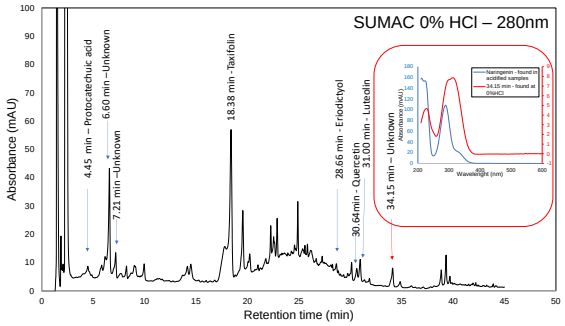
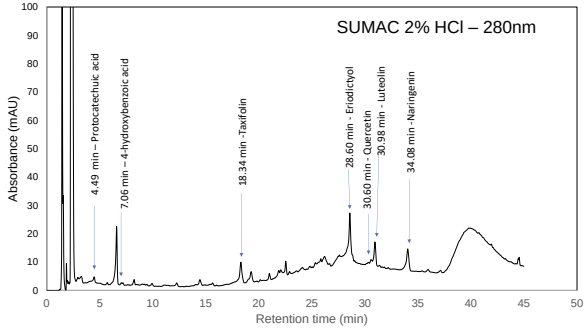
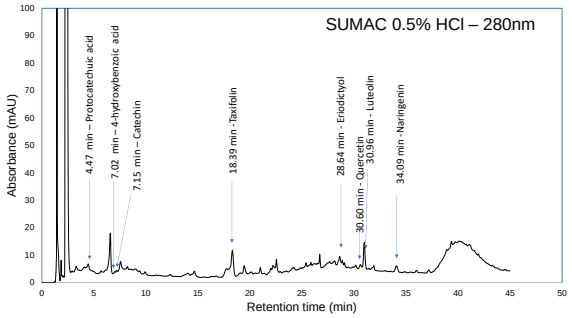
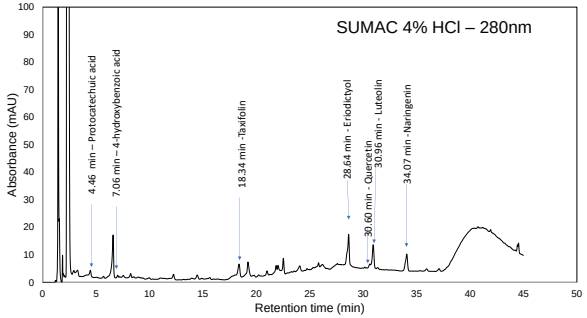
Supplementary material 1

Figure S1.1. The chromatograms at 280 nm registered for Sumac extracts









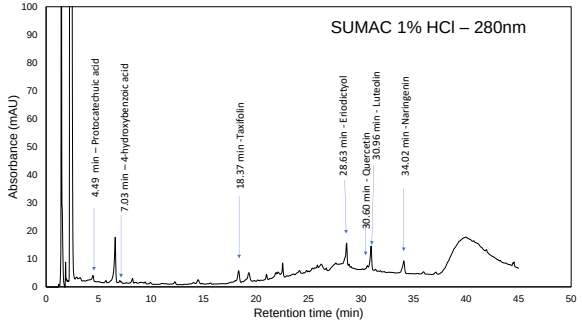
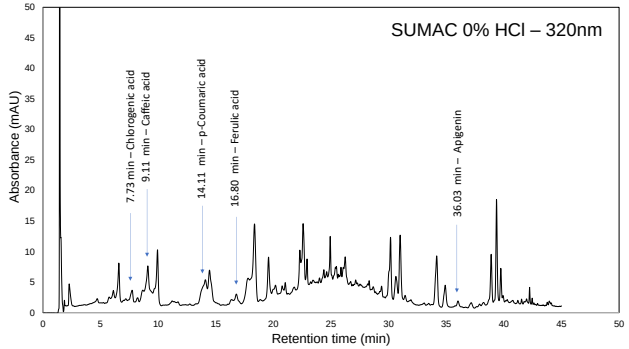
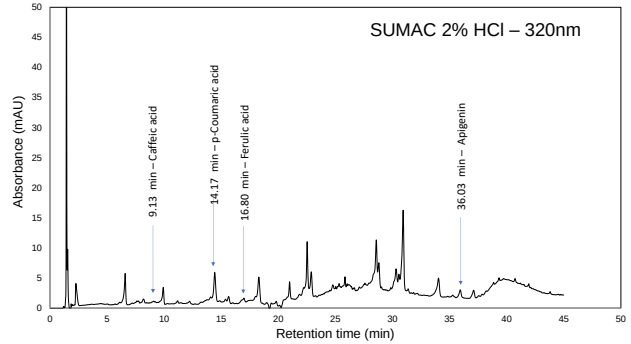
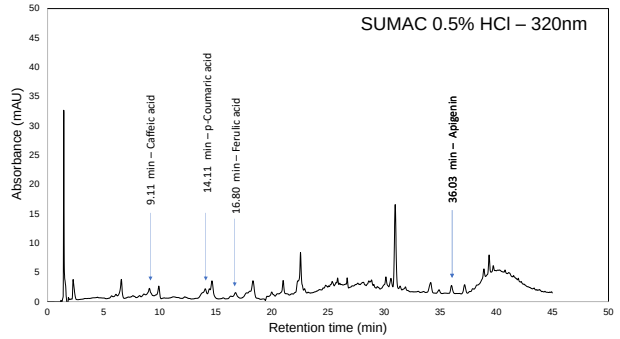
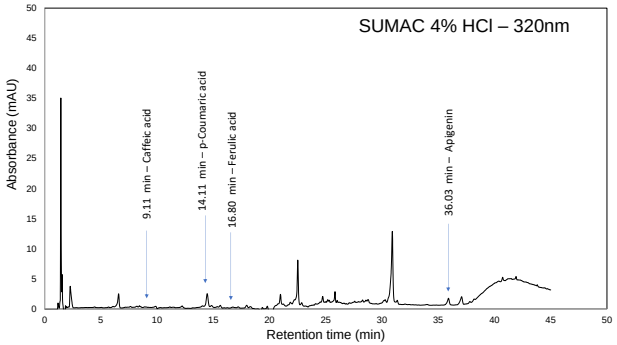


Figure S1.2. The chromatograms at 320 nm registered for Sumac extracts









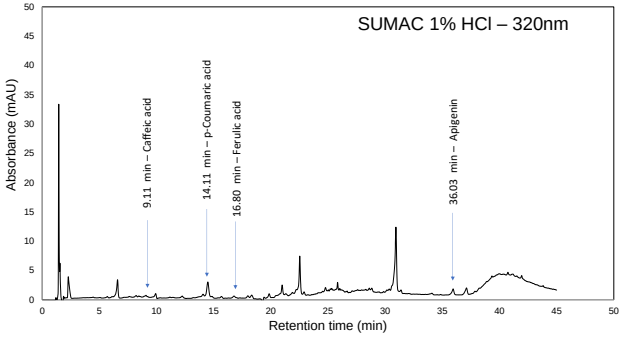
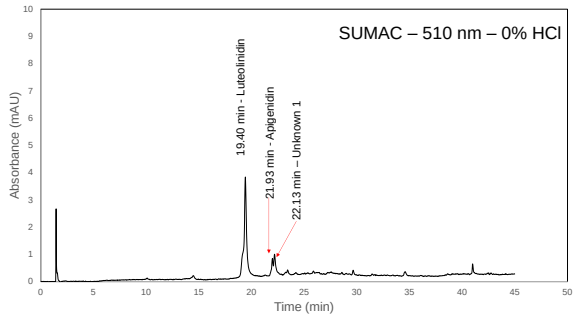
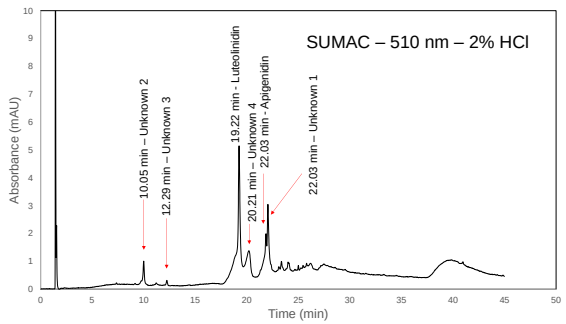
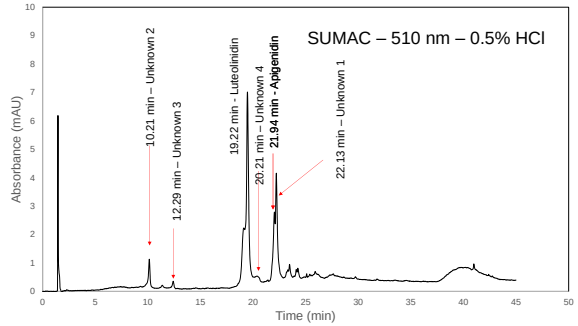
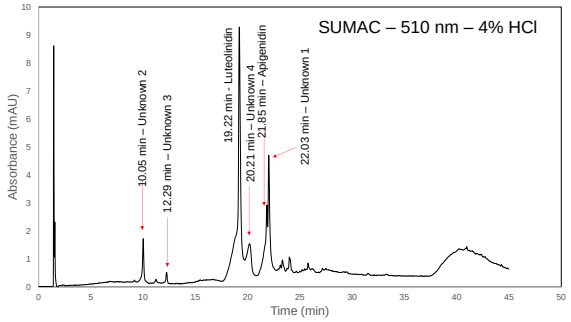


Figure S1.3. The chromatograms at 510 nm registered for Sumac extracts









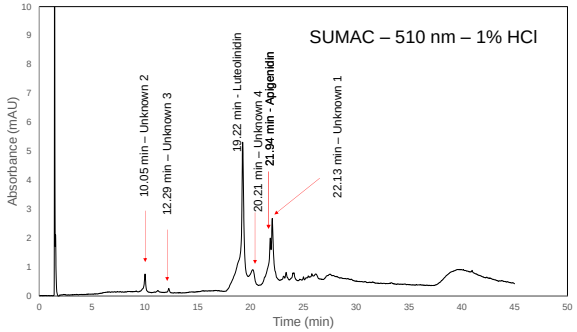
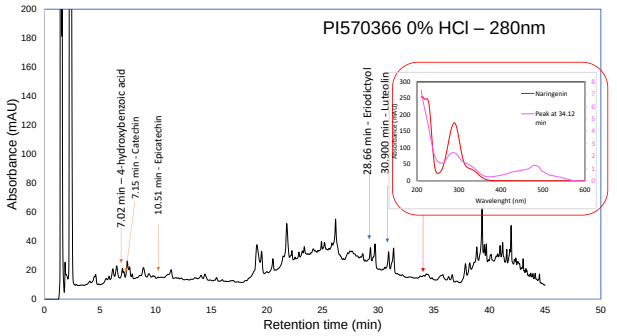
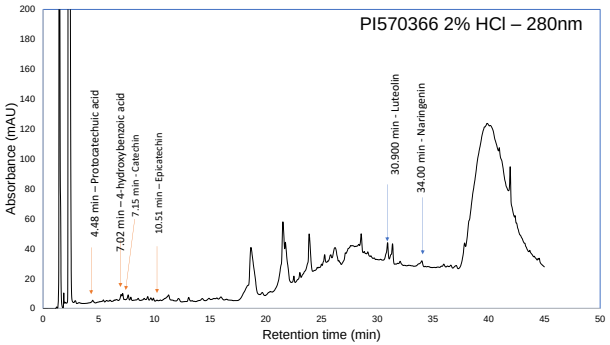
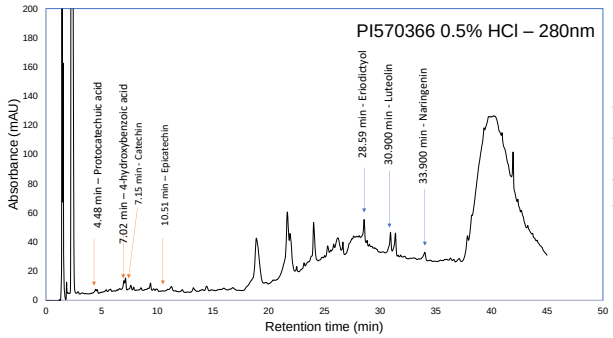
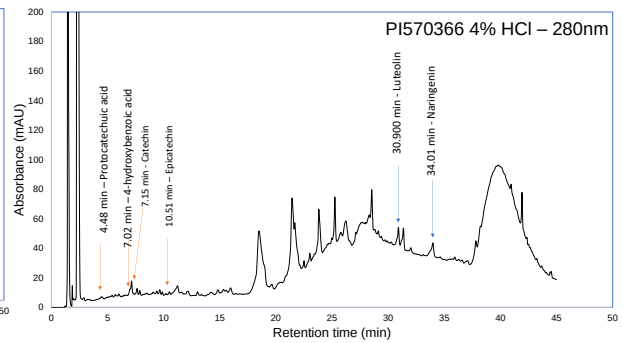


Figure S1.4 The chromatograms at 280 nm registered for PI570366 extracts









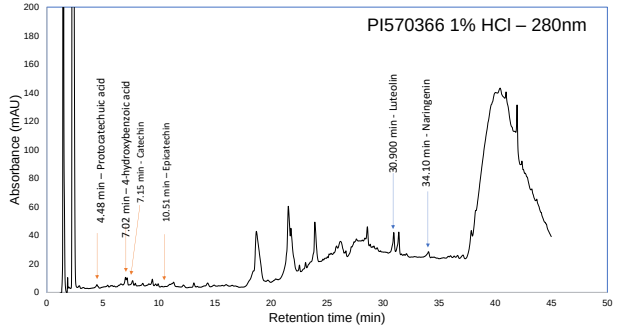


Figure S1.5. The chromatograms at 320 nm registered for PI570366 extracts

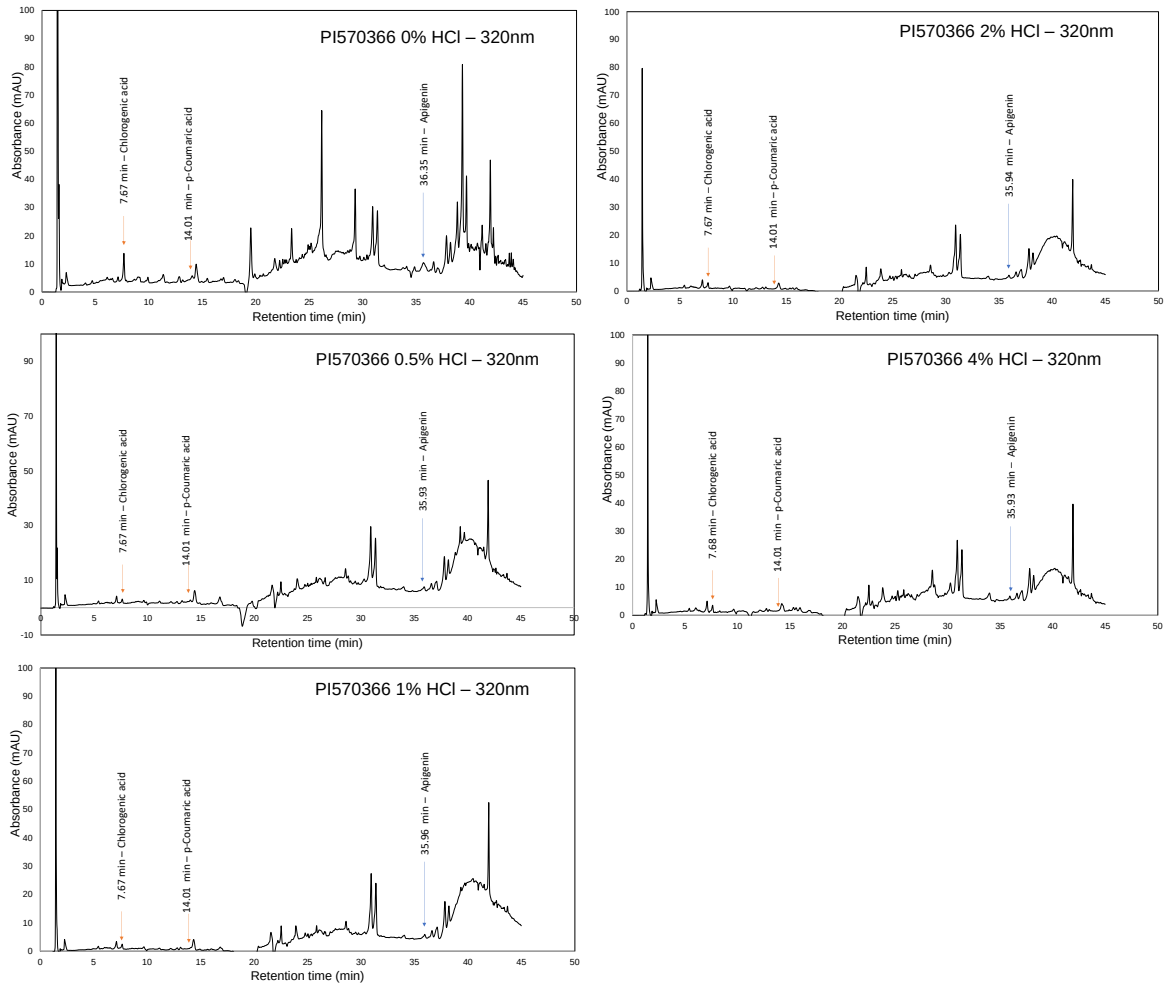


Figure S1.6. The chromatograms at 510 nm registered for PI570366 extracts

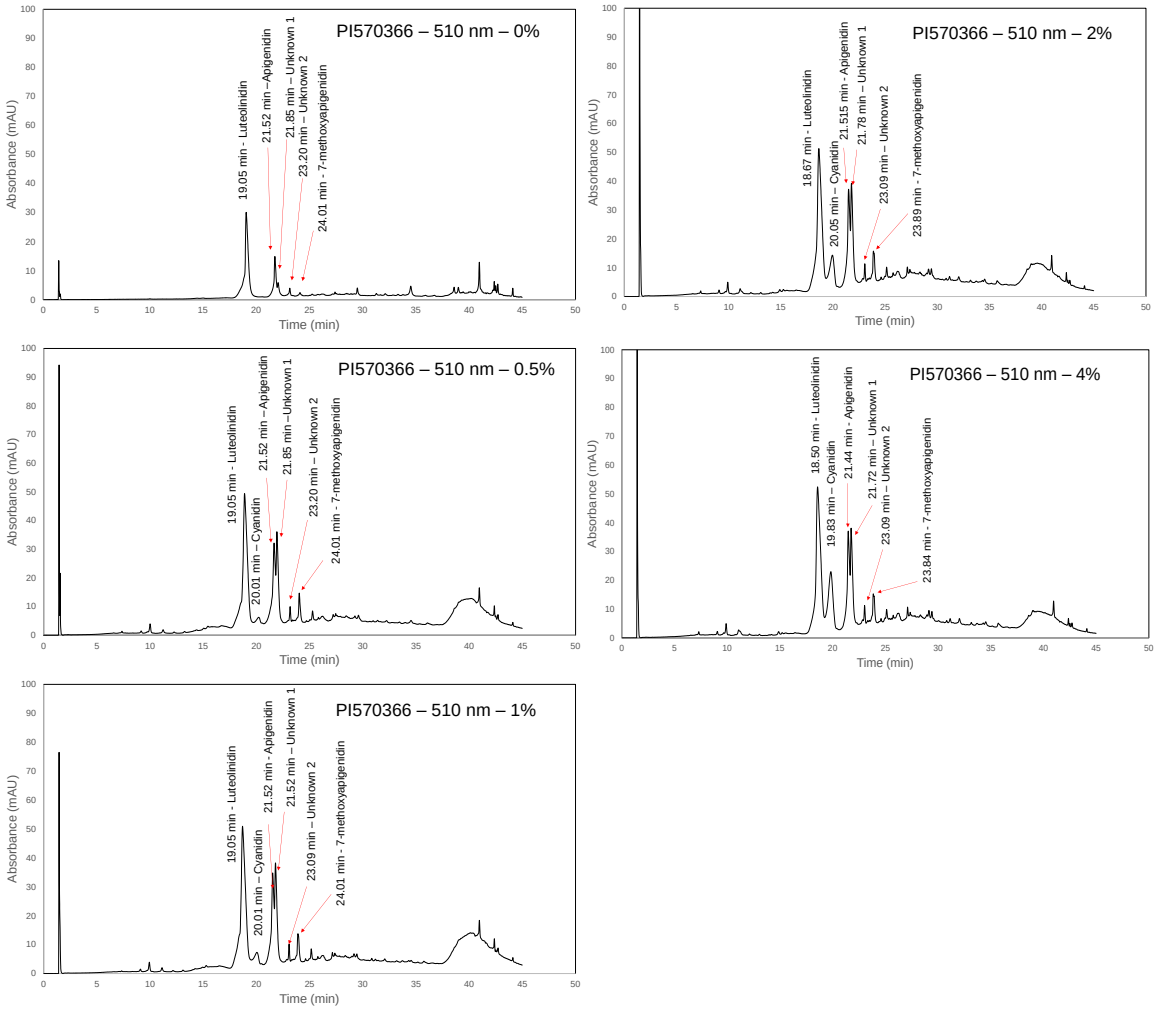


Figure S1.7. The chromatograms at 280 nm registered for SC991 extracts

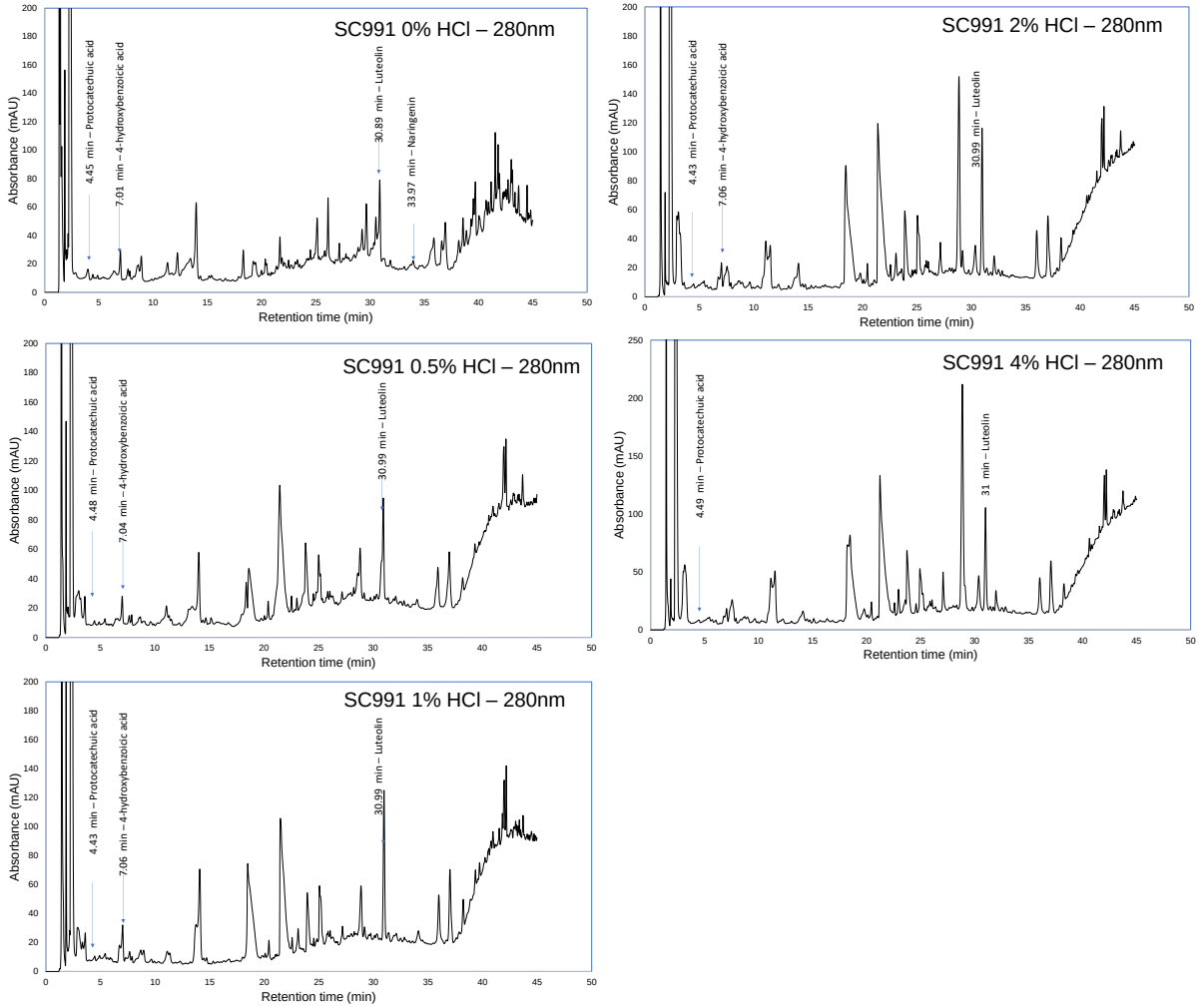


Figure S1.8. The chromatograms at 320 nm registered for SC991 extracts

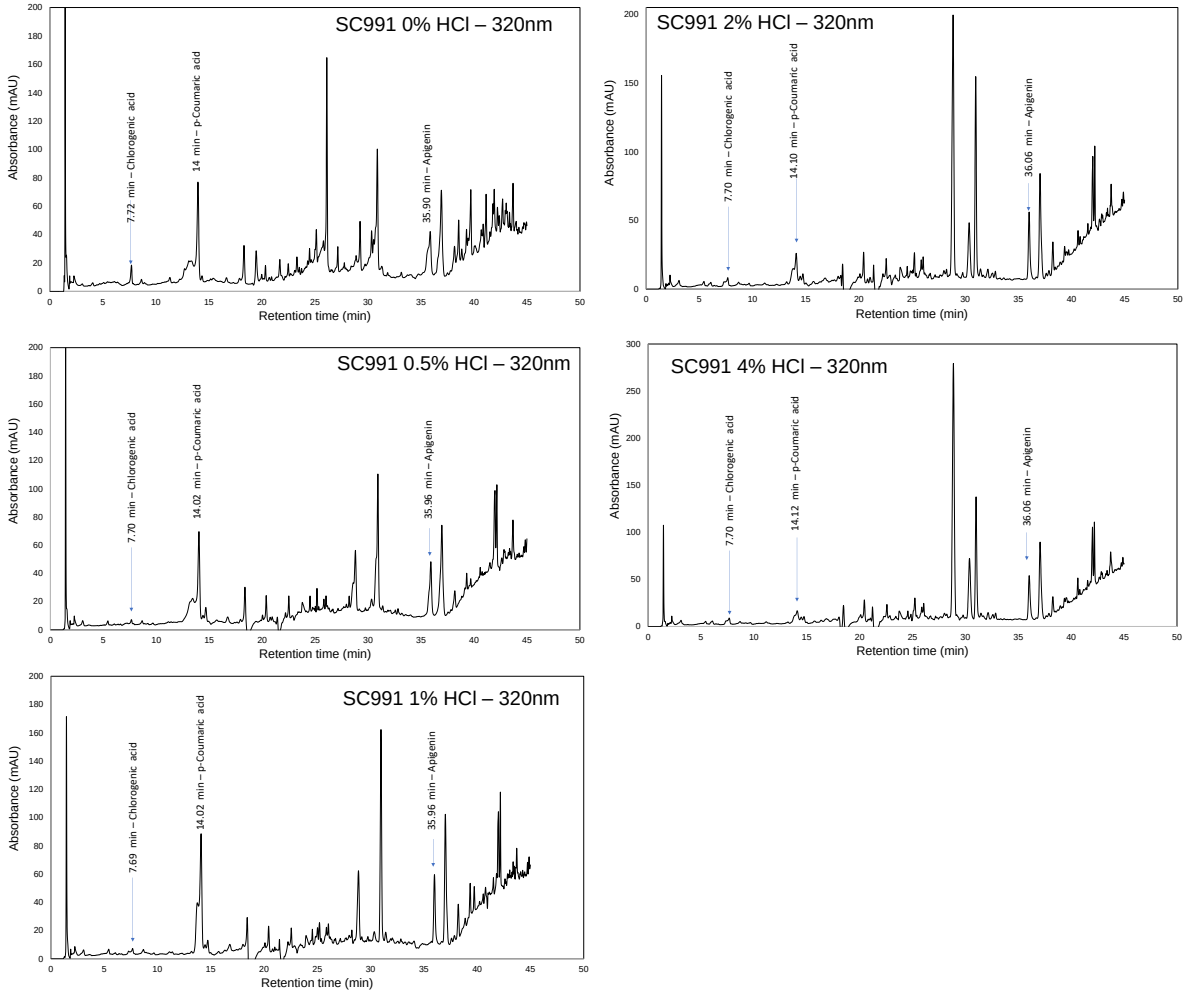


Figure S1.9. The chromatograms at 510 nm registered for SC991 extracts

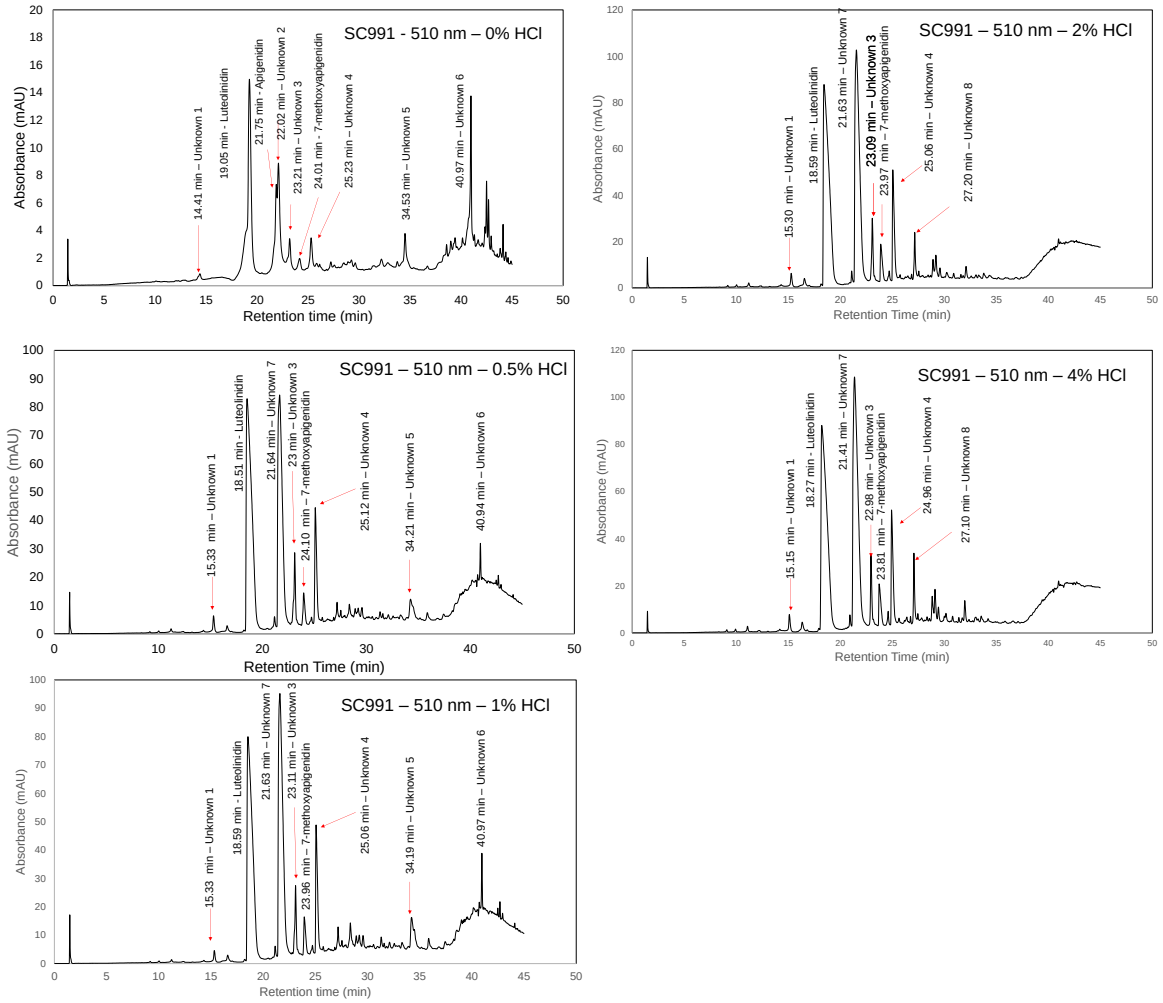
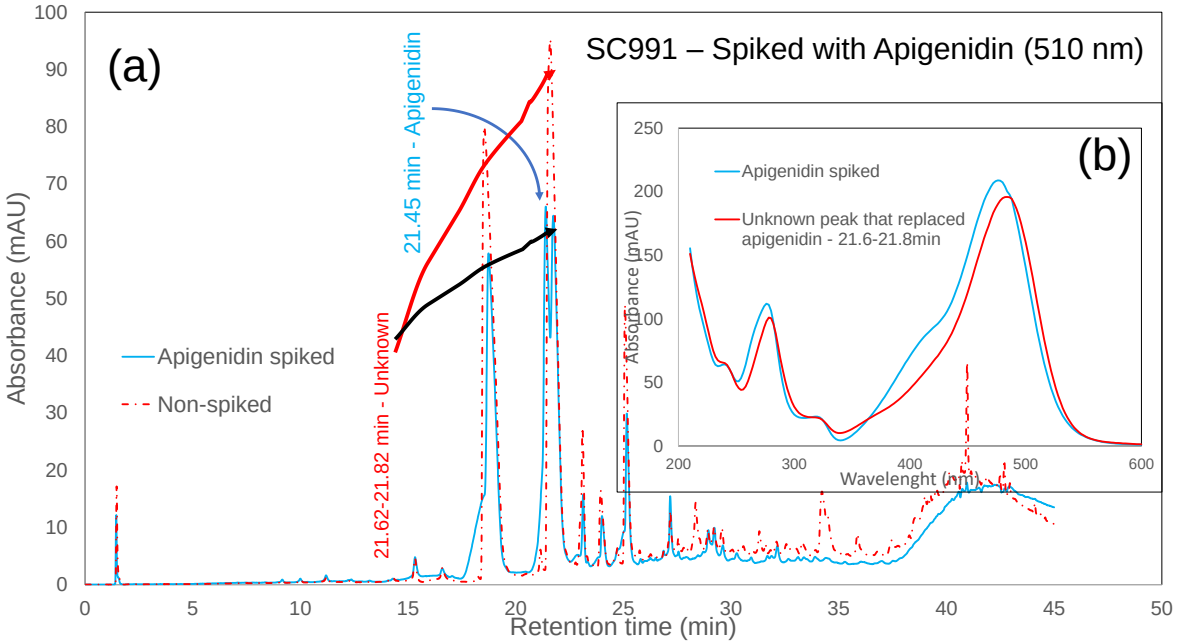


Figure S1.10. The acidified extract of SC991 (1%HCl) before and after apigenidin (0.045mg/mL) spiking (a) and the comparisons within the spectra (b)



Supplementary material 2

Supplementary material 2: HPLC method validation

S.2.1 Sample preparation and standards

- Extracts at 0% and 1% HCl of all genotypes were mixed at equal volumes. From this stock, 0.25 mL was dried under nitrogen and used for HPLC analysis.
- Sixteen standards were used: apigenidin, apigenin, luteolinidin, luteolin, 7-methoxyapigenidin, cyanidin, p-coumaric acid, protocatechuic acid, 4-hydroxybenzoic acid, caffeic acid, chlorogenic acid, naringenin, taxifolin, ferulic acid, quercetin and catechin.
- The solvent used to solubilize standards and sample was methanol:ethanol (90:10, v/v).

S.2.2 Validation

- The method was validated in terms of linearity, accuracy, within-day and between day precision.
- Linearity was evaluated by analyzing mixtures of standards solutions and by spiking sample with known concentration of standards. Standards were analyzed at the concentrations of: (0.00045, 0.0009, 0.0045, 0.009 and 0.09 mg/mL)
- Calibration curves were plotted by the ratio of peak area of the spiked analyte to the peak area of external standard at 0.09 mg/mL (non-spiked) versus concentration of standard.
- Limit of detection (LOD, in µg/g) was calculated as:

Limit of detection (LOD, in µg/g) was calculated as:

$$LOD(\mu g/g) = \frac{3.3 \times Standard\ deviation\ of\ intercept\ y}{Curve\ slope}$$

Limit of quantitation (LOQ, in µg/g) was calculated as:

$$LOQ(\mu g/g) = \frac{10 \times Standard\ deviation\ of\ intercept\ y}{Curve\ slope}$$

S.2.3 Precision and accuracy

- Accuracy, calculated as percentage recovery, was calculated by dividing the concentration of compound detected in the spiked sample to the concentration of standard added.

- Recovery rate (%): $\left(\frac{\text{Concentration of standard detected in sample,in } \mu g/g}{Concentration\ of\ standard\ added\ to\ the\ sample,in\ \mu g/g}\right) \times 100$

- Precision was calculated as relative standard deviation (RSD). The RSD was calculated by dividing the standard deviation of concentrations measured to the mean value.

- RSD (%): $\left(\frac{\text{Standard deviation of concentration ,in } \mu g/g}{Mean\ value\ of\ concentration\ measured,in\ \mu g/g}\right) \times 100$

- For accuracy and precision spiked extracts at three concentrations were tested 2.25, 12.5, 22.5 μg/g. Within day detection data were obtained analyzing 3 biological replicates of spiked samples in the same day. Between day detection data were obtained analyzing 2 biological replicates of spiked samples per 3 consecutive days.

Table S2.1. The calibration curve, correlation factor (R^2), limit of detection (LOD) and limit of quantification (LOQ).

Compound	Calibration curve	R^2	LOD (µg/g)	LOQ (µg/g)
Luteolinidin	(0.089±0.005)x+(0.117±0.009)	0.9995	0.3220	0.9759
7-methoxyapigenidin	(0.0587±0.001)x+(0.0601±0.0023)	0.9995	0.1312	0.3975
Cyanidin	(0.0551±0.0014)x+(-0.00955±0.0029)	0.9996	0.1736	0.5262
Apigenidin	(0.07±0.003)x+(0.038±0.0006)	0.9998	0.0266	0.0806
Protocatechuic acid	(0.044±0.0007)x+(0.0017±0.002)	0.9999	0.1482	0.4490
4-hydroxybenzoic acid	(0.0423±0.002)x+(0.024±0.002)	0.9993	0.1710	0.5182
Taxifolin	(0.05855±0.0002)x+(0.0246±0.0014)	0.9999	0.0797	0.2415
Quercetin	(0.0441±0.0001)x+(0.0031±0.003)	0.9998	0.2011	0.6093
Luteolin	(0.025±0.001)x+(0.0512±0.003)	0.9993	0.3889	1.1785
Naringenin	(0.0456±0.0008)x+(0.003±0.0009)	0.9999	0.0665	0.2016
Catechin	(0.034±0.002)x+(-0.0016±0.0013)	0.9976	0.1250	0.3788
Caffeic acid	(0.0534±0.001)x+(-0.0024±0.0025)	0.9999	0.1529	0.4635
Ferulic acid	(0.0452±0.0003)x+(0.0018±0)	0.9998	0.1314	0.3982
p-Coumaric acid	$(0.045\pm7\times10^{-5})x+(0.021\pm0.0004)$	0.9999	0.0314	0.0950
Chlorogenic acid	(0.0488±0.028)x+(0.0159±0.023)	0.9949	1.5493	4.6947
Apigenin	(0.0453±0.0021)x+(0.014±0.0017)	0.9996	0.1236	0.3746

Table S2.2. Within day and between day precision evaluated at (2.25, 12.5, 22.5 µg/g) in sorghum.

	Within-day precision (n=3 × 1 day)						Between-day precision (n=2 × 3 days)					
	2.25µg/g		*12.5µg/g*		*22.5µg/g*		*2.25µg/g*		*12.5µg/g*		*22.5µg/g*	
Compound	***RSD***	***Recovery***	***RSD***	***Recovery***	***RSD***	***Recovery***	***RSD***	***Recovery***	***RSD***	***Recovery***	***RSD***	***Recovery***
Luteolinidin	14.11	100.67	5.12	99.76	1.14	100.05	23.89	135.19	13.31	87.33	2.51	102.81
7-methoxyapigenidin	20.55	106.96	8.12	97.49	1.75	100.56	84.21	101.08	34.03	102.10	7.76	99.53
Cyanidin	18.87	111.88	7.94	95.72	1.67	100.95	4.87	125.54	2.43	90.81	0.48	102.04
Apigenidin	41.32	81.43	11.35	106.68	2.73	98.51	30.63	99.59	10.97	100.14	2.44	99.97
Protocatechuic acid	23.93	94.28	4.98	98.59	1.99	99.42	11.99	120.97	5.65	92.45	1.14	101.68
4-hydroxybenzoic acid	4.16	113.25	21.98	95.23	4.60	101.06	43.61	107.81	17.42	97.19	3.74	100.62
Taxifolin	32.77	76.41	8.31	108.49	2.04	98.11	18.84	94.01	9.45	104.20	1.30	99.59
Quercetin	20.06	113.21	8.58	95.24	1.80	101.06	2.65	92.97	1.32	61.82	2.08	72.64
Luteolin	8.61	101.43	3.18	99.49	0.69	100.11	20.56	135.16	8.38	85.53	5.62	100.78
Naringenin	14.22	115.36	6.25	94.47	1.30	101.23	14.98	117.81	6.79	93.59	1.39	101.43
Catechin	ND	ND	19.94	128.27	6.07	93.72	ND	ND	87.30	117.69	30.30	96.07
Caffeic acid	15.61	108.82	7.63	92.91	4.18	98.75	13.36	118.25	6.09	93.43	1.25	101.46
Ferulic acid	7.01	130.57	3.70	89.00	0.71	102.45	17.42	128.53	8.98	89.73	1.75	102.28
p-Coumaric acid	21.62	90.53	6.81	103.41	1.58	99.24	14.27	97.75	4.98	100.81	1.12	99.82
Chlorogenic acid	40.55	112.25	17.14	95.59	3.61	100.98	3.78	127.03	1.91	90.27	0.38	102.16
Apigenin	21.03	89.32	6.51	103.84	1.52	99.15	13.80	90.84	4.37	103.29	1.01	99.27

RSD - relative standard deviation (%).

Recovery rate (%)

ND – Not detected, under the equipment and methodology used.

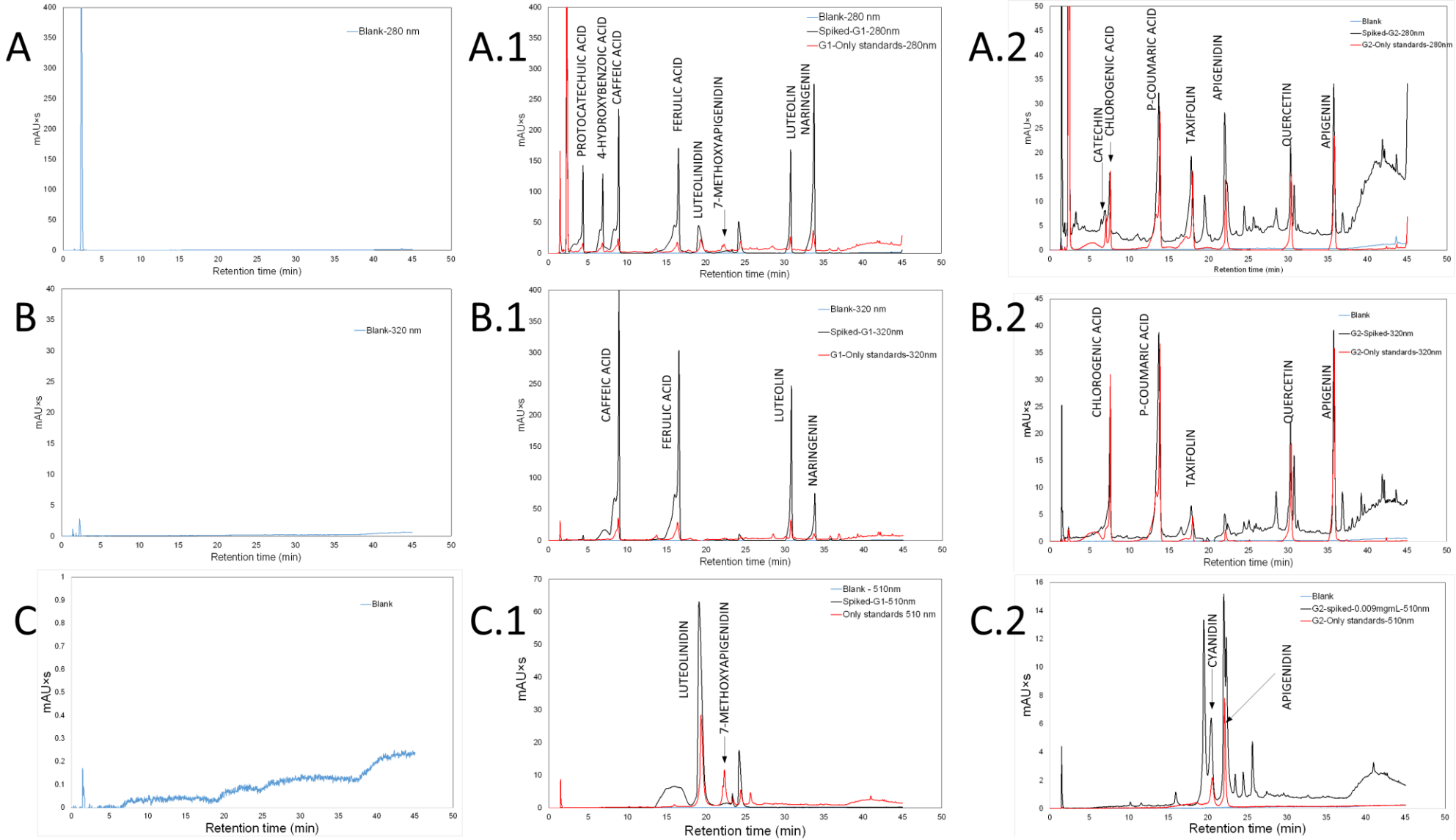


Figure S2.1 The representative chromatograms of blanks, the spiked extracts and the external standards recorded at 280 nm (A, A.1 and A.2), 320 nm (B, B.1 and B.2) and 510 nm (C, C.1 and C.2). Group 1 (or G1) of standards consisted of: protocatechuic acid, 4-hydroxybenzoic acid, caffeic acid, ferulic acid, luteolinidin, 7-methoxyapigenidin, luteolin and naringenin. Group 2 (or G2) of standards consisted of: catechin, chlorogenic acid, p-coumaric acid, taxifolin, cyanidin, apigenidin, quercetin, and apigenin. The concentration of standards was 0.009 mg/mL.

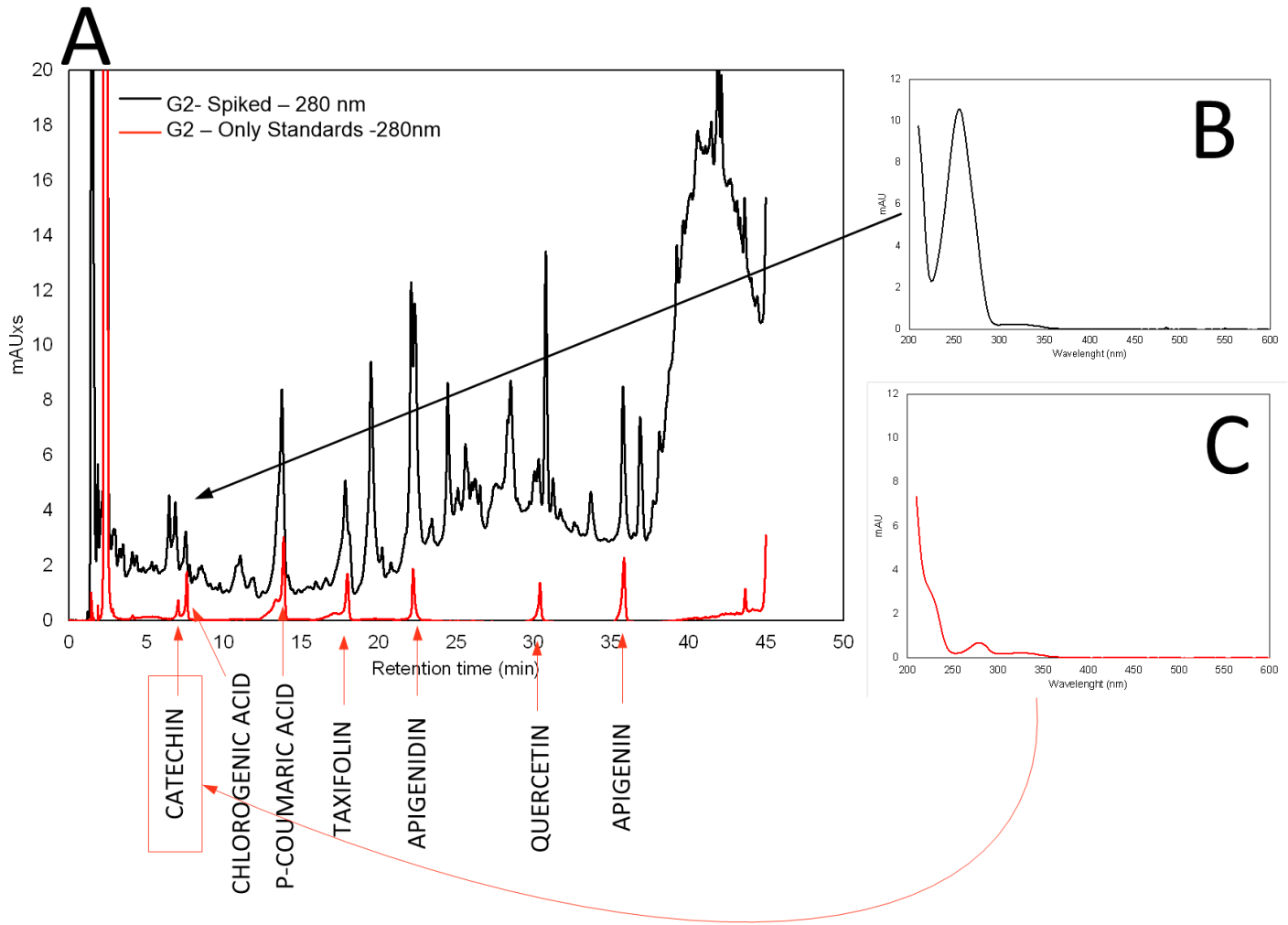


Figure S2.2 The representative chromatograms of spiked extracts and the external standards recorded at 280 nm for the Group 2 of standards (A) the spectrum of 4-hydroxybenzoic acid ((B) detected in the spiked sample and the spectrum of catechin standard (C). Group 2 (or G2) of standards consisted of: catechin, chlorogenic acid, p-coumaric acid, taxifolin, cyanidin, apigenidin, quercetin, and apigenin. The concentration of standards was 2.25µg/g.

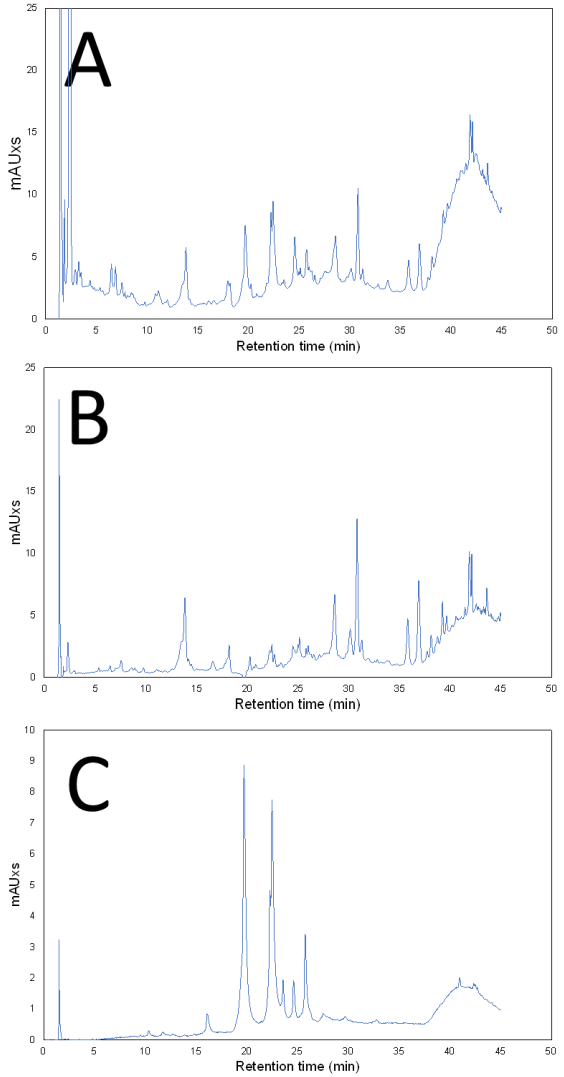


Figure S2.3 The representative chromatograms of non-spiked extract mixture used for method validation at 280 nm (A), 320 nm (B) and 510 nm (C).

S.2.4. Discussion and comparison with literature

For all studied compounds, the sorghum extracts spiked with phenolics presented linear behavior with correlation factor (R^2) of 0.999 (Table S2.1), which is consistent with the previous findings [23,24]. For p-coumaric acid, protocatechuic acid, ferulic acid and 4-hydroxybenzoic acid, the LOQ and LOD ranged around 0.031-0.131 µg/g and 0.095-0.518 µg/g, which is within the range observed by Irakli and coworkers [23]. The RSD of luteolin at 22.5 µg/g was lower than the 1.07-2.37% detected for the paprika samples spiking at 12.50-25 µg [25].

Recovery of chlorogenic acid during within- and between day was by approximately 2-fold higher than the 55.7 and 56.8% recovered by Parkes et al., respectively. Also, the within- and between day precision for quercetin observed by Parkes et al at resulted on recoveries of 21.3% and 33.1% [40].

At lowest concentration (2.25 µg/g, Table S2.2), there was not enough peak separation between catechin (spiked) and 4-hydroxybenzoic acid in sample (non-spiked). This can be visualized by 4-hydroxybenzoic acid over with spiked catechin (Figure S2.2B). By injecting external standards separately, we observed that catechin was detected by our equipment at 2.25 µg/g (Figure S2.2C). Representative chromatograms of non spiked extract mixture are available in Figure S2.3.

The main goal of our manuscript was to detect 3-deoxyanthocyanidins unique to sorghum among cereal crops. With respect to the 3-deoxyanthocyanidins, literature about validation of HPLC method for these substances is scarce, to the best of authors knowledge. Xiong et al. [2] , who validated HPLC coupled with mass spectrometry to detect phenolics in Australian sorghum genotypes for repeatability found the following RSD after 50-200 µg/mL spiking: 0.11-3.28% for luteolinidin, 0.07-4.82% for apigenidin, and 3.52-4.79% 7-methoxyapigenidin, which is comparable to our results after spiking at 12.5 µg/g and 22.5 µg/g (Table S2.2).

References (as mentioned in manuscript):

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24 Xiong, Y.; Zhang, P.; Warner, R.D.; Shen, S.; Johnson, S. HPLC-DAD-ESI-QTOF-MS/MS qualitative analysis data and HPLC-DAD quantification data of phenolic compounds of grains from five Australian sorghum genotypes. *Data Br.* **2020**, *33*, 106584.

25 Bae, H.; Jayaprakasha, G.K.; Jifon, J.; Patil, B.S. Extraction efficiency and validation of an HPLC method for flavonoid analysis in peppers. *Food Chem.* **2012**, *130*, 751–758, doi:https://doi.org/10.1016/j.foodchem.2011.07.041.

40. Parkes, R.; McGee, D.; McDonnell, A.; Gillespie, E.; Touzet, N. Rapid screening of phenolic compounds in extracts of photosynthetic organisms separated using a C18 monolithic column based HPLC-UV method. *J. Chromatogr. B* **2022**, *1213*, 123521, doi:https://doi.org/10.1016/j.jchromb.2022.123521.