SUPPLEMENTARY MATERIAL

Detection of flavonoids from *Spinacia oleracea* leaves using HPLC-ESI-QTOF-MS/MS and UPLC-QqQ$_{LIT}$-MS/MS techniques

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Abstract: *Spinacia oleracea* L. (Spinach) is a leafy vegetable which is considered to have a high nutritional value. Flavonoids in spinach were reported to act as antimutagenic property. Rapid detection of these flavonoids in Spinach was achieved by using HPLC-ESI-QTOF-MS/MS. Thirty six compounds were tentatively identified based on their retention times, accurate mass and MS/MS spectra. The fragmentation patterns of known compounds were applied to elucidate the structure of their corresponding derivatives having the same basic skeleton. Out of thirty six peaks, three peaks were assigned as patuletin and six peaks were assigned as spinacetin derivatives. Twelve compounds were first time identified following the fragmentation pattern of known compounds. Five of the identified compounds i.e., spinacetin, 5,3',4'-trihydroxy-3-methoxy-6,7-methylenedioxyflavone, protocatechuic acid, ferulic acid and coumaric acid were simultaneously quantified in spinach leaves by a validated UPLC-ESI-MS/MS method under MRM mode

Keywords: Flavonoids, HPLC-ESI-QTOF-MS/MS, Spinach, UPLC-ESI-QqQ$_{LIT}$-MS/MS

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SUPPLEMENTARY MATERIALS

Supplementary material relating to this article is available online, alongside Figures S1-S8 and Tables S1-S4.

1. Experimental

1.1. Materials and Reagents

LC/MS grade solvents (Sigma-Aldrich) were used throughout the study. Ultra-pure water was produced by a Direct-Q system (Millipore, Milford, MA, USA). 5,3',4'-Trihydroxy-3-methoxy-6,7-methylenedioxyflavone and spinacetin were isolated from the leaves of Spinach and their structures were unambiguously characterized by direct comparison of their H^1- and C^{13}-NMR spectral data with those reported in the literature. Their purities were determined to be over 95% by HPLC/UV analysis. The standard reference samples of coumaric acid, ferulic acid and protocatechuic acid were purchased from Sigma Aldrich (St. Louis, MO, USA).

1.2. Extraction and Sample Preparation

Fresh plant material of *Spinacia oleracea* Linn. (plant material code: CDRI plant code No. 2492) was purchased from the local market of Lucknow, Uttar Pradesh, India and was identified by L. B. Chaudhary, Principal Scientist, Plant Diversity, Systematics and Harbarium Division, CSIR-National Botanical Research Institute, Lucknow. 1 g powder of dried plant materials of *spinach* leaves were suspended with 20 mL ethanol (100%), sonicated for 30 min at 25°C in an ultrasonic water bath (Bandelin SONOREX, Berlin) and left for 24 h at room temperature. The extract was collected and filtered through filter paper (Whatman No. 1) and the residue was re-extracted three times with fresh solvent following the same procedure. The combined filtrates of each sample was concentrated using a Buchi rotary evaporator (Flawil, Switzerland) under reduced pressure at 20-50 kPa at 40°C. 1 mg/mL solution of the dried ethanolic extract was prepared in methanol and filtered through a 0.22-µm polyvinylidene difluoride (PVDF) membrane (MILLEX GV filter unit, Merck Millipore, Darmstadt, Germany) prior to LC/MS analysis.

1.3. Preparation of standard solutions

Stock solutions of five reference standards (5,3',4'-trihydroxy-3-methoxy-6,7-methylenedioxyflavone, spinacetin, coumaric acid, ferulic acid and protocatechuic acid) were prepared separately in methanol (1.0 mg/mL). Then, methanol stock solution containing the

mixture of five analytes was prepared and diluted in appropriate concentration to yield a series of concentrations. The calibration curves were constructed by plotting the value of peak areas versus the value of concentrations of each analyte. All stock solutions were stored in the refrigerator at -20°C until use.

1.4. HPLC-ESI-QTOF-MS/MS analysis for qualitative study

Qualitative analysis was performed with an Agilent 6520 quadrupole time-of-flight (QTOF) mass spectrometer connected with Agilent 1200 HPLC system via Dual electrospray ionization (ESI) interface (Agilent technologies, USA). The HPLC separation was carried out on a Supelco Discovery HS C18 column (15 cm × 4.6 mm, 3μm) operated at 25°C. The mobile phase consisted of 0.1% formic acid aqueous solution (A) and acetonitrile (B) with flow rate of 0.6 mL/min under the gradient program of 5 to 10% (B) for initial 6 min, 10–30% (B) from 6 to 15 min, 30–40% (B) from 15 to 20 min, 40–45% (B) from 20 to 30 min, 45–70% (B) from 25 to 30 min, 70–70% (B) from 30 to 35 min followed by initial 5% (B) in 35–40 min. The sample injection volume was 2 μL. The UV spectra were obtained by scanning the samples in the range of 200–400 nm.

Mass spectrometric analysis was performed in negative ESI mode. The resolving power of QTOF analyser was set above 10,000 (FWHM, full width at half maximum) and spectra were acquired within a mass range of *m/z* 50–2000. Nitrogen was used as nebulising, drying and collision gas. Capillary temperature was set to 350°C and nebuliser pressure to 40 psi and the drying gas flow rate was 10 L/min. Ion source parameters such as VCap, fragmentor, skimmer and octapole radio frequency (rf) peak voltage were set to 3500 V, 150 V, 65 V and 750 V, respectively. The MS/MS analyses were acquired by auto fragmentation where the three most intense mass peaks where fragmented. Collision energy values for MS/MS experiments were fixed at 15-40 eV for all the selected masses.

1.5. UPLC-ESI-QqQ_{LIT}-MS/MS analysis for quantitative study

Quantitative analysis of selected five analytes was performed on a 4000 QTRAP™ MS/MS system (Applied Biosystem; Concord, ON, Canada), hyphenated with a Waters ACQUITY UPLC™ system (Waters; Milford, MA, USA) via an electrospray ion source (Turbo V™ source with TurboIonSpray™ probe and APCI probe) interface. Chromatographic separation of compounds was obtained with an ACQUITY UPLC BEH™ C18 column (100 mm × 2.1 mm, 1.7μm) operated at 25°C. The mobile phase, which consisted of 0.1% formic acid aqueous

solution (A) and acetonitrile (B), was delivered at a flow rate of 0.4 mL/ min under a gradient program: 5-90% (B) initial to 2.5 min, maintained at 90% (B) from 2.5 min to 3.0 min, back to initial condition from 3.0 min to 3.5 min and maintained at 5% (B) from 3.5 min to 4.0 min. The sample injection volume used was 2 μL.

The compound-dependent parameters such as declustering potential (DP), entrance potential (EP), collision energy (CE) and cell exit potential (CXP) were optimized for each compound by direct infusion of 50 ng/mL solutions of the each analyte using a Harvard '22' syringe pump (Harvard Apparatus, South Natick, MA, USA) in negative ionization mode. The results are shown in **Table S3**. Quadrupole 1 and quadrupole 2 were maintained at unit resolution. Quantitative analysis was performed using multiple-reaction monitoring (MRM) mode. The optimized source dependent parameters were as follows: the Ion Spray voltage (IS) was set to −4200 V; the turbo spray temperature (TEM), 450°C; nebulizer gas (GS 1), 50 psi; heater gas (GS 2), 50 psi; the curtain gas (CUR), 20 psi; the collision-activated dissociation gas (CAD) was set as medium and the interface heater was on. High-purity nitrogen was used for all the processes. AB Sciex Analyst software version 1.5.1 was used to control the LC–MS/MS system and for data acquisition and processing.

1.6. Validation of quantitative method

The developed MRM method was validated for linearity, lower limits of detection (LOD), limits of quantification (LOQ), precisions, stability, recovery and robustness according to the International Conference on Harmonization (ICH, Q2R1; 2014) guidelines. The linearity of calibration curve was performed by the analyte peak area ratio versus the nominal concentration. The calibration curves were constructed on at least five experiments of each reference compound and evaluated with a weighting ($1/x^2$) factor by least-squared linear regression. The LOD and LOQ were defined as a signal-to-noise ratio (S/N) equal to 3 and 10, respectively. The intra- and inter-day precisions were determined by analyzing known concentrations of the five analytes in the nine replicates during a single day and by triplicating the experiments on five successive days while repeatability was examined on six individual samples within a day. For the recovery test, three spike levels were set as 50%, 100% and 200% of each reference standard. For comparison an unspiked sample was concurrently prepared and analyzed. In order to evaluate the robustness of the method, the influence of small variations of analytical parameters on retention time and peak area of selected analytes were studied. In developing this method, mobile phase composition, flow rate and temperature were

taken into consideration. Only one parameter was changed at a time while the others were kept constant.

1.6.1. Linearity, precision recovery and robustness results of the validated method

MRM extracted ion chromatogram of analytes are shown in **Fig. S8**. The calibration curve showed good linearity with correlation coefficient (r^2) of ≥0.9982 over the tested concentration range (0.2-1000 ng/mL) (**Table S4**). The LODs and LOQs were in the range of 0.01-0.38 ng/mL and 0.03-1.15 ng/mL, respectively. Relative standard deviation (RSD) values for precision were in the range of 0.20–1.69% for intraday assays, 0.45–1.10% for interday assays and 0.39-2.14% for repeatability assays. The RSD values for stability and recovery were found ≤ 1.20% and ≤ 1.76%, respectively. The recoveries of the analytes were 97.62–102.35% (n=5), evaluated by calculating the ratio of amount detected versus the amount added. The % RSD of retention time and peak area counts were calculated to assess the robustness of the method and were found to be in agreement with the methods (**Table S5**).

Reference

International Conference on Harmonization (ICH) Guidelines, *Validation of Analytical Procedures: Text and Methodology Q2 (R1).* International Federation of Pharmaceutical Manufacturers and Associations: Geneva, 2005. http://www.ich.org/products/guidelines/quality/quality/single/article/validation-of-analytical procedures-textand-methodology.html [Accessed in February, 2014].

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Table S5- Robustness testing for the investigated components (n = 3)

Figure S1- A) HPLC-DAD chromatogram @λ_{max} 280.16 nm and **B)** Total Ion Chromatogram (TIC) of ethanolic extract of Spinach leaves

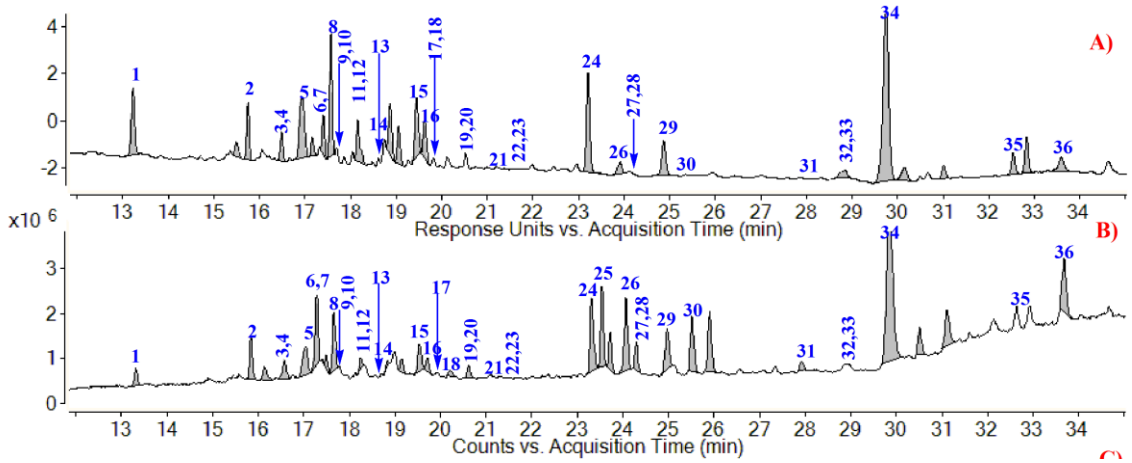


Figure S2- Chemical Structure of identified compounds in Spinach leaves

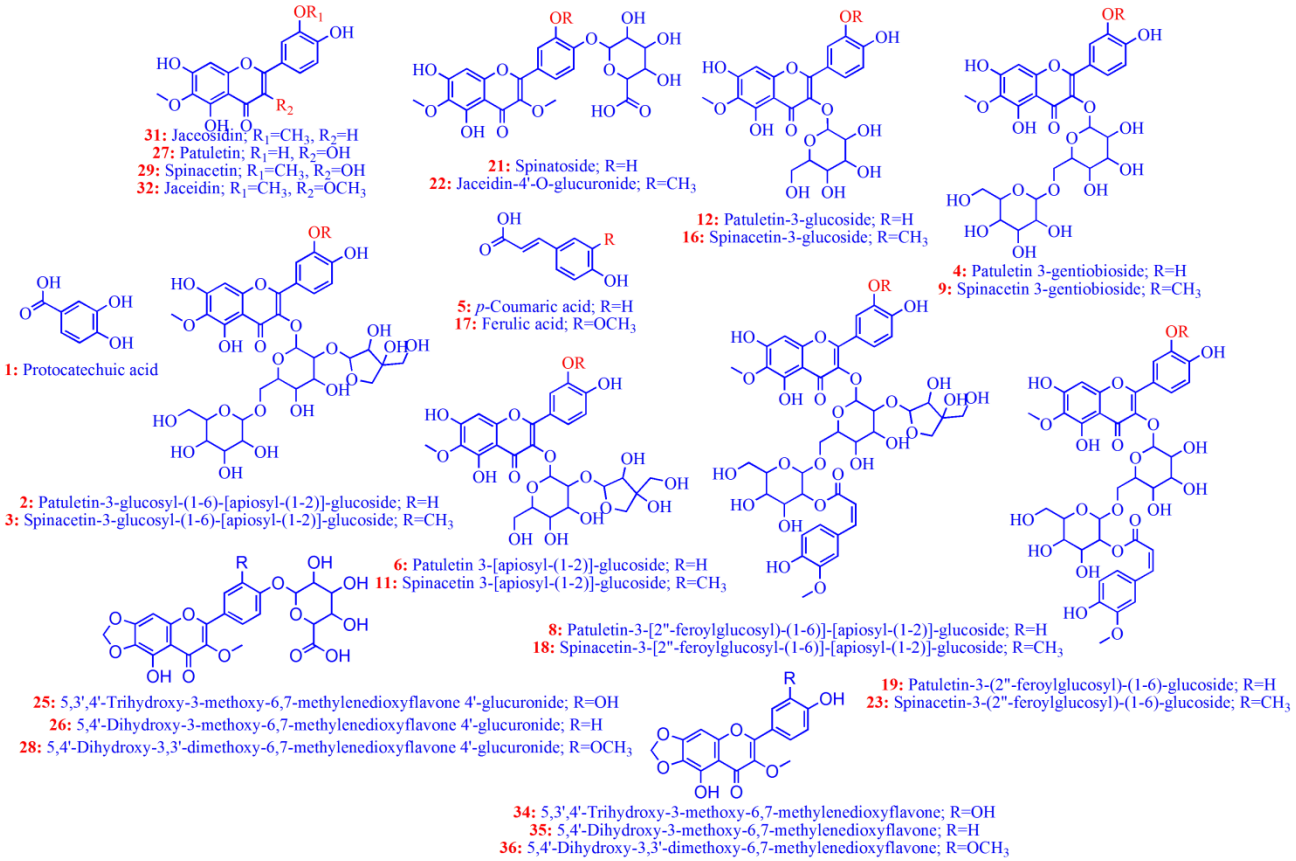


Figure S3- *(-)*-ESI-MS/MS spectra of flavonoids

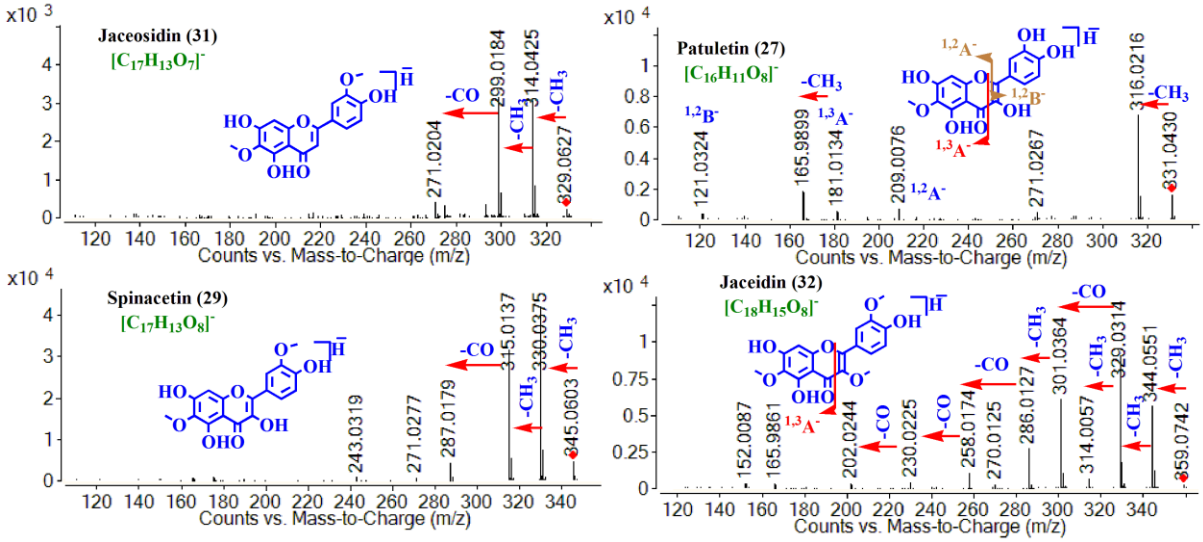


Figure S4- *(-)*-ESI-MS/MS spectra of flavone disaccharides, flavone glucosides and flavone glucuronides

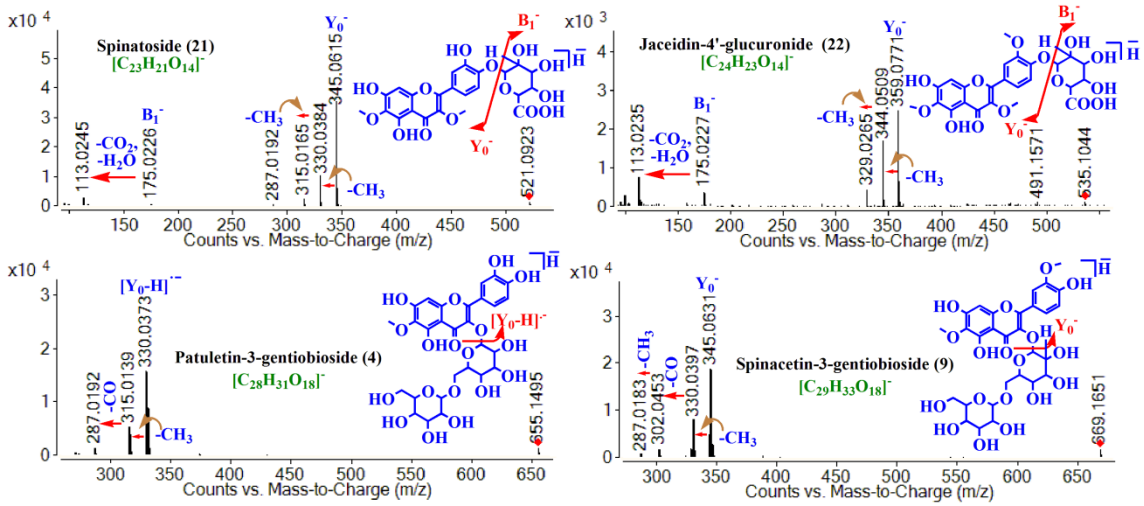


Figure S5- *(-)*-ESI-MS/MS spectra of flavone glucoside and flavone disaccharide derivatives

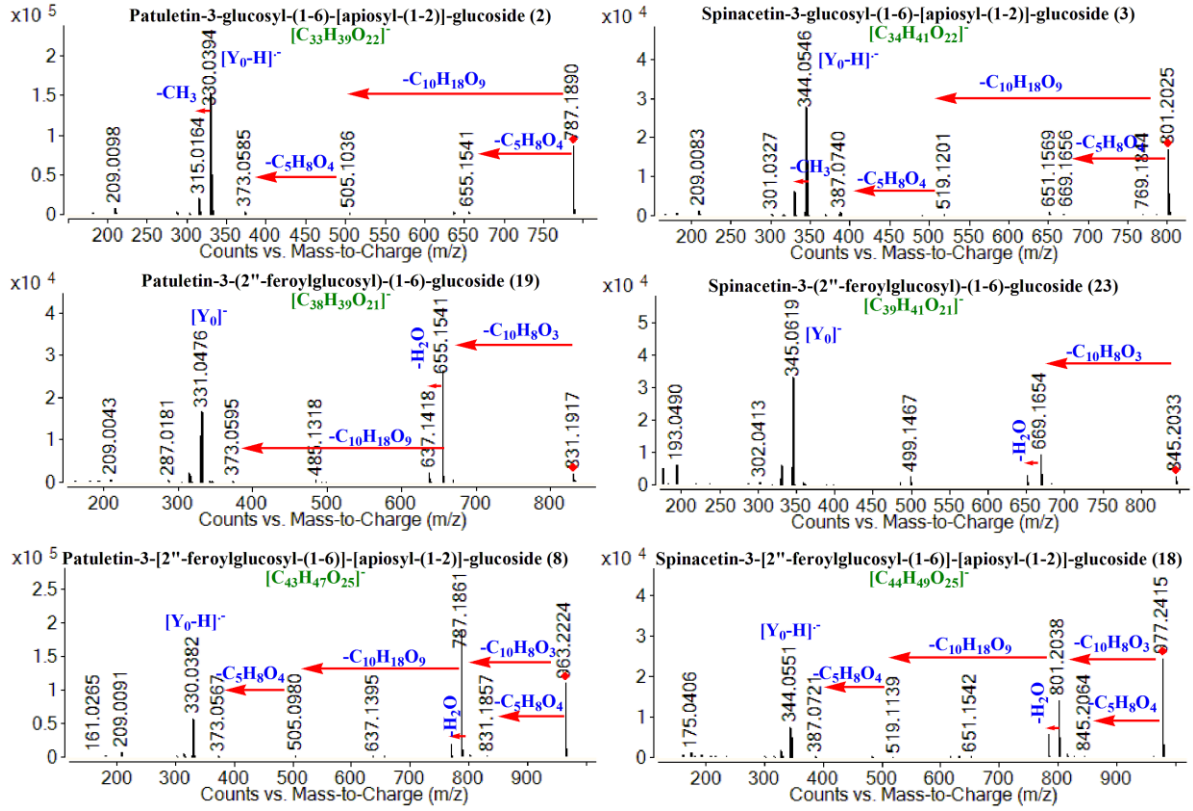


Figure S6- *(-)*-ESI-MS/MS spectra of methylenedioxyflavones and methylenedioxyflavone glucuronides

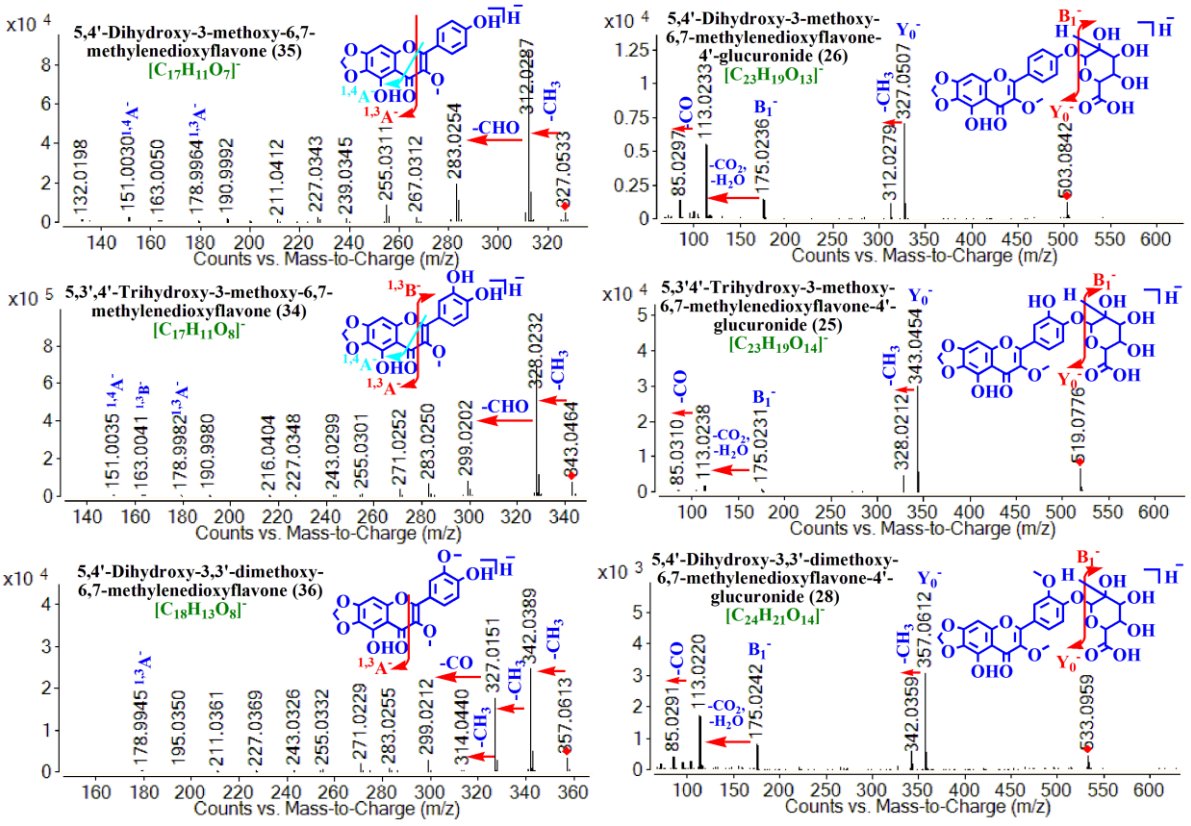


Figure S7- *(-)*-ESI-MS/MS spectra of Unidentified patulein and spinacetin derivatives

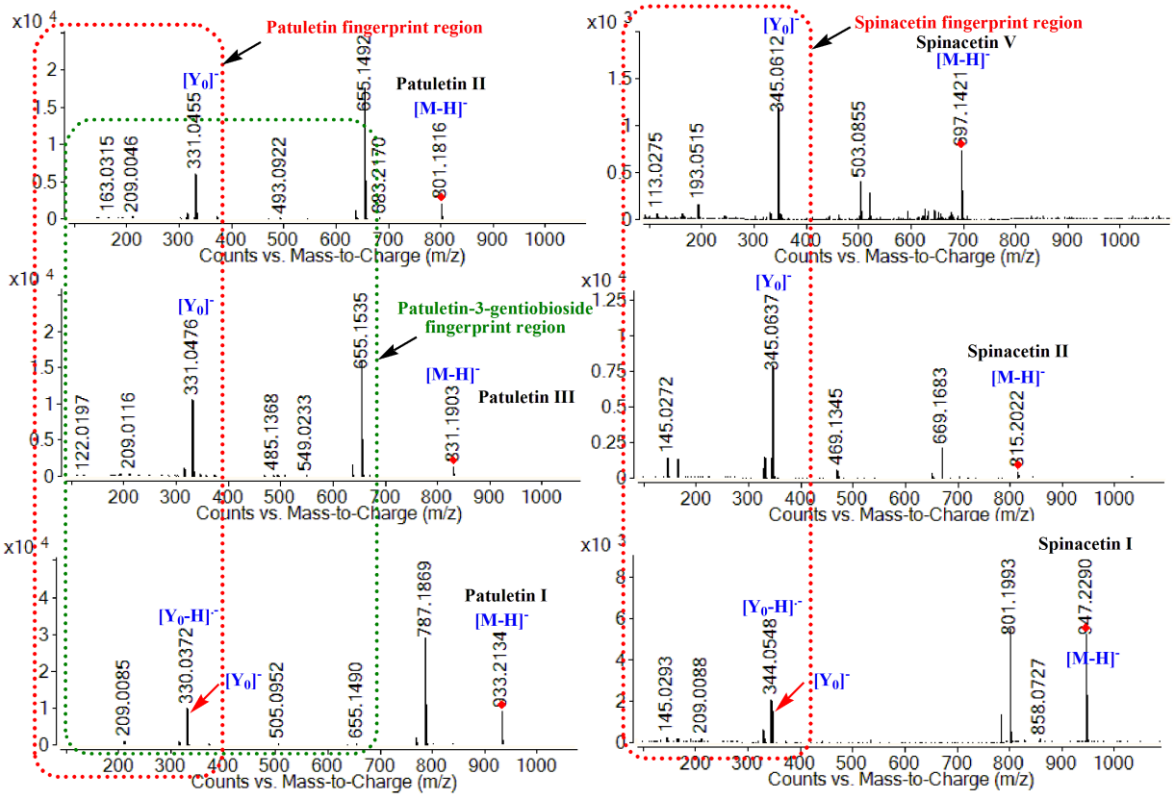
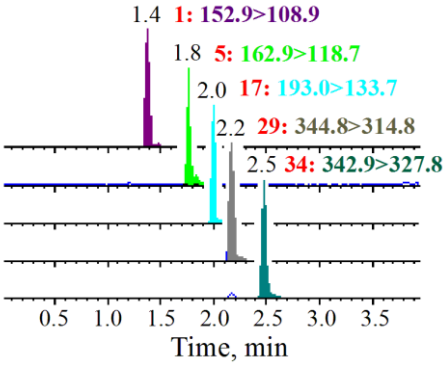


Figure S8- MRM extracted ion chromatogram of selected analytes



Scheme S1- Proposed fragmentation pattern of flavone glucoside and flavone disaccharide derivatives

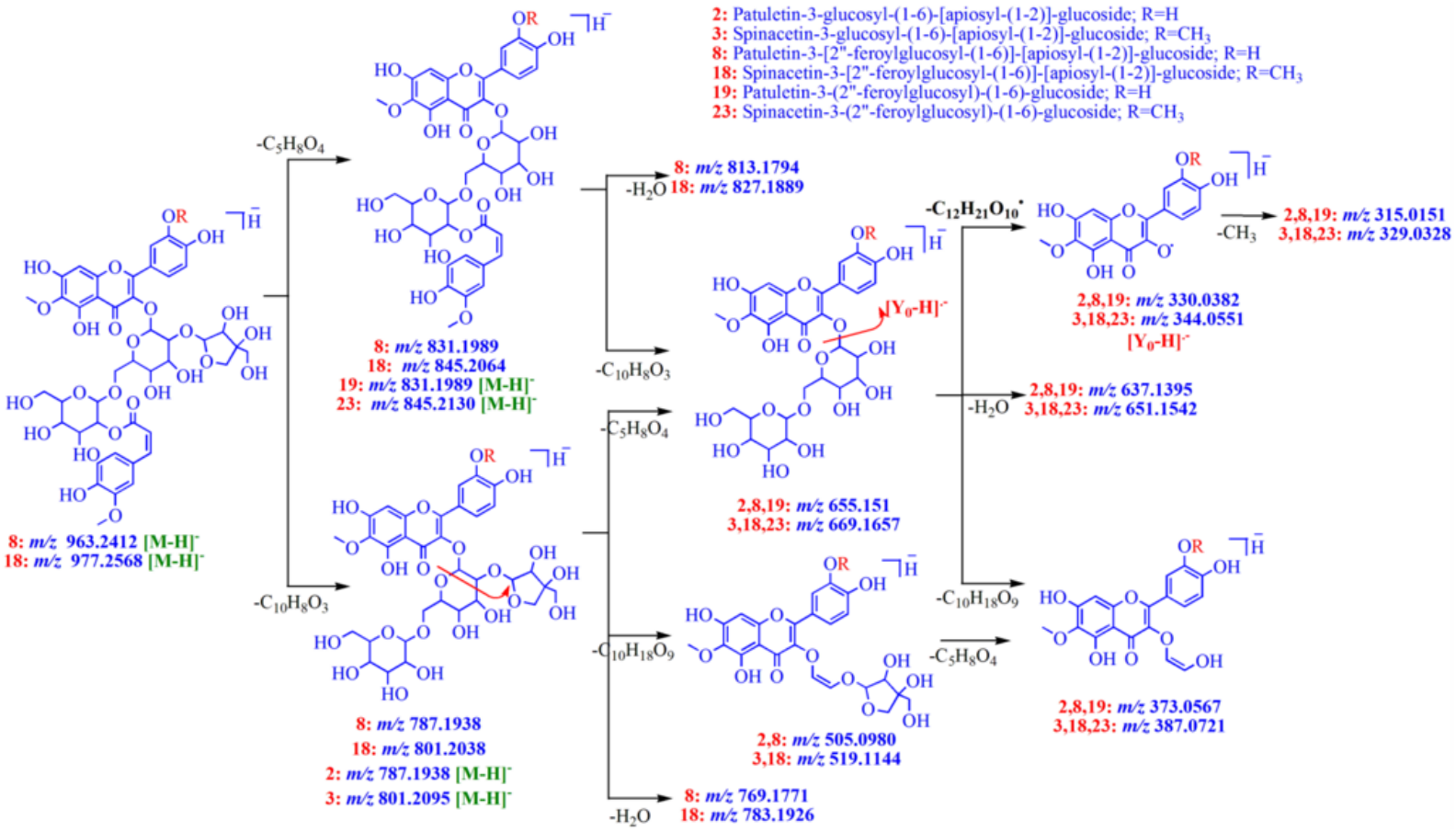


Table S1- Mass spectral characteristics of phytochemicals identified in ethanolic extract of *Spinacia oleracea*

Peak No.	RT (min)	Molecular Formula	$[M-H]^-$ (Calc)	$[M-H]^-$ (Exp)	Mass Error (Δppm)	CE (eV)	*(-)*-ESI-MS/MS fragment ions	Identification
1	13.2	$C_7H_6O_4$	153.0193	153.019	1.96	20	109.0962 (100)	Protocatechuic acid
2	16	$C_{33}H_{40}O_{22}$	787.1938	787.192	2.29	40	655.1542 (2), 637.1387 (2), 505.1039 (1), 373.0587 (2), 330.0394 (100), 315.0162 (13), 287.0210 (2), 209.0100 (5), 181.0113 (2)	Patuletin-3-glucosyl-(1-6)-[apiosyl-(1-2)]-glucoside
3	16.8	$C_{34}H_{42}O_{22}$	801.2095	801.2058	4.62	40	669.1656 (1), 651.1569 (4), 519.1201 (3), 387.0740 (3), 344.0546 (100), 329.0312 (23), 301.0327 (2), 209.0083 (4), 181.0123 (2)	Spinacetin-3-glucosyl-(1-6)-[apiosyl-(1-2)]-glucoside
4	16.9	$C_{28}H_{32}O_{18}$	655.1516	655.1518	-0.31	40	331.0429 (57), 330.0373 (100), 316.0208 (26), 315.0139 (38), 287.0192 (8)	Patuletin-3-gentiobioside
5	17.2	$C_9H_8O_3$	163.0401	163.0398	1.84	15	62.8396 (34), 119.0504 (100)	p-Coumaric acid
6	17.4	$C_{27}H_{30}O_{17}$	625.1405	625.1406	-0.16	35	330.0394 (100), 315.0153 (21), 287.0198 (3), 209.0112 (2)	Patuletin-3-[apiosyl-(1-2)]-glucoside
8	17.8	$C_{43}H_{48}O_{25}$	963.2412	963.2398	1.45	40	831.1857 (1), 813.1794 (1), 801.2042 (2), 787.1861 (100), 769.1771 (10), 655.1540 (1), 637.1395 (1), 505.0980 (1), 373.0567 (1), 330.0382 (30), 315.0151 (3), 287.0245 (1), 303.0486 (1), 209.0091 (4), 193.0511 (1), 181.0116 (1), 175.0390 (1), 161.0265 (1)	Patuletin-3-[2"-feruloylglucosyl-(1-6)]-[apiosyl-(1-2)]-glucoside
9	18	$C_{29}H_{34}O_{18}$	669.1672	669.1667	0.75	35	345.0631 (100), 344.0574 (26), 330.0397 (44), 329.0332 (10), 302.0453 (9), 301.0313 (3), 287.0183 (4)	Spinacetin-3-gentiobioside
11	18.3	$C_{28}H_{32}O_{17}$	639.1561	639.1566	-0.78	35	344.0553 (100), 329.0318 (45), 286.0081 (3), 229.9251 (3)	Spinacetin-3-[apiosyl-(1-2)]-glucoside
12	18.4	$C_{22}H_{22}O_{13}$	493.0988	493.0988	0.00	25	331.0449 (92), 330.0392 (100), 316.0239 (17), 315.0179 (34), 288.0257 (5), 287.0186 (3), 270.0149 (4)	Patuletin-3-glucoside
16	19.7	$C_{23}H_{24}O_{13}$	507.1144	507.1145	-0.20		345.0629 (89), 344.0571 (100), 330.0301 (35), 329.0328 (8), 302.0451 (10), 301.0318 (2), 287.0191 (3)	Spinacetin-3-glucoside
17	19.8	$C_{10}H_{10}O_4$	193.0506	193.051	-2.07		178.0312, 149.036, 134.0401 (100)	Ferulic acid
18	20.4	$C_{44}H_{50}O_{25}$	977.2568	977.2509	6.04	40	845.2064 (1), 827.1889 (1), 815.2183 (3), 801.2038 (58), 783.1926 (23), 769.1823 (1), 669.1657 (1), 651.1542 (1),	Spinacetin-3-[2″-feroylglucosyl-(1-6)]-[apiosyl-(1-2)]-glucoside

							639.1502 (1), 631.1845 (1), 617.1751 (1), 519.1139 (1), 499.1442 (1), 483.1544 (1), 394.8422 (1), 387.0721 (1), 359.0784 (1), 344.0551 (31), 329.0328 (7), 317.0705 (1), 301.0353 (1), 286.0105 (1), 235.0592 (1), 217.0514 (1), 209.0114 (2), 193.0508 (3), 181.0179 (9), 175.0406 (4), 161.0232 (2)	
19	20.6	$C_{38}H_{40}O_{21}$	831.1989	831.1976	1.56	40	655.1541 (100), 637.1418 (9), 331.0476 (63), 330.0398 (42), 316.0219 (6), 315.0196 (8)	Patuletin-3-(2''-feroylglucosyl)-(1-6)-glucoside
21	20.8	$C_{23}H_{22}O_{14}$	521.0937	521.0948	-2.11	15	345.0618 (100), 330.0390 (14), 315.0129 (2), 175.0237 (3), 157.0126 (0.5), 113.0230 (10)	Spinatoside
22	21.5	$C_{24}H_{24}O_{14}$	535.1093	535.1081	2.24	20	359.0771 (100), 344.0509 (68), 329.0265 (17), 175.0227 (15), 113.0235 (63), 99.0070 (11), 85.0284 (33), 75.0074 (8), 59.0142 (21)	Jaceidin-4'-glucuronide
23	21.7	$C_{39}H_{42}O_{21}$	845.2146	845.213	1.89	40	669.1654 (28), 651.1527 (8), 499.1467 (8), 345.0619 (100), 344.0432 (16), 330.0384 (19), 329.0338 (5), 193.0490 (18), 175.0393 (15)	Spinacetin-3-(2′′-feroylglucosyl)-(1-6)-glucoside
25	23.5	$C_{23}H_{20}O_{14}$	519.078	519.0786	-1.16	15	343.0454 (100), 328.0212 (16), 283.0185 (1), 175.0231 (3), 113.0238 (6), 85.0310 (2)	5,3',4'-Trihydroxy-3-methoxy-6,7-methylenedioxyflavone 4'-glucuronide
26	24.2	$C_{23}H_{20}O_{13}$	503.0831	503.084	-1.79	15	327.0507 (100), 312.0279 (20), 175.0236 (26), 113.0233 (98), 103.0015 (6), 99.0088 (9), 95.0144 (7), 85.0297 (24), 59.0146 (7)	5,4'-Dihydroxy-3-methoxy-6,7-methylenedioxyflavone 4'-glucuronide
27	24.3	$C_{16}H_{12}O_{8}$	331.0459	331.0463	-1.21	20	316.0216 (100), 271.0267 (7), 209.0076 (10), 193.9899 (9) 181.01334 (7), 165.9899 (27), 139.0002 (4), 121.0324 (6)	Patuletin
28	24.3	$C_{24}H_{22}O_{14}$	533.0937	533.0955	-3.38	15	357.0612 (100), 342.0359 (21), 327.0140 (4), 175.0242 (26), 113.0220 (56), 103.0032 (8), 95.0155 (7), 85.0291 (13), 71.0163 (6), 59.0146 (4)	5,4'-Dihydroxy-3,3'-dimethoxy-6,7-methylenedioxyflavone 4'-glucuronide
29	25.0	$C_{17}H_{14}O_{8}$	345.0616	345.0613	0.87	25	330.0390 (47), 315.0158 (100), 287.0210 (25), 271.0254 (3), 259.0256 (3), 243.0303 (2), 231.0255 (2), 214.9271 (3), 189.0183 (2), 175.0032 (6), 149.0250 (3)	Spinacetin
31	27.9	$C_{17}H_{14}O_{7}$	329.0667	329.0672	-1.52	20	314.0425 (98), 299.0184 (100), 292.9010 (12), 274.8897 (11), 271.0204 (13)	Jaceosidin

32	28.8	$C_{18}H_{16}O_8$	359.0772	359.0781	-2.51	25	344.0551 (61), 329.0314 (100), 314.0057 (7), 301.0364 (67), 286.0127 (30), 258.0174 (12), 230.0225 (5), 202.0244 (4), 180.0079 (2), 165.9861 (3), 152.0087 (3)	Jaceidin
34	30.1	$C_{17}H_{12}O_8$	343.0459	343.0463	-1.17	20	328.0232 (100), 299.0202 (12), 283.0250 (9), 271.0252 (5), 255.0301 (2), 243.0299 (1), 227.075 (1), 190.9980 (1), 178.9982 (1), 163.0041 (1), 151.0035 (2)	5,3',4'-Trihydroxy-3-methoxy-6,7-methylenedioxyflavone
35	32.6	$C_{17}H_{12}O_7$	327.0533	327.0531	0.61	20	312.0287 (100), 283.0254 (23), 267.0254 (22), 267.0312 (3), 255.0311 (10), 239.0345 (2), 227.0343 (3), 211.0412 (1), 190.9992 (2), 178.9964 (1), 163.0050 (1), 151.0030 (3)	5,4'-Dihydroxy-3-methoxy-6,7-methylenedioxyflavone
36	33.5	$C_{18}H_{14}O_8$	357.0616	357.062	-1.12	20	342.0389 (100), 327.0151 (70), 314.0440 (1), 299.0212 (11), 283.0255 (3), 271.0229 (8), 255.0332 (2), 243.0326 (1), 227.0369 (1), 211.0361 (1), 178.9945 (1)	5,4'-Dihydroxy-3,3'-dimethoxy-6,7-methylenedioxyflavone

Table S2- Mass spectral characteristics of phytochemicals (patuletin and spinacetin derivatives) unidentified in ethanolic extract of *Spinacia oleracea*

Peak No.	**RT (min)**	**$[M-H]^-$ (Exp)**	**CE (eV)**	***(-)*-ESI-MS/MS fragment ions**	**Assignments**
7	17.4	933.2122	40	839.1802 (1), 801.1823 (1), 787.1869 (100), 769.1759 (7), 655.1490 (1), 637.1553 (1), 505.0952 (1), 373.0624 (1), 330.0372 (34), 315.0120 (4), 209.0085 (4)	Patuletin I
10	18	947.229	40	858.0727 (3), 801.1993 (100), 783.199 (24), 345.0604 (28), 344.0548 (38), 329.0289 (11), 209.0088 (3), 165.9873 (5), 145.0293 (5)	Spinacetin I
13	18.7	801.1816	35	655.1492 (100), 637.1437 (6), 373.058 (1), 331.0455 (35), 330.0424 (18), 316.0232 (3), 315.0195 (4), 209.0046 (1)	Patuletin II
14	18.9	831.1903	40	655.1535 (100), 637.1423 (10), 485.1368 (1), 494.0907 (1), 373.0511 (1), 331.0476 (67), 330.0402 (35), 316.0278 (8), 315.0147 (5), 209.0116 (2), 193.0477 (2), 181.0088 (1)	Patuletin III
15	19.6	815.2022	40	669.1686 (26), 651.1598 (4), 469.1345 (7), 345.0637 (100), 344.0512 (17), 330.0362 (18), 329.0292 (9), 163.0366 (16), 145.0272 (18)	Spinacetin II
20	20.6	521.0932	15	345.0618 (100), 330.0390 (14), 175.0237 (4), 113.0230 (9)	Spinacetin III
24	23.3	1039.1497	20	935.1854 (1), 519.0819 (100), 343.0456 (8), 175.0208 (1)	Spinacetin IV
30	25.5	697.1421	15	521.0945 (24), 503.0855 (34), 345.0612 (100), 330.0412 (6), 193.0515 (13), 113.0275 (5), 59.0137 (8)	Spinacetin V
33	28.9	695.1257	20	519.0824 (14), 201.0671 (29), 457.0774 (2), 439.0688 (2), 343.0473 (100), 328.0231 (9), 193.0477 (2), 175.0337 (1), 157.0117 (1), 99.0067 (3)	Spinacetin VI

Table S3- Optimized compound dependent parameters for investigated components

Peak No.	RT (min)	Compound	Precursor ion Q1 (Da)	Product ion Q3 (Da)	DP (V)	EP (V)	CE (eV)	CXP (V)
1	1.37	Protocatechuic acid	152.9	108.9	-64	-5	-22	-9
5	1.75	Coumaric acid	162.9	118.7	-108	-10	-19	-22
17	2.00	Ferulic acid	193.0	133.7	-58	-5	-25	-6
29	2.19	Spinacetin	344.8	314.8	-75	-12	-35	-24
34	2.50	5,3',4'-Trihydroxy-3-methoxy-6,7-methylenedioxyflavone	342.9	327.8	-146	-4	-27	-19

Table S4- Linearity, LOD, LOQ, precisions, stability and recovery results of investigated components

Analytes	Regression equation	R^2	Linear Range (ng/ml)	LOD (ng/ml)	LOQ (ng/ml)	Precision RSD (%)			Stability RSD (%) (n=5)	Recovery	
						Intraday (n=9)	Interday (n=15)	Repeatability (n=6)		Mean (n=5)	RSD (%)
Protocatechuic acid	y = 6020*x + 2170	0.9998	0.25-100	0.01	0.03	0.42	1.10	0.39	0.99	102.05	1.17
***p*-Coumaric acid**	y = 58.6*x - 948	0.9986	1.5-1000	0.38	1.15	1.12	0.45	2.14	1.20	98.71	1.52
Ferulic acid	y = 1300*x + 208	0.9998	0.5-1000	0.07	0.21	1.69	0.61	0.98	1.17	101.40	1.62
Spinacetin	y = 3880*x + 954	0.9988	0.5-1000	0.11	0.33	0.31	0.89	1.28	0.23	102.35	1.76
5,3',4'-Trihydroxy-3-methoxy-6,7-methylenedioxyflavone	y = 69.7*x – 10.4	0.9982	0.2-500	0.01	0.03	0.20	0.75	0.54	1.19	97.62	1.16

Table S5- Robustness testing for the investigated components (n = 3; %RSD)

Parameter	Protocatechuic acid		*p*-Coumaric acid		Ferulic acid		Spinacetin		5,3',4'-Trihydroxy-3-methoxy-6,7-methylenedioxyflavone	
	RT	Peak area	RT	Peak area	RT	Peak area	RT	Peak area	RT	Peak area
Mobile phase composition	1.14	0.39	1.48	0.58	1.99	1.68	2.46	2.01	2.55	0.87
Flow Rate	0.97	0.76	1.01	0.75	1.23	0.68	1.75	1.54	1.98	1.21
Column temperature	0.28	0.34	0.71	0.98	0.78	0.87	0.81	0.74	0.56	1.08