysteine & Glutamic acid. (B), Resolution b/w L-Cysteine & Alanine. (C), Resolution b/w L Cysteine & Aspartic acid. (D), Resolution b/w Cystine & Valine. (E), RT of L-Cysteine.

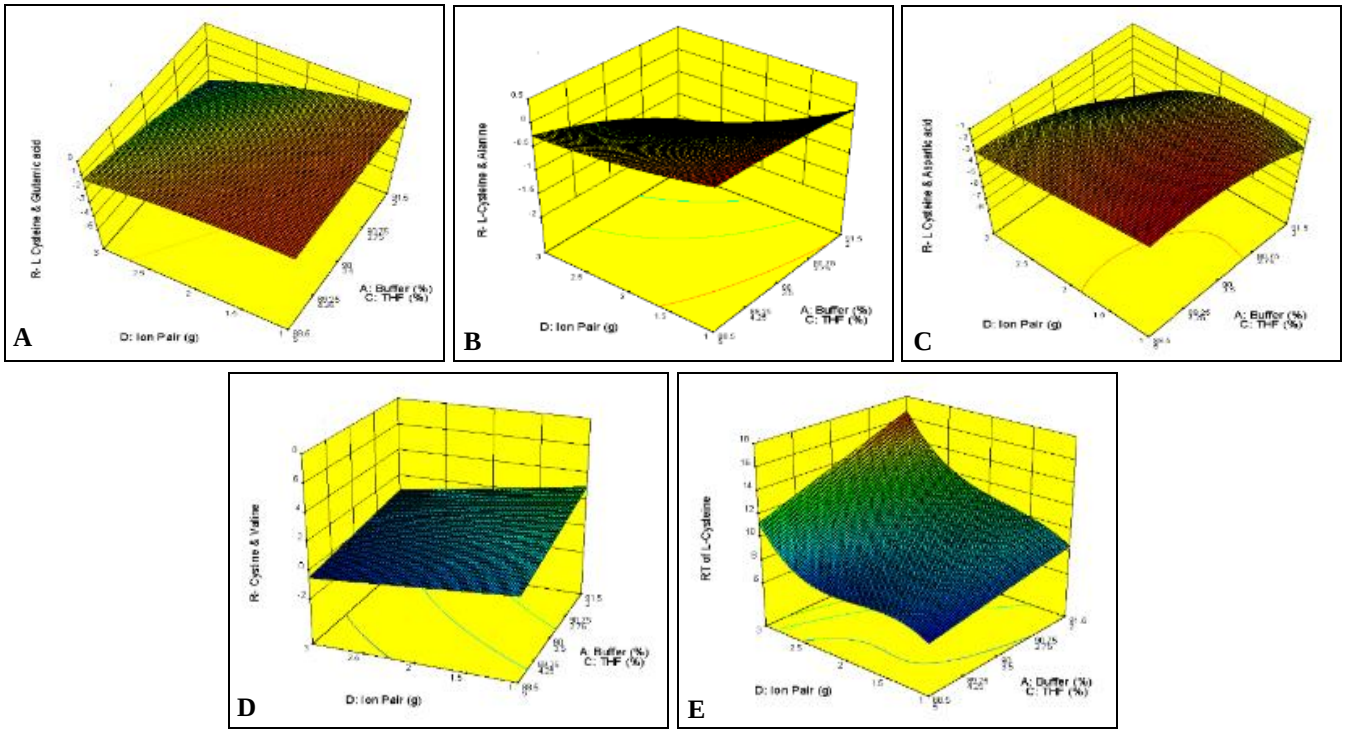

FIG. 5: 3D MIX PROCESS PLOTS R-1 TO R-5. (A), Resolution b/w L Cysteine & Glutamic acid. (B), Resolution b/w L-Cysteine & Alanine. (C), Resolution b/w L Cysteine & Aspartic acid. (D), Resolution b/w Cystine & Valine. (E), RT of L-Cysteine.

Design Validation with Predicted and Selected Solutions: The target of selected and weights are given when they are incorporated into the design.

TABLE 4: CONSTRAINT SELECTION

Names	Goal Type	Lower-Limit	Upper-Limit	Lower-Weight	Upper-Weight	Imp.
A	is in. range	85.0	95.0	1.0	1.0	3.0
B	is in. range	3.0	10.0	1.0	1.0	3.0
C	is in. range	2.0	5.0	1.0	1.0	3.0
D	is in. range	1.0	3.0	1.0	1.0	3.0
R1	is in. range	-4.412	-1.55	1.0	1.0	3.0
R2	minimize	-1.693	-1.51	1.0	1.0	3.0
R3	is in. range	-7.671	-1.53	1.0	1.0	3.0
R4	is in. range	1.54	7.422	1.0	1.0	3.0
R5	is in. range	6.44	16.99	1.0	1.0	3.0

A, Buffer (Octane-1 Sulphonic acid sodium salt monohydrate). B, Methanol. C, Tetrahydrofuran. D, Ion Pair reagent concentration. R1, Resolution b/w L Cysteine & Glutamic acid. R2, Resolution b/w L-Cysteine & Alanine. R3, Resolution b/w L Cysteine & Aspartic acid. R4, Resolution b/w Cystine & Valine. R5, RT of L-Cysteine.

The design optimizes the finalized solutions; in this design, DoE predicted 24 types of solutions with variations in each factor and variable in each run.

TABLE 5: DESIGN PREDICTED SOLUTIONS

S. no.	A	B	C	D	R1	R2	R3	R4	R5
1	95.0	3.0	2.0	2.2	-3.10	-1.69	-5.97	1.66	12.51
2	95.0	3.0	2.0	2.2	-3.20	-1.72	-6.16	1.60	12.58
3	94.8	3.1	2.0	2.3	-3.17	-1.70	-6.03	1.59	12.56
4	94.4	3.5	2.0	2.3	-3.20	-1.69	-5.98	1.50	12.64
5	94.9	3.0	2.0	2.3	-3.30	-1.75	-6.32	1.52	12.67
6	95.0	3.0	2.0	2.3	-3.27	-1.75	-6.31	1.55	12.64
7	95.0	3.0	2.0	2.2	-3.15	-1.71	-6.07	1.63	12.55
8	94.6	3.3	2.0	2.3	-3.23	-1.71	-6.10	1.52	12.63
9	95.0	3.0	2.0	2.2	-3.12	-1.70	-6.01	1.65	12.53
10	94.7	3.2	2.0	2.3	-3.19	-1.71	-6.06	1.56	12.59

11	95.0	3.0	2.0	2.3	-3.23	-1.73	-6.23	1.58	12.60
12	95.0	3.0	2.0	2.2	-3.17	-1.72	-6.11	1.61	12.56
13	94.7	3.2	2.0	2.3	-3.15	-1.69	-5.97	1.56	12.54
14	95.0	3.0	2.0	2.3	-3.30	-1.76	-6.37	1.53	12.66
15	94.8	3.1	2.0	2.2	-3.14	-1.69	-5.96	1.60	12.54
16	94.6	3.3	2.0	2.3	-3.21	-1.71	-6.07	1.53	12.61
17	94.5	3.4	2.0	2.3	-3.19	-1.69	-5.97	1.51	12.62
18	94.8	3.1	2.0	2.3	-3.24	-1.73	-6.20	1.54	12.62
19	94.7	3.2	2.0	2.3	-3.27	-1.73	-6.20	1.50	12.67
20	95.0	3.0	2.0	2.3	-3.35	-1.77	-6.48	1.50	12.71
21	95.000	3.0	2.0	2.186	-2.92	-1.63	-5.61	1.78	12.43
22	93.5	4.4	2.0	2.380	-2.95	-1.55	-5.27	1.50	12.53
23	94.9	3.0	2.0	2.066	-2.65	-1.52	-5.08	1.98	12.36
24	93.3	4.6	2.0	2.3	-2.88	-1.51	-5.09	1.50	12.50

A, Buffer (Octane-1 Sulphonic acid sodium salt monohydrate). B, Methanol. C, Tetrahydrofuran. D, Ion Pair reagent concentration. R1, Resolution b/w L Cysteine & Glutamic acid. R2, Resolution b/w L-Cysteine & Alanine. R3, Resolution b/w L Cysteine & Aspartic acid. R4, Resolution b/w Cystine & Valine. R5, RT of L-Cysteine.

Out of the given solutions by the design, 17 and 24 are selected for the experimentation. These solutions are done for further evaluation, and the design validation is done for the selected solutions. **Fig. 2** illustrates the Trace plots for all the responses, **Fig. 3** illustrates the contour plots for all the responses, **Fig. 4** illustrates the mix process plots for all the responses, **Fig. 5** illustrates the 3D Mix Process plots for all the responses. **Table 6** describes the effect of each response in Mix Process plots.

TABLE 6: OBSERVATIONS FOR MIX PROCESS PLOTS

Responses	Buffer	Methanol	THF	Ion pair Concentration
R 1	+	+	-	+
R 2	+	+	-	+
R 3	+	+	-	+
R 4	+	+	-	-
R 5	+	+	-	+

+, Positive effect. -, Negative effect. R1, Resolution b/w L Cysteine & Glutamic acid. R2, Resolution b/w L-Cysteine & Alanine. R3, Resolution b/w L Cysteine & Aspartic acid. R4, Resolution b/w Cystine & Valine. R5, RT of L-Cysteine.

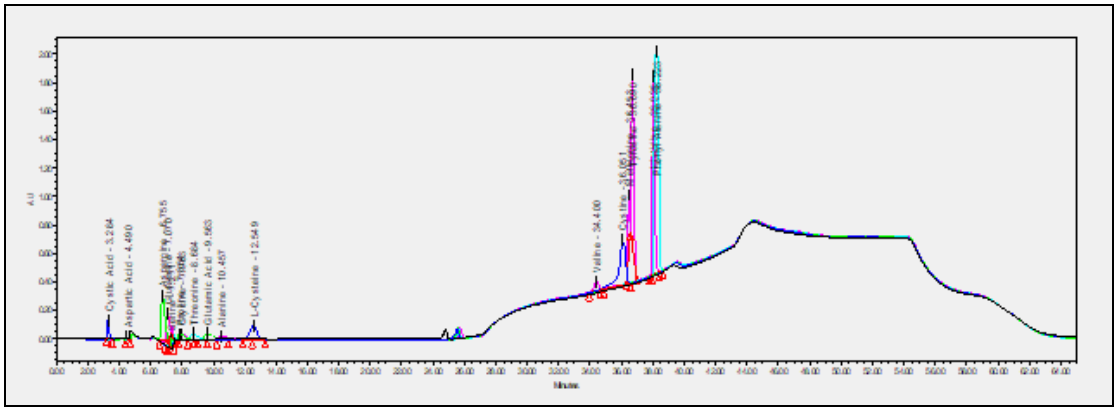

FIG. 6: OVERLAY PLOT OF SOLUTION 17

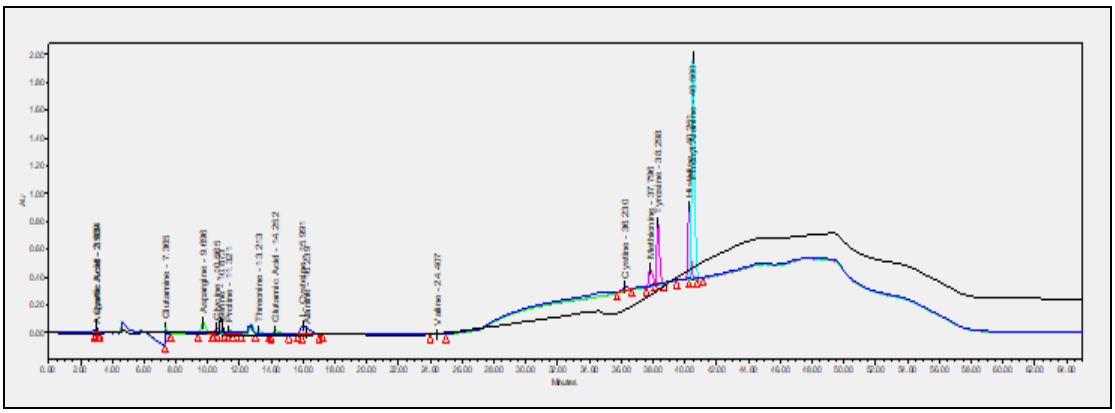

FIG. 7: CHROMATOGRAPH OF SOLUTION 24

CONCLUSION: These two solutions are performed in the lab, and the DoE predicted values are closely matching with that of the experimental results that which indicates that the design is validated and the solutions given by the DoE are having an adequate effect. Solution-17 is considered the final method for the separation of the amino acids. By the scope of the total study using the Design-Expert Software, the developed method can be used for the routine analysis of the amino acids in the laboratory in reverse phase chromatography.

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