

SUPPLEMENTARY MATERIAL

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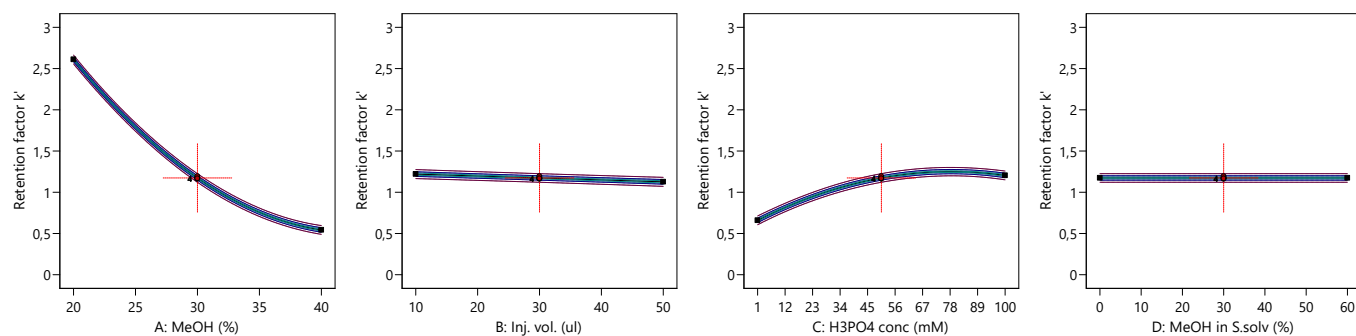
Table S1. Experimental matrix and results obtained for the Plackett–Burman Design (PBD).

		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	Response 4	Response 5
Std	Run	A:MeOH %	B:Flow ml/min	C:Oven temp C deg.	D:Inj. vol. ul	E:H3PO4 conc mM	F:Detection	G:MeOH in S.solv %	H:Dummy2	J:Dummy3	K:Dummy4	L:Dummy5	RT min	Plates	Resolution	Tail.F. 1	Tail. F. 2
2	1	20	0.9	50	10	100	360	40	-1	-1	-1	1	6.798	10922	9.392	1.843	1.254
4	2	20	0.9	25	50	100	220	40	1	1	-1	-1	6.717	4637	5.29	2.096	1.191
7	3	40	0.7	25	10	100	220	40	1	-1	1	1	3.545	7327	6.805	1.597	1.349
11	4	40	0.7	50	50	100	220	10	-1	1	-1	1	3.178	8343	6.853	1.648	1.316
3	5	40	0.7	50	50	1	360	40	1	-1	-1	-1	3.13	4177	6.303	2.982	1.433
9	6	40	0.9	50	10	1	220	40	-1	1	1	-1	2.44	7805	7.698	2.216	1.367
5	7	20	0.7	50	10	100	360	10	1	1	1	-1	7.348	11950	9.788	1.887	1.234
6	8	20	0.7	25	50	1	360	40	-1	1	1	1	9.103	4495	7.69	7.51	1.186
8	9	40	0.9	25	10	1	360	10	1	1	-1	1	2.725	8577	8.44	2.144	1.377
1	10	40	0.9	25	50	100	360	10	-1	-1	1	-1	2.821	8910	6.473	1.95	1.407
10	11	20	0.9	50	50	1	220	10	1	-1	1	1	6.07	12054	8.816	6.152	1.288
12	12	20	0.7	25	10	1	220	10	-1	-1	-1	-1	9.149	10402	10.417	3.643	1.309

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Table S2. Results of ANOVA for the reduced quartic model of response R1: retention factor k' .

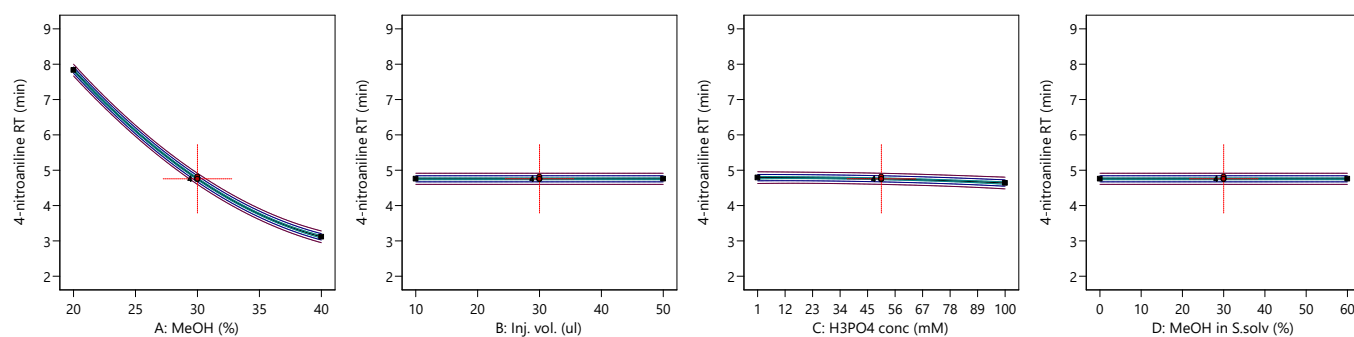
Source	Sum of Squares	df	Mean Square	F-value	p-value
Model	14.40	15	0.9602	7536.21	< 0.0001 significant
A-MeOH	8.55	1	8.55	67078.12	< 0.0001
B-Inj. vol.	0.0177	1	0.0177	138.91	< 0.0001
C-H ₃ PO ₄ conc	0.5941	1	0.5941	4662.29	< 0.0001
AB	0.0074	1	0.0074	58.45	< 0.0001
AC	0.1660	1	0.1660	1302.67	< 0.0001
AD	0.0020	1	0.0020	16.02	0.0015
BC	0.0023	1	0.0023	17.92	0.0010
A ²	0.5004	1	0.5004	3927.37	< 0.0001
C ²	0.1801	1	0.1801	1413.16	< 0.0001
A ² C	0.0179	1	0.0179	140.53	< 0.0001
AC ²	0.0936	1	0.0936	734.33	< 0.0001
B ² D	0.0010	1	0.0010	7.60	0.0163
BD ²	0.0032	1	0.0032	24.83	0.0003
A ² C ²	0.0095	1	0.0095	74.51	< 0.0001
B ² D ²	0.0008	1	0.0008	6.13	0.0279
Residual	0.0017	13	0.0001		
Lack of Fit	0.0010	9	0.0001	0.6766	0.7130 not significant
Pure Error	0.0007	4	0.0002		
Cor Total	14.41	28			

**Fig. S1.** Effects of individual factors on the response R1: retention factor k' .

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Table S3. Results of ANOVA for the reduced quadratic model of response R2: retention time of 4-nitroaniline peak as an analysis time.

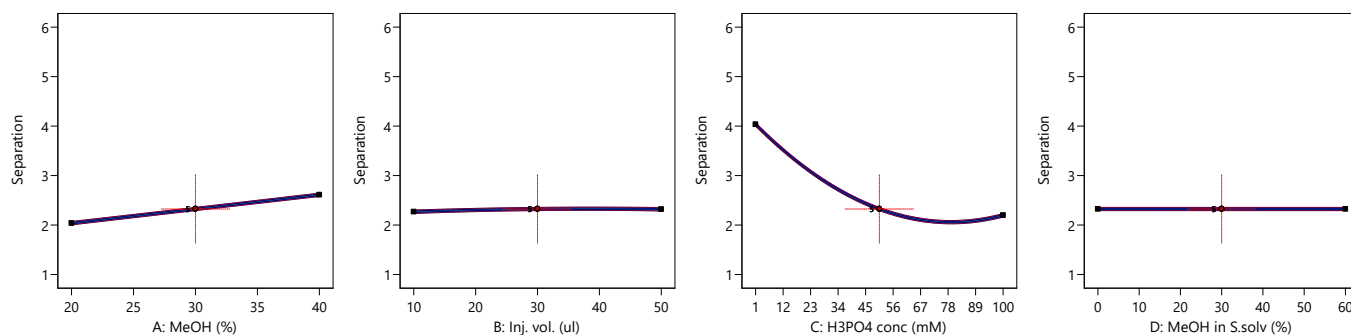
Source	Sum of Squares	df	Mean Square	F-value	p-value
Model	70.60	5	14.12	8825.73	< 0.0001 significant
A-MeOH	66.78	1	66.78	41740.48	< 0.0001
C-H ₃ PO ₄ conc	0.0696	1	0.0696	43.51	< 0.0001
AC	0.0559	1	0.0559	34.96	< 0.0001
A ²	3.55	1	3.55	2221.68	< 0.0001
C ²	0.0127	1	0.0127	7.97	0.0096
Residual	0.0368	23	0.0016		
Lack of Fit	0.0328	19	0.0017	1.74	0.3158 not significant
Pure Error	0.0040	4	0.0010		
Cor Total	70.64	28			

**Fig. S2.** Effects of individual factors on the response R2: retention time of 4-nitroaniline peak as an analysis time.

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Table S4. Results of ANOVA for the reduced quartic model of response R3: separation factor between L-alanine-4-nitroanilide and 4-nitroaniline peaks.

Source	Sum of Squares	df	Mean Square	F-value	p-value
Model	18.98	19	0.9990	23243.83	< 0.0001 significant
A-MeOH	0.6575	1	0.6575	15299.13	< 0.0001
B-Inj. vol.	0.0026	1	0.0026	59.78	< 0.0001
C-H ₃ PO ₄ conc	3.37	1	3.37	78473.51	< 0.0001
AC	0.3652	1	0.3652	8497.85	< 0.0001
AD	0.0008	1	0.0008	19.69	0.0016
BC	0.1092	1	0.1092	2539.88	< 0.0001
BD	0.0021	1	0.0021	49.96	< 0.0001
CD	0.0030	1	0.0030	70.81	< 0.0001
B ²	0.0047	1	0.0047	110.08	< 0.0001
C ²	3.06	1	3.06	71247.61	< 0.0001
A ² B	0.0006	1	0.0006	14.70	0.0040
A ² C	0.0328	1	0.0328	763.12	< 0.0001
AC ²	0.2206	1	0.2206	5133.17	< 0.0001
B ² C	0.0004	1	0.0004	8.92	0.0153
B ² D	0.0019	1	0.0019	43.32	0.0001
BC ²	0.1049	1	0.1049	2441.92	< 0.0001
C ² D	0.0063	1	0.0063	146.71	< 0.0001
A ² C ²	0.0291	1	0.0291	677.85	< 0.0001
A ² D ²	0.0029	1	0.0029	67.03	< 0.0001
Residual	0.0004	9	0.0000		
Lack of Fit	0.0003	5	0.0001	3.24	0.1387 not significant
Pure Error	0.0001	4	0.0000		
Cor Total	18.98	28			

**Fig. S3.** Effects of individual factors on the response R3: separation factor between L-alanine-4-nitroanilide and 4-nitroaniline peaks.

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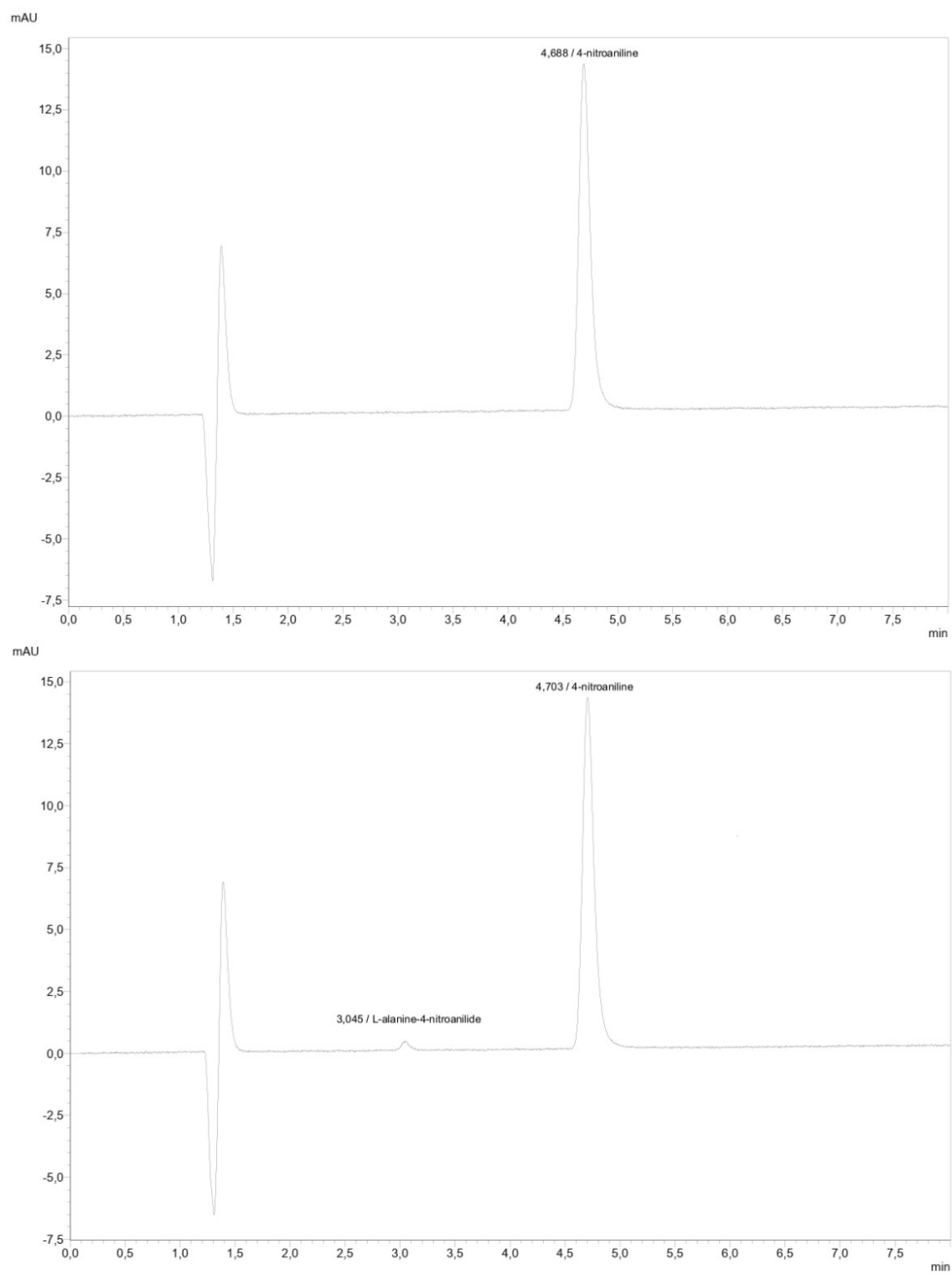


Fig. S4. Typical chromatograms of Reference (upper) and Test (lower) solutions at 380 nm wavelength.

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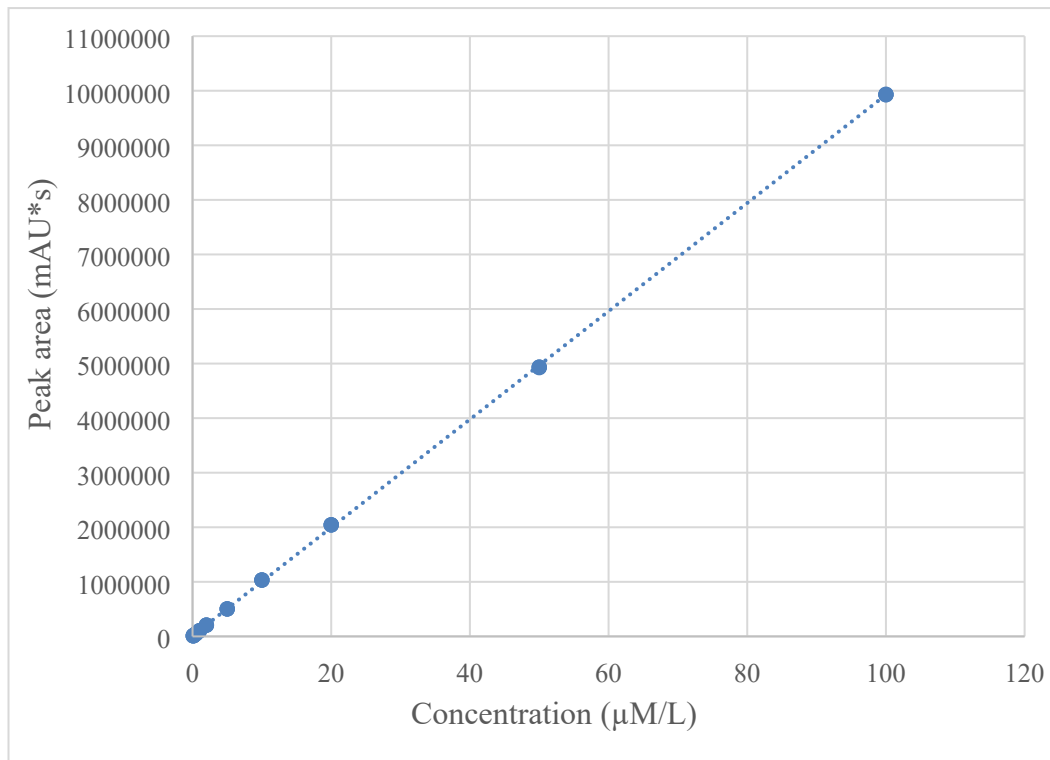


Fig. S5. Calibration curve for 4-nitroaniline: peak area vs. concentration (linearity testing).