

Supplementary Material

S-1. Coded and experimental points of Box-Behnken design.

Run Number	Coded Variables			Actual Variables		
STD	X ₁	X ₂	X ₃	%ACN _s	%ACN _m	Flow Rate (μL min ⁻¹)
1	+1	-1	0	28	44	150
2	0	-1	-1	25	44	100
3	-1	-1	0	22	44	150
4	0	-1	+1	25	44	200
5	+1	0	+1	28	47	200
6	-1	0	+1	22	47	200
7	-1	0	-1	22	47	100
8	+1	0	-1	28	47	100
9	0	+1	0	22	50	150
10	0	+1	+1	25	50	200
11	+1	+1	0	28	50	150
12	0	+1	-1	25	50	100
C-1	0	0	0	25	47	150
C-2	0	0	0	25	47	150
C-3	0	0	0	25	47	150
C-4	0	0	0	25	47	150
C-5	0	0	0	25	47	150

S-2. Factor levels for Box Behnken design for each factor

Factors	Low level (-1)	Center point (0)	High Level (+1)
SGP (Arb)	33	35	38
ISGP (Arb)	0	1	2
AGP (Arb)	10	15	20
CT (°C)	250	300	350
VT (°C)	325	375	425
CGP (mTorr)	1.5	2.0	2.5
SV (V)	2500	2750	3000

S-3. Box Behnken Experimental Design for optimization of LC-MS/MS operating conditions.

Run Number	SGP (Arb)	ISGP (Arb)	AGP (Arb)	CT (°C)	VT (°C)	CGP (Arb)	SV (V)
1	35	1	10	350	375	2	2500
2	32	2	15	350	375	2	2750
3	35	1	10	350	375	2	3000
4	35	1	15	250	325	1.5	2750
5	35	1	20	250	375	2	3000
6	35	0	20	300	375	2.5	2750
7	35	1	15	250	325	2.5	2750
8	35	0	10	300	375	2.5	2750
9	38	1	15	300	375	2.5	3000
10	32	1	15	300	375	2.5	3000
11	35	0	15	300	425	2	3000
12	38	1	20	300	325	2	2750
13	35	1	20	350	375	2	3000
14	35	0	20	300	375	1.5	2750
15	32	1	10	300	325	2	2750
16	32	0	15	250	375	2	2750
17	32	2	15	250	375	2	2750
18	35	1	15	250	425	2.5	2750
19	32	1	20	300	325	2	2750
20	38	2	15	250	375	2	2750
21	38	1	15	300	375	1.5	3000
22	35	0	10	300	375	1.5	2750
23	35	1	20	250	375	2	2500
24	35	1	10	250	375	2	3000
25	35	1	15	250	425	1.5	2750
26	35	1	15	350	325	1.5	2750
27	32	1	15	300	375	2.5	2500
28	32	0	15	350	375	2	2750
29	38	1	10	300	425	2	2750
30	35	2	15	300	325	2	2500
31	32	1	20	300	425	2	2750
32	38	1	10	300	325	2	2750
33	35	1	15	350	425	1.5	2750
34	38	0	15	350	375	2	2750
35	38	1	15	300	375	1.5	2500
36	35	2	10	300	375	1.5	2750
37	35	1	15	350	325	2.5	2750
38	35	1	20	350	375	2	2500
39	38	1	15	300	375	2.5	2500
40	35	2	15	300	425	2	3000

41	35	2	15	300	325	2	3000
42	38	1	20	300	425	2	2750
43	35	2	15	300	425	2	2500
44	35	2	20	300	375	2.5	2750
45	38	2	15	350	375	2	2750
46	32	1	10	300	425	2	2750
47	32	1	15	300	375	1.5	3000
48	35	0	15	300	325	2	3000
49	32	1	15	300	375	1.5	2500
50	38	0	15	250	375	2	2750
51	35	2	10	300	375	2.5	2750
52	35	0	15	300	325	2	2500
53	35	1	10	250	375	2	2500
54	35	0	15	300	425	2	2500
55	35	2	20	300	375	1.5	2750
56	35	1	15	350	425	2.5	2750
C1*	35	1	15	300	375	2	2750
C2*	35	1	15	300	375	2	2750
C3*	35	1	15	300	375	2	2750
C4*	35	1	15	300	375	2	2750
C5*	35	1	15	300	375	2	2750
C6*	35	1	15	300	375	2	2750

*C: centre point

S-4 Regression analysis and quadratic equations for different hormones

for peak symmetry (PS)

$$Y_{PSE1} = - 246.26 + 15.46322X_1 + 6.201013X_2 - 0.155584X_3 - 0.324824X_1X_2 - 0.01002X_1X_3 - 0.00012X_2X_3 - 0.29722X_1^2 - 0.02204 X_2^2 - 5.9.10^{-6}X_3^2 + 0.006328X_1^2X_2 + 0.000168X_1^2X_3 \quad \text{Eq.2}$$

$$Y_{PSE2} = - 492.878 + 47.50023X_1 + 10.7824X_2 - 0.71428X_3 - 1.18659X_1X_2 + 0.053843X_1X_3 + 0.001263X_2X_3 - 0.95077X_1^2 + 0.040647 X_2^2 - 3.2.10^{-6}X_3^2 + 0.023717X_1^2X_2 + -0.00107X_1^2X_3 \quad \text{Eq 3}$$

$$Y_{PSE3} = - 441.752 + 36.10214X_1 + 10.13496X_2 - 0.05877X_3 - 0.82847X_1X_2 + 0.011562X_1X_3 - 0.00124X_2X_3 - 0.73982X_1^2 - 0.0008 X_2^2 - 7.7.10^{-6}X_3^2 + 0.017091X_1^2X_2 + - 0.00027X_1^2X_3 \quad \text{Eq.4}$$

$$Y_{PSEE2} = - 141.667 + 18.38937X_1 - 0.5126X_2 + 0.498594X_3 - 0.24048X_1X_2 - 0.04091X_1X_3 - 0.00028X_2X_3 - 0.39681X_1^2 + 0.036328 X_2^2 - 0.000112X_3^2 + 0.004887X_1^2X_2 + 0.000807X_1^2X_3 \quad \text{Eq.5}$$

S-5. Observed and predicted values of peak symmetries and resolution factors of hormones.

Run No:	X ₁	X ₂	X ₃	Peak Symmetry								Resolution factor					
				E1		E2		E3		EE2		E3		E2		EE2	
				Observed	Predicted	Observed	Predicted	Observed	Predicted	Observed	Predicted	Observed	Predicted	Observed	Predicted	Observed	Predicted
1	22	44	150	2.24	2.12	2.00	1.82	2.10	2.08	2.00	2.04	3.95	3.80	1.09	1.08	0.39	0.47
2	28	44	150	1.91	2.03	1.41	1.59	1.79	1.80	2.00	1.96	3.73	3.70	1.29	1.21	0.59	0.53
3	22	50	150	2.41	2.29	3.00	2.82	1.62	1.60	1.61	1.65	2.70	2.72	0.62	0.69	0.48	0.53
4	28	50	150	1.78	1.90	2.38	2.56	2.25	2.26	1.75	1.71	2.05	2.20	0.57	0.57	0.54	0.47
5	22	47	100	1.82	1.94	1.65	1.83	1.64	1.65	1.36	1.31	4.05	4.24	1.08	1.07	0.59	0.56
6	28	47	100	2.32	2.20	1.69	1.51	2.46	2.45	1.42	1.46	3.07	3.14	0.93	0.99	0.45	0.54
7	22	47	200	2.71	2.83	1.75	1.93	2.00	2.01	2.33	2.29	2.50	2.43	0.74	0.70	0.51	0.41
8	28	47	200	2.22	2.10	1.92	1.75	1.60	1.59	2.06	2.11	3.10	2.91	0.78	0.80	0.39	0.43
9	25	44	100	1.86	1.86	1.86	1.86	2.00	2.00	2.50	2.50	4.50	4.46	1.25	1.30	0.75	0.71
10	25	50	100	1.58	1.58	1.19	1.19	1.44	1.44	2.00	2.00	3.05	2.83	0.79	0.77	0.65	0.63
11	25	44	200	2.14	2.14	2.62	2.62	2.36	2.36	2.67	2.67	2.90	3.11	0.91	1.00	0.49	0.51
12	25	50	200	1.79	1.79	2.70	2.70	1.06	1.06	2.00	2.00	2.11	2.15	0.47	0.49	0.53	0.57
C1	25	47	150	1.64	2.06	1.85	1.81	1.74	1.74	1.80	1.68	3.93	3.53	0.88	0.89	0.46	0.54
C2	25	47	150	2.59	2.06	1.71	1.81	1.82	1.74	1.93	1.68	3.70	3.53	1.03	0.89	0.57	0.54
C3	25	47	150	1.92	2.06	1.94	1.81	1.86	1.74	1.32	1.68	3.21	3.53	0.72	0.89	0.54	0.54
C4	25	47	150	1.92	2.06	1.94	1.81	1.57	1.74	1.69	1.68	3.47	3.53	1.08	0.89	0.52	0.54
C5	25	47	150	2.21	2.06	1.59	1.81	1.73	1.74	1.68	1.68	3.33	3.53	0.89	0.89	0.63	0.54
SRD				4	12	10	35	9	8	7	12	10	13	5	11	18	40

S-6 Regression analysis and quadratic equations for different hormones

for resolution factor (RF)

$$Y_{RFE2} = -1.07978 + 0.26869X_1 + 0.075353X_2 - 0.01237X_3 - 0.00669X_1X_2 + 0.000316X_1X_3 + 3.47 \cdot 10^{-5} X_2X_3 \quad \text{Eq. 6}$$

$$Y_{RFE3} = -51.3902 + 0.26869X_1 + 2.339036X_2 - 0.10904X_3 - 0.01194X_1X_2 + 0.002635X_1X_3 + 0.001107X_2X_3 - 0.02107X_1^2 - 0.02577X_2^2 - 6.3 \cdot 10^{-5}X_3^2 \quad \text{Eq. 7}$$

$$Y_{RFE2} = 2.170366 + 0.616001X_1 - 0.34164X_2 - 0.01582X_3 - 0.0036X_1X_2 + 4.67 \cdot 10^{-5}X_1X_3 + 0.000226X_2X_3 - 0.00907X_1^2 - 0.004225X_2^2 - 9.09 \cdot 10^{-6}X_3^2 \quad \text{Eq. 8}$$

S-7. Observed and predicted values of resolution factor and peak symmetry

at the experimental points, which are different than design points.

Response	%ACN _s : 25		%ACN _s : 25	
	%ACN _m : 48		%ACN _m : 44	
	Flow rate: 145 μL min ⁻¹		Flow rate: 175 μL min ⁻¹	
	Observed	Predicted	Observed	Predicted
RF E3	3.16	3.33	3.05	3.57
RF E2	0.717	0.817	1.00	1.08
RF EE2	0.521	0.552	0.588	0.540
PS E1	1.94	1.97	2.06	2.084
PS E2	1.85	1.73	1.93	2.49
PS E3	1.62	1.59	2.67	2.29
PS EE2	1.45	1.62	2.36	2.41

S-8 Regression analysis and quadratic equations for different hormones for peak area

$$Y_{IAE1} = 172463.6 + 2696.783X_1 - 5943.64X_2 + 1313.332X_3 - 20611.1X_4 - 63301.3 X_5 - 1278.89X_6 - 10495X_7 + 3123.884 X_1X_2 + 22051.21X_1X_3 + 252.3905X_1X_4 + 18513.91 X_1X_5 - 535.769 X_1X_6 + 1921.665 X_1X_7 - 324.297 X_2X_3 - 4448.24 X_2X_4 + 2913.445 X_2X_5 - 1160.33 X_2X_6 + 2342.146 X_2X_7 + 2240.425 X_3X_4 + 15702.7 X_3X_5 - 3881.99 X_3X_6 - 2076.36 X_3X_7 + 12118.37 X_4X_5 + 3311.824 X_4X_6 + 8374.847 X_4X_7 + 1435.664 X_5X_6 - 2806 X_5X_7 + 2618.567 X_6X_7 + 6011.336 X_1^2 - 971.644 X_2^2 - 93.3027X_3^2 - 13418X_4^2 - 64789.2 X_5^2 - 12216.1 X_6^2 - 25650.7 X_7^2$$

Eq.9

$$Y_{IAE2} = 42673.08 + 1496.183X_1 - 3189.39X_2 - 279.06X_3 - 9877.67X_4 - 17206.5X_5 - 1851.56X_6 - 3949.05X_7 + 367.206X_1X_2 + 4768.905X_1X_3 + 386.5508X_1X_4 + 4094.343X_1X_5 + 308.2959X_1X_6 - 704.53X_1X_7 - 813.049X_2X_3 - 616.791X_2X_4 + 2146.758X_2X_5 + 1336.474X_2X_6 + 543.0506X_2X_7 - 129.756X_3X_4 + 5787.002X_3X_5 - 1.0186X_3X_6 - 723.366X_3X_7 + 5664.571X_4X_5 - 1.3249X_4X_6 + 3739.379X_4X_7 + 687.1358X_5X_6 + 1122.957X_5X_7 + 1278.819X_6X_7 + 974.9913X_1^2 + 854.6614X_2^2 + 309.9898X_3^2 - 3054.52X_4^2 - 16137.7X_5^2 - 2746.48X_6^2 - 6714.027X_7^2$$

Eq.10

$$Y_{IAE3} = 75588.75 + 1969.335X_1 - 3706X_2 + 289.9176X_3 - 5215.27X_4 - 27668.8X_5 - 1267.68X_6 - 5276.8X_7 + 265.28 X_1X_2 + 8073.63X_1X_3 + 2165.158X_1X_4 + 6921.138X_1X_5 + 1576.173X_1X_6 + 3019.263X_1X_7 - 2415.97 X_2X_3 - 1449.26X_2X_4 - 722.698X_2X_5 + 2584.524X_2X_6 + 1860.682X_2X_7 + 725.704X_3X_4 + 5086.341X_3X_5 - 297.02X_3X_6 + 363.4076X_3X_7 + 2492.89X_4X_5 + 63.2076X_4X_6 + 2718.584X_4X_7 + 1705.947X_5X_6 - 789.546X_5X_7 + 430.395X_6X_7 + 3019.214X_1^2 - 176.116X_2^2 - 383.987X_3^2 - 5945.89X_4^2 - 28368.1X_5^2 - 5241.74X_6^2 - 12386.7X_7^2$$

Eq.11

$$Y_{IAEE2} = 26534.69 + 370.2664X_1 - 2019.15X_2 - 98.3677X_3 - 4739.28X_4 - 10228X_5 - 787.092X_6 - 2635.8X_7 + 2527.171X_1X_2 + 1524.091X_1X_3 - 310.661X_1X_4 + 4104.024X_1X_5 + 526.8978X_1X_6 + 1095.008X_1X_7 - 52.0269X_2X_3 + 148.2908X_2X_4 + 1573.113X_2X_5 - 578.253X_2X_6 + 1985.744X_2X_7 + 407.2529X_3X_4 + 2979.356X_3X_5 - 1492.05X_3X_6 + 495.9503X_3X_7 + 2598.758X_4X_5 + 297.5233X_4X_6 + 1212.984X_4X_7 + 1072.538X_5X_6 + 814.9745X_5X_7 - 779.864X_6X_7 + 450.7009X_1^2 + 1199.041X_2^2 - 577.002X_3^2 - 2007.78X_4^2 - 10214.5X_5^2 - 2774.85X_6^2 - 5172.58X_7^2$$

Eq.12

S-9. Observed and predicted integration areas of hormones at several experimental conditions for LC-MS/MS conditions

Run number	EE2		E2		E3		E1	
	Observed	Predicted	Observed	Predicted	Observed	Predicted	Observed	Predicted
1	13588.77	11810.39	25162.33	24259.36	47411.46	47719.62	112476.4	109093.1
2	30343.71	31260.73	53520.67	56020.48	74880.79	74446.85	187925	184097.5
3	24233.51	24653.79	40832.53	41595.58	66486.81	72049.57	157771.2	162763.3
4	24439.69	26946.42	47506.97	50945.74	68900.91	68373.19	166362.3	172044.8
5	12965.73	12843.02	27712.36	28565.82	50585.06	48300.64	129583.8	127370.2
R ²	0.958713		0.946327		0.938551		0.943031	
Adeq Precision	16.72297		13.59588		13.55452		13.18931	

S-10. Verification studies for Box-Behnken design for LC-MS/MS conditions.

Run	SGP (arb)	ISGP (arb)	AGP (arb)	CT (°C)	VT (°C)	CGP (mTorr)	SV (V)	Area (EE2) Predicted/ Observed
prediction-1	38	1.5	12	325	350	1.5	2750	20375/ 22623
prediction-2	34	0.5	19	340	400	2.2	3000	4941/ 3769
prediction-3	33	0.4	17	254	352	1.9	2740	38023/ 39840