Supplementary Material

Validation of an RP-HPLC Method for Simultaneously Quantifying Three UV Filters Encapsulated into Nanostructured Lipid Carriers

Andressa C. Schneid[a†]. Hatylas Azevedo[a]. Margarete M. de Araújo[a†]*

1. Method Development

Table S1. Values of peak area and purity found in DHHB chromatogram.

Solução	Área	Pureza	%
Standard	112875027	1.00	100.00
Standard	112972317	1.00	100.09
Standard	112739741	1.00	99.88
Ethanol	0	0.00	0.00
Acidificated Water:Ethanol (25:75)	0	0.00	0.00
Diluent (EtOH+DMF)	0	0.00	0.00
Acetonitrile	0	0.00	0.00
Blank sample	0	0.00	0.00
Blank sample (Filtered)	0	0.00	0.00

Table S2. Values of peak area and purity found in BEMT chromatogram.

Solução	Área	Pureza	%
Standard	499038084	1.00	100.00
Standard	499160585	1.00	100.02
Standard	498276065	1.00	99.85
Ethanol	2306718	0.00	0.46
Acidificated Water:Ethanol (25:75)	2418133	0.00	0.48
Diluent (EtOH+DMF)	2755972	0.00	0.55
Acetonitrile	2684339	0.00	0.54
Blank sample	3361542	0.00	0.67
Blank sample (Filtered)	2534416	0.00	0.51

Table S3. Values of peak area and purity found in EHT chromatogram.

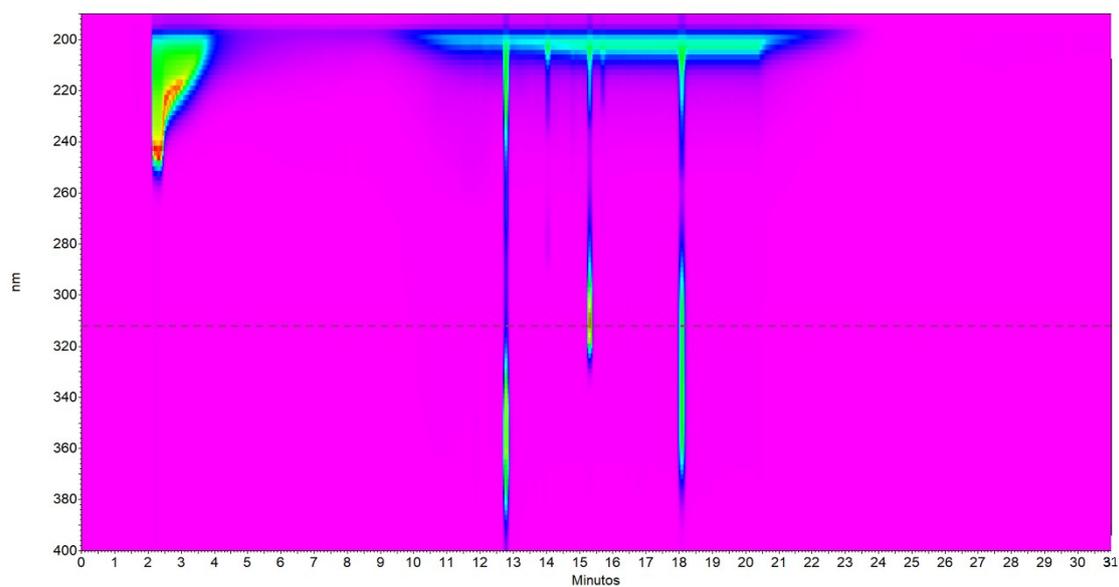| Solução	Área	Pureza	%
Standard	491844734	1.0000	100.00
Standard	491961352	1.0000	100.02
Standard	489778120	1.0000	99.58
Ethanol	2835850	1.0000	0.58
Acidificated Water:Ethanol (25:75)	2749282	1.0000	0.56
Diluent (EtOH+DMF)	3037644	1.0000	0.62
Acetonitrile	3520873	1.0000	0.72
Blank sample	3619452	1.0000	0.74
Blank sample (Filtered)	3085810	1.0000	0.63

Figure S1. Counter map of light absorption for the standard solution (containing DHHB. BEMT and EHT) detected by DAD in the range of 400 - 800 nm within the time window of 31 minutes.

2. Method Validation

2.1. Linearity

2.1.1. Diethylamino hydroxybenzoyl hexyl benzoate (DHHB)

Table S4. Values concentration and peak area obtained in each level imployed in the linearity study for DHHB.

| Sample	Observation	Theoretical Concentration (µg/mL)	Area
Linearity - 1	1	6.0960	15035016
	2	6.0960	15059745
	3	6.0960	15058464
Linearity - 2	4	12	28583242
	5	12.0000	28635730
	6	12.0000	28635730
Linearity - 3	7	24.0000	56550514
	8	24.0000	57495099
	9	24.0000	56044887
Linearity - 4	10	48.0000	114510769
	11	48.0000	113500383
	12	48.0000	112871500
Linearity - 5	13	60.0000	142599948
	14	60.0000	144725994
	15	60.0000	143220791
Linearity - 6	16	72.0000	170231462
	17	72.0000	172094837
	18	72.0000	172518112

$y = 2389176.4344x - 397822.1008$
$R^2 = 0.9998$
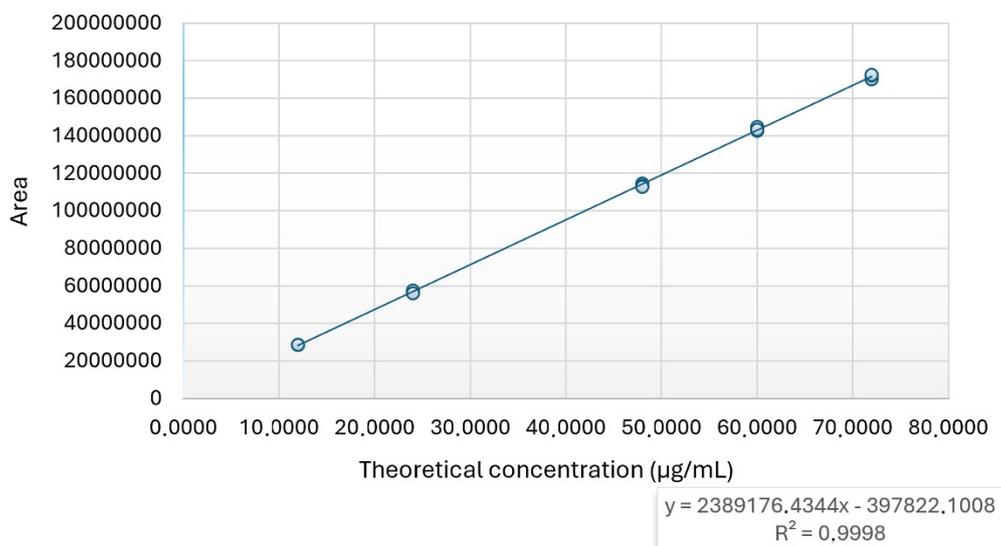
Figure S2. Linear fitting for DHHB data obtained in five concentration levels.

Table S5. Data of regression statistics and Cochran Test for DHHB.

Intercept		28922.97741
Inclination		2381630.18
Correlation Coefficient:		0.999903
Residual Variance		7.01098E+11
Standard Error		863085.7127
Residual Sum of Squared		1.19187E+13
Confidence Interval (95%)	*Inferior Limit*	-1726171.4253
	Superior Limit	1726171.4253
	Residual Average	-5.69142E-09
	Standard Deviation of Residues	837316.1311
	Standardized Inferior Limit	-2.0616
	Standardized Superior Limit	2.0616
Number		18
	N	16
Cochran Test		
Greater variance of the residuals		1.48002E+12
Residuals Variance Sum		3.90209E+12
Cochran Statistics		0.379
Cochran Critical		0.616
Result		homocedastic

Table S6. Residue analysis data obtained for the model fitting of DHHB.

| Observation	Y predicted	Residue	Standard Residue	Variance
1	14547340.56	487675.445	0.5824	
2	14547340.56	512404.445	0.6120	193828851.000
3	14547340.56	511123.445	0.6104	
4	28608485.14	-25243.138	-0.0301	
5	28608485.14	27244.862	0.0325	918330048.000
6	28608485.14	27244.862	0.0325	
7	57188047.3	-637533.299	-0.7614	
8	57188047.3	307051.701	0.3667	541835721716.333
9	57188047.3	-1143160.299	-1.3653	
10	114347171.6	163597.380	0.1954	
11	114347171.6	-846788.620	-1.0113	683929425174.333
12	114347171.6	-1475671.620	-1.7624	
13	142926733.8	-326785.781	-0.3903	
14	142926733.8	1799260.219	2.1488	1195192282662.330
15	142926733.8	294057.219	0.3512	
16	171506295.9	-1274833.941	-1.5225	
17	171506295.9	588541.059	0.7029	1480016056458.330
18	171506295.9	1011816.059	1.2084	
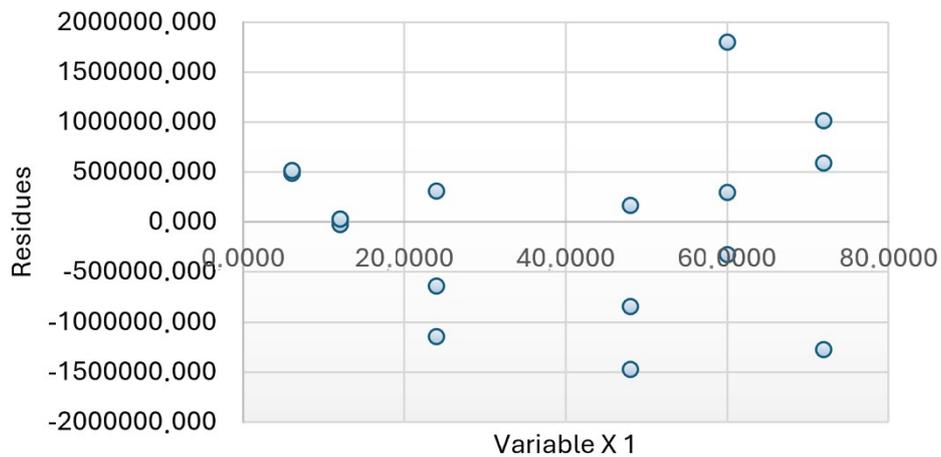
Figure S3. Plot of residues obtained for each data point from their fitting model for DHHB.

Figure S4. Evaluation of residues dispersion data for DHHB.

Table S7. Outlier evaluation using Z values of each sample. employing Grubb's test for DHHB data.

Media	2393155.855
Standard Deviation	39363.55528
$Z_{critical}$	1.96310
Factor	*Z*
2466374.016	1.8600
2470430.610	1.9631
2470220.472	1.9578
2381936.833	0.2850
2386310.833	0.1739
2386310.833	0.1739
2356271.417	0.9370
2395629.125	0.0628
2335203.625	1.4722
2385641.021	0.1909
2364591.313	0.7257
2351489.583	1.0585
2376665.800	0.4189
2412099.900	0.4813
2387013.183	0.1560
2364325.861	0.7324
2390206.069	0.0749
2396084.889	0.0744

2.1.2. Bis-ethylhexyloxyphenol methoxyphenyl triazine (BEMT)

Table S8. Values concentration and peak area obtained in each level employed in the linearity study for BEMT.

| Sample	Observation	Theoretical Concentration (µg/mL)	Area
Linearity - 1	1	4.0600	68170985
	2	4.0600	68586504
	3	4.0600	68277541
Linearity - 2	4	8.0000	125749366
	5	8.0000	127011498
	6	8.0000	127011498
Linearity - 3	7	16.0000	251252987
	8	16.0000	254160158
	9	16.0000	244287700
Linearity - 4	10	32.0000	499897055
	11	32.0000	497102551
	12	32.0000	492460988
Linearity - 5	13	40.0000	622195704
	14	40.0000	632820979
	15	40.0000	625475386
Linearity - 6	16	48.0000	751077254
	17	48.0000	750181197
	18	48.0000	751537867
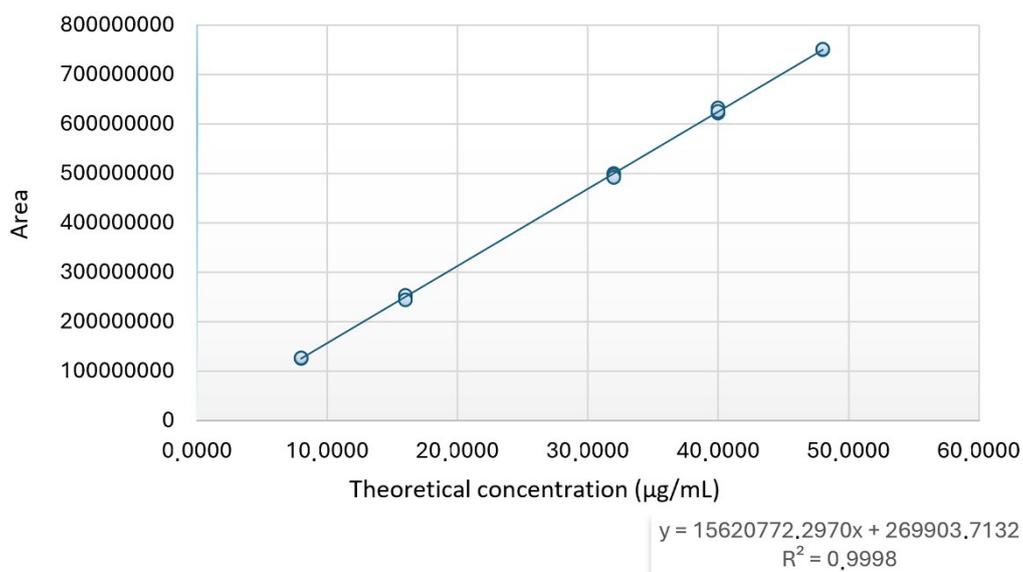
Figure S5. Linear fitting for BEMT data obtained in five concentration levels.

Table S9. Data of regression statistics and Cochran Test for BEMT.

		Intercept	2515797.334
		Inclination	15561197.86
		Correlation Coefficient:	0.999900
		Residual Variance	1.382E+13
		Standard Error	3831942.3011
		Residual Sum of Squared	2.34941E+14
Confidence Interval (95%)		Inferior Limit	-7663884.6023
		Superior Limit	7663884.6023
		Residual Average	-4.01504E-08
		Standard Deviation of Residues	3717530.084
		Standardized Inferior Limit	-2.0616
		Standardized Superior Limit	2.0616
Number			18
	N		16
Cochran Test			
Greater variance of the residuals			2.96018E+13
Residuals Variance Sum			7.05021E+13
Cochran Statistics			0.420
Cochran Critical			0.616
Result			homocedastic

Table S10. Residue analysis data obtained for the model fitting of BEMT.

| Observation	$Y_{predicted}$	Residue	Standard Residue	Variance
1	65694260.65	2476724.350	0.6662	46578059311.000
2	65694260.65	2892243.350	0.7780	
3	65694260.65	2583280.350	0.6949	
4	127005380.2	-1256014.223	-0.3379	530992395141.333
5	127005380.2	6117.777	0.0016	
6	127005380.2	6117.777	0.0016	
7	251494963.1	-241976.113	-0.0651	25738715529562.300
8	251494963.1	2665194.887	0.7169	
9	251494963.1	-7207263.113	-1.9387	
10	500474128.9	-577073.892	-0.1552	14108075352912.300
11	500474128.9	-3371577.892	-0.9069	
12	500474128.9	-8013140.892	-2.1555	
13	624963711.8	-2768007.782	-0.7446	29601753228066.300
14	624963711.8	7857267.218	2.1136	
15	624963711.8	511674.218	0.1376	
16	749453294.7	1623959.328	0.4368	475939328653.000
17	749453294.7	727902.328	0.1958	
18	749453294.7	2084572.328	0.5607	
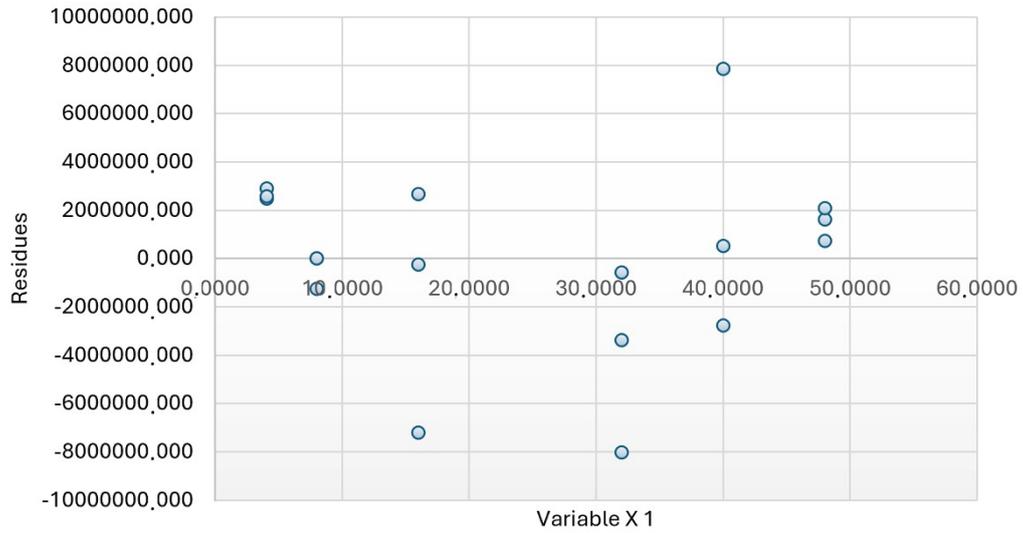
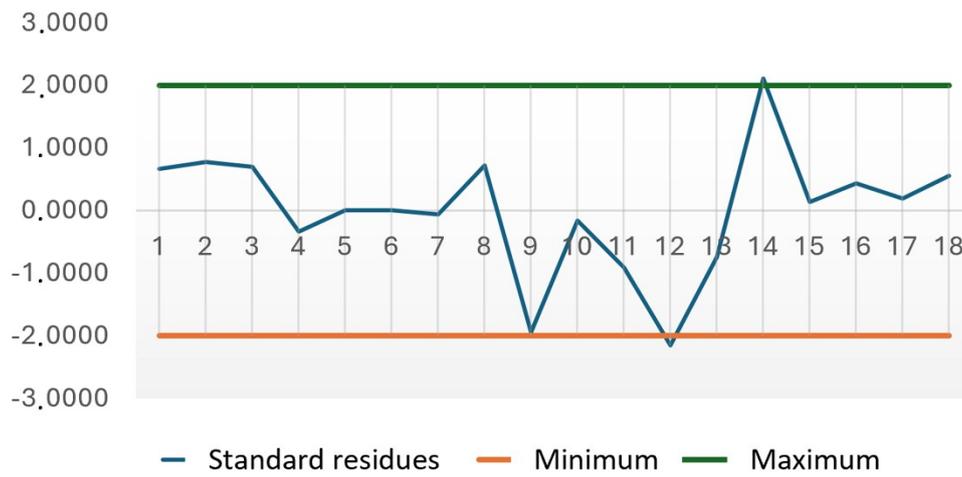**Figure S6. Plot of residues obtained for each data point from their fitting model for BEMT.**

Figure S7. Evaluation of residues dispersion data for BEMT.

Table S11. Outlier evaluation using Z values of each sample. employing Grubb's test for BEMT data.

Media	15851127.16
Standard Deviation	479792.3093
$Z_{critical}$	2.17198
Factor	*Significance*
16790883.005	1.9587
16893227.586	2.1720
16817128.325	2.0134
15718670.750	0.2761
15876437.250	0.0528
15876437.250	0.0528
15703311.688	0.3081
15885009.875	0.0706
15267981.250	1.2154
15621782.969	0.4780
15534454.719	0.6600
15389405.875	0.9623
15554892.600	0.6174
15820524.475	0.0638
15636884.650	0.4465
15647442.792	0.4245
15628774.938	0.4634
15657038.896	0.4045

2.2.3. Ethylhexyl triazone (EHT)

Table S12. Values concentration and peak area obtained in each level employed in the linearity study for EHT.

| Sample	Observation	Theoretical Concentration (μg/mL)	Area
Linearity - 1	1	2.0300	70102853
	2	2.0300	70993856
	3	2.0300	70686250
Linearity - 2	4	4.0000	128881423
	5	4.0000	130396028
	6	4.0000	130396028
Linearity - 3	7	8.0000	255481127
	8	8.0000	259458541
	9	8.0000	250291076
Linearity - 4	10	16.0000	508005323
	11	16.0000	505068901
	12	16.0000	500271395
Linearity - 5	13	20.0000	631220282
	14	20.0000	643415347
	15	20.0000	633346412
Linearity - 6	16	24.0000	760175508
	17	24.0000	759425267
	18	24.0000	760956635
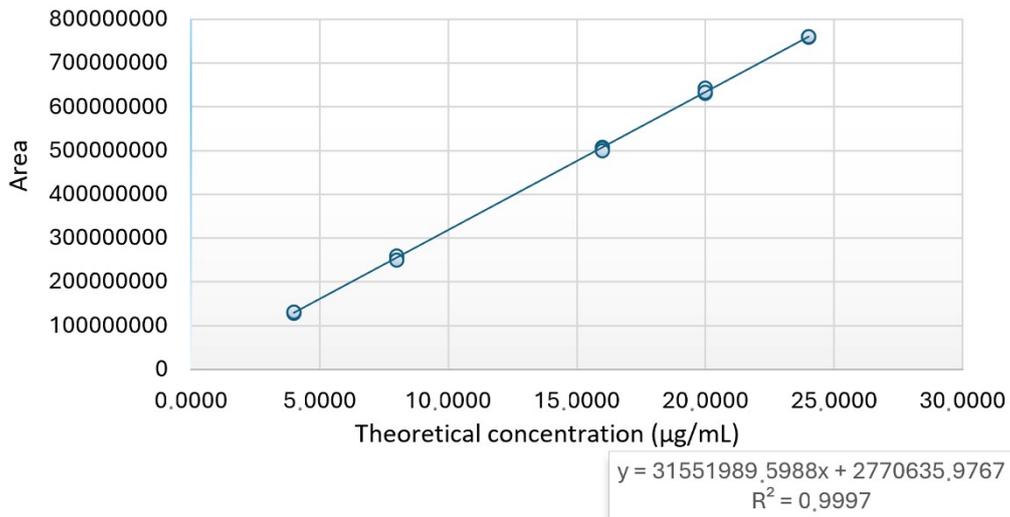
Figure S8. Linear fitting for EHT data obtained in five concentration levels.

Table S13. Data of regression statistics and Cochran Test for EHT.

Intercept			4591151.375
Inclination			31455407.84
Correlation Coefficient:			0.999903
Residual Variance			1.36366E+13
Standard Error			3806433.2729
Residual Sum of Squared			2.31823E+14
Confidence Interval (95%)	*Inferior Limit*		-7612866.5459
	Superior Limit		7612866.5459
	Residual Average		9.10627E-09
	Standard Deviation of Residues		3692782.692
	Standardized Inferior Limit		-2.0616
	Standardized Superior Limit		2.0616
Number			18
	N		16
Teste de Cochran			
Greater variance of the residuals			4.24372E+13
Residuals Variance Sum			8.03683E+13
Cochran Statistics			0.528
Cochran Critical			0.616
Result			homocedastic

Table S14. Residue analysis data obtained for the model fitting of EHT.

| Observation	$Y_{predicted}$	Residue	Standard Residue	Variance
1	68445629.29	1657223.709	0.4488	204809976142.334
2	68445629.29	2548226.709	0.6901	
3	68445629.29	2240620.709	0.6068	
4	130412782.7	-1531359.736	-0.4147	764676102008.333
5	130412782.7	-16754.736	-0.0045	
6	130412782.7	-16754.736	-0.0045	
7	256234414.1	-753287.097	-0.2040	21133144339370.300
8	256234414.1	3224126.903	0.8731	
9	256234414.1	-5943338.097	-1.6094	
10	507877676.8	127646.180	0.0346	15242046715217.300
11	507877676.8	-2808775.820	-0.7606	
12	507877676.8	-7606281.820	-2.0598	
13	633699308.2	-2479026.181	-0.6713	42437248527558.300
14	633699308.2	9716038.819	2.6311	
15	633699308.2	-352896.181	-0.0956	
16	759520939.5	654568.458	0.1773	586351483272.333
17	759520939.5	-95672.542	-0.0259	
18	759520939.5	1435695.458	0.3888	
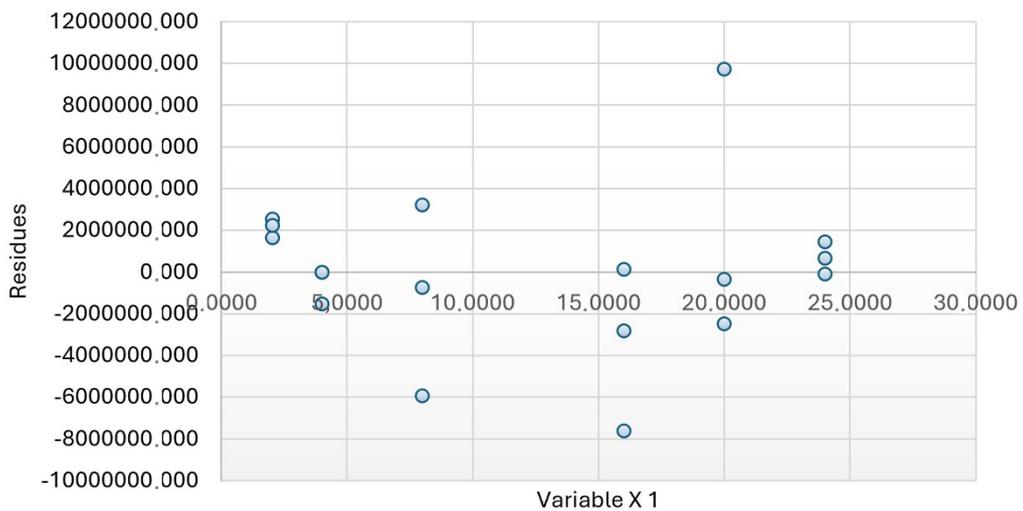
Figure S9. Plot of residues obtained for each data point from their fitting model for EHT.

Figure S10. Evaluation of residues dispersion data for EHT.

Table S15. Outlier evaluation using Z values of each sample. employing Grubb's test for EHT data.

Media	31871909.43
Standard Deviation	435919.8777
$Z_{critical}$	1.66796
Factor	*Significance*
32220355.750	0.7993
32599007.000	1.6680
32599007.000	1.6680
31935140.875	0.1451
32432317.625	1.2856
31286384.500	1.3432
31750332.688	0.2789
31566806.313	0.6999
31266962.188	1.3877
31561014.100	0.7132

32170767.350	0.6856
31667320.600	0.4693
31673979.500	0.4541
31642719.458	0.5258
31706526.458	0.3794

2.2.3. LOQ and LOD

Table S16. Signal-to-noise ratio values obtained from the chromatograms for DHHB, BEMT and EHT

Analyte	Signal/Noise
DHHB	360±18
BEMT	1014±45
EHT	1854±69

Table S17. LOQ and LOD obtained for DHHB.

Results	Curve 1	Curve 2	Curve 3	Media	Standard Deviation	LOQ	LOD
Intercept do axis y	103.967.74	230332.6744	2694980.771		1460810.669	6.0909	1.8273
Inclination	2.370.167.11	2396313.455	2428603.986	2398361.517			

Table S 19. LOQ and LOD obtained for BEMT.

Results	Curve 1	Curve 2	Curve 3	Media	Standard Deviation	LOQ	LOD
Intercept do axis y	1.151.083.02	2602903.442	-11448057.34		7727393.042	4.9242	1.4773
Inclination da reta	15.586.228.83	15612929.62	15879074.78	15692744.41			

Table S 20. LOQ and LOD obtained for EHT.

Results	Curve 1	Curve 2	Curve 3	Media	Standard Deviation	LOQ	LOD
Intercept do axis y	3.096.170.70	5101720.581	-7036179.314		6506603.838	2.0543	0.6163
Inclination da reta	31.503.927.91	31559103.9	31956032.87	31673021.56			

2.2.4. Precision (Intra and Inter runs)

Table S21. Precision Intra-Runs DHHB.

Sample	Concentration (µg/mL)
Precision Intra 1	44.820
Precision Intra 2	45.519
Precision Intra 3	45.886
Precision Intra 4	47.077
Precision Intra 5	45.834
Precision Intra 6	44.958
Media	**45.682**
Standard Deviation	**0.8130**
Variance	**0.6609**
DPR %	**1.78**

Table S22. Precision Intra-Runs for BEMT.

Sample	Concentration (µg/mL)
Precision Intra 1	28.926
Precision Intra 2	28.402
Precision Intra 3	29.342
Precision Intra 4	28.12
Precision Intra 5	29.527
Precision Intra 6	26.405
Media	**28.453667**
Standard Deviation	**1.1380**
Variance	**1.2951**
DPR %	**4.00**

Table S23. Precision Intra-Runs for EHT.

Sample	Concentration (µg/mL)
Precision Intra 1	15.1990
Precision Intra 2	15.3260
Precision Intra 3	15.6130
Precision Intra 4	15.6880
Precision Intra 5	15.9490
Precision Intra 6	14.7000
Media	**15.412500**
Standard Deviation	**0.4392**
Variance	**0.1929**
DPR %	**2.85**

Table S24. Precision Inter - Runs for DHHB.

Sample	Concentration (µg/mL)
Precision Intra 1	44.820
Precision Intra 2	45.519
Precision Intra 3	45.886
Precision Intra 4	47.077
Precision Intra 5	45.834
Precision Intra 6	44.958
Precision Inter 1	48.0610
Precision Inter 2	46.8510
Precision Inter 3	47.3520
Precision Inter 4	47.4250
Precision Inter 5	47.6410
Precision Inter 6	49.5680

Media	46.7493
Standard Deviation	1.3956
Variance (Inter)	0.8919
DPR %	2.99

Table S25. Precision Inter - Runs for BEMT.

Sample	Concentration (µg/mL)
Precision Intra 1	15.1990
Precision Intra 2	15.3260
Precision Intra 3	15.6130
Precision Intra 4	15.6880
Precision Intra 5	15.9490
Precision Intra 6	14.7000
Precision Inter 1	16.0020
Precision Inter 2	15.5930
Precision Inter 3	15.7960
Precision Inter 4	15.8860
Precision Inter 5	15.9090
Precision Inter 6	16.9980
Media	15.7216
Standard Deviation	0.5502
Variance (Inter)	0.2438
DPR %	3.50

Table S26. Precision Inter - Runs for EHT.

Sample	Concentration (µg/mL)
Precision Intra 1	15.1990
Precision Intra 2	15.3260
Precision Intra 3	15.6130
Precision Intra 4	15.6880
Precision Intra 5	15.9490
Precision Intra 6	14.7000
Precision Inter 1	16.0020
Precision Inter 2	15.5930
Precision Inter 3	15.7960
Precision Inter 4	15.8860
Precision Inter 5	15.9090
Precision Inter 6	16.9980
Media	**15.7216**
Standard Deviation	**0.5502**
Variance (Inter)	**0.2438**
DPR %	**3.50**

2.2.5. Accuracy

Table S27. Accuracy for DHHB in NLC.

Sample	Interval	Standard weight (mg)	Theoretical Concentration (µg/mL)	Experimental concentration (µg/mL)	Recover (%)
Accuracy 1A		75	38.4000	38.9090	101.33
Accuracy 2A	LOW 80%	75	38.4000	39.2010	102.09
Accuracy 3A		75	38.4000	37.2620	97.04
Accuracy 1B		75	48.0000	48.7980	101.66
Accuracy 2B	AVERAGE 100%	75	48.0000	47.7340	99.45
Accuracy 3B		75	48.0000	48.2290	100.48
Accuracy 1C		75	57.6000	56.9020	98.79
Accuracy 2C	HIGH 120%	75	57.6000	59.0510	102.52
Accuracy 3C		75	57.6000	58.0510	100.78
				Media	**100.46**
				Standard Deviation	**1.76**
				DPR (%)	**1.75**

Table S28. Accuracy for BEMT in NLC

Sample	Interval	Standard weight (mg)	Theoretical Concentration (µg/mL)	Experimental concentration (µg/mL)	Recover (%)
Accuracy 1A	LOW 80%	50	25.6000	26.0420	101.73
Accuracy 2A		50	25.6000	27.6750	108.11
Accuracy 3A		50	25.6000	24.9200	97.34
Accuracy 1B	AVERAGE 100%	50	32.0000	33.4860	104.64
Accuracy 2B		50	32.0000	32.7490	102.34
Accuracy 3B		50	32.0000	32.5600	101.75
Accuracy 1C	HIGH 120%	50	38.4000	37.9080	98.72
Accuracy 2C		50	38.4000	40.2040	104.70
Accuracy 3C		50	38.4000	38.9050	101.32
				Media	**102.29**
				Standard Deviation	**3.24**
				DPR (%)	**3.17**

Table S29. Accuracy for EHT in NLC.

Sample	Interval	Standard weight (mg)	Theoretical Concentration (µg/mL)	Experimental concentration (µg/mL)	Recover (%)
Accuracy 1A	LOW 80%	25	12.8000	13.0250	101.76
Accuracy 2A		25	12.8000	13.8440	108.16
Accuracy 3A		25	12.8000	12.4930	97.60
Accuracy 1B	AVERAGE 100%	25	16.0000	16.7150	104.47
Accuracy 2B		25	16.0000	16.2260	101.41
Accuracy 3B		25	16.0000	16.2300	101.44
Accuracy 1C	HIGH 120%	25	19.2000	19.0200	99.06
Accuracy 2C		25	19.2000	19.9920	104.13
Accuracy 3C		25	19.2000	19.3980	101.03
				Media	102.12
				Standard Deviation	3.12
				DPR (%)	3.06

2.2.6. Robustness

Table S30. Results of Robustness – DHHB.

Parameters	Analytical method	Changes	k'	Simmetry	Theoretical plates (USP)	Conc. (µg/mL)	Recover
Reference values	Alteration	-	6.16	1.18	144408	49.014	102.1
Filter	Filtered	Non Filtered in Filter PVDF 0.45 µm	6.16	1.10	143371	49.7670	103.7
Stability	0h	6 days	6.49	1.17	139857	44.5170	92.7
Temperature	35°C	34°C	6.16	1.16	143952	48.961	102.0
		36°C	6.14	1.13	142165	48.934	101.9
Mobile Phase flow	0.7mL/min	0.6mL/min	6.58	1.10	153771	48.616	101.3
		0.8 mL/min	5.83	1.21	132627	48.523	101.1
Mobile phase composition	Acidificated Water (H_3PO_4)+EtOH-Scharlau	Acidificated Water (H_3PO_4)+EtOH-Biograde	6.19	1.18	146560	47.540	99.0

Table S 31. Results de Robustness – BEMT

Parameters	Analytical method	Changes	k'	Simmetry	Theoretical plates (USP)	Conc. (µg/mL)	Recover
Reference	Alteration	-	9.01	1.11	146045	28.6550	89.5

Parameters	Analytical method	Changes	k'	Simmetry	Theoretical plates (USP)	Conc. (µg/mL)	Recover
values							
Filter	Filtered	Non Filtered in Filter PVDF 0.45 µm	9.01	1.13	146623	28.4410	88.9
Stability	0h	6 days	9.47	1.18	147081	28.4570	88.9
Temperature	35°C	34°C	9.06	1.13	139750	28.624	89.5
		36°C	8.95	1.15	150734	28.598	89.4
Mobile Phase flow	0.7mL/min	0.6mL/min	7.94	1.18	254333	34.286	107.1
		0.8 mL/min	7.31	1.22	296689	34.168	106.8
Mobile phase composition	Acidificated Water (H$_3$PO$_4$)+EtOH-Scharlau	Acidificated Water (H$_3$PO$_4$)+EtOH-Biograde	7.62	1.21	266963	33.825	105.7

Table S 32. Results de Robustness – EHT

Parameters	Analytical method	Changes	k'	Simmetry	Theoretical plates (USP)	Conc. (µg/mL)	Recover
Reference value	Alteration	-	7.58	1.16	277891	16.3690	102.3
Filter	Filtered	Non Filtered no Filter PVDF 0.45 µm	7.58	1.18	278802	16.2360	101.5
Stability	0h	6 days	7.98	1.19	277363	24.4490	152.8

Temperature	35°C	34°C	7.58	1.23	275378	16.342	102.1
		36°C	7.56	1.23	271510	16.306	101.9
Mobile Phase flow	0.7mL/min	0.6mL/min	9.58	1.15	1442270	14.087	88.0
		0.8 mL/min	8.56	1.14	160150	14.057	87.9
Mobile phase composition	Acidificated Water (H₃PO₄)+EtOH-Scharlau	Acidificated Water (H₃PO₄)+EtOH-Biograde	9.20	1.12	126220	13.697	85.6

2.2.7. System Suitability for Robustness

Table S 33 – System Suitability for Robustness do DHHB

System Suitability for Robustness		
Parameter	**Minimum**	**Maximum**
Capacity factor (k´)	4.31	8.01
Assimmetry	0.83	1.53
Theoretical plates	101086	187730
Concentration (%)	46.56	51.46

Table S34. System Suitability for Robustness do BEMT.

Parameter	**Minimum**	**Maximum**

Capacity factor (k´)	6.31	11.71
Assimmetry	0.78	1.44
Theoretical plates	102232	189859
Concentration (%)	27.22	30.09

Table S35. System Suitability for Robustness do EHT.

Parameter	Minimum	Maximum
Capacity factor (k´)	5.31	9.85
Assimmetry	0.81	1.51
Theoretical plates	194524	361258
Concentration (%)	15.55	17.19

3.   Response Surface for models of Extraction Content and Encapsulation Efficiency
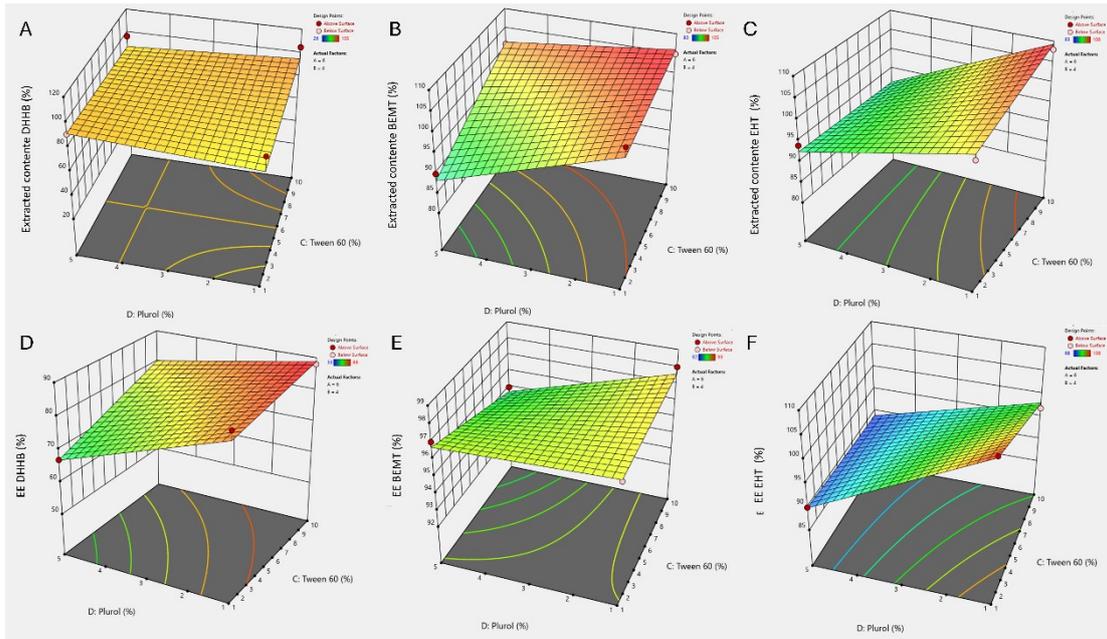
Figure S11. Response surfaces obtained from prediction models obtained for extraction content of (A) DHHB, (B) BEMT, (C) EHT, and encapsulation efficiency of (D) DHHB, (E) BEMT, (F) EHT.