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Research Article

Validated UHPLC-HRMS method for simultaneous quantification of six saponins from the roots of the wild spinach (*Chenopodium bonus-henricus* L.)

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Abstract

An UHPLC-HRMS method for simultaneous quantification of six saponins from the roots of *Chenopodium bonus-henricus* L. was developed and validated. All calibration curves showed very good linear regressions and the correlation coefficients were $R^2 > 0.99$. The limits of detection and quantitation limits ranged from 0.20 to 0.61 ng/mL and from 0.61 to 1.85 ng/mL, respectively. A good agreement between the spiked and determined concentrations indicated acceptable accuracy. Besides, the related compounds showed overall recoveries ranging from 95.38% to 103.47% with RSD ranging from 0.64% to 4.25%. The intra-day and inter-day precision were determined by analyzing the retention times and recovery of the calibrants. The saponins of medicagenic acid (**3**), 2β-hydroxygypsogenin (**4**), and bayogenin (**2**) were the predominant compounds and reached 15.01%, 3.87%, 2.41% in the crude EtOH extract and 43.69%, 16.16%, 10.07% in the purified EtOH extract, respectively.

Keywords

Amaranthaceae, *Chenopodium bonus-henricus*, quantification, saponins, UHPLC-HRMS

Introduction

The genus *Chenopodium* (Amaranthaceae) numbers a wide range of species (more than 200) and is native to all the continents with exception of Antarctica as well as in some distant archipelagoes (such as Juan Fernandez, New Zealand, and Hawaii) (Nedialkov and Kokanova-Nedialkova 2021). *Chenopodium bonus-henricus* L. (Amaranthaceae) is a perennial herbaceous plant and is spread in the mountainous regions of Bulgaria (Grozeva 2011). The leaves and flowering tops of Good King Henry are used as a vegetable in the same manner as spinach in some European countries. In Bulgarian folk medicine the extracts of the roots have been used for the treatment of bronchitis, laryngitis, rheumatism, gout, constipation, dermatitis, and eczema. A decoction of the roots of *C. bonus-henricus* (also known as "chuven") is used in the food industry to produce "tahin" and "white halva" (Kokanova-Nedialkova et al. 2019a). Recently phytochemical investigation of the roots of *C. bonus-henricus* L. led to the isolation of six saponins of phytolaccagenin, 2β-hydroxyoleanoic acid, bayogenin, 2β-hydroxygypsogenin and medicagenic acid (Kokanova-Nedialkova et al. 2019a). The MeOH extract together with the saponins of

a title plant have exerted hepatoprotective and antioxidant activities comparable to those of silymarin in an *in vivo* and *in vitro* models of CCl_4-induced liver damage, respectively (Kokanova-Nedialkova et al. 2019b). The MeOH extract and saponins showed moderate or marginal cytotoxicity on five leukemic cell lines (HL-60, SKW-3, Jurkat E6-1, BV-173, and K-562) and stimulatory effects on interleukin-2 production in PHA/PMA stimulated Jurkat E6-1 cells (Kokanova-Nedialkova et al. 2019a). The application of the roots of *C. bonus-henricus* in Bulgarian folk medicine and the food industry for the production of "tahin" and "white halva" stimulated us for creating a UHPLC-HRMS method for simultaneous quantification of the main saponins.

Material and methods

Apparatus, materials, and chemicals

UHPLC-HRMS analysis was performed using a Thermo Scientific Dionex Ultimate 3000 RSLC (Germering, Germany) consisting of 6-channel degasser SRD-3600, high-pressure gradient pump HPG-3400RS, autosampler WPS-3000TRS, and column compartment TCC-3000RS coupled to Thermo Scientific Q Exactive Plus (Bremen, Germany) mass spectrometer. Column chromatography (CC) was achieved on Diaion HP-20 (Supelco, USA). All the reagents used were of analytical grade. The main saponins (purity 95–96%), Bonushenricoside A (3-O-α-L-arabinopyranosyl-phytolaccagenin-28-O-β-D-glucopyranosyl ester) (**1**), 3-O-β-glucuronopyranosyl-bayogenin-28-O-β-glucopyranosyl ester (**2**), 3-O-β-glucuronopyranosyl-medicagenic acid-28-β-xylopyranosyl(1→4)-α-rhamnopyranosyl(1→2)-α-arabinopyranosyl ester (**3**), 3-O-β-glucuronopyranosyl-2β-hydroxygypsogenin-28-O-β-glucopyranosyl ester (**4**), 3-O-L-α-arabinopyranosyl-bayogenin-28-O-β-glucopyranosyl ester (**5**), Bonushenricoside B (3-O-β-D-glucuronopyranosyl-2β-hydroxyoleanoic acid-28-O-β-D-glucopyranosyl ester)(**6**) were previously isolated from the roots of *C. bonus-henricus* L. (Kokanova-Nedialkova et al. 2019a).

Plant material

The roots of *Chenopodium bonus-henricus* L. were collected from Beglica, Western Rhodopes, Bulgaria in September 2017. The plant was identified by P. Nedialkov and a voucher specimen from the plant population (No. SOM-Co-169848) was deposited at the National Herbarium, Bulgarian Academy of Sciences, Sofia, Bulgaria.

Preparation of crude EtOH extract (CEE)

The roots of *C. bonus-henricus* were dried in the shade, and the powdered plant material (200 mg) was extracted with 70 vol. % EtOH (2×30 mL) by ultrasonic-assisted extraction. After filtration, the EtOH extracts were diluted to 100 mL 70 vol. % EtOH. The resulting solution was filtered again and the first 10 mL were removed. An aliquot (10 mL) of this solution was evaporated to dryness, then dissolved in water and further purified by solid-phase extraction over RP_{18}. The sorbent was first washed with H_2O, then eluted with 70 vol. % EtOH (12 × 500 μL) in a 10.0 mL volumetric flask and diluted to the nominal volume with the same solvent (solution A). Subsequently, 1 mL of solution A was diluted to 100 mL 70 vol. % EtOH (solution B). Solution B was used for LC-MS quantification of saponins in the crude EtOH extract (CEE).

Preparation of purified EtOH extract (PEE)

The powdered plant material (40 g) was extracted subsequently with absolute EtOH (5 × 300 mL) and 70 vol. % EtOH (5 × 300 mL) by ultrasonic-assisted extraction. After filtration, the EtOH extracts were combined and the solvent was evaporated under reduced pressure to give 20.43 g white-yellow residue. For further purification, EtOH extract was dissolved in 200 mL H_2O and then subjected to CC over Diaion HP-20 (30 × 4 cm) with H_2O (1 L) and 90 vol. % MeOH (1 L). The 90% MeOH eluate was evaporated to dryness in a rotary evaporator to give 6.70 g whitish to creamy powder referred to as a PEE that was further used for LC-MS quantification of saponins. PEE (50 mg) was dissolved in 50 mL 70 vol. % EtOH (solution A). Subsequently, 5 mL of solution A was diluted to 25 mL 70 vol. % EtOH (solution B). Further, 1 mL of solution B was diluted to 100 mL 70 vol. % EtOH (solution C). Solution C was used for LC-MS quantification of saponins.

UHPLC chromatographic conditions

UHPLC separations were performed on a Kromasil Eternity XT C18 column (AkzoNobel, Sweeden) (2.1 × 100 mm, 1.8 μm) equipped with precolumn SecurityGuard ULTRA UHPLC EVO C18 (Phenomenex, USA) at 40 °C. Each chromatographic run was carried out with a binary mobile phase consisting of water containing 0.1% (v/v) formic acid (A) and acetonitrile also with 0.1% (v/v) formic acid (B). A gradient program was used as follows: 0–1 min, 15% B; 1–3 min, 15–25% B; 3–13 min, 25–40% B; 13–15 min, 40–95% B; 15–18 min, 95% B. The flow rate was 0.3 mL. min^{-1} and the sample injection volume was 2 μL.

High resolution electrospray ionization mass spectrometry (HRESIMS) conditions

Operating conditions for the HESI source used in a negative ionization mode were: -2.5 kV spray voltage, 320 °C capillary and probe heater temperature, sheath gas flow rate 38 a.u., auxiliary gas flow 12 a.u. (a.u. refer to arbitrary values set by the Exactive Tune software) and S-Lens RF

level 50.00. Nitrogen was used for sample nebulization and collision gas in HCD cell. The full MS mode was used as an MS experiment where the resolution, AGC target, max. IT and mass range were 70000 (at *m/z* 200), 3e6, 200 ms, and *m/z* 300–1500, respectively. Xcalibur software ver. 4.0 was used for data acquisition and processing.

Method validation

The quantification of saponins was carried out using the external standard method. The amounts of saponins are relative and not absolute. Each of the saponins was dissolved in 10 mL 70 vol. % EtOH (primary solutions). The stock standard solution of six saponins was prepared by combining the aliquots (1 mL) of each primary solution and dilution to 10 mL with 70 vol. % EtOH. It was stored in the refrigerator at 4 °C. The working standard solutions of appropriate concentration were prepared by diluting the stock standard solution with 70 vol. % EtOH. External standard calibrations were established on five data points covering the concentration range of 2.016–1260 ng/mL for (**1**) and (**2**), 2–1250 ng/mL for (**3**), 1.792–1120 ng/mL for (**4**), 1.984–1240 ng/mL for (**5**), and 2.048–1280 ng/mL for (**6**).

The limit of detection (LOD) of an analytical procedure is the lowest analytical concentration at which an analyte(s) could be detected qualitatively. Typically, peak heights are two or three times the noise level. The quantitation limit (LOQ) is also the lowest concentration at that level analyte can be quantitated with acceptable precision, requiring peak heights 10 to 20 times higher than the baseline noise. This signal-to-noise ratio is a good rule of thumb. Limits of detection (LODs) were calculated according to the expression 3.3 σ/S, where σ was the standard deviation of the response and S the slope of the calibration curve. Limits of quantification (LOQs) were established from the expression 10 σ/S (ICH 2005; Kazusaki et al. 2012).

Accuracy is the closeness of the analytical results obtained by the analyses to the true values and usually presented as a percent of nominal (ICH 2005; Kazusaki et al. 2012). The accuracy of analytes was evaluated by applying the entire extraction procedure to a control plant matrix that had been spiked with a standard solution of analytes at three concentrations close to that expected in the real plant samples. The accuracy data was recorded as percent recovery of the spiked concentration with relative standard deviations. Each solution was tested in triplicate.

The precision of an analytical method is the amount of variation in the results obtained from multiple analyses of the homogeneous samples. Intra-day precision (repeatability), defines the precision obtained using the same operating conditions over a designated short period (typically ≤1 day). Inter-day precision (intermediate precision), defines the precision obtained using the same operating conditions, typically within the same laboratory, over a designated period (typically ≥1 day) (ICH 2005; Borman and Elder 2017). The intra-day and inter-day precision were determined by analyzing the calibration samples during a single day and on three different days, respectively. The intra-day variation was determined by analyzing the nine replicates on the same day and the inter-day variation was determined on three consecutive days. The retention times (RT) and recovery were obtained for the assayed compounds. The relative standard deviation (RSD) was taken as a measure of precision.

Results and discussion

Ultra-high performance liquid chromatography – high-resolution mass spectrometry (UHPLC-HRMS) was used to detect the saponins in the crude and purified EtOH extracts of *C. bonus-henricus* roots in this work.

The efficiency of the extraction procedure was tested by using different solvents (80 vol. % MeOH, 50 vol. % MeOH, 70 vol. % EtOH, and 50 vol. % EtOH) and techniques (ultrasonic-assisted and magnetic stirring extractions). The results showed that the highest extraction efficiency was achieved using ultrasonic-assisted extraction with 70% EtOH.

The excellent selectivity of solid-phase extraction over RP_{18} and column chromatography over Diaion HP-20 for the preparation of crude and purified EtOH extracts helped to improve the LC-MS analysis.

The chromatographic conditions were optimized to achieve effective separation, symmetric peak shape, and short run time. Two chromatographic columns, Kromasil Eternity XT C18 column (2.1 × 100 mm, 1.8 μm) and Phenomenex Kinetex EVO C18 (100 × 2.1 mm, 1.7 μm) were pretested, and the best separation efficiency was obtained with the Kromasil Eternity XT C18 column. Acetonitrile was selected as the mobile phase due to its improved separation, and reduced column backpressure compared with methanol. The addition of formic acid in the mobile phase improved the peak shape, sensitivity, and retention time of individual saponins, which was consistent with the previous report (Kokanova-Nedialkova et al. 2020).

Quantitative determination of the main saponins in the roots of *C. bonus-henricus* L. was performed by the method of the external standard. Six previously isolated saponins from a title plant were used as external standards (Kokanova-Nedialkova et al. 2019a) (Fig. 1).

The calibration curves were linear over the concentration range of 2.016–1260 ng/mL for (**1**) and (**2**), 2–1250 ng/mL for (**3**), 1.792–1120 ng/mL for (**4**), 1.984–1240 ng/mL for (**5**), and 2.048–1280 ng/mL for (**6**). All

Table 1. Linearity of calibration curve for the six saponins.

Marker compound	Linear range (ng/mL)	Regression equations	R^2	LOD (ng/mL)	LOQ (ng/mL)
1	2.016–1260	Y = 70407.4 + 53568.2X	0.9976	0.20	0.61
2	2.016–1260	Y = -188231 + 62786.2X	0.9996	0.21	0.64
3	2.000–1250	Y = -174018 + 23140.9X	0.9986	0.61	1.85
4	1.792–1120	Y = -94568 + 43512.4X	0.9987	0.42	1.28
5	1.984–1240	Y = 121137 + 66095.8X	0.9993	0.53	1.62
6	2.048–1280	Y = -41183.7+13884.9X	0.9997	0.53	1.60

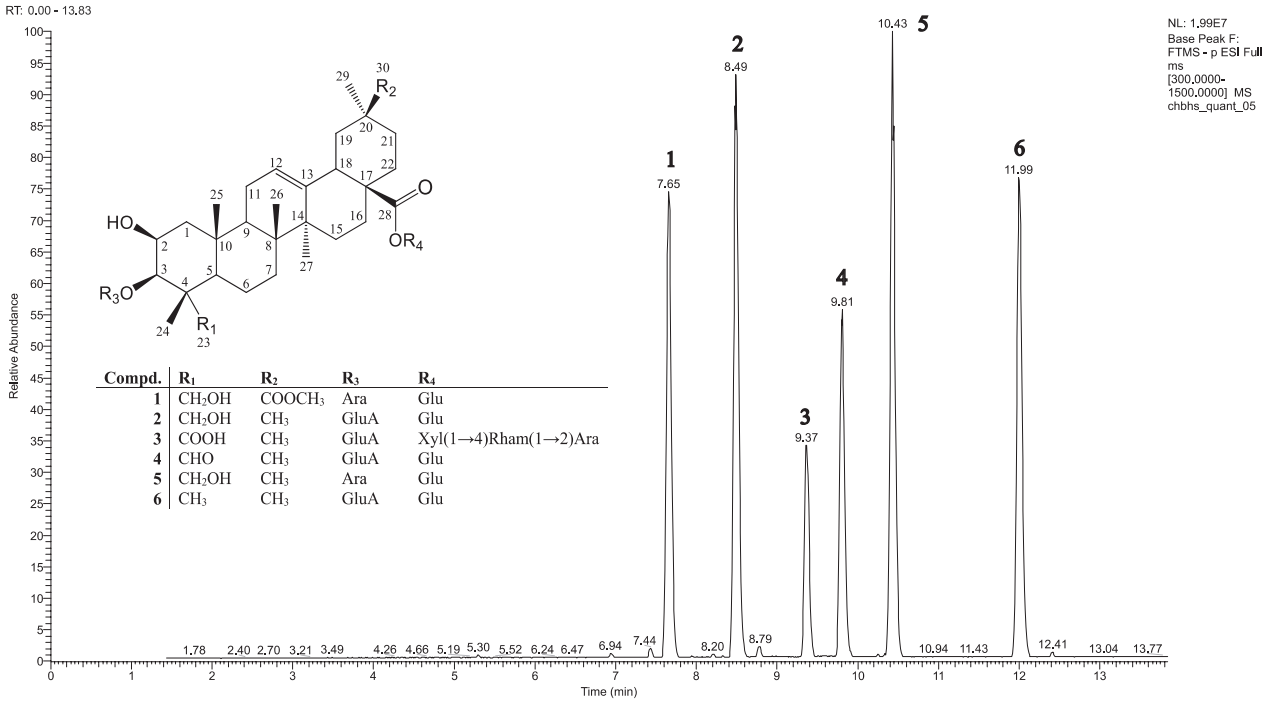

Figure 1. A chromatogram of the standard mixture of saponins.

calibration curves showed very good linear regressions and the correlation coefficients were $R^2 > 0.99$ (Table 1).

The method showed that LODs and LOQs were 0.20 ng/mL and 0.61 ng/mL (**1**), 0.21 ng/mL and 0.64 ng/mL (**2**), 0.61 ng/mL and 1.85 ng/mL (**3**), 0.42 ng/mL and 1.28 ng/mL (**4**), 0.53 ng/mL and 1.62 ng/mL (**5**), and 0.53 ng/mL and 1.60 ng/mL (**6**), respectively (Table 1).

The accuracy of the analytes (**1**–**6**) was checked by addition of a standard solution mixture at three concentrations (126, 252, and 378 ng/mL for (**1**) and (**2**); 125, 250 and 375 ng/mL for (**3**); 112, 224 and 336 ng/mL for (**4**); 124, 248 and 372 ng/mL for (**5**); 108, 216 and 324 ng/mL for (**6**)) close to that expected in the real plant samples. Blank samples from the same unspiked plant material were analyzed at the same time as the spiked samples and the measured values were subtracted. Besides, the related compounds showed overall recoveries ranging from 95.38% to 103.47% with RSD ranging from 0.64% to 4.25%. A good agreement between the spiked and determined concentrations indicated acceptable accuracy (Table 2).

The precision of the retention times was determined by analyzing the calibration samples during a single day and on three different days, respectively. The RSDs of retention times of the analytes were ≤ 0.10 for intra-day and ≤ 0.11 for inter-day precision assays, respectively. Also, the related compounds showed overall recoveries ranging from 95.02% to 98.71% (for intra-day and inter-day precision assays) with RSDs from 1.57% to 3.18%. (Tables 3, 4).

Table 2. Accuracy of the UHPLC-HRMS method.

Saponins	Added (ng/mL)	Found[a] (ng/mL)	Recovery[a] (%)	RSD (%)
1	126	120.18 ± 0.77	95.38 ± 0.61	0.64
	252	241.18 ± 6.29	95.70 ± 2.50	2.61
	378	383.20 ± 3.44	101.38 ± 0.91	0.90
2	126	124.76 ± 5.30	99.02 ± 4.20	4.25
	252	241.24 ± 4.49	95.73 ± 1.78	1.86
	378	373.41 ± 4.94	98.79 ± 1.31	1.32
3	125	128.83 ± 2.92	103.07 ± 2.34	2.27
	250	258.00 ± 3.08	103.20 ± 1.23	1.19
	375	374.82 ± 3.56	99.95 ± 0.95	0.95
4	112	113.68 ± 4.11	101.50 ± 3.67	3.61
	224	218.79 ± 7.32	97.67 ± 3.27	3.35
	336	347.65 ± 7.34	103.47 ± 2.18	2.11
5	124	121.40 ± 1.65	97.90 ± 1.33	1.36
	248	242.44 ± 4.29	97.76 ± 1.73	1.77
	372	374.02 ± 7.19	100.54 ± 1.93	1.92
6	108	103.90 ± 1.84	96.20 ± 1.70	1.77
	216	207.56 ± 4.82	96.09 ± 2.23	2.32
	324	322.35 ± 7.13	99.49 ± 2.20	2.21

[a] Values are the mean ± SD (n = 3).

Table 3. Evaluation of intra-day precision (repeatability) of the UHPLC-HRMS method.

Compds.	Intra-day precision (repeatability)			
	RT ± SD (min)	RSD	Recovery ± SD (%)	RSD (%)
1	7.68 ± 0.008	0.10	97.38 ± 2.68	2.75
2	8.51 ± 0.006	0.07	96.36 ± 2.66	2.76
3	9.38 ± 0.007	0.07	95.25 ± 2.22	2.33
4	9.83 ± 0.004	0.04	95.07 ± 2.68	2.82
5	10.45 ± 0.006	0.05	98.71 ± 2.61	2.64
6	12.03 ± 0.007	0.06	96.71 ± 2.69	2.78

Table 4. Evaluation of inter-day precision (intermediate precision) of the UHPLC-HRMS method.

Compds.	Inter-day precision (intermediate precision)			
	RT±SD (min)	RSD	Recovery ± SD (%)	RSD (%)
1	7.67 ± 0.007	0.09	95.89 ± 1.90	1.98
2	8.51 ± 0.008	0.10	95.59 ± 3.04	3.18
3	9.38 ± 0.010	0.11	95.29 ± 2.66	2.79
4	9.83 ± 0.008	0.08	95.02 ± 2.81	2.95
5	10.45 ± 0.008	0.08	96.74 ± 1.52	1.57
6	12.02 ± 0.006	0.05	95.17 ± 1.63	1.72

Table 5. Content of saponins in the crude EtOH extract (CEE) and purified EtOH extract (PEE) from the roots of *C. bonus-henricus* L.

Compds.	Saponins	Content of saponins (%)	
		CEE	PEE
1	**Bonushenricoside A** (3-O-α-L-arabinopyranosyl-phytolaccagenin-28-O-β-D-glucopyranosyl ester)	1.66 ± 0.004	6.19 ± 0.21
2	3-O-β-glucuronopyranosyl-bayogenin-28-O-β-glucopyranosyl ester	2.41 ± 0.03	10.07 ± 0.39
3	3-O-β-glucuronopyranosyl-medicagenic acid-28-β-xylopyranosyl(1→4)-α-rhamnopyranosyl(1→2)-α-arabinopyranosyl ester	15.01 ± 0.12	43.69 ± 1.79
4	3-O-β-glucuronopyranosyl-2β-hydroxygypsogenin-28-O-β-glucopyranosyl ester	3.87 ± 0.005	16.16 ± 0.65
5	3-O-L-α-arabinopyranosyl-bayogenin-28-O-β-glucopyranosyl ester	0.75 ± 0.007	2.46 ± 0.03
6	**Bonushenricoside B** (3-O-β-D-glucuronopyranosyl-2β-hydroxyoleanoic acid-28-O-β-D-glucopyranosyl ester)	1.20 ± 0.01	5.46 ± 0.13
The total content of saponins (%)		24.90	84.02

The developed UHPLC-HRMS method was applied for quantification of the main saponins (**1**–**6**) in the crude and purified EtOH extracts from the roots of *C. bonus-henricus* L. The results show that the saponins of medicagenic acid (**3**), 2β-hydroxygypsogenin (**4**), and bayogenin (**2**) were the predominant compounds and reached 15.01%, 3.87%, 2.41% in the crude EtOH extract and 43.69%, 16.16%, 10.07% in the purified EtOH extract, respectively (Table 5). The glycosides of phytolaccagenin (**1**) and 2β-hydroxyoleanoic acid (**6**) were found in smaller quantities. The content of saponins (**1**) and (**6**) were from 1.66% and 1.20% in CEE to 6.19% and 5.46% in PEE, respectively. The saponin (**5**) was found in the smallest amount (0.75% in CEE and 2.46% in PEE). The total amount of assayed saponins was estimated to be 24.90% in CEE and 84.02% in PEE (Table 5).

Conclusions

A novel UHPLC-HRMS method for simultaneous quantification of six saponins from the roots of the wild spinach (*Chenopodium bonus-henricus* L.) was developed. The optimized method was validated for specificity, the limit of detection and quantitation limit, linearity, accuracy, and precision. The saponins of medicagenic acid (**3**), 2β-hydroxygypsogenin (**4**), and bayogenin (**2**) were the predominant compounds. The total amount of assayed saponins was estimated to be 24.90% in the crude EtOH extract and 84.02% in the purified EtOH extract, respectively. The results demonstrate that the proposed method can be readily utilized for the quantitative determination of saponins in *C. bonus-henricus* roots and could be useful for the assurance of the quality of the herbal drug used in the food industry in the production of "tahin" and "white halva".

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