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Research Article

Validated UHPLC-HRMS method for simultaneous quantification of flavonoid contents in the aerial parts of *Chenopodium bonus-henricus* L. (wild spinach)

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Abstract

A UHPLC-HRMS method for simultaneous quantification of flavonoid contents in the aerial parts of *Chenopodium bonus-henricus* L. was developed and validated. The amount of 12 detected flavonoids was calculated relative to external standard hyperoside. The calibration curve of hyperoside showed very good linear regressions and the correlation coefficient was $R^2 > 0.9979$. The limits of detection and quantitation limits were 0.39 ng/mL and 1.17 ng/mL, respectively. The UHPLC-HRMS method showed acceptable accuracy. At three different concentrations the recoveries of hyperoside ranging from 99.63% to 100.70% with RSD from 1.58% to 2.31%. The intra-day and inter-day precision were determined by analyzing the retention times and recovery of the external standard. The glycosides of spinacetin and patulenin (**1**) were the predominant compounds in the wild spinach which contents ranging from 1.79 to 4.41 mg g^{-1} D.W., calculated as hyperoside. The total amount of flavonoids was found to be 15.12 mg g^{-1} D.W.

Keywords

Amaranthaceae, *Chenopodium bonus-henricus*, flavonoids, quantification, 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. is widespread in Europe, Western Asia, and North America. The leaves and flowering tops of Good King Henry (*C. bonus-henricus* L.) are used as a vegetable similar to spinach in some European traditional cuisines. In Italy, Spain, and England it is used in soups or stews and roughly in salads. In Turkey, it is known as "yabanı ispanak" (wild spinach). Canadians have also cultivated the plant as a daily vegetable. The shoots and flower clusters are eaten like asparagus and broccoli, respectively (Kokanova-Nedialkova et al. 2017). Nine flavonol glycosides of patuletin, 6-methoxykaempferol, and spinacetin were isolated from the aerial part of *C. bonus-henricus*. All flavonoids (100 μM), compared to silibinin (100 μM), significantly reduced the cellular damage caused by CCl_4 in rat hepatocytes, preserved cell viability and GSH level, de-

creased LDH leakage, and reduced lipid damage. High concentrations of compounds showed marginal or no cytotoxicity on the HepG2 cell line (Kokanova-Nedialkova et al. 2017). Besides, these flavonoids possessed DPPH (2,2-diphenyl-1-picrylhydrazyl) and ABTS (2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)) radical-scavenging activity as well as significantly inhibited the lipid peroxidation in a linoleic acid system by the ferric thiocyanate method (Kokanova-Nedialkova and Nedialkov 2017). Additionally, the glycosides of patuletin, 6-methoxykaempferol, and spinacetin from *C. bonus-henricus* L. were investigated for neuroprotective, anti-α-glucosidase, and lipase activities. All tested flavonoids (100 μM) showed statistically significant neuroprotective activities on isolated rat brain synaptosomes using a 6-hydroxydopamine *in vitro* model. They preserved synaptosome viability as well as the reduced glutathione level. Anti-α-glucosidase and lipase activities of the tested compounds were established by measuring the levels of the released 4-nitrophenol using LC-MS. Patuletin glycosides possessed similar activity to acarbose. All flavonoids exhibited prolipase activity and could be used in the treatment of cachexia. The most active were flavonoids, which contain esterified ferulic acid (Kokanova-Nedialkova et al. 2020). Recently, a UHPLC-HRMS profiling method was used for a comprehensive study of flavonoid and saponin-rich fractions from the aerial parts of wild spinach (*C. bonus-henricus* L.). Thirty-six compounds, respectively, 22 saponins of eight sapogenins, together with 12 flavonoid glycosides of 6-methoxykaempferol, isorhamnetin, patuletin, spinacetin as well as two ecdysteroids were detected (Kokanova-Nedialkova et al. 2021). The application of the aerial parts of *C. bonus-henricus* L. as food in the same manner as spinach stimulated us for creating a UHPLC-HRMS method for simultaneous quantification of the flavonoid contents.

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. All the reagents used were of analytical grade. Hyperoside (≥ 97%, HPLC) was purchased from Sigma-Aldrich (Taufkirchen, Germany).

Plant material

The aerial parts of *Chenopodium bonus-henricus* L. were collected from Beglica, Western Rhodopes, Bulgaria in June 2019. The plant was identified by P. Nedialkov and a voucher specimen from the plant population (No. SOM-Co-169849) was deposited at the National Herbarium, Bulgarian Academy of Sciences, Sofia, Bulgaria.

Preparation of MeOH extract

The aerial parts of *C. bonus-henricus* L. were dried in a shade, and the powdered plant material (200.08 mg) was extracted with 80 vol. % MeOH (60 mL) by ultrasonic-assisted extraction for 30 minutes. The MeOH extract was diluted to 100 mL 80 vol. % MeOH. The resulting solution was filtered, 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 80 vol. % MeOH (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 10 mL 80 vol. % MeOH (solution B). Solution B was used for LC-MS quantification of flavonoid contents.

UHPLC chromatographic conditions

UHPLC separations were performed on a Kromasil Eternity XT C18 column (AkzoNobel, Sweden) (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: 0–0.5 min, 10% B; 0.5–1 min, 10–12% B; 1–7 min, 12–14% B; 7–13 min, 14–17% B; 13–16 min, 17–28% B, 16–17.50 min, 28–95% B, 17.50–19.50 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 positive ionization mode were as follows: +3.5 kV spray voltage, 320 °C capillary and probe heater temperature, sheath gas flow rate 36 a.u., auxiliary gas flow 11 a.u., spare gas flow 1 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 as well as collision gas in the HCD cell. The All Ion Fragmentation (AIF) mode was used as an MS experiment where the resolution, automatic gain control (AGC) target, maximum inject time (IT) and mass range were 70000 (at *m/z* 200), 3e6, 200 ms, and *m/z* 200–1200, respectively. The product ions at *m/z* 303.0499 (for hyperoside), 317.0656 (for **3**, **4**, **6**, **7**, **10** and **12**), 333.0605 (for **1**, **2** and **9**) and 347.0761 (for **5**, **8** and **11**) with 5.0 ppm isolation window were used as quantifiers. Xcalibur software ver. 4.0 was used for data acquisition and processing.

Method validation

The quantification of flavonoids was carried out using the external standard method. The amount of 12 previously detected flavonoids (Kokanova-Nedialkova et al. 2021) was calculated relative to external standard hyperoside. The amount of hyperoside is relative and not absolute. The external standard was dissolved in 100 mL 80 vol. % MeOH (primary solution). The stock standard solution of hyperoside was prepared by taking an aliquot (1 mL) of a primary solution and dilution to 25 mL with 80 vol. % MeOH. It was stored in the refrigerator at 4 °C. The working standard solution of appropriate concentration was prepared by diluting the stock standard solution with 80 vol. % MeOH. External standard calibration was established on six data points covering the concentration range of 12.588–402.8 ng/mL.

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 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. 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. The 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 is usually presented as a percent of nominal (ICH 2005; Kazusaki et al. 2012). The accuracy of hyperoside was evaluated at three different concentrations and recorded as percent recoveries 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 hyperoside 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 external standard hyperoside. 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 flavonoids in the aerial parts of *C. bonus-henricus* (Fig. 1) in this work.

Figure 1. *Chenopodium bonus-henricus* L.

The efficiency of the extraction procedure was tested by using different solvents (80 vol. % MeOH, 70 vol. % MeOH and 50 vol. % MeOH) and techniques (ultrasonic-assisted and magnetic stirring extractions). The results showed that the highest extraction efficiency was achieved using ultrasonic-assisted extraction with 80% MeOH. The excellent selectivity of solid-phase extraction over RP_{18} for the preparation of MeOH extract 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 flavonoids, which was consistent with the previous report (Kokanova-Nedialkova et al. 2021).

Quantitative determination of flavonoid contents in the aerial parts of *C. bonus-henricus* L. was performed by

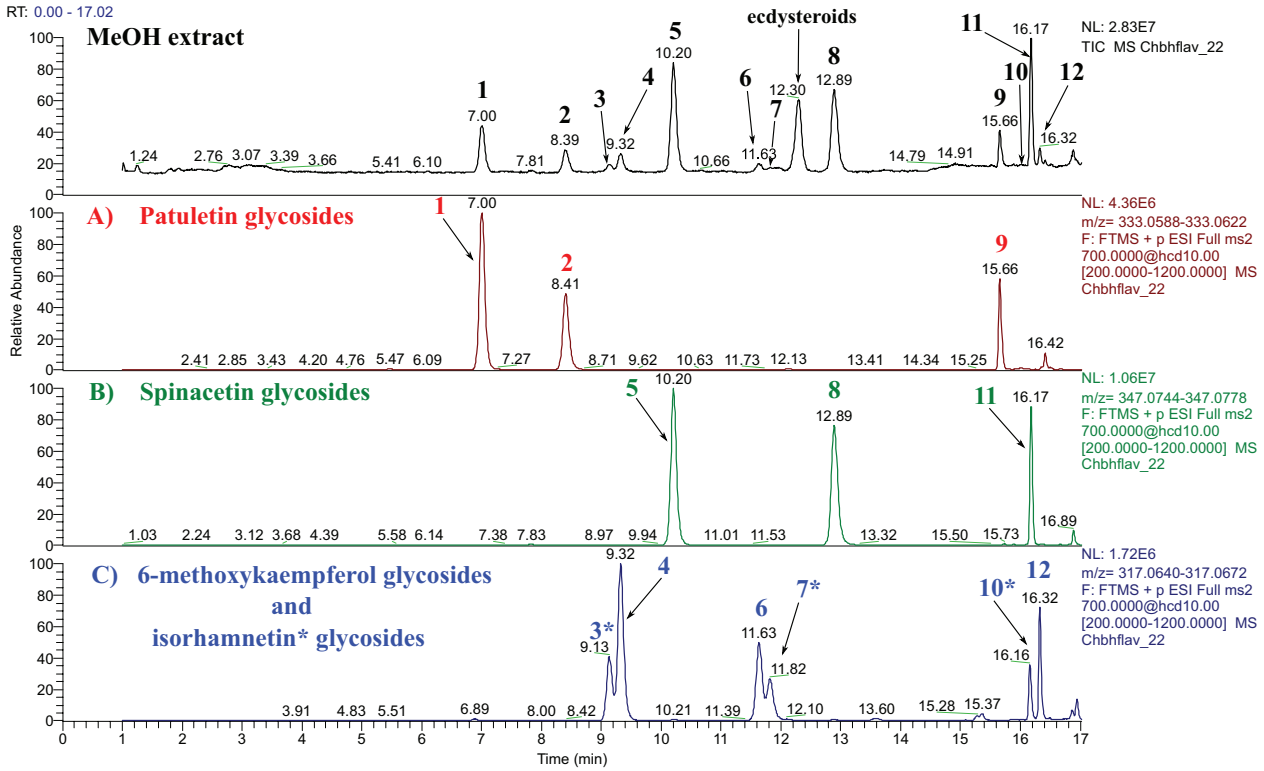

Figure 2. Flavonoids in the MeOH extract from the aerial parts of *C. bonus-henricus* L. and detected glycosides of patuletin (**A**), spinacetin (**B**), 6-methoxykaempferol, and isorhamnetin (**C**).

the method of the external standard. The amount of 12 previously detected flavonoids (Kokanova-Nedialkova et al. 2021) (Fig. 2) was calculated relative to external standard hyperoside.

The calibration curve of hyperoside was linear over the concentration range from 12.588 to 402.8 ng/mL and showed very good linear regression. The correlation coefficient was $R^2 = 0.9979$. The method showed that LOD and LOQ were 0.39 ng/mL and 1.17 ng/mL, respectively (Table 1).

Table 1. Linearity of calibration curve for the hyperoside.

External standard	Linear range (ng/mL)	Regression equation	R^2	LOD (ng/mL)	LOQ (ng/mL)
Hyperoside	12.588–402.8	Y = -391191+110556*X	0.9979	0.39	1.17

The accuracy of hyperoside was checked at three different concentrations (40.08, 80.16, and 160.32 ng/mL). The external standard showed overall recoveries ranging from 99.63% to 100.70% with RSD from 1.58% to 2.31% (Table 2).

Table 2. Accuracy of the UHPLC-HRMS method.

External standard	Added (ng/mL)	Found[a] (ng/mL)	Recovery[a] (%)	RSD (%)
Hyperoside	40.08	40.36 ± 0.82	100.70 ± 2.05	2.03
	80.16	80.47 ± 1.86	100.39 ± 2.32	2.31
	160.32	159.73 ± 2.53	99.63 ± 1.58	1.58

[a] Values are the mean ± SD (n = 4).

The precision of the retention times was determined by analyzing the calibration sample during a single day and on three different days, respectively. The RSDs of retention times of hyperoside were 0.07 for intra-day and 0.09 for inter-day precision assays. Also, the external standard showed recoveries at 98.04% (for intra-day precision assay) and 99.23% (for inter-day precision assay) with RSDs at 2.38% and 1.32% respectively (Tables 3).

Table 3. Evaluation of intra-day (repeatability) and inter-day (intermediate precision) precision of the UHPLC-HRMS method applied on hyperoside.

Precision type	RT ± SD (min)	RSD (%)	Recovery ± SD (%)	RSD (%)
Intra-day	10.50 ± 0.008	0.07	98.04 ± 2.34	2.38
Inter-day	10.49 ± 0.009	0.09	99.23 ± 1.31	1.32

The results (Table 4) show that the glycosides of spinacetin (**5**, **8,** and **11**), and patuletin (**1**) were the main components of the flavonoid mixture which content ranging from 1.79 to 4.41 mg g^{-1} D.W., calculated as hyperoside. The glycosides of patuletin (**2** and **9**) and 6-methoxykaempferol (**4**) were found in small quantities (equivalent to 0.70–0.82 mg g^{-1} D.W. hyperoside). The quantity of the remaining flavonoids (**3**, **6**, **7**, **10,** and **12**) was very small ranging from 0.20 to 0.34 mg g^{-1} D.W., calculated as hyperoside. The total amount of flavonoids was found to be 15.12 mg g^{-1} D.W.

Table 4. Content of flavonoids in the aerial parts of *C. bonus-henricus* L.

No.	t_R (min)	Flavonoids	mg g^{-1} D.W. ± SD
1	7.00	patuletin-3-O-[β-apiofuranosyl (1→2)]-β-glucopyranosyl (1→6)-β-glucopyranoside	1.79 ± 0.0007
2	8.39	patuletin-3-O-gentiobioside	0.82 ± 0.0036
3	9.13	isorhamnetin-Hex-Hex-Pent	0.32 ± 0.0006
4	9.32	6-methoxykaempferol-3-O-[β-apiofuranosyl (1→2)]-β-glucopyranosyl (1→6)-β-glucopyranoside	0.74 ± 0.0010
5	10.20	spinacetin-3-O-[β-apiofuranosyl (1→2)]-β-glucopyranosyl (1→6)-β-glucopyranoside	4.41 ± 0.0075
6	11.63	6-methoxykaempferol-3-O-gentiobioside	0.34 ± 0.0035
7	11.82	isorhamnetin-Hex-Hex	0.20 ± 0.0017
8	12.89	spinacetin-3-O-gentiobioside	3.19 ± 0.0104
9	15.66	patuletin-3-O-(5″′-O-E-feruloyl)-β-D-apiofuranosyl(1→2) [β-D-glucopyranosyl (1→6)]-β-D-glucopyranoside	0.70 ± 0.0022
10	16.16	isorhamnetin-Hex-Hex-Pent-FA	0.20 ± 0.0016
11	16.17	spinacetin-3-O-(5″′-O-E-feruloyl)-β-D-apiofuranosyl (1→2) [β-D-glucopyranosyl (1→6)]-β-D-glucopyranoside	2.07 ± 0.0031
12	16.32	6-methoxykaempferol-3-O-(5″′-O-E-feruloyl)-β-D-apiofuranosyl (1→2) [β-D-glucopyranosyl (1→6)]-β-D-glucopyranoside	0.34 ± 0.0036

Conclusions

A novel UHPLC-HRMS method for simultaneous quantification of flavonoid contents from the aerial parts 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 glycosides of spinacetin (**5**, **8,** and **11**), and patulenin (**1**) were the predominant compounds in the aerial parts of *C. bonus-henricus* L. The results show that the proposed method can be easily used to quantify the total content of flavonoids in plant substances to control their quality, as well as to facilitate the isolation of potentially phytochemically interesting molecules.

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