

# **Supporting Information**

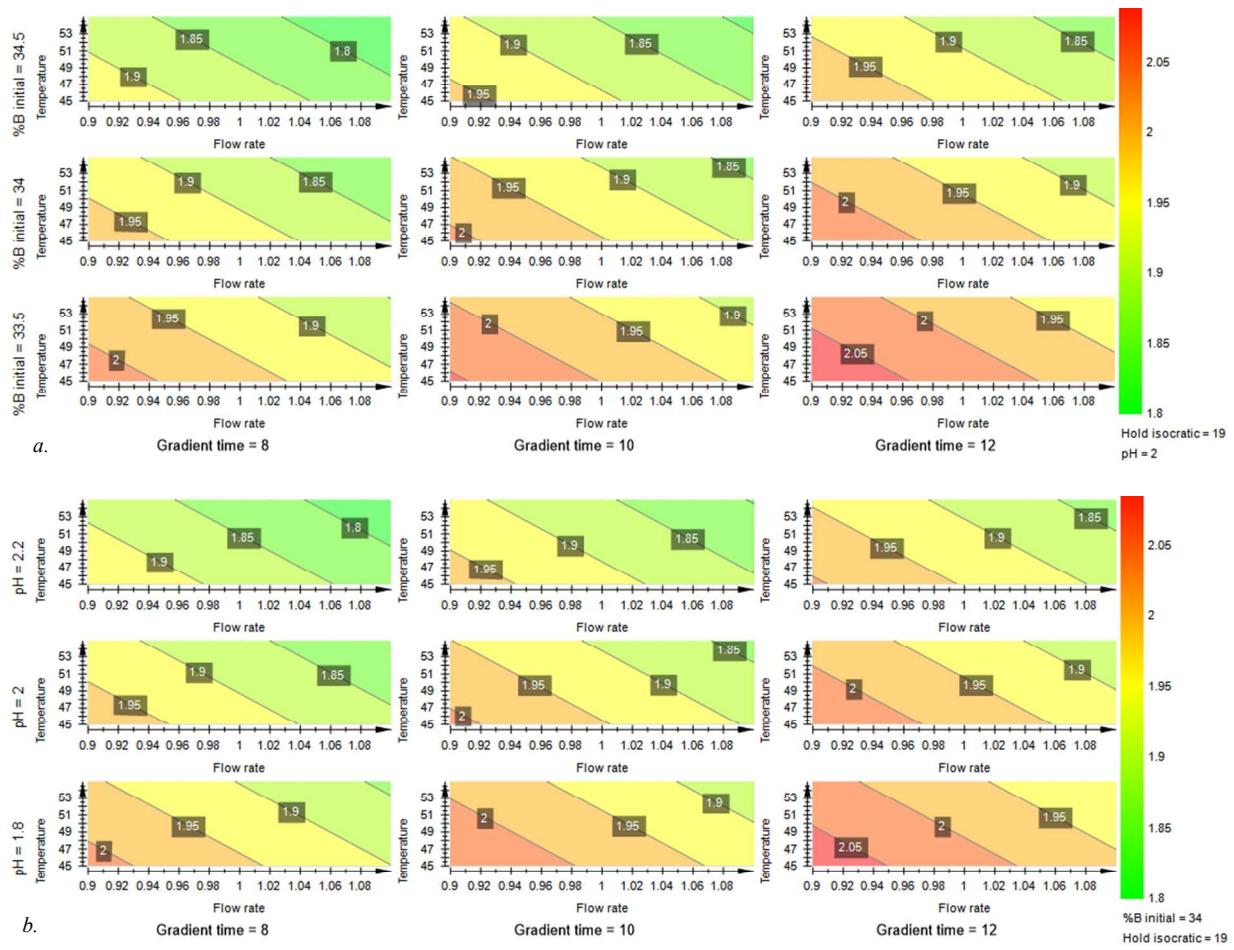
## **A Validated Method for the Analysis of**

## **Frangulins A and B and Glucofrangulins A and B**

## **Using HPLC and UHPLC**

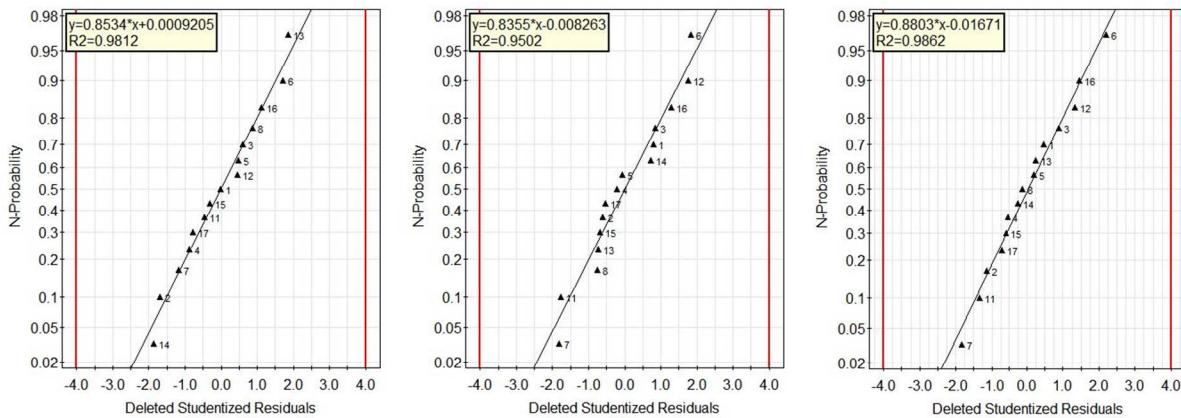
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Phytopharmacy, Einsiedlerstrasse 31, CH-8820 Wädenswil, Switzerland

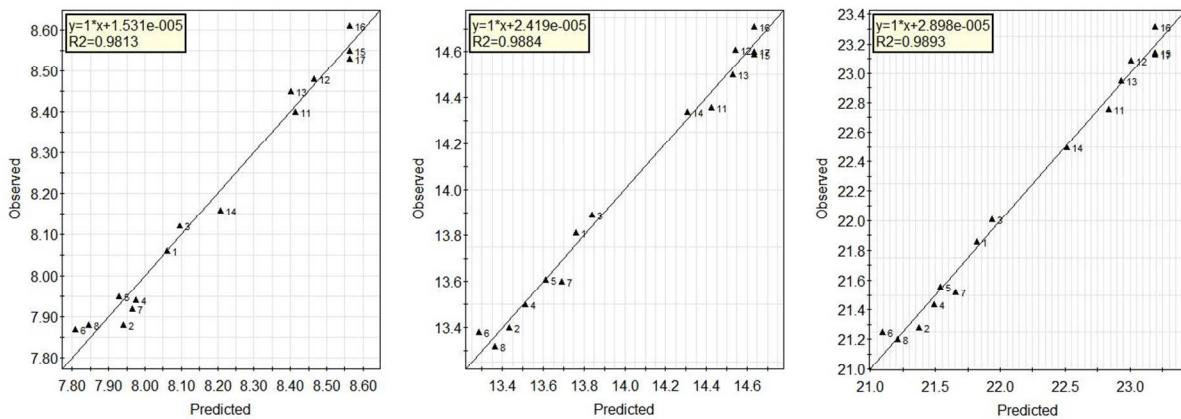


**Figure S 1.** 4-D contour Plots for the resolution  $R_s$  between compounds **4** and **3**. <sup>a</sup> pH Constant at pH=2; <sup>b</sup> %B initial constant at 34%

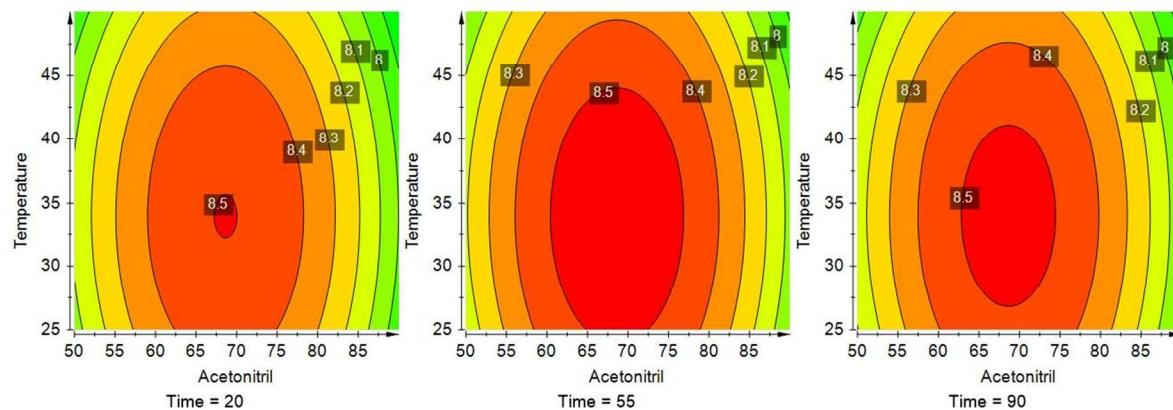
To obtain a valid model, the residuals have to follow a normal distribution. The assumption is fulfilled as the data points in [Figure S 2](#)[Figure S 2](#) show satisfactory linearity. Model validity is checked by comparing predicted values with observed values. As [Figure S 3](#)[Figure S 3](#) shows a straight line with high  $r^2$  for all responses, all three models are considered valid. An  $r^2$  of 0.98 means that more than 98% of the deviation of the response from the mean is explained by the model.



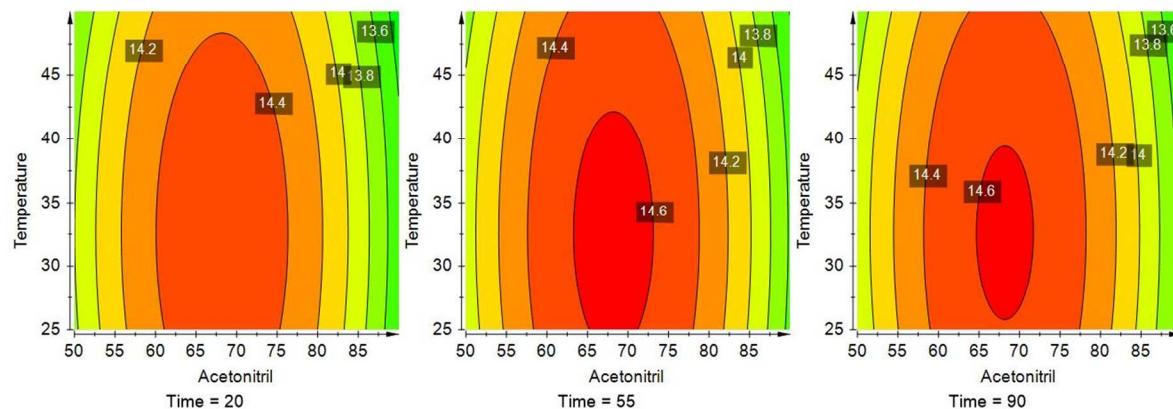
**Figure S 2.** Residual N-Plots (left: sum compounds **4, 2**; middel: sum compounds **3, 1**, right: sum of compounds **1-4**)



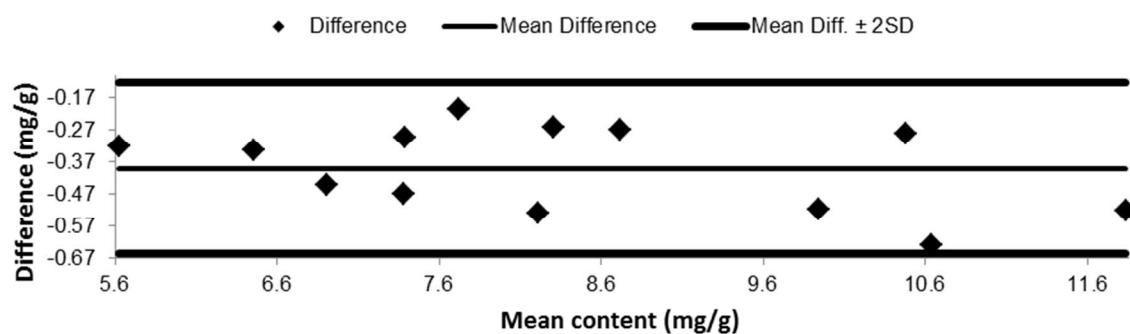
**Figure S 3.** Observed vs. Predicted plots (left: sum compounds **4, 2**; middel: sum compounds **3, 1**; right: sum of compounds **1-4**)



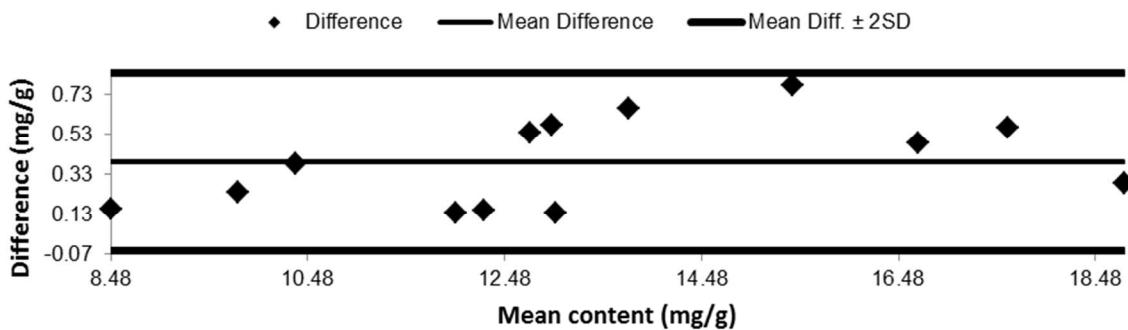
**Figure S 4.** 4-D contour plot calculated with Equation 1 for the Sum of Compounds **2** and **4** (mg/g)



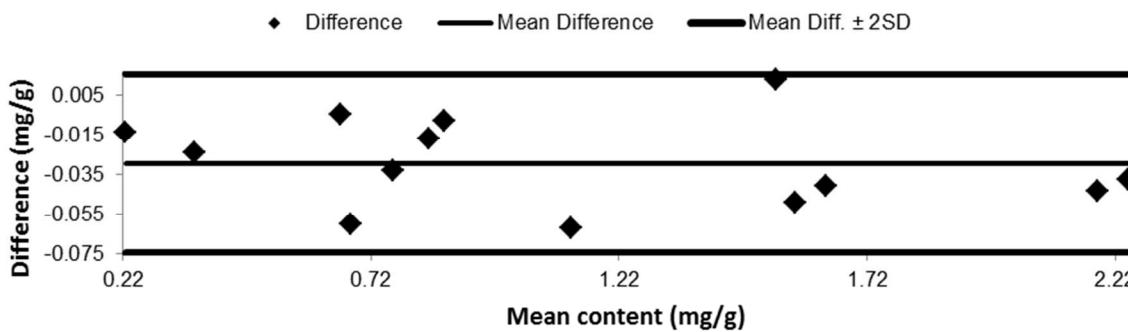
**Figure S 5.** 4-D contour plot calculated with Equation 3 for the sum of Compounds **1** and **3** (mg/g)



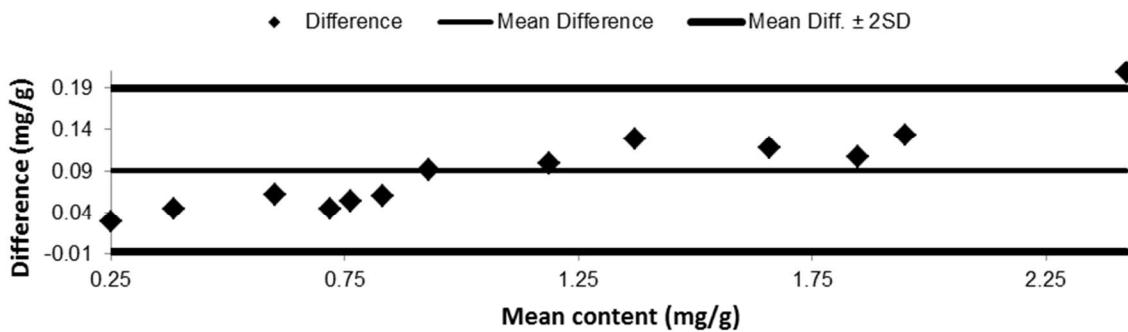
**Figure S 6.** Comparision of HPLC and UHPLC of the content of compound **2** by the Bland-Altman plot



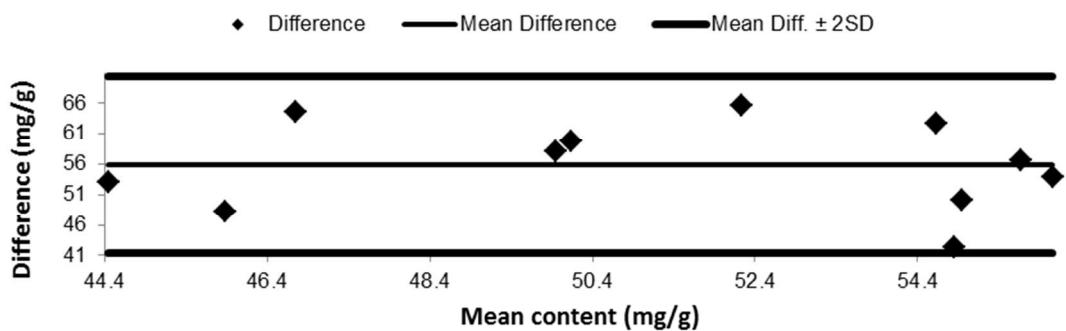
**Figure S 7.** Comparision of HPLC and UHPLC of the content of compound 1 by the Bland-Altman plot



**Figure S 8.** Comparision of HPLC and UHPLC of the content of compound 4 by the Bland-Altman plot



**Figure S 9.** Comparision of HPLC and UHPLC of the content of compound 3 by the Bland-Altman plot.



**Figure S 10.** Bland-Altman plot of the photometric assay and the HPLC method (sum of compounds 1-4)

**Table S 11. Linearity of the Detector Response, Concentration Range of the Standard Solutions, LOD and LOQ.**

	range ( $\mu\text{g/mL}$ )	slope (a) (IU/(mg/mL))	intercept (b) (IU)	$r^2$	LOD <sup>a</sup> ( $\mu\text{g/L}$ )	LOQ <sup>b</sup> ( $\mu\text{g/L}$ )
<b>4</b>	0.460-9.2	38076905	-2438	0.99999	77.7	155.3
<b>3</b>	0.475-9.5	33227066	-1397	0.99997	61.4	122.9

<sup>a</sup>LOD=b + 3 SE<sub>b</sub>. <sup>b</sup>LOQ=b + 6 SE<sub>b</sub>

**Table S 12. Concentrations of compound 4 in the Samples used for the Recovery Test to demonstrate the accuracy of the method**

No.	SW (mg)	content 4 ( $\mu\text{g}/\text{mg}$ )	total 4 in SW ( $\mu\text{g}$ )	calculated 4 from matrix ( $\mu\text{g}$ )	4 found ( $\mu\text{g}$ )	4 spike ( $\mu\text{g}$ )	recovery 4 (%)
1	74.9	<sup>a</sup> 0.965	72.3	71.1		0.0	-
2	80.5	<sup>a</sup> 0.932	75.1	76.4		0.0	-
3	74.6	1.904	142.1	70.8	71.3	76.0	93.8
4	76.9	1.894	145.6	72.9	72.7	76.0	95.6
5	80.3	2.736	219.7	76.2	143.5	152.0	94.4
6	77.2	2.800	216.2	73.2	143.0	152.0	94.0
<b>mean</b>						<b>94.5</b>	

<sup>a</sup>mean of both values is 0.9486  $\mu\text{g}/\text{mg}$  and was used to calculate “4 from matrix” by multiplication with SW.

**Table S 13. Concentrations of compound 3 in the Samples used for the Recovery Test to demonstrate the accuracy of the method**

No.	SW (mg)	content 3 ( $\mu\text{g}/\text{mg}$ )	total 3 in SW ( $\mu\text{g}$ )	calculated 3 from matrix ( $\mu\text{g}$ )	3 found ( $\mu\text{g}$ )	3 spike ( $\mu\text{g}$ )	recovery 3 (%)
1	74.9	<sup>a</sup> 0.922	69.0	67.9		0.0	
2	80.5	<sup>a</sup> 0.893	71.9	73.0		0.0	
3	74.6	1.875	139.9	67.7	72.2	73.6	98.1
4	79.9	1.794	143.3	72.5	70.9	73.6	96.3
5	80.3	2.746	220.5	72.8	147.6	147.2	100.3
6	77.2	2.801	216.2	70.0	146.2	147.2	99.3
<b>mean</b>						<b>98.5</b>	

<sup>a</sup>mean of both values is 0.9072  $\mu\text{g}/\text{mg}$  and was used to calculate “3 from matrix” by multiplication with SW.

**Table S 14. Repeatability of the Method (n = 6)**

	<b>2</b>	<b>1</b>	<b>4</b>	<b>3</b>	<b><math>\Sigma 1-4</math></b>	<b><math>t_R 2</math></b>	<b><math>t_R 1</math></b>	<b><math>t_R 4</math></b>	<b><math>t_R 3</math></b>
	content (mg/g)					time (min)			
MEAN	6.95	12.04	0.95	0.97	20.90	12.84	15.19	24.35	25.25
SD	0.02	0.04	0.01	0.01	0.06	0.18	0.21	0.05	0.05
% RSD	0.31	0.30	0.55	0.84	0.29	1.39	1.37	0.21	0.20

**Table S 15. Inter-day Precision of the Contents of the Anthranoids 1-4 and the Sum of 1-4 over Three Different Points in Time (n = 3).**

	<b>2</b>	<b>1</b>	<b>4</b>	<b>3</b>	<b><math>\Sigma 1-4</math></b>	
	content (mg/g)					
$t_1$	MEAN	7.00	12.09	0.95	0.97	21.01
$t_2$	MEAN	6.95	12.04	0.95	0.97	20.90
$t_3$	MEAN	6.88	11.90	0.95	0.94	20.67
	MEAN	6.94	12.01	0.95	0.96	20.86
	SD	0.13	0.21	0.02	0.02	0.35
	% RSD	1.85	1.73	1.60	2.00	1.70
ANOVA	<i>p</i> value	0.277	0.270	0.977	<sup>a</sup> 0.037	0.257

<sup>a</sup>significant at  $\alpha=0.05$ ;  $t_1$  = day 0;  $t_2$  = day 4;  $t_3$  = day 4

Except for **3**, the ANOVA does not indicate a significant difference between the groups. Even though a *p*-value of 0.037 indicates a significant difference, the lower content of **3** at  $t_3$  does not diminish the method's inter-day precision as such differences occur occasionally in plant analytics, especially when the content of the analyte is relatively low.

**Table S 16. Inter-day Precision the Retention Times of Compounds 1-4 over Three Different Points in Time (n = 3).**

		t <sub>R</sub> 2	t <sub>R</sub> 1	t <sub>R</sub> 4	t <sub>R</sub> 3
				time (min)	
t <sub>1</sub>	MEAN	12.79	15.14	24.42	25.32
t <sub>2</sub>	MEAN	12.84	15.19	24.35	25.25
t <sub>3</sub>	MEAN	12.79	15.14	24.38	25.28
	MEAN	12.81	15.16	24.38	25.28
total	SD	0.14	0.16	0.04	0.04
	% RSD	1.10	1.08	0.17	0.17
ANOVA	p value	0.814	0.850	<sup>a</sup> 0.013	<sup>a</sup> 0.008

<sup>a</sup>significant at  $\alpha=0.05$ ; t<sub>1</sub> = day 0; t<sub>2</sub> = day 4; t<sub>3</sub> = day 4

**Table S 17. Experimental Results of the Responses of the Fractional Factorial Design**

No.	k' 2	k' 1	k' 4	k' 3	2 (IU)	1 (IU)	4 (IU)	3 (IU)	Rs
1	13.05	15.74	28.11	29.22	414005	653296	71886	75767	1.92
2	10.93	13.13	26.72	27.54	412322	649000	72342	75928	1.88
3	18.01	21.46	28.92	30.00	411260	650764	72291	75923	1.94
4	15.77	18.77	27.89	28.63	411566	652899	72923	76750	1.91
5	15.38	18.33	26.90	27.64	337368	539383	59409	62508	1.84
6	12.73	15.11	28.05	29.12	339111	535290	59215	62196	1.86
7	10.95	13.21	26.08	26.84	337348	534302	59159	62176	1.88
8	9.774	11.77	27.18	28.26	335183	535253	59326	62406	1.81
9	11.98	14.33	27.36	28.28	371072	591611	65733	68651	1.95
10	12.10	14.50	27.40	28.33	372490	593617	65729	68792	1.96
11	11.93	14.28	27.37	28.29	369434	588112	65231	68317	1.99

**Table S 18. p values of the Responses for the Examined Factors.**

	t <sub>R</sub> 2 (min)	t <sub>R</sub> 1 (min)	t <sub>R</sub> 4 (min)	t <sub>R</sub> 3 (min)	2 (IU)	1 (IU)	4 (IU)	3 (IU)	Rs
%B	0.155	0.155	0.501	0.647	0.917	0.923	0.273	0.386	0.215
Hold	0.756	0.737	0.374	0.551	0.615	0.493	0.758	0.916	0.315
Flow	0.129	0.129	<sup>a</sup> 0.004	<sup>a</sup> 0.004	<sup>a</sup> <0.001	<sup>a</sup> <0.001	<sup>a</sup> <0.001	<sup>a</sup> <0.001	0.115
pH	0.285	0.302	0.674	0.867	0.878	0.489	0.330	0.391	0.259
t <sub>G</sub>	0.529	0.525	<sup>a</sup> 0.002	<sup>a</sup> 0.001	0.982	0.700	0.239	0.370	0.346
Temp	<sup>a</sup> 0.017	<sup>a</sup> 0.019	<sup>a</sup> 0.004	<sup>a</sup> 0.004	0.870	0.715	0.714	0.917	0.285

<sup>a</sup>indicates factors that are significant on  $\alpha=0.05$

**Table S 19. Regression Coefficients and Analysis of the Model for Three Response Variables.**

	4+2 (mg/g)		3+1 (mg/g)		$\Sigma$ 1-4 (mg/g)		
	coeff.	p value	coeff.	p value	coeff.	p value	
b <sub>0</sub>	Constant	8.563	<0.0001	14.633	<0.0001	23.196	<0.0001
b <sub>1</sub>	ACN	-0.060	0.0107	-0.164	0.0003	-0.222	0.0004
b <sub>2</sub>	Time	0.018	0.2597	0.039	0.1047	0.057	0.1039
b <sub>3</sub>	Temp	-0.065	0.0020	-0.074	0.0079	-0.138	0.0021
b <sub>11</sub>	ACN*ACN	-0.441	<0.0001	-0.909	<0.0001	-1.351	<0.0001
b <sub>22</sub>	Time*Time	-0.055	0.0300	-0.066	0.0621	-0.120	0.0270
b <sub>33</sub>	Temp*Temp	-0.115	0.0005	-0.095	0.0143	-0.209	0.0015

**Table S 20. Predicted Optimum Condition and Experimental Values of the Responses under these Conditions**

response	r <sup>2</sup>	r <sup>2</sup> adj	F-value	p-value	predicted	observed	Δ %
<b>2 + 4</b>	0.981	0.967	70.06	<0.0001	8.57	8.36	2.45
<b>1 + 3</b>	0.988	0.980	114.07	<0.0001	14.66	14.52	0.95
total	0.989	0.981	123.65	<0.0001	23.22	22.87	1.51

**Table S 21. Frangulin Contents of the Analyzed Samples of *F. alnus* with HPLC (*n* = 3)**

Lot		2	1	4 content (mg/g)	3	$\Sigma$ 1-4
11732	MEAN	7.24	13.01	1.09	1.24	22.58
11732	SD	0.09	0.18	<0.01	<0.01	0.27
11732	% RSD	1.29	1.37	0.21	0.12	1.20
1806602	MEAN	9.69	16.92	1.55	1.72	29.88
1806602	SD	0.04	0.08	0.01	0.01	0.13
1806602	% RSD	0.38	0.45	0.53	0.59	0.42
2008.04.0881	MEAN	7.15	12.35	0.65	0.74	20.89
2008.04.0881	SD	0.18	0.34	0.05	0.03	0.59
2008.04.0881	% RSD	2.53	2.73	7.71	4.09	2.83
18103247	MEAN	5.47	8.56	0.22	0.27	14.51
18103247	SD	0.09	0.14	0.01	0.01	0.23
18103247	% RSD	1.71	1.57	2.10	1.95	1.60
2293402	MEAN	6.69	12.06	0.75	0.79	20.29
2293402	SD	0.06	0.09	0.01	0.01	0.15
2293402	% RSD	0.83	0.73	0.73	0.84	0.75
2287202	MEAN	7.95	13.07	0.35	0.41	21.77
2287202	SD	0.04	0.10	0.005	0.01	0.12
2287202	% RSD	0.51	0.80	1.53	3.24	0.56
17730349	MEAN	10.32	18.91	1.62	1.90	32.75
17730349	SD	0.16	0.31	0.02	0.04	0.53
17730349	% RSD	1.52	1.62	1.48	2.09	1.61
11001253	MEAN	6.29	9.89	0.83	0.86	17.88
11001253	SD	0.19	0.33	0.01	0.02	0.54
11001253	% RSD	3.01	3.31	1.74	2.23	3.04
2013.01.1534	MEAN	10.34	15.80	1.54	1.44	29.12
2013.01.1534	SD	0.34	0.55	0.05	0.05	0.99
2013.01.1534	% RSD	3.32	3.46	3.48	3.36	3.40
12000068	MEAN	11.58	17.87	2.23	2.02	33.69
12000068	SD	0.23	0.40	0.06	0.05	0.74
12000068	% RSD	1.99	2.21	2.85	2.55	2.19
507/13/O	MEAN	7.62	10.55	0.66	0.63	19.46
507/13/O	SD	0.054	0.09	0.01	0.02	0.12
507/13/O	% RSD	0.71	0.82	2.13	3.76	0.59
506/13/O	MEAN	8.17	13.25	0.87	0.98	23.26
506/13/O	SD	0.14	0.28	0.01	0.01	0.43
506/13/O	% RSD	1.69	2.08	1.21	1.16	1.87
505/13/O	MEAN	8.58	14.08	2.16	2.53	27.35
505/13/O	SD	0.15	0.25	0.03	0.04	0.38
505/13/O	% RSD	1.73	1.78	1.29	1.53	1.39

**Table S 22. Frangulin Contents of the Analyzed Samples of *F. alnus* with UHPLC (*n* = 3)**

Lot		2	1	4	3	$\Sigma$ 1-4
		content (mg/g)				
11732	MEAN	7.53	12.47	1.15	1.14	22.30
11732	SD	0.08	0.13	0.01	0.01	0.20
11732	% RSD	1.08	1.00	0.53	0.56	0.90
1806602	MEAN	10.20	16.44	1.60	1.60	29.84
1806602	SD	0.06	0.07	0.01	0.01	0.14
1806602	% RSD	0.61	0.41	0.34	0.66	0.47
2008.04.0881	MEAN	7.62	12.20	0.71	0.70	21.22
2008.04.0881	SD	0.05	0.12	0.01	0.01	0.17
2008.04.0881	% RSD	0.63	0.99	1.37	1.70	0.81
18103247	MEAN	5.79	8.40	0.23	0.24	14.66
18103247	SD	0.14	0.21	0.00	0.00	0.35
18103247	% RSD	2.43	2.49	1.25	1.16	2.39
2293402	MEAN	7.13	11.92	0.78	0.74	20.56
2293402	SD	0.07	0.08	0.01	0.01	0.16
2293402	% RSD	0.96	0.65	1.50	0.69	0.76
2287202	MEAN	8.48	12.93	0.38	0.36	22.14
2287202	SD	0.02	0.10	0.00	0.00	0.12
2287202	% RSD	0.21	0.79	1.10	1.04	0.55
17730349	MEAN	10.95	18.63	1.66	1.80	33.03
17730349	SD	0.20	0.41	0.03	0.03	0.65
17730349	% RSD	1.81	2.19	1.69	1.89	1.98
11001253	MEAN	6.62	9.65	0.85	0.80	17.92
11001253	SD	0.20	0.30	0.02	0.02	0.53
11001253	% RSD	3.07	3.13	1.84	2.29	2.95
2013.01.1534	MEAN	10.62	15.02	1.53	1.31	28.48
2013.01.1534	SD	0.24	0.35	0.03	0.02	0.64
2013.01.1534	% RSD	2.22	2.34	1.94	1.78	2.24
12000068	MEAN	12.10	17.31	2.27	1.88	33.55
12000068	SD	0.25	0.40	0.07	0.05	0.77
12000068	% RSD	2.09	2.31	3.17	2.61	2.30
507/13/O	MEAN	7.83	10.17	0.66	0.57	19.23
507/13/O	SD	0.11	0.11	0.02	0.03	0.18
507/13/O	% RSD	1.34	1.08	2.20	4.37	0.95
506/13/O	MEAN	8.43	12.67	0.87	0.88	22.86
506/13/O	SD	0.16	0.31	0.01	0.01	0.49
506/13/O	% RSD	1.89	2.48	1.11	0.93	2.13
505/13/O	MEAN	8.85	13.42	2.21	2.32	26.79
505/13/O	SD	0.17	0.25	0.02	0.04	0.41
505/13/O	% RSD	1.88	1.85	1.01	1.69	1.54