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VALIDATION OF AN LC-MS/MS METHOD FOR THE QUANTIFICATION OF POLYPHENOLIC COMPOUNDS IN CLOVER SEEDS

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Abstract: This study aimed to validate an LC-MS/MS method for the determination of 43 polyphenolic compounds in clover seeds. Compounds were extracted using ultrasound-assisted extraction with methanol–water and analysed using an Agilent 1200 HPLC system coupled to an Agilent 6410 triple quadrupole mass spectrometer in positive and negative electrospray ionization modes. Separation was performed on a C18 column, with optimized multiple reaction monitoring transitions and collision energies.

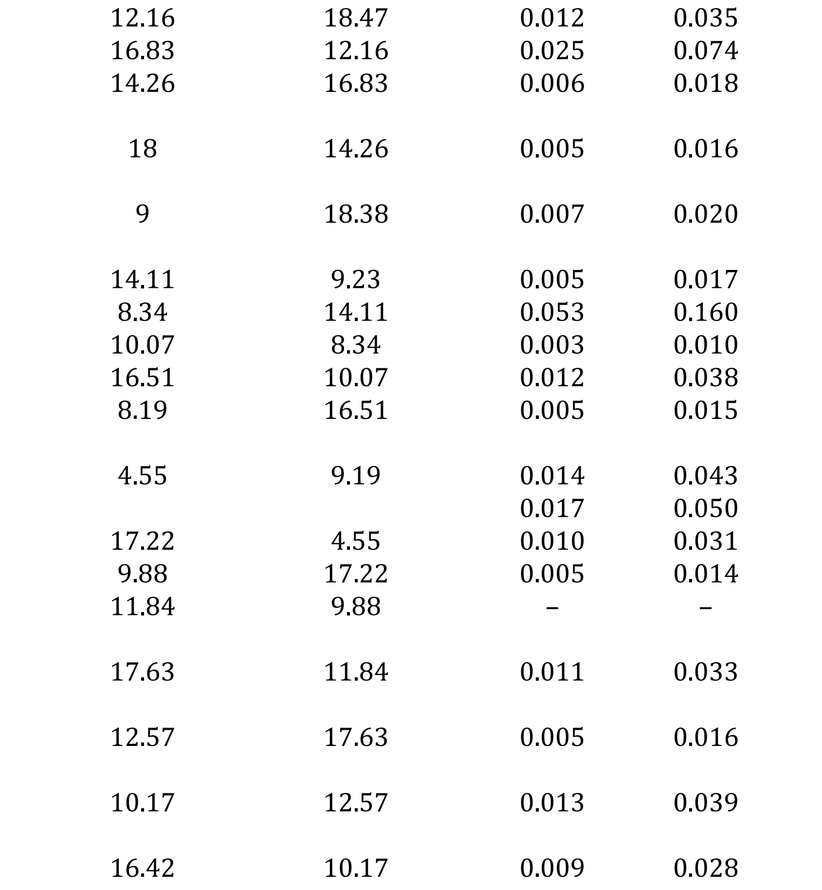
Material and method

The homogenized clover seeds (1 g) was soaked in 10 mL of MeOH and sonicated for 60 min at 55 °C. The supernatant was removed after the centrifugation (4000 rpm for 5 min). The aliquot was evaporated to dryness and reconstituted in 0.5 mL of the mobile phase and inject in the LC-MS/MS.

Results and discussions: The linearity: 9 concentration levels in the range of 0.010–2.5 µg/mL for the compounds determined in ESI− and at up to five concentration levels in the range of 0.025–1.0 µg/mL for the compounds in ESI+. Quadratic regression was applied to the compounds determined in ESI− mode, and linear regression to those determined in ESI+ mode. All compounds showed R^2 values ≥ 0.993, confirming a good fit of the calibration models within the investigated concentration ranges. For the compounds determined in ESI- mode, LOD and LOQ were estimated by linear regression over the lower part of the calibration range (0.010-0.25 µg/mL, five levels), since the residuals of the full-range curves are dominated by the highest calibration levels, whereas the full linear calibration curves were used for the compounds determined in ESI+ mode. The LOD values ranged from 0.003 to 0.088 µg/mL and the LOQ values from 0.010 to 0.268 µg/mL.

Conclusions: An LC-MS/MS method for the simultaneous determination of 43 polyphenolic compounds in clover seeds was developed and validated. Optimization of the MRM transitions in both negative and positive electrospray ionization modes enabled selective identification of phenolic acids, flavonoids, isoflavones, stilbenes, coumarins and anthocyanins within a single 25-min chromatographic run. The calibration curves showed a good fit (R^2 ≥ 0.993) over the investigated concentration ranges, confirming the suitability of the method for quantitative analysis. Combined with a simple ultrasound-assisted extraction, the proposed method can be applied to the chemical characterization of clover seeds and to comparative studies of the phenolic composition of different *Trifolium* species, cultivars and growing conditions.

Compound	Prec Ion	Prod Ion	Frag (V)	CE (V)	Ret Time (min)	Repeatability (RSD, %)	LOD (µg/mL)	LOQ (µg/mL)
Negative ionization mode (ESI−)								
4-Hydroxybenzoic acid	137	93	100	10	16.20	16.20	0.007	0.023
Apigenin	268.9	151	135	36	12.14	12.14	0.005	0.015
	268.9	117	135	44				
Benzoic acid	121	77	100	12	3.63	3.63	0.058	0.174
Caffeic acid	179	135	100	10	18.06	18.06	0.022	0.066
	179	107	100	20				
Catechin	289	245	100	10	15.56	16.47	0.018	0.054
	289	203	100	10				
Chlorogenic acid	353	191	100	10	10.64	15.56	0.010	0.029
Ellagic acid	301	145	100	35	14.19	10.64	0.011	0.034
Epicatechin	289	245	100	10	8.33	14.19	0.013	0.041
	289	109.2	104	20				
Epigallocatechin	304.9	179	135	20	17.97	8.33	0.013	0.038
	304.9	124.9	135	26				
Epigallocatechin gallate	440.9	289	135	28	17.86	17.97	–	–
	440.9	169	135	22				
Esculetin	339.1	176.7	100	20	18.47	17.86	0.026	0.079
Gallic acid	169	125	100	10	12.16	18.47	0.012	0.035
Juglone	189	161	100	20	16.83	12.16	0.025	0.074
Kaempherol	285	285	100	0	14.26	16.83	0.006	0.018
	285	239	100	20				
Luteolin	285.1	217	100	20	18	14.26	0.005	0.016
	285.1	133.2	100	20				
Myricetin	317	179	100	20	9	18.38	0.007	0.020
	317	151	100	20				
Naringenin	271	151	100	16	14.11	9.23	0.005	0.017
Naringin	579	271.1	140	10	8.34	14.11	0.053	0.160
p-Coumaric acid	163	119	100	9	10.07	8.34	0.003	0.010
Protocatechuic acid	153	109	100	9	16.51	10.07	0.012	0.038
Quercetin	301	179	100	15	8.19	16.51	0.005	0.015
	301	151	100	15				
Rasveratol	227	185	100	20	4.55	9.19	0.014	0.043
Resveratrol	227	143	100	30			0.017	0.050
Rutin	609	300	100	42	17.22	4.55	0.010	0.031
Salicylic acid	137	93	100	20	9.88	17.22	0.005	0.014
Sinapinic acid	223	208	100	17	11.84	9.88	–	–
	223	193	100	17				
Syringic acid	197	182	100	7	17.63	11.84	0.011	0.033
	197	181.9	100	20				
Taxifolin	303.7	284.8	100	14	12.57	17.63	0.005	0.016
	302.7	124.9	100	26				
trans-Cinnamic acid	147	103	100	14	10.17	12.57	0.013	0.039
	147	77	100	28				
trans-Ferulic acid	193	178	100	18	16.42	10.17	0.009	0.028
	193	134	100	11				
Vanillic acid	167	152	100	18	12.35	12.35	0.023	0.070
	167	108	100	15				
Positive ionization mode (ESI+)								
Cyanidin	287	213	100	42	9.17	9.17	0.043	0.129
	287	185	100	43				
Cyanidin-3-rutinoside	595.2	287.1	100	36	11.98	11.98	0.031	0.095
	595.2	137.1	100	92				
Daidzin	417.1	255	100	20	10.48	10.48	**0.045**	0.136
	417.1	199	100	52				
Delphinidin	303	229	100	43	17.09	17.09	**0.013**	**0.038**
	303	201	100	43				
	303	173	100	43				
Delphinidin-3-rutinoside	611.2	303.1	100	32	18.07	**18.07**	**0.013**	**0.041**
	611.2	229.1	100	92				
Genistin	433.1	271	96	20	14.88	**14.88**	**0.035**	**0.105**
	433.1	153	96	60				
Glycitil	447.2	285	101	12	10.11	**10.11**	**0.051**	**0.154**
	447.2	270	101	52				
Malvidin-3-glucoside	493.1	331.1	116	24	**13.12**	**13.12**	**0.014**	**0.044**
Naringin	581	272.8	120	21	**16.07**	**16.07**	**0.053**	**0.160**
	581	152.8	120	65				
Petunidin- 3-glucoside	479.1	317.1	108	24	**8.99**	**8.99**	**0.034**	**0.105**
	479.1	302.1	108	52				
Umbeliferone	163	107	100	40	**5.41**	**5.41**	**0.029**	**0.088**



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