Supplementary Information For

Structure characterization and quantification of isomeric bioactive flavonoid C-glycosides utilizing high-performance liquid chromatography coupled with diode array detection and mass spectrometry

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List of content

1. Methodology

1.1. Extraction and fractionation

Air-dried aerial parts of the *Cajanus scarabaeoides* (1 kg) were extracted 3 times with (1:1 v/v) (EtOH: H_2O) for 2 hours at 80°C (3x8L). The extract was filtered through a fresh cotton bed and finally with vacuum filtration. The filtrate was concentrated with a rotary evaporator at a low temperature (40°C) and reduced pressure to produce 157 g of concentrated Aqueous-ethanol extract (15.7%w/w). The Aqueous-ethanol extract (50 g) was further subjected to partitioning by suspending in a mixture of methanol-water (5: 200 mL) with sonication of 5 minutes and partitioned with ethyl acetate (7 x 300 mL). Add 2.5g anhydrous sodium sulfate

(Na_2SO_4) to the separated organic phase and filter through a cotton bed. Evaporation of the solvent from the organic phase afforded the 3.94 g of ethyl acetate residue (7.88%, w/w).

1.2. Isolation and purification

The HPLC analysis and purification of the dried ethyl acetate residue were performed using a Waters e2695 Alliance HPLC system, equipped with a 2998 PDA multi-wavelength detector and an automotive Waters Fraction Collector III. The system was fitted with a LiChroCART Purosphere® STAR RP-18e semi-preparative column (250 x 10 mm, 5 µm), which was operated at 35 °C. For the separation of targeted compounds, an isocratic separation method was developed with a flow rate of 4 mL/min. The mobile phases consisted of 5% acetonitrile in water as solvent A and acetonitrile as solvent B, mixed in a 1:9 ratio, and the run time was set for 80 minutes. For purification, the sample was prepared by dissolving 20 mg of dried ethyl acetate residue in 1 ml of (1:1 v/v) methanol-water. The multiple injections of 50 µL sample were injected into the semipreparative HPLC column, with a total loading of 1 mg per run through an Alliance autosampler operating at 20 °C. The compounds were purified using minimum intensity threshold (MIT) and UV absorbance at 350 nm. The FractionLynx parameters were set as follows; solvent front delay (400 sec), split/collector delay (68 sec), minimum fraction width (40 sec), maximum fraction width (200 sec), PDA span (2 nm), PDA Minimum Threshold *(*6000.00), PDA Maximum Threshold (300000000.00) and wavelength trigger was set at 350 nm. Data acquisition and processing were carried out using MassLynx V4.1 SCN 683 software. The simulated HPLC chromatogram of compounds are shown in **Figure S1.**

The collected peak fractions were evaporated to dryness under reduced pressure and low temperature (40 °C). Finally, the powder was obtained after freeze-drying and was subjected to further analysis.

1.3. HPLC/PDA analysis

The HPLC purity of the purified peak fractions was tested using the same HPLC/PDA system that was utilized for isolating the targeted compounds. Apart from this, the developed analytical conditions were used as a chromatographic condition for purity analysis (**manuscript section 2.4.2**). Each isolated compound was injected at a concentration of 100 ppm using a 10 µL injection volume. The compounds were analysed at wavelengths ranging from 190 to 450 nm, and the eluted compounds were monitored specifically at their highest UV absorbance (λ_{max}).

2. Figures

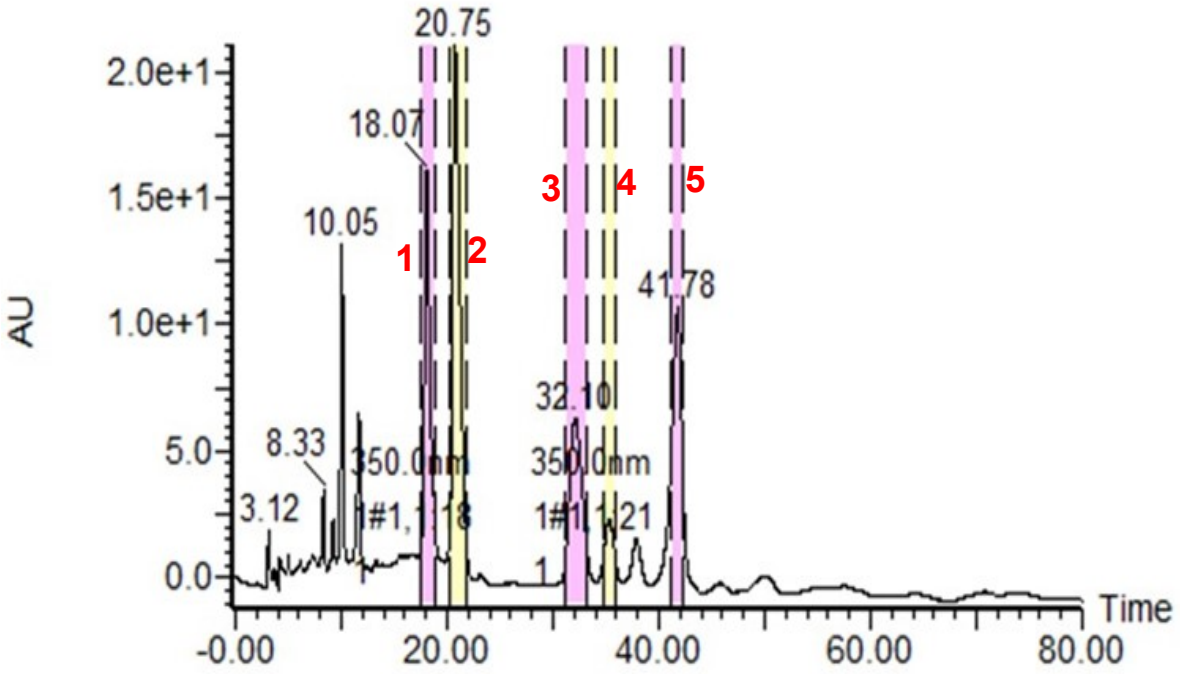

Figure S1; The simulated HPLC chromatogram of flavonoids on LiChroCART Purosphere® STAR RP-18e 250 x 10 mm, 5 µm semipreparative column.

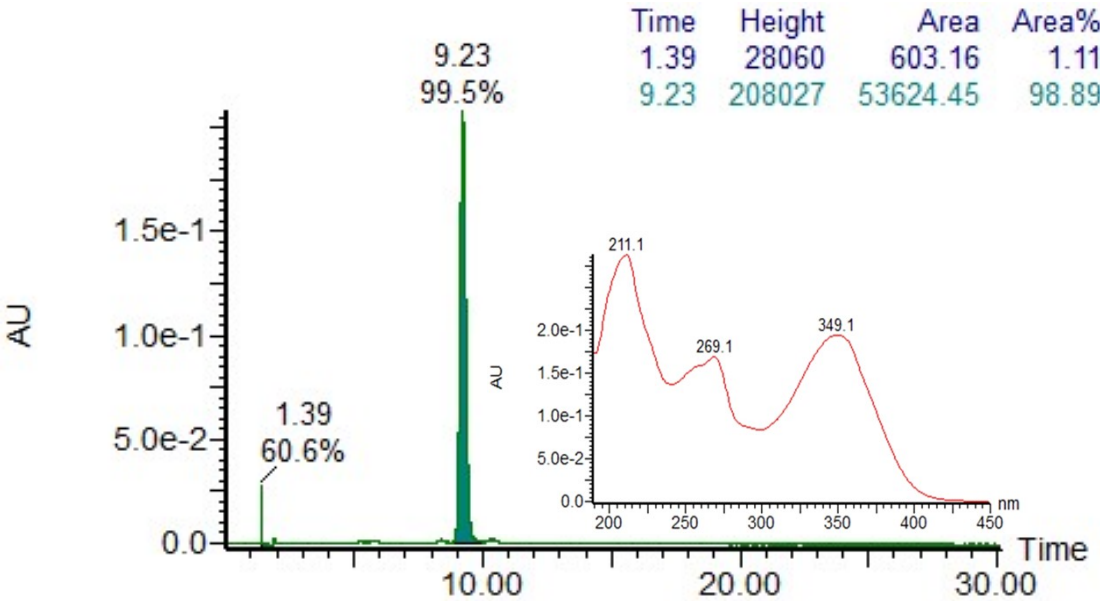

Figure S2; HPLC/PDA chromatogram of compound 1 (isoorientin) at 349 nm, showing λmax at 211,269,349 nm and peak purity 99.5%.

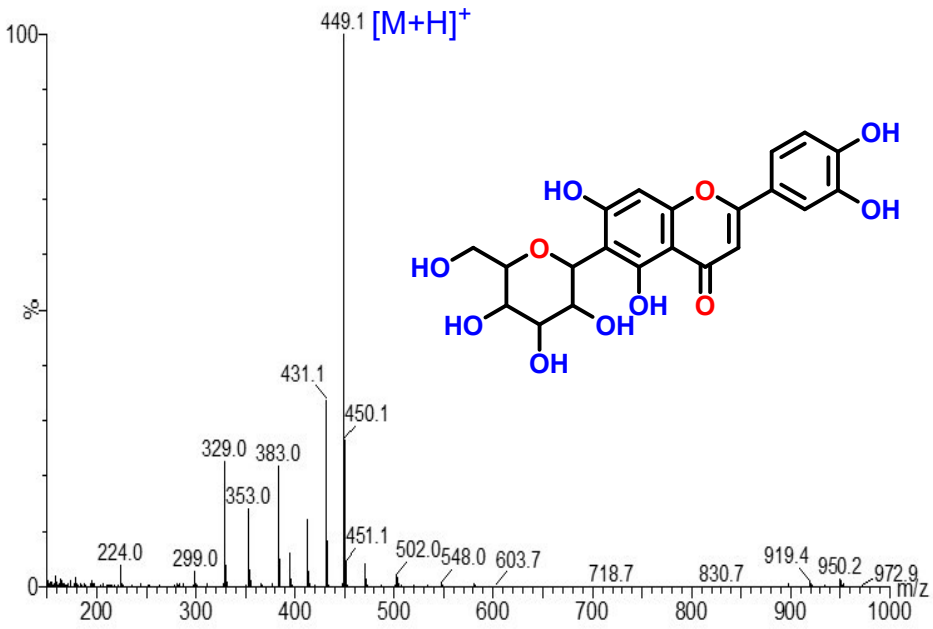

Figure S3; Full scan LC-ESI (+) MS spectra of compound 1 (isoorientin) with adduct ion of $[M+H]^+$ *m/z* 449 and $[2M+Na]^+$ *m/z* 919.

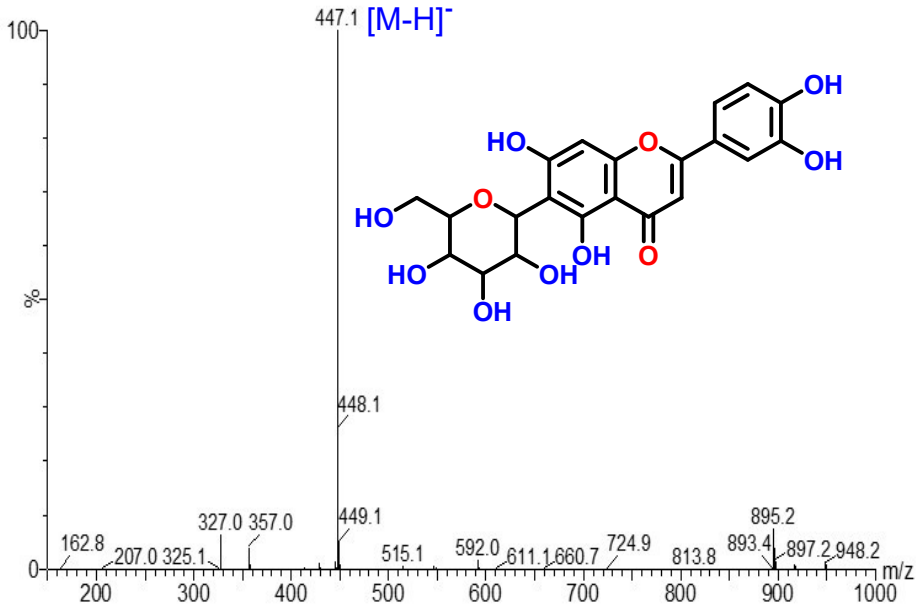

Figure S4; Full scan LC-ESI (-) MS spectra of compound 1 (isoorientin) with adduct ion of $[M-H]^-$ *m/z* 447 and $[2M-H]^-$ *m/z* 895.

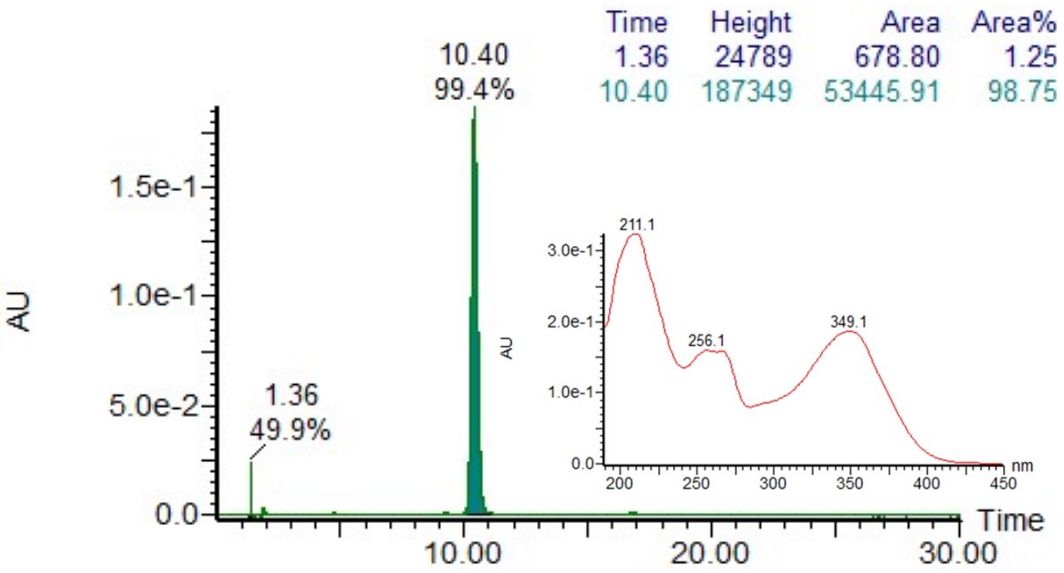

Figure S5; HPLC/PDA chromatogram of compound 2 (orientin) at 349 nm, showing λmax at 211,256,349 nm and peak purity 99.4%.

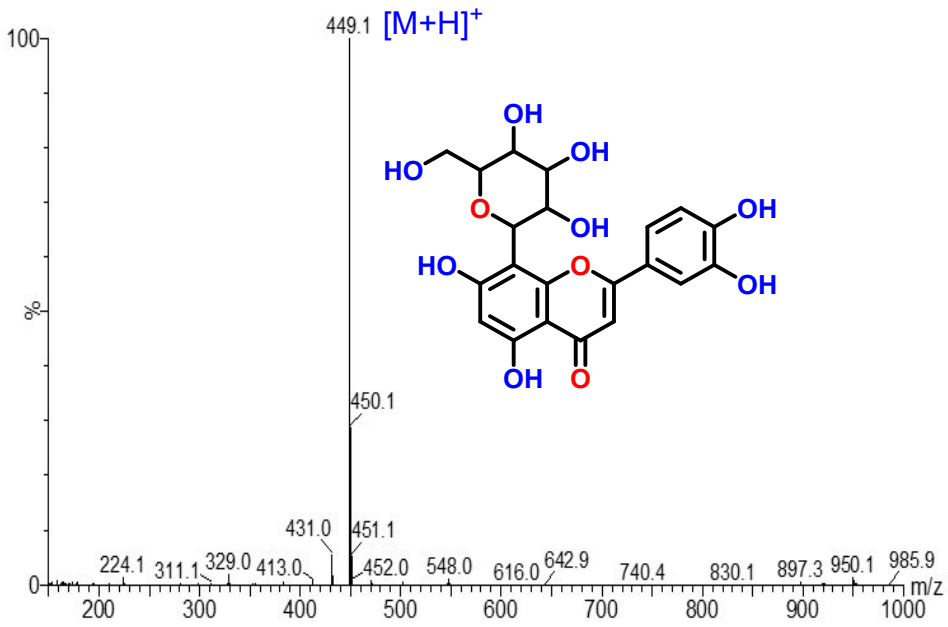

Figure S6; Full scan LC-ESI (+) MS spectra of compound 2 (orientin) with adduct ion of $[M+H]^+$ *m/z* 449 and $[2M+H]^+$ *m/z* 897.

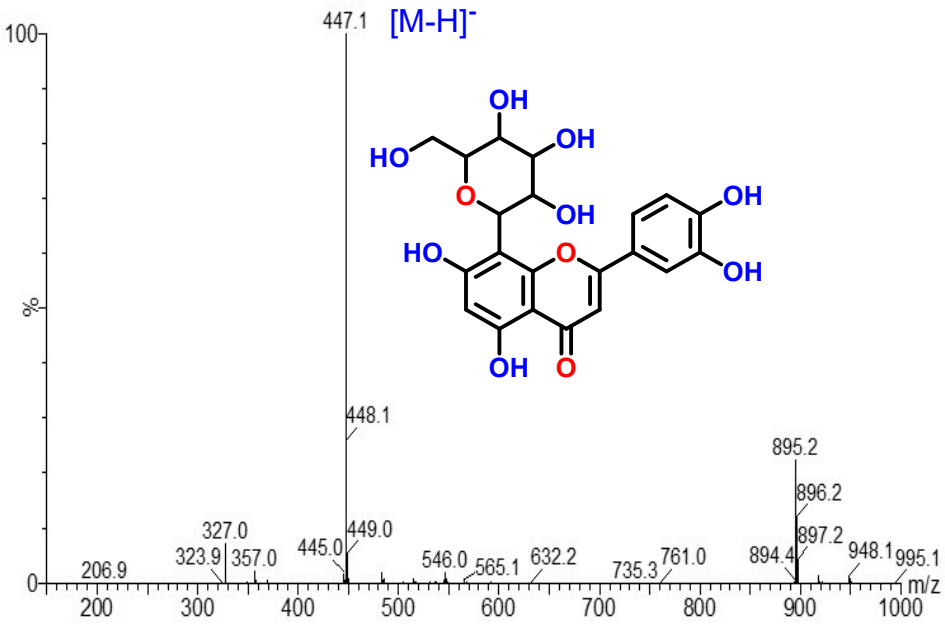

Figure S7; Full scan LC-ESI (-) MS spectra of compound 2 (orientin) with adduct ion of $[M-H]^-$ *m/z* 447 and $[2M-H]^-$ *m/z* 895.

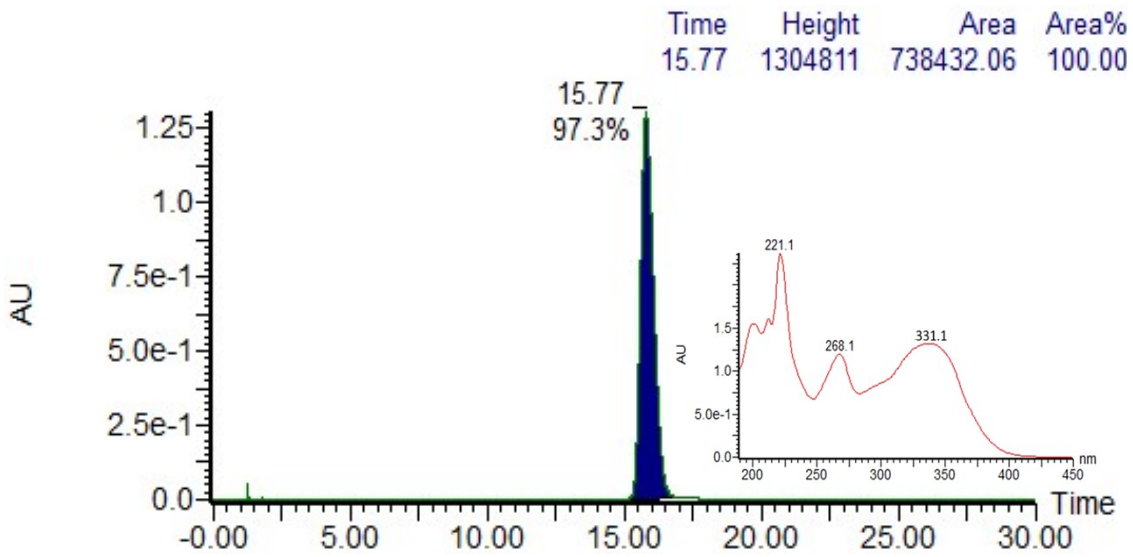

Figure S8; HPLC/PDA chromatogram of compound 3 (vitexin) at 331 nm, showing λmax at 221,268,331 nm and peak purity 97.3%.

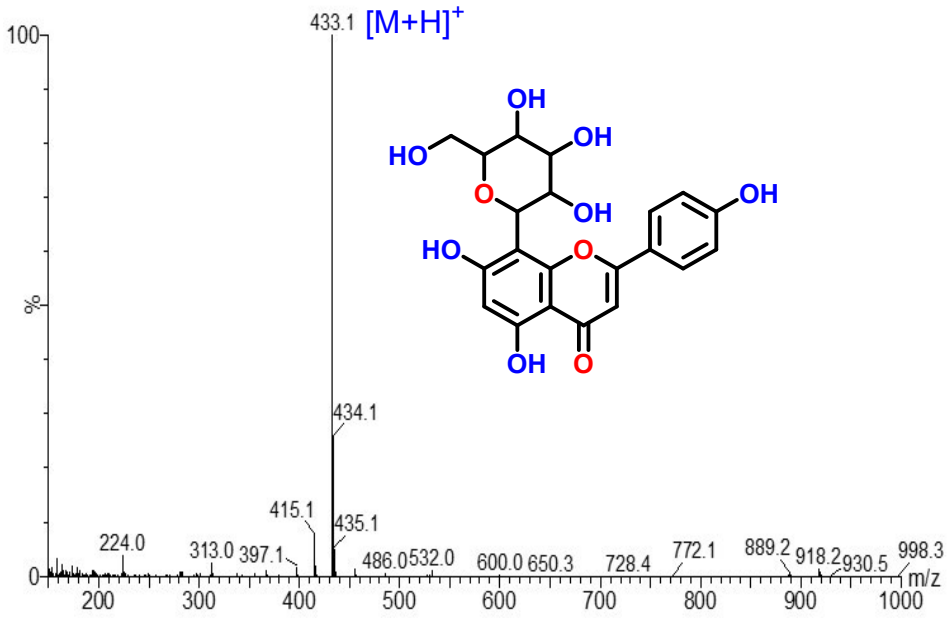

Figure S9; Full scan LC-ESI (+) MS spectra of compound 3 (vitexin) with adduct ion of $[M+H]^+$ *m/z* 433.

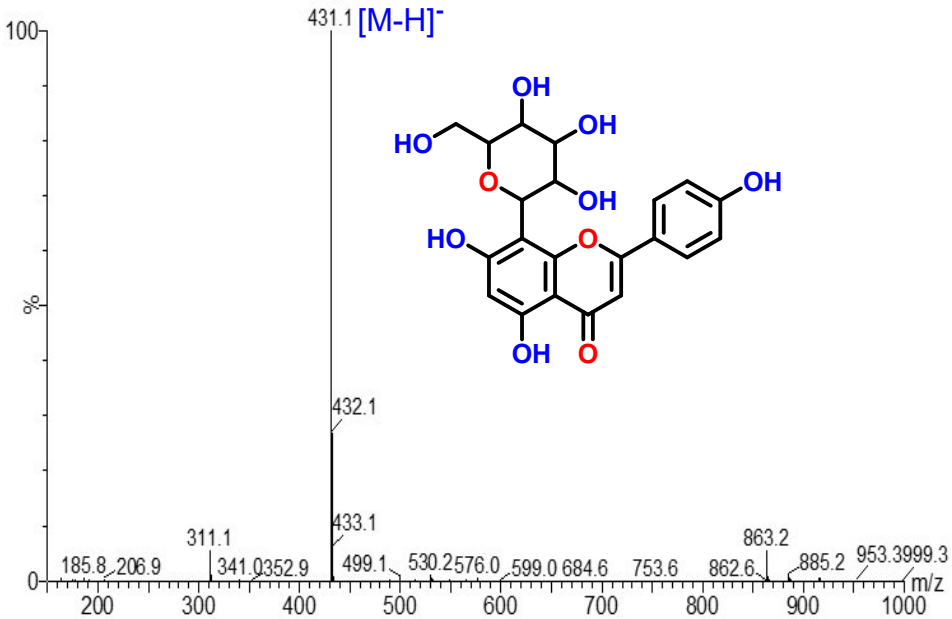

Figure S10; Full scan LC-ESI (-) MS spectra of compound 3 (vitexin) with adduct ion of $[M-H]^-$ *m/z* 431 and $[2M-H]^-$ *m/z* 863.

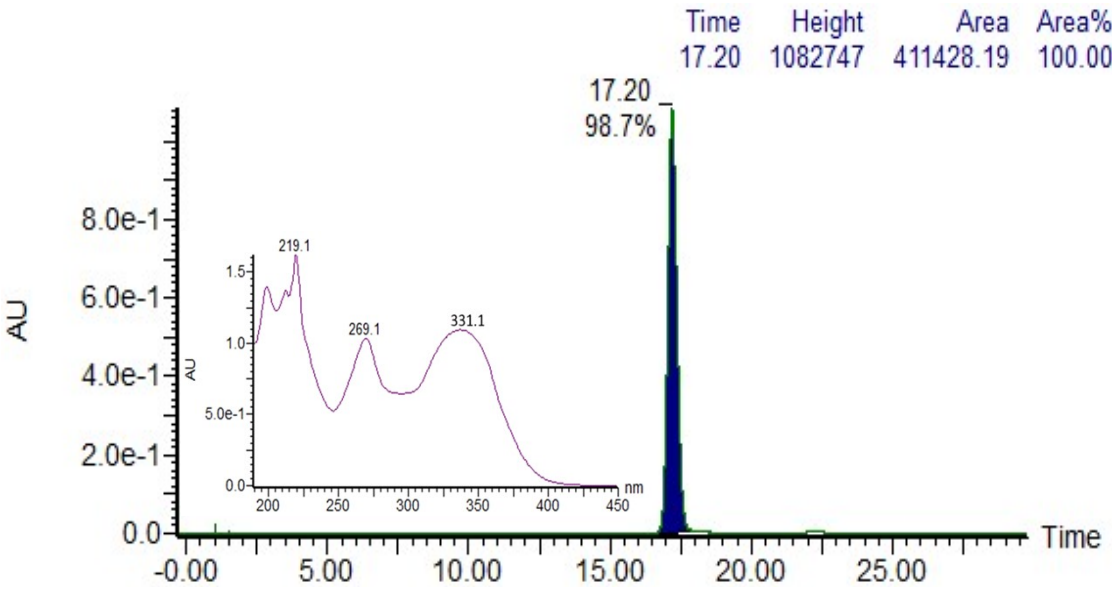

Figure S11; HPLC/PDA chromatogram of compound 4 (isovitexinn) at 331 nm, showing λmax at 219,269,331 nm and peak purity 98.7%.

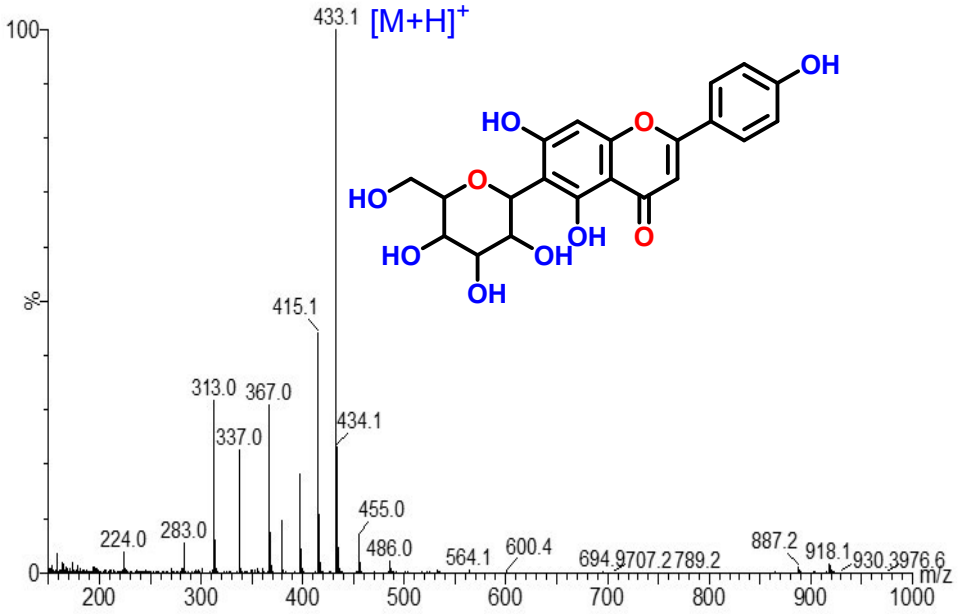

Figure S12; Full scan LC-ESI (+) MS spectra of compound 4 (isovitexin) with adduct ion of $[M+H]^+$ *m/z* 433, $[M+Na]^+$ *m/z* 455 and $[2M+Na]^+$ *m/z* 887.

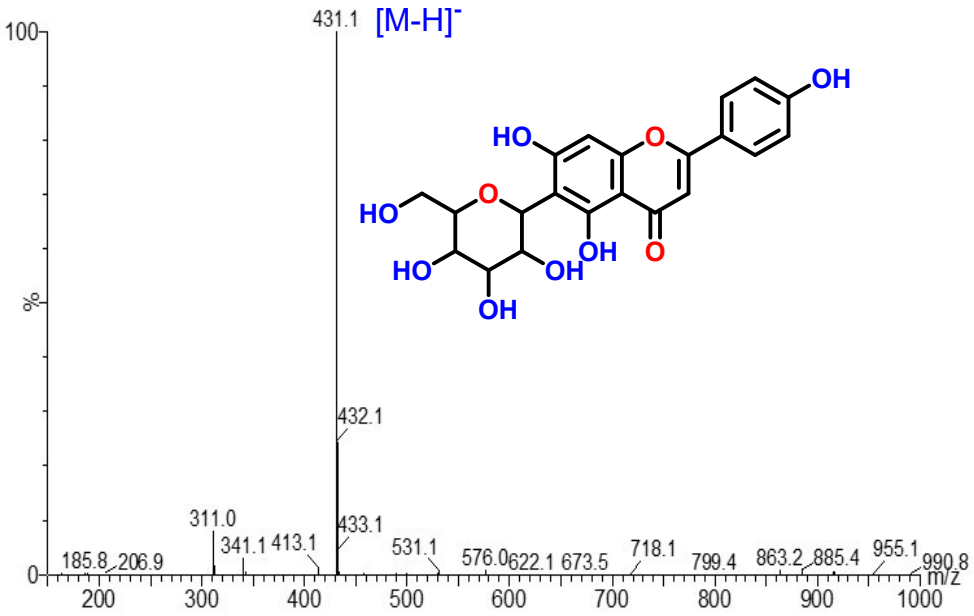

Figure S13; Full scan LC-ESI (-) MS spectra of compound 4 (isovitexin) with adduct ion of $[M-H]^-$ *m/z* 431 and $[2M-H]^-$ *m/z* 863.

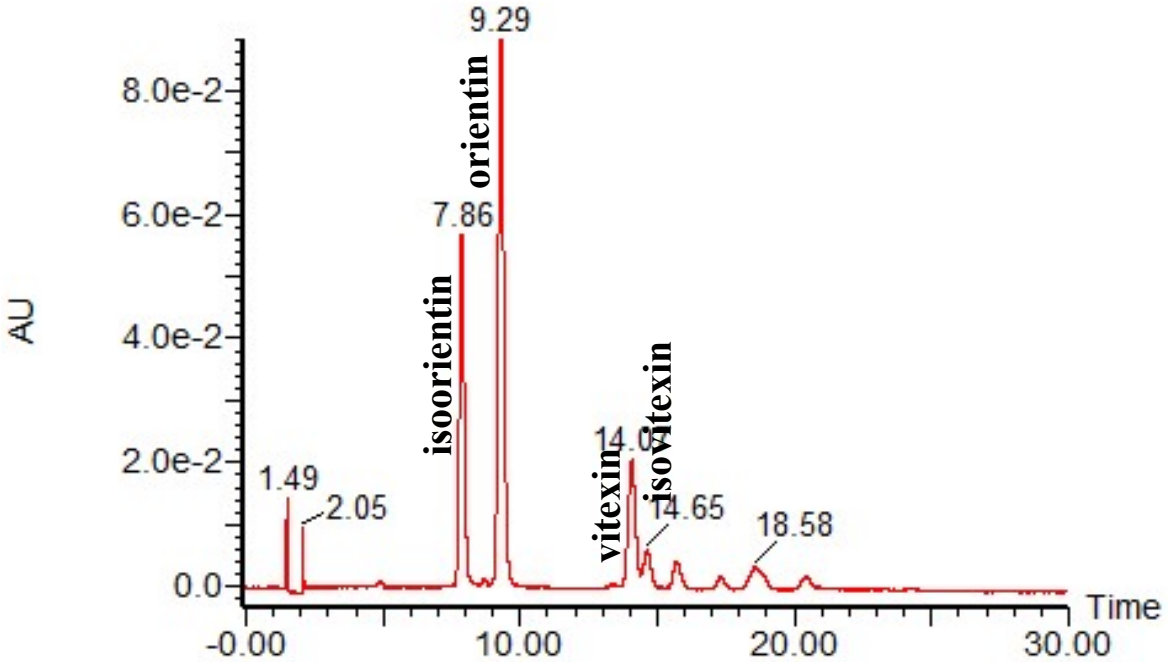

Figure S14; The peaks of targeted analytes in preliminary method development trials with acetonitrile and 0.1% formic acid aqueous buffer (prepared in 5% acetonitrile-water) in a ratio of 10:90 %v/v with a flow rate of 1.0 ml/min.

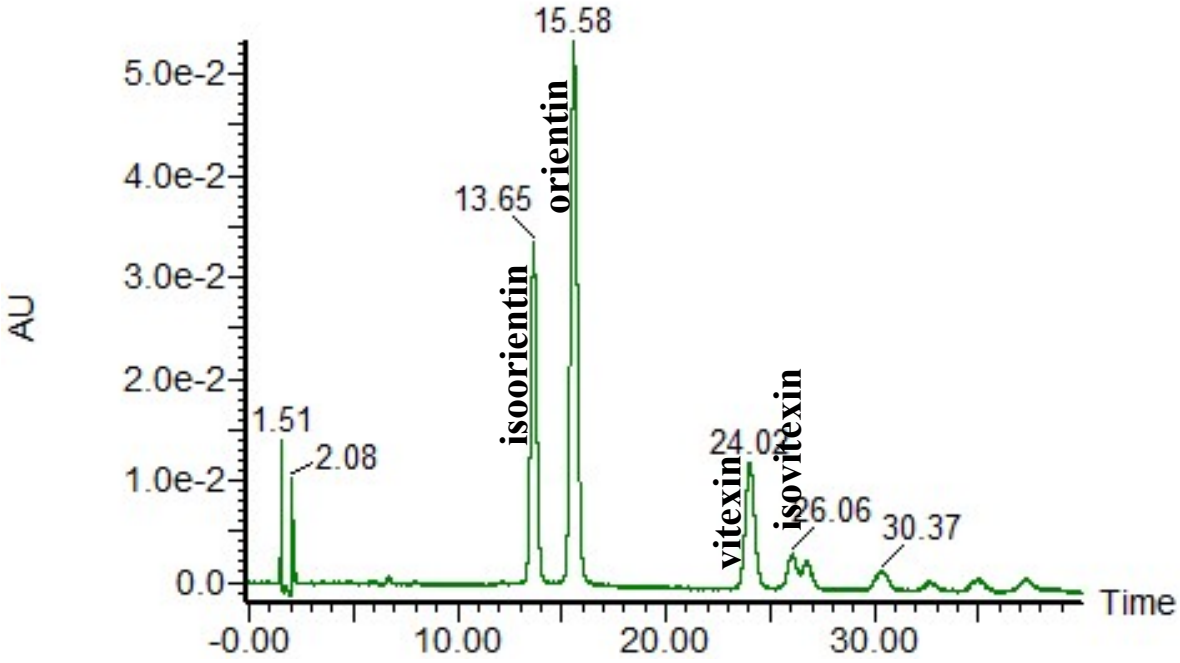

Figure S15; The peaks of targeted analytes in preliminary method development trials with acetonitrile and 0.1% formic acid aqueous buffer (prepared in 5% acetonitrile-water) in a ratio of 8:92 %v/v with a flow rate of 1.0 ml/min.

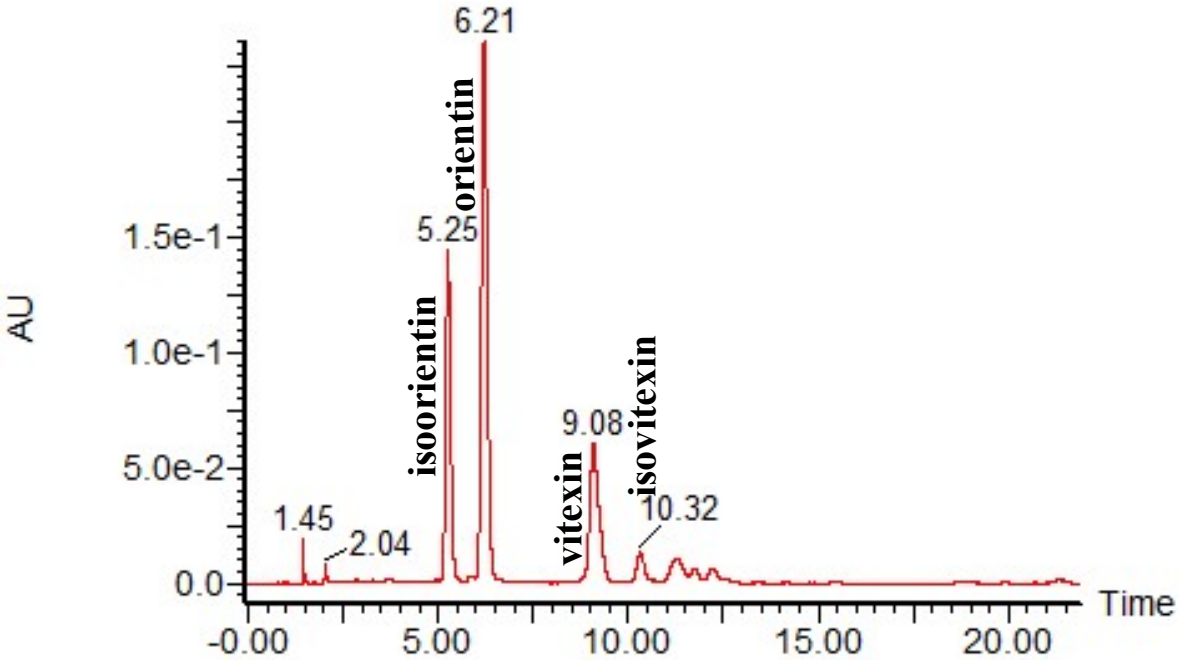

Figure S16; The peaks of targeted analytes in preliminary method development trials with acetonitrile and 0.1% formic acid aqueous buffer (prepared in 5% methanol-water) in a ratio of 15:85 %v/v with a flow rate of 1.0 ml/min.

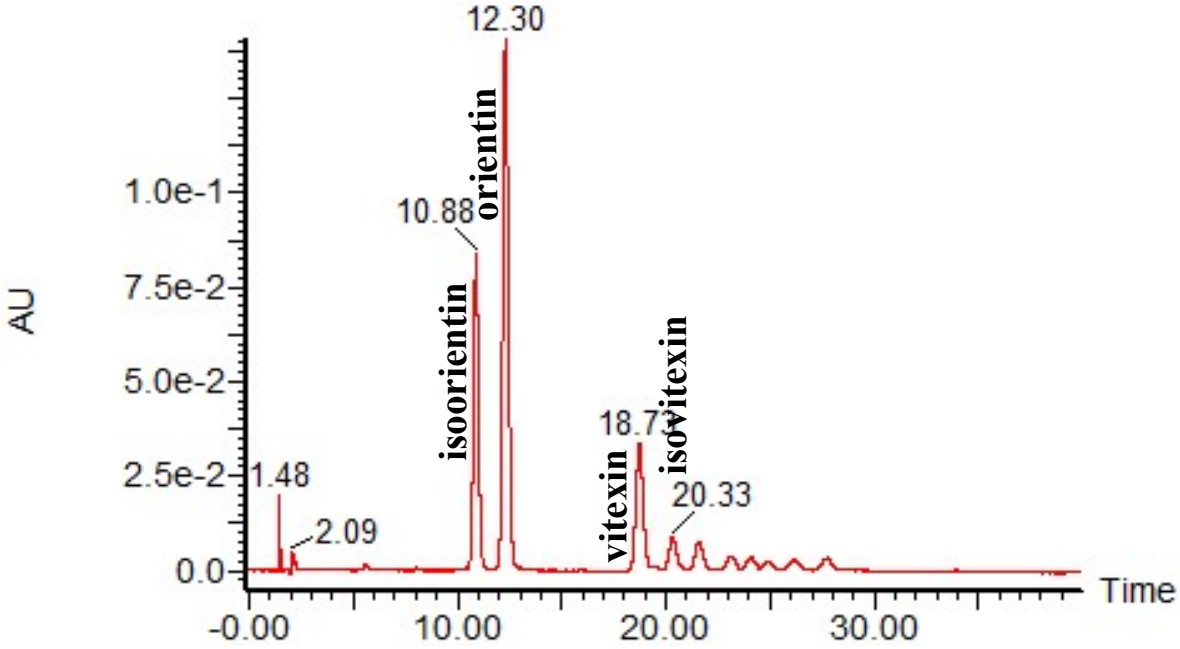

Figure S17; The peaks of targeted analytes in preliminary method development trials with acetonitrile and 0.1% formic acid aqueous buffer (prepared in 5% methanol-water) in a ratio of 12:88 %v/v with a flow rate of 1.0 ml/min.

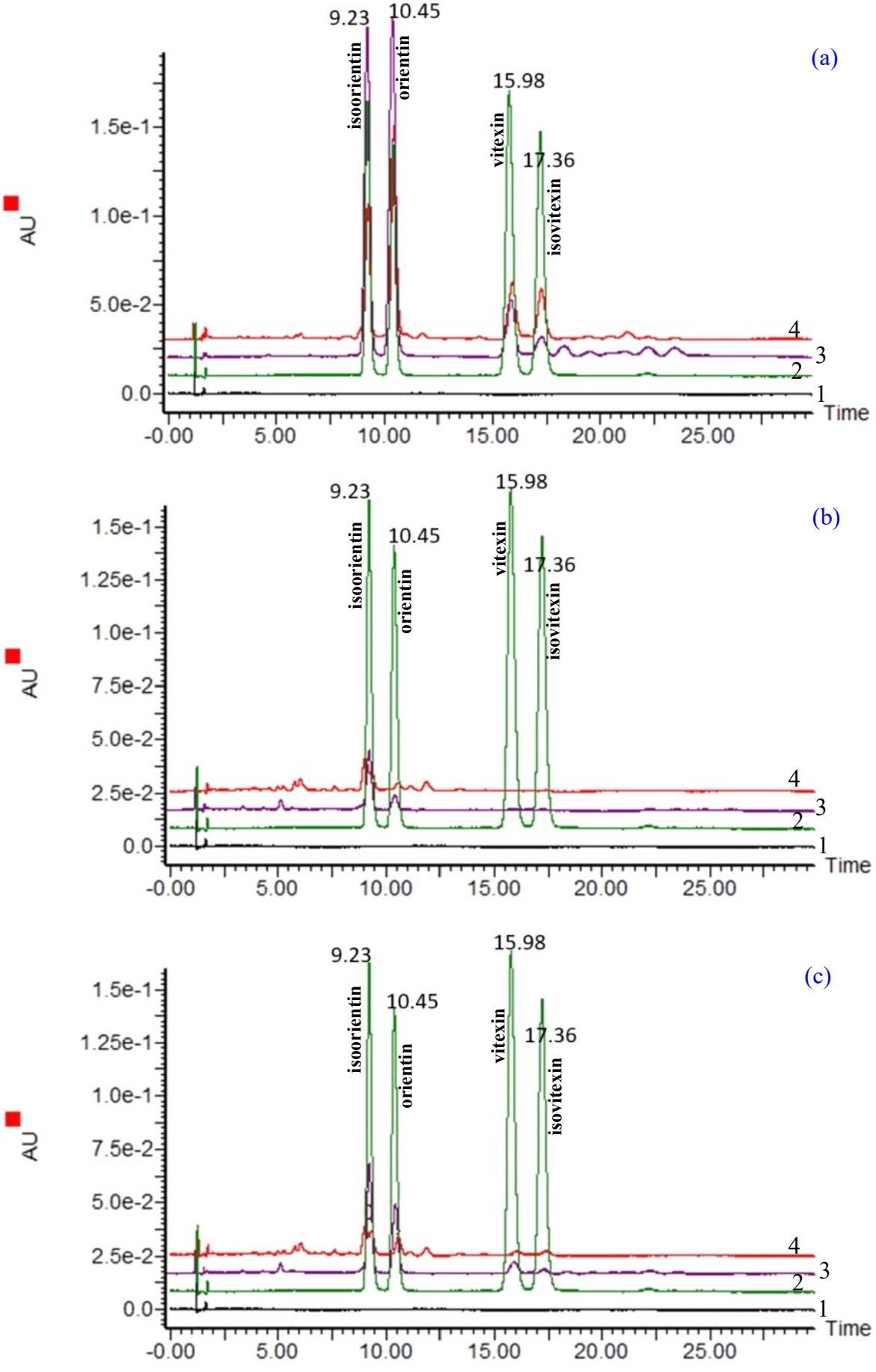
(a)
9.23
10.45
15.98
17.36
isoorientin
orientin
vitexin
isovitexin
AU
1.5e-1
1.0e-1
5.0e-2
0.0
-0.00
5.00
10.00
15.00
20.00
25.00
Time
4
3
2
1
(b)
9.23
10.45
15.98
17.36
isoorientin
orientin
vitexin
isovitexin
AU
1.5e-1
1.25e-1
1.0e-1
7.5e-2
5.0e-2
2.5e-2
0.0
-0.00
5.00
10.00
15.00
20.00
25.00
Time
4
3
2
1
(c)
9.23
10.45
15.98
17.36
isoorientin
orientin
vitexin
isovitexin
AU
1.5e-1
1.25e-1
1.0e-1
7.5e-2
5.0e-2
2.5e-2
0.0
-0.00
5.00
10.00
15.00
20.00
25.00
Time
4
3
2
1

Figure. S18; Overlay chromatograms showing specificity at 349 nm: a-1 blank, a-2 working standard solution, a-3 ethyl acetate fraction (matrix) of CS, a-4 ethyl acetate fraction (matrix) of RM. b-1 blank, b-2 working standard solution, b-3 water fraction (matrix) of CS, b-4 water fraction (matrix) of RM: c-1 blank, c-2 working standard solution, c-3 aq-ethanolic extract (matrix) of CS, a-4 aq-ethanolic extract (matrix) of RM.

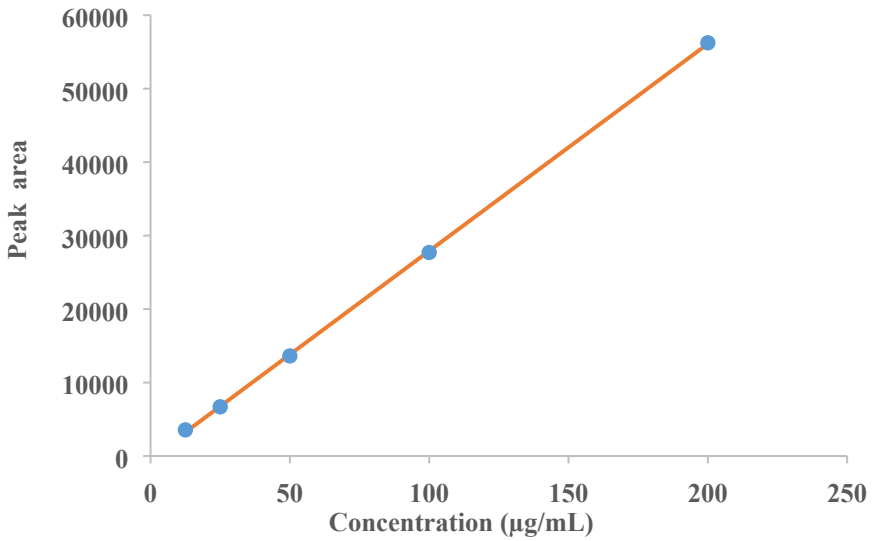

Figure S19; Calibration curve for isoorientin**.**

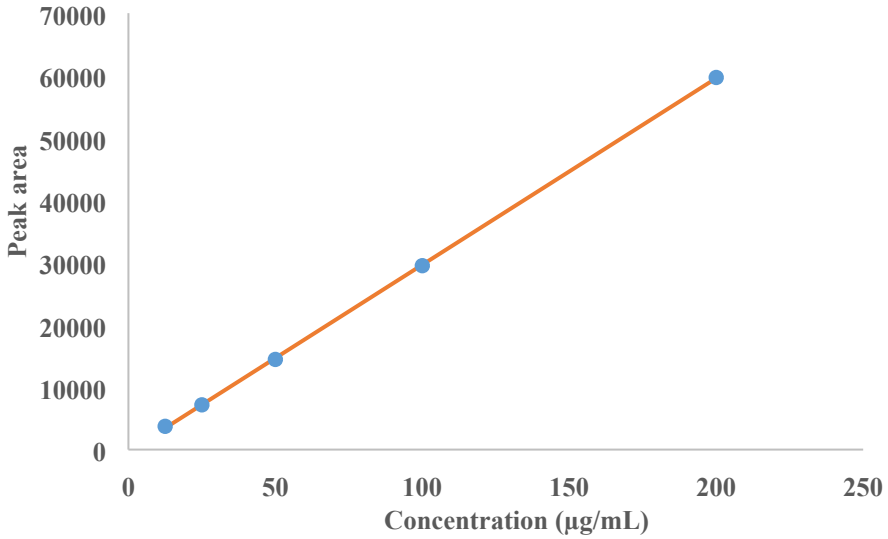

Figure S20; Calibration curve for orientin**.**

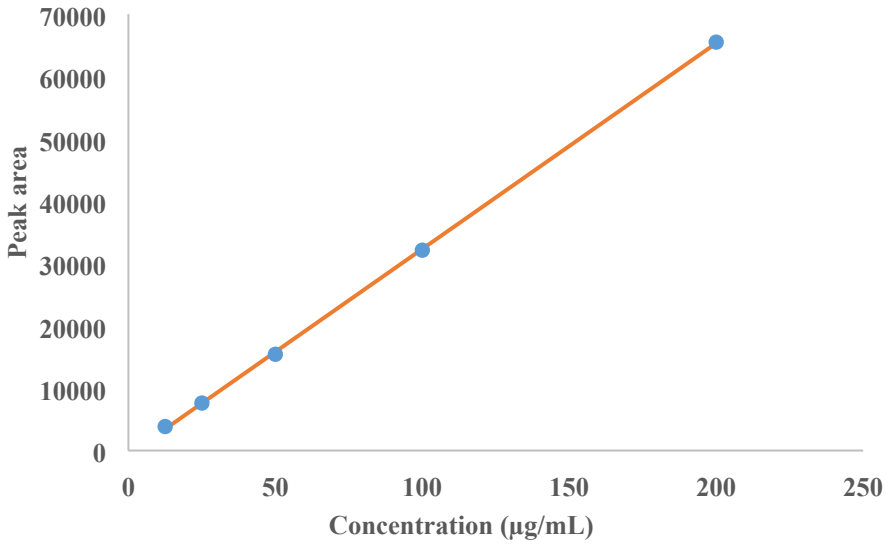

Figure S21; Calibration curve for vitexin**.**

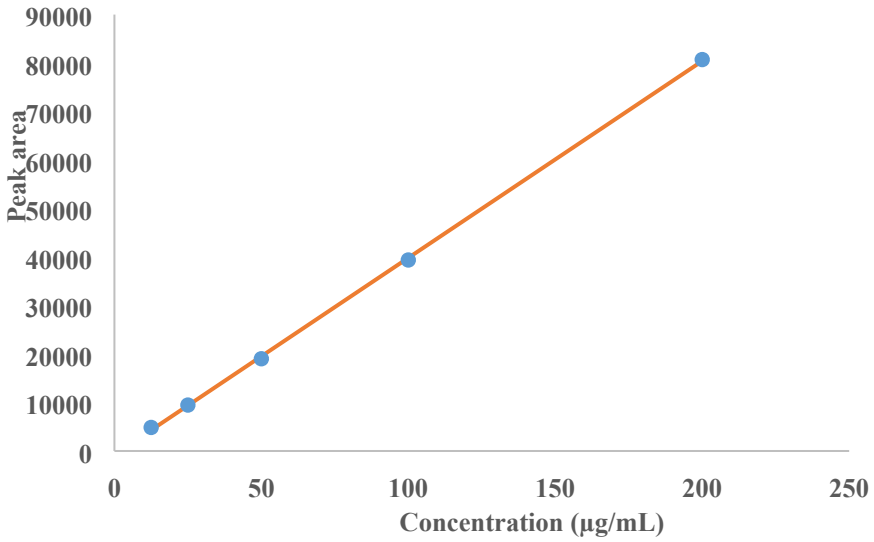

Figure S22; Calibration curve for isovitexin**.**

3. Tables

Table S1. Compared ESI (+) MS/MS data for compounds 1 and 2 with reference TMS database

Standard isoorientin MS/MS at CE 20eV m/z (relative intensity%)	Compound **1** MS/MS at CE 18ev m/z (relative intensity%)	Standard orientin MS/MS at CE 20eV m/z (relative intensity%)	Compound **2** MS/MS at CE 18eV m/z (relative intensity%)
85(2.42)	85(1.0)	85(2.1)	-
287(4.2)	287(5.68)	217(4.48)	217(3.35)
299(88.67)	**299**(100.0)	243(5.39)	243(4.02)
300(8.67)	-	259(2.78)	259(2.56)
311(4.4)	311(3.52)	261(2.35)	-
325(2.23)	325(3.5)	299(31.85)	299(33.78)
327(2.23)	-	300(5.44)	-
-	328(2.63)	311(7.61)	311(7)
329(100)	329(85.27)	323(2.15)	-
330(12.68)	-	-	327(2.2)
339(7.41)	339(9.46)	328(3.99)	328(2.59)
-	341(1.04)	**329**(100)	329(89.71)
-	349(0.83)	330(10.39)	-
-	352(1.15)	339(8.4)	339(11.12)
353(91.28)	353(59.61)	353(28.97)	353(21.9)
354(8.19)	-	359(12.71)	359(8.73)
365(11.91)	365(11.26)	360(3.96)	-
367(5.55)	367(2.71)	367(8.45)	367(6.53)
377(8.92)	377(9.53)	-	371(2.94)
378(2.47)	-	383(19.95)	383(19.53)
383(42.2)	383(27.53)	395(14.31)	395(12.02)
384(3.73)	-	412(2.86)	-
395(37.16)	395(19.71)	413(47.39)	413(42.87)
396(4.75)	-	414(2.85)	-
413(38.52)	413(24.4)	431(18.34)	431(36.52)
414(2.99)	-	-	448(3.84)
431(10.97)	431(12.41)	449(15.64)	**449**(100)
432(2.06)	-	450(3.41)	450(4.03)
449(10.91)	449(5.86)		

Table S2. Compared ESI (+) MS/MS data for compound **3** and **4** with reference TMS database

Standard vitexin MS/MS at CE 20eV m/z (relative intensity%)	Compound **3** MS/MS at CE 18ev m/z (relative intensity%)	Standard isovitexin MS/MS at CE 20eV m/z (relative intensity%)	Compound **4** MS/MS at CE 18eV m/z (relative intensity%)
121(7.46)	-	85(2.1)	-
217(4.52)	217(2.27)	103(2.98)	-
243(6.47)	243(2.7)	109(1.27)	-
259(2.29)	-	271(5.28)	271(7.73)
271(5.25)	-	-	282(1.56)
283(77.95)	283(38.44)	**283**(100)	**283**(100)
284(22.39)	-	284(9.88)	-
295(11.95)	295(7.82)	295(3.54)	295(3.61)
297(14.68)	297(2.92)	-	297(1.37)
309(2.8)	-	309(9.29)	309(3.99)
311(3.67)	311(2.86)	-	311(2.31)
-	312(2.41)	-	312(1.37)
313(100)	313(99.93))	313((71.02)	313(84.95)
314(9.63)	-	314(6.87)	314(6.87)
323(23.63)	323(8.47)	323(14.44)	323(9.15)
324(3.43)	-	324(1.63)	-
325(5.76)	325(2.05)	333(2.28)	333(1.83)
327(2.57)	-	337(43.86)	337(55.76)
337(20.69)	337(26.82)	338(5.48)	-
343(7.78)	343(9.87)	349(14.12)	349(9.99)
351(7.59)	351(8.02)	350(1.92)	-
355(2.35)	355(2.26)	351(1.62)	351(4.03)
367(8.67)	367(24.79)	361(13.83)	361(14.3)
379(9.23)	379(13.74)	362(2.09)	-
397(14.56)	397(44.52)	367(6.17)	367(25.89)
415(3.31)	415(42.82)	379(14.57)	379(26.43)
-	432(4)	380(2.35)	-
-	**433**(100)	397(8.65)	397(19.1)
		398(1.2)	-
		-	415(7.29)
		-	433(6.59)

TableS3. System suitability test parameters.

Analyte	**Parameters**		**%RSD**
Isoorientin	Retention time (min)	9.23 ± 0.07	0.72
	Peak area (mAU)	39119 ± 86	0.22
	Tailing factor	0.18 ± 0.02	12.83

	Theoretical plates	8512 ± 13	0.15
Orientin	Retention time (min)	10.46 ± 0.09	0.81
	Peak area (mAU)	37127 ± 44	0.12
	Tailing factor	0.14 ± 0.01	12.37
	Theoretical plates	8494 ± 5	0.0006
Vitexin	Retention time (min)	16.00 ± 0.13	0.80
	Peak area (mAU)	62195 ± 33	0.53
	Tailing factor	0.32 ± 0.04	12.44
	Theoretical plates	11204 ± 9	0.08
Isovitexin	Retention time (min)	17.35 ± 0.10	0.57
	Peak area (mAU)	56113 ± 80	0.14
	Tailing factor	0.37 ± 0.01	3.12
	Theoretical plates	12073 ± 2	0.02

Table S4. Results of the precision and accuracy (n=3).

	Isoorientin (ug/mL)			**Orientin (ug/mL)**			**Vitexin (ug/mL)**			**Isovitexin (ug/mL)**		
Intraday	**LQC**	**MQC**	**HQC**	**LQC**	**MQC**	**HQC**	**LQC**	**MQC**	**HQC**	**LQC**	**MQC**	**HQC**
Day-1	12.5	50	200	12.5	50	200	12.5	50	200	12.5	50	200
Mean ±SD	13.98 ± 0.04	47.76 ± 0.01	199.87 ± 1.12	13.84 ± 0.02	47.91 ± 0.18	200.06 ± 1.15	14.41 ± 0.18	47.65 ± 0.02	200.25 ± 0.83	14.46 ± 0.09	47.99 ± 0.07	200.83 ± 0.28
Precision	0.27	0.01	0.56	0.16	0.39	0.58	1.25	0.04	0.41	0.65	0.15	0.14
Accuracy	111.90	95.52	99.94	110.75	95.81	100.03	115.22	95.31	100.12	115.72	95.99	100.41
Day-2	12.5	50	200	12.5	50	200	12.5	50	200	12.5	50	200
Mean ± SD	14.01 ± 0.09	50.50 ± 0.52	200.90 ± 0.82	13.85 ± 0.15	50.43 ± 0.49	200.92 ± 2.12	14.13 ± 0.10	49.34 ± 0.57	201.09 ± 1.62	14.69 ± 0.17	49.15 ± 0.46	201.43 ± 1.53
Precision	0.61	1.04	0.41	1.12	0.98	1.05	0.69	1.16	0.80	1.16	0.94	0.76
Accuracy	112.11	101.01	100.45	110.84	100.87	100.46	113.00	98.68	100.55	117.50	98.31	100.71
Day-3	12.5	50	200	12.5	50	200	12.5	50	200	12.5	50	200
Mean ± SD	12.81 ± 0.06	49.62 ± 0.23	200.65 ± 0.46	12.41 ± 0.76	49.34 ± 0.19	200.08 ± 1.98	12.40 ± 0.04	49.44 ± 0.28	200.10 ± 0.06	12.13 ± 0.05	49.12 ± 0.68	199.77 ± 1.32
Precision	0.47	0.45	0.23	1.40	0.38	0.99	0.36	0.57	0.03	0.44	1.39	0.66
Accuracy	102.44	99.24	100.32	99.27	98.69	100.04	99.18	98.87	100.05	97.05	98.24	99.88
Interday	12.5	50	200	12.5	50	200	12.5	50	200	12.5	50	200
Mean ± SD	13.60 ± 0.06	49.29 ± 0.25	200.48 ± 0.80	13.37 ± 0.12	49.23 ± 0.29	200.35 ± 1.75	13.64 ± 0.11	48.81 ± 0.29	200.48 ± 0.84	13.76 ± 0.11	48.76 ± 0.40	200.67 ± 1.04
Precision	0.45	0.50	0.40	0.89	0.58	0.87	0.77	0.59	0.41	0.75	0.83	0.52
Accuracy	108.82	98.59	100.24	106.95	98.46	100.18	109.13	97.62	100.24	110.09	97.51	100.33

Table S5. Stability of analytes at 20 °C, (n=3).

Table S5. Stability of analytes at 20 °C, (n=3).												
Time	**Isoorientin (ug/mL)**			**Orientin (ug/mL)**			**Vitexin (ug/mL)**			**Isovitexin (ug/mL)**		
	LQC	**MQC**	**HQC**	**LQC**	**MQC**	**HQC**	**LQC**	**MQC**	**HQC**	**LQC**	**MQC**	**HQC**
0.0 h	12.5	50	200	12.5	50	200	12.5	50	200	12.5	50	200
Mean	13.98 ± 0.04	47.76 ± 0.01	199.87 ± 1.12	13.84 ± 0.02	47.91 ± 0.18	200.06 ± 1.15	14.41 ± 0.18	47.65 ± 0.02	200.25 ± 0.83	14.46 ± 0.09	47.99 ± 0.07	200.83 ± 0.28
%RSD	0.27	0.01	0.56	0.16	0.39	0.58	1.25	0.04	0.41	0.65	0.15	0.14
24.0 h	12.5	50	200	12.5	50	200	12.5	50	200	12.5	50	200
Mean	14.01 ± 0.09	50.50 ± 0.52	200.90 ± 0.82	13.85 ± 0.15	50.43 ± 0.49	200.92 ± 2.12	14.13 ± 0.10	49.34 ± 0.57	201.09 ± 1.62	14.69 ± 0.17	49.15 ± 0.46	201.43 ± 1.53
%RSD	0.61	1.04	0.41	1.12	0.98	1.05	0.69	1.16	0.80	1.16	0.94	0.76

Table S6. Recovery of analytes.

Analyte	Spiked level (µg/mL)	Recovery[a] (%)				
		1	**2**	**3**	**Mean (%) ±SD**	**RSD (%)**
Isoorientin	20	112.83	110.96	112.54	112.11 ± 1.00	0.89
	40	98.36	98.29	100.38	99.01 ± 1.19	1.20
	60	99.20	98.20	98.14	98.51 ± 0.60	0.61
Orientin	20	116.03	116.93	117.84	116.93 ± 0.91	0.78
	40	95.79	95.62	97.91	96.43 ± 1.27	1.32
	60	95.09	94.62	93.24	94.32 ± 0.96	1.02
Vitexin	20	106.22	105.80	106.48	106.17 ± 0.34	0.32
	40	103.06	102.07	105.14	103.4 2 ± 1.57	1.51
	60	102.25	101.91	100.80	101.66 ± 0.76	0.75
Isovitexin	20	99.94	98.47	100.52	99.64 ± 1.06	1.06
	40	103.03	103.85	104.62	103.84 ± 0.79	0.76
	60	98.57	98.86	96.85	98.10 ± 1.09	1.11

[a]The values are mean values calculated by carrying the triplicate analysis.

Table S7. Robustness of the analytical method.

Parameter	Theoretical Plates				Tailing Factor				Capacity Factor			
Flow rate (mL/min)	**Iso-orientin**	**Orientin**	**Vitexin**	**Iso-vitexin**	**Iso-orientin**	**Orientin**	**Vitexin**	**Iso-vitexin**	**Iso-orientin**	**Orientin**	**Vitexin**	**Iso-vitexin**
1	7729	7638	9853	11204	1.26	1.05	0.99	1.04	5.92	7.61	14.10	15.60
1.2	8486	8526	10386	11628	1.30	1.04	0.98	1.07	5.71	7.28	14.70	16.00
1.4	7704	7567	9061	10433	1.39	1.27	1.02	1.12	6.07	7.61	18.50	19.62
Mobile Phase (A: B)												
87:13	7540	7367	9714	110920	1.52	1.55	1.00	1.17	4.29	5.64	11.41	12.16
88:12	8486	8526	10386	11628	1.30	1.04	0.98	1.07	5.71	7.28	14.70	16.00
89:11	9739	9359	11433	13277	1.34	1.28	1.03	1.00	8.00	8.90	19.37	21.75
pH												
3.3	7303	7203	9023	10114	1.24	1.30	1.00	1.09	4.58	5.23	11.83	13.00
3.72	8177	7951	9751	11056	1.28	1.27	1.02	1.07	5.46	6.26	15.00	16.50
4.52	7659	7563	9261	10479	1.40	1.29	1.01	1.03	5.71	7.35	14.70	16.2
Column temperature (°C)												
30	7919	7607	9091	10616	1.37	1.04	1.00	1.04	6.25	7.48	17.47	19.23
35	8486	8526	10386	11628	1.30	1.04	0.98	1.07	5.71	7.28	14.70	16.00
40	7314	7343	9216	10325	1.39	1.39	1.05	1.17	4.80	5.60	12.70	14.00
Injection Volume (µ)												
5	9575	9187	10191	11571	1.52	1.29	1.01	1.05	5.46	6.93	15.00	16.40

10	8486	8526	10386	11628	1.30	1.04	0.98	1.07	5.71	7.28	14.70	16.00
15	4445	4632	7163	8159	1.22	1.22	1.04	0.99	5.78	7.35	14.70	16.10
Wavelength (nm)												
λ_{max} = 346 & 328 nm	8598	8398	10529	12259	1.29	1.22	1.00	1.01	5.64	6.54	14.50	16.00
λ_{max} = 349 & 331 nm	8486	8526	10386	11628	1.30	1.04	0.98	1.07	5.71	7.28	14.70	16.00
λ_{max} = 352 & 334 nm	8113	7875	9990	11552	1.47	1.36	1.18	1.21	5.60	7.20	15.10	16.55