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Ultrasound-assisted extraction and (U)HPLC-DAD-ESI-MS^n analysis of phenolic compounds from black chokeberries cultivated in Turkey

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Summary

Phenolic compounds from two black chokeberry cultivars 'Viking' and 'Nero' grown in Turkey were analyzed by high performance liquid chromatography-diode array detection-electrospray ionization-multistage mass spectrometry (HPLC-DAD-ESI-MS^n). In the first step, five different solvents were compared to efficiently isolate phenolic compounds by ultrasound-assisted extraction. Three sequential extraction cycles using methanol/formic acid (95:5, v/v) sufficed for exhaustive extraction of anthocyanins, hydroxycinnamic acid derivatives, and flavonol glycosides from black chokeberry within merely 60 sec. A total of four cyanidin glycosides, two hydroxycinnamic acids, and five quercetin mono- and di-glycosides were detected in both cultivars. Total anthocyanins (425-438 mg/100 g of fresh weight, FW), hydroxycinnamic acids (173-179 mg/100 g of FW), and flavonols (37 mg/100 g of FW) were determined in a similar range for both cultivars. Complementary, a rapid ultra-high performance liquid chromatography (UHPLC)-DAD method was developed, permitting a high throughput screening of chokeberry phenolics. The established methods were validated considering extraction recoveries, intra- and inter-day repeatability, calibration linearity, limit of detection (LOD), and limit of quantitation (LOQ). UHPLC provided a 2.3 times faster compound separation (30 min) and less solvent consumption than HPLC (68 min).

Keywords: *Aronia*, Viking, Nero, anthocyanins, hydroxycinnamic acids, flavonols, method validation, chromatography

Introduction

Chokeberry or Aronia is a deciduous shrub of the Rosaceae family. Native to North America, it spread to Europe and Russia (Janick and Paull, 2001). The plant bears edible, cherry-like fruits that are commonly used for the production of juice, jam, tea, and wine. Black chokeberry is currently of particular interest due to its phenolic composition, being rich in anthocyanins along with several phenolic acids, flavonols, and proanthocyanidins (Sidor and Gramza-Michałowska, 2019). These compounds are potent antioxidants, possibly exerting health-promoting effects as extensively reviewed by SIDOR et al. (2019). Amongst others, chokeberry consumption has been associated with diminution of risk factors leading to the development of the metabolic syndrome, such as hypertension (Pokimica et al., 2019), obesity (Kim et al., 2018), glucose metabolism disorders (Milutinović et al., 2019), and pro-inflammatory conditions (Gajic et al., 2020). Moreover, Aronia concentrates have been demonstrated to inhibit NF-κB and synergize with selenium to suppress the release of pro-inflammatory mediators in macrophages (Appel et al., 2015). Owing to its high anthocyanin content, black chokeberry has a large potential in the food industry, e.g., as a natural colorant (Espín et al., 2000). Aronia berries have been proposed as promising raw material for manufacturing healthy snack foods (Oniszczuk et al., 2019), functional food packaging (Staroszczyk et al., 2020), as well as antimicrobial and antioxidant agents applicable in meat products (Anton et al., 2019).

Aronia melanocarpa (Michx.) Elliot (black), *A. arbutifolia* (L.) Pers. (red), and *A. prunifolia* (Marshall) Rehder (purple) are three well-known chokeberry species. Among them, cvs. 'Viking' and 'Nero' are two prominent European cultivars of economic importance. These commercial cultivars are considered as *A. melanocarpa* in many scientific studies, however, are also separated as a fourth species, *A. mitschurinii* Skvortsov et Majjtulina. The above two cultivars have been proposed to be the intergeneric hybrid between rowan (*Sorbus aucuparia* L.) and *A. melanocarpa*, backcrossed with *A. melanocarpa*. They possess superior phenotypic characteristics by producing larger and sweeter berries compared to those of North American wild types of *A. melanocarpa* (Brand, 2010). These features of European cultivars have been achieved by breeding (Smolik et al., 2011), the exact details of which remain unclear (Brand, 2010; Leonard et al., 2013). Recently, these cultivars were introduced to Turkey, where their cultivation started in 2012 (see section "plant material"). Geographic location, agronomic practices, and seasonal differences have been reported to largely affect the accumulation of phenolic compounds in berries (Kulling and Rawel, 2008). For instance, a previous study has already shown the differences in the phenolic contents of black chokeberries grown in Germany and Poland (Rodríguez-Werner et al., 2019). The phenolic composition of black chokeberries cultivated in Turkey has been neglected in previous studies.

Extraction is a critical step for the analysis of phenolic compounds as their physicochemical properties largely differ, ranging from highly water-soluble anthocyanins to mid-polar quercetins (Naczk and Shahidi, 2004). A comprehensive approach aiming at the exhaustive extraction of all individual phenolic compounds is a prerequisite for their accurate quantitation. As an alternative to the commonly applied stirring- and high-shear force homogenization-based methods, ultrasound-assisted extraction is often the method of choice providing fast extraction and low solvent consumption (Tiwari, 2015). Most recently, a report about ultrasound-assisted extraction followed by determination of total phenolic and anthocyanin contents has become available (Vázquez-Espinosa, 2019). However, this promising method for screenings has not been considered for the quantitation of individual phenolic compounds from black chokeberry.

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The present study aimed at establishing a fast and exhaustive ultrasound-assisted extraction method for the subsequent liquid chromatographic determination of phenolic compounds from black chokeberry cultivated in Turkey. Different solvent systems of varying polarity and the number of required extraction cycles for exhaustive recovery of black chokeberry phenolics were tested. An HPLC and a complementary UHPLC method were developed and validated. Two commercial varieties, 'Viking' and 'Nero', recently introduced to Turkey, were analyzed applying the established workflow. The established methods may be highly instrumental for quality control in the food industry and for screening of black chokeberries, e.g., from different cultivars and growing regions.

Materials and methods

Chemicals

The authentic standards cyanidin 3-*O*-galactoside chloride (≥97%), cyanidin 3-*O*-glucoside chloride (≥96%), cyanidin 3-*O*-arabinoside chloride (≥95%), quercetin 3-*O*-galactoside (≥98%), quercetin 3-*O*-rutinoside (≥99%), chlorogenic acid (≥99%), and neochlorogenic acid (≥99%) were purchased from Extrasynthèse (Genay, France). Analytical grade formic acid, acetone, ethanol (EtOH), and hydrochloric acid (HCl, 37%) were from Merck and HPLC grade methanol (MeOH) from VWR (both Darmstadt, Germany). Purified water was prepared using an Arium 611 ultrapure water system (Sartorius, Göttingen, Germany).

Plant material

Two different black chokeberry cultivars (cvs. 'Viking' and 'Nero') were grown in an orchard of Atatürk Horticultural Central Research Institute, Yalova, Turkey (GPS coordinates: 40°39'46.6"N 29°18'19.0"E). The soil type of the plantation was loamy, and a drip irrigation system was used during the vegetation period. Nitrogen fertilizer (20 kg N/ha) was applied to the plantation as ammonium sulfate (21% nitrogen) in two doses with 15 days intervals in April. Since the soil contained sufficient phosphorus and potassium based on the soil test results, further fertilizers were not needed. Pesticide treatments were not required as Aronia is innately resistant to pathogens. Berries were harvested by hand in September 2017 from a five-year-old plantation according to the optimum harvest period determined previously (Poyraz Engin and Mert, 2020). Berries were then manually separated from leaves, stalks and other impurities, washed, and immediately frozen by immersing in liquid nitrogen after removal of residual washing water. Frozen berries were transferred to the University of Hohenheim, Stuttgart, Germany in a special packaging for frozen products (Koroplast Cooler Bag, Istanbul, Turkey) within 12 h. Subsequently, they were freeze-dried for 72 h using a VaCo-10-II-E (Zirbus Technology, Bad Grund, Germany) following grinding with an A11 laboratory mill (IKA, Staufen, Germany), and stored at -20 °C until analysis within 6 months. In addition to obtaining freeze-dried samples, a portion of frozen but not freeze-dried chokeberries (ca. 200 g) were milled using a laboratory blender (Conair Waring blender, Stamford, CT, USA) and then immediately subjected to the extraction procedure. These samples are referred to as "fresh samples" below. Black chokeberry cv. 'Viking' was used throughout method development and validation. Dry matter contents of the fresh berries were determined with an infrared moisture analyzer MA 40 (Sartorius, Göttingen, Germany).

Ultrasound-assisted extraction of phenolic compounds from black chokeberry

An aliquot of fresh (200.0 ± 1.0 mg) or freeze-dried (50.0 ± 0.5 mg) ground chokeberry was weighed into a 10-mL conical glass centrifuge tube. Following admixture of 4 mL of the below-mentioned extraction solvents, the samples were probe-sonicated for 20 sec at 75% amplitude (MS 72 micro tip, Sonopuls UW 3100, Bandelin Electronics, Berlin, Germany) while they were immersed in an ice-filled beaker (approx. 0 °C) to prevent sample heating. Subsequently, the samples were centrifuged at 3,112 × *g* for 4 min (Heraeus Labofuge 400R; Hanau, Germany). Supernatants were collected and the solid residues were re-extracted up to four times as described above. The combined supernatants were evaporated to dryness *in vacuo* at 30 °C using a rotary evaporator (Heidolph Laborota 4000, Schwabach, Germany).

For the selection of the highest yielding extraction solvent, five different solvent mixtures, i.e., methanol/formic acid (95:5, v/v), methanol/water/formic acid (70:25:5, v/v/v), ethanol/water/formic acid (70:25:5, v/v/v), acetone/water/formic acid (70:25:5, v/v/v), and methanol/water/formic acid (50:45:5, v/v/v), were compared. After that, the effect of up to four repeated extractions was examined by collecting and analyzing each fraction separately. The extraction efficiency of the individual solvents was evaluated based on the total peak area of anthocyanins (520 nm), hydroxycinnamic acids (320 nm), and flavonols (360 nm).

Chromatographic determinations

HPLC-DAD-ESI-MS^n analysis

Dried extracts prepared as described above were re-dissolved in 2 mL of pure methanol containing 5% (v/v) formic acid and filtered through a cellulose filter (0.45 µm pore size, Macherey-Nagel, Düren, Germany) into amber glass vials prior to HPLC analysis.

HPLC analysis was performed using an Agilent 1100 series HPLC system (Agilent, Waldbronn, Germany), equipped with a G1315A diode array detector (DAD). A core-shell column (Kinetex C18, 250 × 4.6 mm, particle size d_p = 5 µm, 100 Å, Phenomenex, Torrance, CA, USA) with a C18 guard column (4.0 mm × 2.0 mm i.d., Phenomenex) was used at a temperature of 35 °C. The mobile phases were water/formic acid (95:5, v/v) and methanol/formic acid (95:5, v/v) as eluents A and B, respectively. The solvent gradient program was as follows: isocratic at 6% B for 15 min, 6 to 15% B in 5 min, 15 to 25% B in 25 min, 25 to 40% B in 10 min, 40 to 100% B in 3 min, isocratic hold at 100% for 4 min, 100 to 6% B in 1 min followed by isocratic conditioning at 6% B for 5 min prior to the next run. The total run time was 68 min at a flow rate of 1 mL/min. The injection volume was 5 µL.

The LC system described above was connected to an Esquire 3000+ ion-trap mass spectrometer (Bruker Daltonics, Bremen, Germany) with an electrospray ionization (ESI) source. Anthocyanins were monitored in positive, non-anthocyanin phenolic compounds in negative ion mode. The scan rate was *m/z* 50-1500 at a scan speed of 13,000 Th/s. Nitrogen was used at a flow rate of 10 L/min at 60 psi both as drying and nebulizing gas. Potential on the capillary was ±2287 V and the nebulizer temperature was 365 °C. Helium was used as collision gas for collision-induced dissociation (CID) applying a fragmentation amplitude of 0.8 V.

UHPLC-DAD analysis

For UHPLC analysis, an Acquity UPLC H-class system with eλ DAD detector (Waters, Milford, MA, USA) equipped with a Kinetex C18 column (100 × 2.1 mm, d_p = 1.7 µm, 100 Å, Phenomenex) was applied. The same mobile phase system and oven temperature were used as detailed for the aforementioned HPLC method (see "HPLC-DAD-ESI-MS^n analysis"). UHPLC separation was achieved using the following gradient: isocratic at 6% B for 1 min, from 6 to 15% B in 5.7 min, from 15 to 25% B in 5.3 min, from 25 to 100% B in 6 min, isocratic at 100% for 3 min, from 100 to 6% B in 1 min fol-

lowed by isocratic conditioning at 6% B for 8 min prior to the next run. The total run time was 30 min at a flow rate of 0.4 mL/min and an injection volume of 0.4 µL. In both UHPLC and HPLC, UV/Vis spectra were recorded in the range of 200–700 nm. Anthocyanins, hydroxycinnamic acids, and flavonols were monitored at 520, 320, and 360 nm, respectively.

Compound identification and quantitation

Compound identification was accomplished by the comparison of retention times (t_R), UV/Vis absorption, and mass spectra to those of authentic standards (see "Chemicals") and data from a previous study (SLIMESTAD et al., 2005). For compound assignment during UHPLC analysis, for which no mass spectral information was available, UV/Vis spectra, elution orders, and relative peak heights were compared to those of the HPLC-DAD-ESI-MS^n analysis when standard compounds were unavailable. Peak resolutions (*Rs*) were calculated as described previously (ERŞAN et al., 2017).

For quantitation, linear calibration curves of authentic external standards of chlorogenic acid, cyanidin 3-*O*-galactoside, quercetin 3-*O*-galactoside, and quercetin 3-*O*-rutinoside were established. Stock solutions of chlorogenic acid (2 g/L), quercetin 3-*O*-galactoside (0.5 g/L), and quercetin 3-*O*-rutinoside (0.5 g/L) were prepared in pure methanol. Cyanidin 3-*O*-galactoside (2 g/L) was dissolved in methanol containing 0.1% (v/v) HCl. Subsequently, the stock solutions were diluted to the respective concentration ranges given in Tab. 1 using methanol/formic acid (95:5, v/v). Three independent external calibration curves each at ten different concentrations were established. For quantitation of compounds other than the aforementioned three standards, calibration curves of structurally related substances were used after applying molecular-weight-correction factors (MWCF), calculated separately for each compound by dividing the molecular weight of the compound of interest by that of the respective authentic standard compound (LIN and HARNLY, 2012). Resulting MWCFs calculated relative to cyanidin 3-*O*-galactoside were 0.93 for cyanidin 3-*O*-arabinoside and cyanidin 3-*O*-xyloside, and 0.98 for quercetin 3-*O*-vicianoside as calculated relative to quercetin 3-*O*-rutinoside. Extraction and quantitation of individual phenolic compounds were executed in six replicates.

Method validation

Both HPLC and UHPLC systems were validated for the determination of phenolic compounds from black chokeberry extracts, based on calibration linearity, the limit of detection (LOD), the limit of quantitation (LOQ), intra-day and inter-day repeatability, and extraction recoveries according to ICH guidelines (ICH, 2005). Calibration linearity was expressed as the coefficient of determination (R^2) after linear regression analysis. The standard deviation of the y-intercepts (σ) and the slope of regression lines (S) were determined from three independent replicates of calibration curves and were used for the calculation of LOD (3.3 σ/S) and LOQ (10 σ/S) values.

Extraction recoveries were established by adding authentic standards at specified high and low levels (Tab. 2) prior to the extraction. Briefly, a methanolic solution containing the calculated amount of standard compound was transferred to an extraction tube and the

Tab. 1: Linear concentration ranges, detection parameters, slope of calibration curves, limit of detection (LOD), and limit of quantitation (LOQ) of reference standards (chlorogenic acid, cyanidin 3-*O*-galactoside, quercetin 3-*O*-galactoside, and quercetin 3-*O*-rutinoside) used for validation of the HPLC- and UHPLC-based methods.

Peak no.	Compound	Conc. range (mg/L) [b]	Slope [a]		LOD (ng on column)		LOQ (ng on column)	
			HPLC (mAU * min * L * mg^{-1})	UHPLC (mV * s * L * mg^{-1})	HPLC	UHPLC	HPLC	UHPLC
2	Chlorogenic acid	2.0-140.0	13.85	3341.4	54.5	0.31	165.1	0.94
3	Cyanidin 3-*O*-galactoside	6.5-454.6	20.44	4042.5	48.4	4.1	146.8	12.5
8	Quercetin 3-*O*-galactoside	1.1-26.3	9.87	2188.7	6.9	0.51	20.9	1.5
11	Quercetin 3-*O*-rutinoside	1.1-26.3	8.98	1890.5	6.5	0.62	19.7	1.9

[a] Means of three independent replicates of standard curve, [b] Injection volumes were 5 µL (HPLC) and 0.4 µL (UHPLC).

Tab. 2: Recovery and repeatability of the developed ultrasound-assisted extraction of phenolic compounds from freeze-dried chokeberry and subsequent HPLC-DAD analysis.

Peak no.	Compound	Original content (µg)	Amount added (µg)	Amount measured [c] (µg)	Recovery (%)	Repeatability CV (%) Intra-day (n = 6) [a]	Inter-day (n = 4) [b]
2	Chlorogenic acid	163.4 ± 7.9 169.9 ± 7.4	139.8 69.1	297.1 ± 21.9 229.4 ± 9.0	97.9 ± 4.7 97.2 ± 1.6	< 3.23	0.67
3	Cyanidin 3-*O*-galactoside	595.5 ± 28.8 608.1 ± 27.0	323.7 152.9	916.6 ± 55.2 758.7 ± 60.9	99.7 ± 2.9 99.6 ± 5.0	< 2.50	2.87
8	Quercetin 3-*O*-galactoside	18.3 ± 0.9 18.7 ± 0.8	17.5 7.6	33.4 ± 1.7 25.9 ± 1.7	93.3 ± 2.6 98.3 ± 3.8	< 3.53	2.93
11	Quercetin 3-*O*-rutinoside	10.0 ± 0.5 10.2 ± 0.5	16.3 7.5	25.1 ± 2.0 17.0 ± 1.0	95.5 ± 5.9 95.9 ± 3.3	< 4.70	3.52

CV: coefficients of variation

[a] Six determinations on one day.

[b] Six determinations each week within a month (n = 4).

[c] The measured amounts were added to approximately 50 mg of freeze-dried chokeberry sample with high and low levels (each n = 3).

Footnote: [d]: Recovery (%) = $\frac{\text{Amount measured}}{\text{Original content + Amount added}} \times 100$

solvent was evaporated under a gentle nitrogen stream. Afterwards, the chokeberry sample was weighed into the extraction tube and the extractions were carried out according to the procedure described above. Recovery experiments were conducted in triplicate.

Intra-day repeatability was evaluated by six independent repeated determinations within one day, while inter-day repeatability was estimated from six determinations each on four different days within a month. Results were expressed as the coefficient of variation means (CV).

Statistical analysis

All results were given as mean ± standard deviation. Data analyses were performed using Minitab® 18.1 (State College, PA, USA), considering $p < 0.05$ as significant. Differences between means were examined using one-way analysis of variance (ANOVA) and Tukey's post-hoc test. The different black chokeberry cultivars were compared by applying a two-sample *t*-test.

Results and discussion

Ultrasound-assisted extraction of phenolic compounds from black chokeberry

Selection of extraction solvent

As methanol, acetone, ethanol and their aqueous mixtures are commonly used solvents for the extraction of phenolic compounds (Naczk and Shahidi, 2004), we first compared their efficiency for the simultaneous extraction of anthocyanins and non-anthocyanin phenolic compounds from freeze-dried black chokeberry powder. As shown in Fig. 1a, flavonols, anthocyanins, and hydroxycinnamic acids were effectively extracted from black chokeberry using different solvent systems with slight, but insignificant differences among methanol/formic acid (95:5, v/v), methanol/water/formic acid (70:25:5 and 50:45:5, v/v/v), ethanol/water/formic acid (70:25:5, v/v/v) and acetone/water/formic acid (70:25:5, v/v/v) ($p > 0.05$). Employing acetone/water/formic acid (70:25:5, v/v/v), extraction yields were vaguely higher by trend than when using other solvents without reaching statistical significance ($p = 0.20$). Adding water to the methanol/formic acid solvent system (methanol/water/formic acid, 70:25:5 and 50:45:5, v/v/v) did not significantly improve the results, although the addition of different proportions of water into alcoholic solvents is often practiced for extraction of phenolic compounds (Erşan et al., 2017). Ultimately, methanol/formic acid (95:5, v/v) was selected as an extraction solvent; and was used in all subsequent experiments, also because this solvent can be rapidly evaporated for concentration prior to HPLC analysis. The methanol-based solvent system also enabled direct injection when applying our HPLC system, without the need of its complete removal. Consequently, time- and resource-consuming sample preparation steps can be omitted, thus reducing personnel expenses. Ethanol/water/formic acid (70:25:5) provides an environmentally friendly and non-toxic alternative among all solvent systems tested, achieving yields of the target analytes in a similar range. Noteworthy, ethanol/water/formic acid (70:25:5) provided the lowest yields for two quercetin diglycosides, i.e., quercetin 3-*O*-rutinoside and quercetin 3-*O*-robinobioside ($p < 0.05$; data not shown). Although water is a readily available non-toxic extraction solvent, it was not included in our solvent comparison, due to the enhanced activity of fruit enzymes, i.e., polyphenol oxidase and peroxidase, in aqueous environments, being detrimental for phenolic compounds (Antolovich et al., 2000). Their activities are suppressed in extraction solutions containing denaturing organic solvents (Gras et al., 2015) like methanol and ethanol, thus preventing chemical modification and degradation of the target analytes.

In contrast to our results, methanol/water (80/20, v/v) provided significantly higher quercetin 3-*O*-galactoside yields from pistachio hull than those of ethanol/water (80:20), methanol/water (50:50) and pure methanol (Erşan et al., 2017). According to Gras et al. (2015), the most exhaustive extraction of cyanidin 3-*O*-glucoside and acylated black carrot anthocyanins has been achieved by using water/methanol/formic acid (75:20:5, v/v/v) compared to methanol/formic acid (95:5, v/v) and water/methanol/formic acid (45:50:5, v/v/v). These variations from our study may be attributed to the differing plant matrices, i.e., pectin-rich Aronia berry tissue versus the outer layers of a stone fruit (pistachio) (Chaharbaghi et al., 2017) or the root vegetable black carrot assessed in these previous studies as well as their differing anthocyanin pattern, e.g., acylated anthocyanins prevail in black carrot root whereas chokeberry merely contains non-acylated anthocyanins.

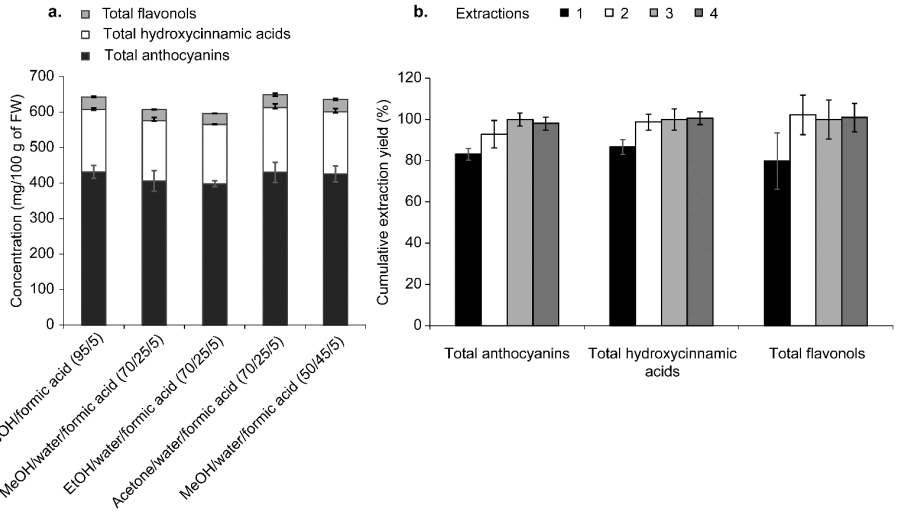

Fig. 1: Extraction yields of phenolic compounds summarized as total flavonols, hydroxycinnamic acids, and anthocyanins from black chokeberry cv. `Viking´ according to extraction solvent (a) and the number of repeated extractions (b)

Footnote-1: Values represent means ± standard deviations of six replicate measurements.

Footnote-2: 1, 2, 3, and 4 in b. indicate the number of repeated extractions.

Footnote-3: The results are based on the HPLC method

Footnote-4: Relative extraction yields are standardized to the maximum concentration achieved for each compound class.

Number of extraction cycles

To ensure the exhaustive isolation of phenolic compounds, an ultrasound-assisted extraction of black chokeberry repeated up to four times using the previously selected solvent mixture of methanol/formic acid (95/5, v/v) was examined. The solid residue was devoid of any blueish color after four cycles, indicating that predominating phenolic compounds, i.e., anthocyanins, were exhaustively extracted. As shown in Fig. 1b, recoveries of total hydroxycinnamic acids, anthocyanins, and flavonols ranged from 76 to 86% after one extraction cycle, and consecutively increased to 95-102 and 98-101% after the second and third extraction cycle, respectively ($p < 0.05$). Applying a fourth extraction cycle did not further increase the recovery. Consequently, three extraction cycles sufficed for exhaustive extraction and thus, were applied for further studies.

Effect of sample pre-treatment

The effect of freeze-drying of black chokeberries prior to extraction of phenolic compounds was evaluated, as adverse effects of this sample pretreatment on contents of antioxidants (Shofian et al., 2011) and phenolic compounds (De Torres et al., 2010) have been previously reported, attributed to the decomposition or chemical modifications during freeze-drying (Abascal et al., 2005). However, in our study, when recovery rates of phenolic compounds from freeze-dried and fresh black chokeberry samples were compared, similar qualitative and quantitative compositions of phenolic compounds were observed. Noteworthy, phenolic contents of extracts of fresh fruits resulted in higher CV values, reaching up to 30%, than those obtained when extracting freeze-dried samples (data not shown). These higher CV values of the fresh fruit extracts may be attributed to the fact that the fresh puree-like samples were less homogeneous than the freeze-dried and milled powdered samples. In addition, activities of the aforementioned fruit enzymes (see "Selection of extraction solvent") might have been deteriorative to the analytes during sample preparation of the freshly pureed samples, although this remains speculative. To conclude, although freeze-drying may be energy- and time-consuming, it was selected for black chokeberry in our study, as it resulted in a more homogeneous sample as compared to fresh or frozen berries.

Final extraction procedure

The optimized method is based on the ultrasound-assisted extraction of freeze-dried black chokeberry using methanol/formic acid (95:5, v/v) at three repetitive extraction cycles. This procedure provided an exhaustive extraction of phenolic compounds from freeze-dried black chokeberry. Compared to the previously developed 60-min maceration (Ćujić et al., 2016) and a one-step 10-min probe-sonication (Vázquez-Espinosa, 2019) of black chokeberries, our optimized method provides a rapid and efficient extraction of the black chokeberry phenolics. However, it should be noted that the whole extraction procedure is not necessarily quicker than the aforementioned one-step 10 min method due to added times for pipetting, centrifuging and phase separation. As reviewed previously (Tiwari, 2015), ultrasound-assisted extraction often provides enhanced extraction yields in short times as a result of acoustic cavitation, which is the creation, expansion, and collapse of microbubbles in a liquid medium during extraction, causing intense matrix disruption and increased diffusion rates.

Comparison of HPLC and UHPLC separations

Chromatographic separations of black chokeberry phenolics on an HPLC and an UHPLC system, both equipped with core-shell C_{18} columns (d_p = 5 and 1.7 µm, resp.), were compared. A representative HPLC chromatogram is shown in Fig. 2. Elution orders of the compounds were comparable during UHPLC and HPLC separation. Our UHPLC method (30 min) is 2.3 times faster than our HPLC method (68 min) and consumed 82% less solvent. Consequently, the novel UHPLC method permits a higher sample throughput, rendering this approach more economical and reduces the environmental impact owing to the significantly reduced eluent consumption. Both HPLC

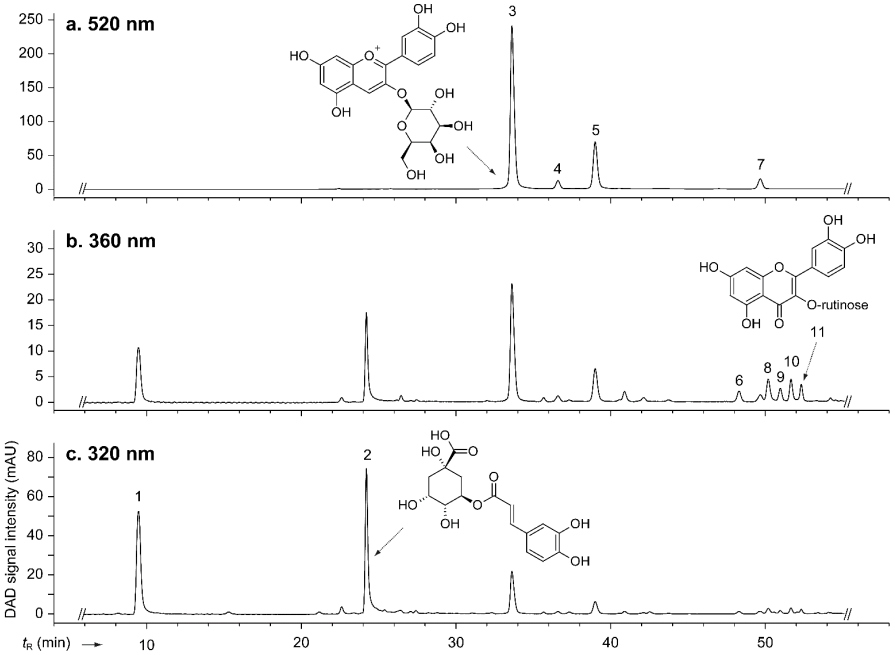

Fig. 2: HPLC separation of methanol/formic acid (95/5, v/v) extract of black chokeberry cv. 'Viking' at wavelengths of 520 nm (a), 360 nm (b), and 320 nm (c). Peak assignments: Neochlorogenic acid (1), chlorogenic acid (2), cyanidin 3-*O*-galactoside (3), cyanidin 3-*O*-glucoside (4), cyanidin 3-*O*-arabinoside (5), quercetin pentosyl-hexoside (6), cyanidin pentoside (7), quercetin 3-*O*-galactoside (8), quercetin deoxyhexosyl-hexoside (9), quercetin 3-*O*-glucoside (10), quercetin 3-*O*-rutinoside (11). Retention times and spectral characteristics are shown in Tab. 3

and UHPLC systems provided similar separation efficiencies with slight differences in *Rs* of peaks 7-11, which were the limiting factors for the further reduction of total run times in both systems. Briefly, *Rs* between peaks 7-11 ranged between 0.6 to 0.9in both chromatographic systems, while it was greater than 1.5 for all remaining peaks. In favor of a good chromatographic separation, being a prerequisite for a proper quantitation by UV detection, the comparatively long analysis times may be accepted. Noteworthy, during UHPLC separation, all peaks eluted within 13.5 min (Tab. 3); however, as applies for the HPLC method, cleaning and flushing back the column to the initial conditions added up to the total analysis time. In summary, both HPLC and UHPLC methods are suitable for the separation of black chokeberry phenolics permitting the simultaneous quantitation of 11 phenolic compounds of three different classes.

HPLC-DAD-ESI-MS^n analysis of individual phenolic compounds

A total of 11 different phenolic compounds, including two hydroxycinnamic acids (1 and 2), four anthocyanins (3-5, and 7), and five flavonols (6, and 8-11), were identified in black chokeberry extracts (Tab. 3). By comparing their HPLC-DAD-ESI-MS^n data to those of the corresponding authentic standard compounds, neochlorogenic acid, chlorogenic acid (both $[M-H]^-$ at *m/z* 353), cyanidin 3-*O*-galactoside ($[M]^+$ at *m/z* 449), cyanidin 3-*O*-glucoside ($[M]^+$ at *m/z* 449), and cyanidin 3-*O*-arabinoside ($[M]^+$ at *m/z* 419), quercetin 3-*O*-galactoside ($[M-H]^-$ at *m/z* 463), quercetin 3-*O*-glucoside ($[M–H]^-$ at *m/z* 463), and quercetin 3-*O*-rutinoside ($[M-H]^-$ at *m/z* 609) were assigned. Compound no. 7 displayed the same spectral characteristics as no. 5 and thus, was also assigned to a cyanidin pentoside, possibly cyanidin 3-*O*-xyloside ($[M]^+$ at *m/z* 419). Based on their UV_{max} at 354 nm, in a range specific to flavonols, and a CID fragment ion at *m/z* 301, corresponding to quercetin aglycone after elimination of a dehydrated pentosyl-hexose moiety, e.g., vicianoside (294 atomic mass units, amu), and a dehydrated deoxyhexosyl-hexose, e.g., robinobioside (308 amu) from the respective precursor ions, two additional quercetin glycosides were assigned to a quercetin pentosyl-hexoside (no. 6) and an additional quercetin deoxyhexosylhexoside (no. 9). They may correspond to quercetin 3-*O*-vicianoside (quercetin 3-*O*-(6"-*O*-β-arabinosyl-β-glucoside, $[M-H]^-$ at *m/z* 595) and quercetin 3-*O*-robinobioside (quercetin 3-*O*-(6"-*O*-β-rhamnosyl-β-galactoside, $[M–H]^-$ at *m/z* 609) that have been previously reported in black chokeberry and unambiguously identified by nuclear magnetic resonance (NMR) spectroscopy (Slimestad et al., 2005).

The composition of phenolic compounds reported herein was consistent with previous studies (Slimestad et al., 2005; Tian et al., 2017; Rodríguez-Werner et al., 2019), except for some minor constituents reported in the literature such as pelargonidin 3-*O*-arabinoside (Wu et al., 2004; Veberic et al., 2015), less abundant phenolic acids, i.e., caffeic, *p*-coumaric, *p*-hydroxybenzoic, salicylic, syringic, and vanillic acid (Szopa et al., 2013), in addition to kaempferol, isorhamnetin, and myricetin derivatives (Mikulic-Petkovsek et al., 2012). These minor constituents were not detected by the diode array detector in our study.

Method validation

All calibration curves displayed excellent signal linearity ($R^2 > 0.99$) in the studied concentration ranges (Tab. 1). The values of LOD and LOQ for the HPLC method ranged between 54.5 and 165.1 ng on the column for chlorogenic acid, and 48.4 and 146.8 ng on the column for cyanidin 3-*O*-galactoside, respectively. Smaller LODs and LOQs were determined for quercetin 3-*O*-galactoside (6.9 and 20.9 ng on column, resp.) and quercetin 3-*O*-rutinoside (6.48 and 19.65 ng on column, resp.) than for those of the other compounds. Smaller LOD and LOQ values were achieved by applying our UHPLC system, and thus, higher sensitivities were obtained than those of our HPLC. LOD and LOQ for cyanidin 3-*O*-galactoside were higher than those reported previously by Erşan et al. (2017) and Gras et al. (2015). For quercetin 3-*O*-galactoside, LOD and LOQ values applying the UHPLC system were lower than those reported by Erşan et al. (2017).

Tab. 3: Retention times, UV/Vis absorption maxima, and HPLC-DAD-ESI-MS^n data of phenolic compounds from black chokeberry.

Peak no.	Analyte	Retention time (min)		UV/Vis abs. max (nm)		HPLC-ESI-MS^n analysis		
		HPLC	UHPLC	HPLC	UHPLC	$[M]^+$	$[M–H]^-$	MS^n *m/z* (% base peak)
1	Neochlorogenic acid [a]	9.5	1.8	326	325		353	[353]: 191 (100), 179 (78), 173 (6), 135 (16) [353→191]: 153 (40), 127 (17), 99 (14), 85 (100)
2	Chlorogenic acid [a]	24.2	4.5	326	325		353	[353]: 191 (100)
3	Cyanidin 3-*O*-galactoside [a]	33.4	7.9	516	515	449		[449]: 287 (100)
4	Cyanidin 3-*O*-glucoside [a]	36.6	9.0	514	515	449		[449]: 287 (100)
5	Cyanidin 3-*O*-arabinoside [a]	39.0	9.5	516	515	419		[419]: 287 (100)
6	Quercetin pentosyl-hexoside (Quercetin 3-*O*-vicianoside [b])	48.3	12.3	354	353		595	[595]: 301 (100)
7	Cyanidin pentoside (Cyanidin 3-*O*-xyloside [b])	49.7	12.4	516	515	419		[419]: 287 (100)
8	Quercetin 3-*O*-galactoside [a]	50.1	12.6	354	352		463	[463]: 301 (100)
9	Quercetin deoxyhexosyl-hexoside (Quercetin 3-*O*-robinobioside [b])	51.0	12.9	354	352		609	[609]: 301 (100)
10	Quercetin 3-*O*-glucoside [a]	51.6	13.2	354	352		463	[463]: 301 (100)
11	Quercetin 3-*O*-rutinoside [a]	52.3	13.5	354	352		609	[609]: 301 (100)

[a] Identity was confirmed by authentic standard compounds.

[b] Tentatively identified based on (i) a comparison of retention times, peak sizes, and UV/Vis and mass spectral data with those reported by Slimestad et al. (2005) and (ii) by being able to exclude possible confusions with other iso-absorptive and isobaric compounds through the use of reference compounds.

Excellent recovery rates ranging from 95.5 to 99.7% for all individual phenolic compounds spiked at high and low concentration ranges, and intra-day and inter-day repeatabilities with CVs of 2.5-4.7% and 0.67-3.52% for HPLC and UHPLC, respectively, were obtained (Tab. 2).

HPLC-DAD analysis of phenolic compounds in two black chokeberry varieties

The developed extraction and HPLC method were used for the determination of phenolic compounds from two different chokeberry varieties 'Viking' and 'Nero' grown in Turkey. Dry matter contents were 25.4 ± 0.3 and 28.9 ± 0.2% for cvs. 'Viking' and 'Nero', respectively. As presented in Tab. 4, the total phenolics were in the range of 641.7 and 647.5 mg/100 g of FW. The phenolic composition was predominated by anthocyanins (67% of total phenolic compounds), followed by hydroxycinnamic acids (ca. 27%), and flavonols (6%). Cyanidin 3-*O*-galactoside was the prevailing phenolic constituent (Tab. 4). Concentrations of the individual phenolic compounds in cvs. 'Nero' and 'Viking' berries were comparable, except those of quercetin 3-*O*-galactoside that was found at elevated concentrations in the first mentioned cultivar. *Vice versa*, quercetin 3-*O*-robinobioside was determined at diminished levels in cv. 'Nero' compared to those in cv. 'Viking' berries (Tab. 4). The concentrations determined herein were comparable to those reported previously (Jakobek et al., 2012; Taheri et al., 2013; Rodríguez-Werner et al., 2019). In contrast to our findings, Jakobek et al. (2012) have reported significant differences between berries from cvs. 'Nero' and 'Viking' cultivated in Croatia in two consecutive years, specifically for their anthocyanin contents. In their study, anthocyanin levels of cv. 'Nero' differed between the two years, indicating that the accumulation of phenolic compounds, and particularly of anthocyanins, in black chokeberries may also be affected by environmental conditions.

Anthocyanin levels in black chokeberries exceeded those of other berries from the *Rosaceae* family, such as blackberry (*Rubus fruticosus* L.) (172.59 mg/100 g of FW), and red raspberry (*Rubus idaeus* L.) (72.5 mg/100 g of FW), but were lower than that of, e.g., black elderberry (*Sambucus nigra* L.) (1316 mg/100 g of FW) from *Adoxaceae* family (Neveu et al., 2010). Considering their flavonol (129-144 mg/100 g of DM) and chlorogenic acid (619-681 mg/100 g of DM) contents, black chokeberries are rich sources compared to, e.g., blackberry (12.77 mg/100 g of FW) (Neveu et al., 2010).

Tab. 4: Quantitation of individual phenolic compounds from black chokeberry cvs. 'Viking' and 'Nero' as determined by HPLC-DAD.

Peak no.	Compound	Conc. (mg/100 g of FW) 'Viking'	'Nero'
1	Neochlorogenic acid	89.0 ± 1.5	91.3 ± 3.2
2	Chlorogenic acid	84.2 ± 3.3	87.8 ± 3.5
3	Cyanidin 3-*O*-galactoside	305.1 ± 15.1	292.3 ± 18.0
4	Cyanidin 3-*O*-glucoside	18.1 ± 0.9	18.2 ± 1.1
5	Cyanidin 3-*O*-arabinoside	91.8 ± 4.4	90.7 ± 5.8
6	Quercetin pentosyl-hexoside	5.5 ± 0.5	5.4 ± 0.6
7	Cyanidin pentoside	22.5 ± 1.2	24.0 ± 1.6
8	Quercetin 3-*O*-galactoside	11.1 ± 1.0 *	14.2 ± 0.8 *
9	Quercetin deoxyhexosyl-hexoside	5.5 ± 0.6 *	4.5 ± 0.3 *
10	Quercetin 3-*O*-glucoside	8.5 ± 0.9	8.0 ± 0.4
11	Quercetin 3-*O*-rutinoside	6.1 ± 0.9	5.4 ± 0.3
	Total anthocyanins [b]	437.6 ± 21.5 (67.6%) [a]	425.3 ± 26.3 (66.3%) [a]
	Total hydroxycinnamic acids [b]	173.2 ± 4.3 (26.8%) [a]	179.1 ± 6.3 (27.9%) [a]
	Total flavonols [b]	36.7 ± 3.7 (5.7%) [a]	37.4 ± 2.3 (5.8%) [a]
	Total phenolics [b]	647.5 ± 27.8	641.7 ± 34.4

Values expressed as the mean ± standard deviation derived from the analyses of six samples ($n = 6$). Dry matter contents used for the calculation of fresh weight (FW) contents were 25.4 ± 0.3 % ('Viking') and 28.9 ± 0.2 % ('Nero'), respectively. Significantly different values ($p < 0.05$) between two cultivars were presented with an asterisk (*).

[a] Percentages of total phenolics (% w/w).

[b] Total anthocyanins, hydroxycinnamic acids, flavonols, and phenolics represent the sum of anthocyanins (no. 3, 4, and 5), hydroxycinnamic acids (no. 1 and 2), flavonols (no. 8, 9, 10, and 11), and all individual constituents, respectively.

Conclusions

An ultrasound-assisted extraction of phenolic compounds from black chokeberries cvs. 'Viking' and 'Nero' cultivated in Turkey was developed and validated, being based on the use of methanol/formic acid (95:5, v/v) as extraction solvent. Then, an HPLC- and UHPLC-based method was developed and validated for the quantitative analysis of 11 phenolic compounds from black chokeberries. Cyanidin glycosides were the most abundant phenolic constituents, followed by chlorogenic acid, and quercetin glycosides. Both cultivars from Turkey displayed similar phenolic compositions that resembled the profile previously reported in literature. Noteworthy, merely a few contributions have assessed Aronia fruit cultivated in a Mediterranean climate. Consequently, from a nutritional point of view, chokeberries from Turkey may be considered equally rich in the studied phenolic compounds as compared to berries from other provenances. The validated extraction and (U)HPLC methods established in this contribution may be applied in the food industry, e.g., for quality control and authentication of black chokeberry products. Future studies may target at establishing extraction protocols and liquid chromatographic methods to complementary assess the phenolic constituents of larger molecular weight contained in Aronia berries such as oligomeric and polymeric procyanidins.

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Conflict of interests

No potential conflict of interest was reported by the authors.

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