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White versus blue: Does the wild 'albino' bilberry (*Vaccinium myrtillus* L.) differ in fruit quality compared to the blue one?

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ABSTRACT

Wild albino and blue bilberry fruit were analyzed to compare different fruit characteristics linked to the composition of primary and secondary metabolites. Compounds were identified using HPLC–MS and standard quality parameters were determined. Albino berries were significantly smaller, accumulated less water and were characterized by 23% higher dry weight. Soluble solids content and pH value were higher in albino bilberry and their surface was lighter and characterized by a yellow hue. Both bilberry forms accumulated identical individual sugars and organic acids; however, the albino form had 33% higher content of total sugars and 9% higher content of total organic acids compared to the blue type. Fifteen anthocyanins were identified in both forms, but in albino bilberries, individual anthocyanins were only detected in traces. Blue bilberry contained 1.6-fold higher levels of flavanols, 2.1-fold higher levels of flavonols, 2.5-fold higher levels of hydroxycinnamic acid derivatives and consequently, 4.6-fold higher total phenolic content.

1. Introduction

Bilberry (*Vaccinium myrtillus* L.), also called European blueberry, is one of the most important wild berries in northern Europe (Riihinen, Jaakola, Kärenlampi, & Hohtola, 2008), because of their potential benefits for human health. The berries are a rich source of various phenolic compounds (Giovanelli & Buratti, 2009; Mikulic-Petkovsek, Schmitzer, Slatnar, Stampar, & Veberic, 2014; Može et al., 2011), which have been linked to their antioxidant potential. Bilberry contains particularly high levels of anthocyanins, possessing antimicrobial, anti-inflammatory and anti-mutagenic properties (Šaponjac, Čanadanović-Brunet, Ćetković, Djilas, & Četojević-Simin, 2015).

Composition and content of flavonoids and other phenolic compounds in bilberry fruits depend on growing conditions (specifically site, sun exposure and altitude) and climatic parameters (Lätti, Riihinen, & Kainulainen, 2008; Mikulic-Petkovsek et al., 2014; Rosłon, Osińska, Pióro-Jabrucka, & Grabowska, 2011). Differences are also found due to seasons, the degree of ripeness and most importantly, among genotypes (Prior et al., 1998). Several authors have studied the composition of bilberry fruits from diverse locations and have determined their content of anthocyanins (Garzón, Narváez, Riedl, & Schwartz, 2010; Giovanelli & Buratti, 2009; Lätti et al., 2008) and other phenolics (Jovančević et al., 2011; Laaksonen, Sandell, & Kallio, 2010). Wild bilberries are also common in Slovenia, and several compositional studies have elucidated their metabolic profile (Mikulic-Petkovsek, Schmitzer, Slatnar, Stampar, & Veberic, 2012; Mikulic-Petkovsek et al., 2014; Može et al., 2011; Veberic, Slatnar, Bizjak, Stampar, & Mikulic-Petkovsek, 2015). Nevertheless, the distribution of primary and secondary metabolites in natural bilberry color mutants is still largely unknown. Jaakola et al. (2002) collected white or pink-colored wild bilberry from the forests of Finland. This rare white type has also been found in Slovenian forests. Apparently, the only difference between these variants is the color of the berries, while the flavor and the size of the fruit are similar (Jaakola et al., 2002). Suppressed expression of anthocyanin biosynthesis genes causes the pale color of the albino mutant, as opposed to the typical intense blue of wild bilberries. Further investigation of the expression of flavonoid pathway genes in bilberry mutants confirmed the activity of anthocyanidin synthase in pink and phenylalanine ammonia-lyase and dihydroflavanol 4-reductase in white bilberry fruit. Similarly, chalcone synthase and transcription factors VmTDR4 and VmMYB2 were down-regulated in white/pink berries compared to the wild blue type (Jaakola et al., 2002, 2010) indicating their different metabolic pathways. Biosynthetic genes may be regulated by a common plant hormone – abscisic acid

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(ABA) (Karppinen et al., 2013; Zifkin et al., 2012). However, according to Ballinger, Maness, Galletta, and Kushman *(1972)* highbush blueberry (*Vaccinium corymbosum*) pink fruit is regulated by a single recessive gene.

The aim of the present study was to determine how different wild bilberry types (albino and blue) differ in terms of fruit quality parameters, with a special emphasis on phenolic composition. Previous studies have focused mainly on the elucidation of genes involved in anthocyanin accumulation. However, little is known on the specific phenolic composition of the albino bilberry mutant. Wild species with different external and internal quality parameters represent an appealing native genetic source for breeders and producers. White strawberry, blackberry, raspberry, and currant hybrids have already been offered to the market, which is constantly in search of novel products. Wild bilberry variability could thus prove interesting for cultivation of new, attractive white and pink cultivars.

2. Materials and methods

2.1. Plant material

Wild blue and albino bilberry fruit were collected at the fully ripe stage on 11 June 2014 from native populations in a forest near Žiri, 40 km west of Ljubljana, Slovenia. Only undamaged fruits were selected for the analysis. Immediately after harvest, fruit characteristics were measured, and 500 g of fruit of each type was frozen in liquid nitrogen and stored for up to one week at −80 °C until chemical analyses.

2.2. Fruit characteristics

To measure the fruit characteristics, five replicates (each replicate containing 20 fruits) were carried out. Fruit fresh weight was recorded and, for the determination of fruit dry weight content, a gravimetric method was used (Liu et al., 2014). Fresh fruit samples were dried at 105 °C until a constant weight was achieved, and then percentage content of water was calculated. Fruit firmness was evaluated on the surface of berries using a penetrometer (T.R. Turoni srl, Forlì, Italy) with a 1 mm-diameter needle (Newton, N). Soluble solids content (SSC) and pH value were evaluated in freshly extracted bilberry juice. SSC (°Brix) were determined using a digital hand-held refractometer (Mettler-Toledo, Greifensee, Switzerland) and pH value was measured with a pH-meter (WTW, Weilheim, Germany). Berry surface color was measured using a portable colorimeter (Konica Minolta, Tokyo, Japan) and values were expressed in L*, a* and b* color parameters. The lightness dimension L* ranges from 0 (pure black) to 100 (the reference white), while negative parameter a* represents green, a* positive red and b* negative blue and b* positive yellow color (McGuire, 1992).

2.3. Extraction and determination of sugars and organic acids

Primary metabolites (sugars and organic acids) were analyzed in whole bilberry fruit. For each bilberry type, ten repetitions were carried out (n = 10); each repetition included several fruits. For the extraction of primary metabolites, 2.5 g of fruit were ground to a fine paste in a mortar, homogenized with 9 mL of double distilled water and left for 30 min at room temperature as reported by Mikulic-Petkovsek et al. (2012). After the extraction, the homogenate was centrifuged (Eppendorf, Hamburg, Germany) at 12.857 g for 7 min at 10 °C. The supernatant was filtered through a 0.20 μm cellulose ester filter (Macherey–Nagel, Düren, Germany) and transferred into a vial. The analysis of primary metabolites was carried out using high performance liquid chromatography (HPLC) of Thermo Separation Products on a Rezex RCM-monosaccharide Ca+ (2%) column (300 mm × 7.8 mm) from Phenomenex (Torrance, USA) (operated at 65 °C). The mobile phase was double distilled water, and the flow rate maintained at 0.6 mL min^{-1}. Total run time was 30 min, and a refractive index (RI) detector was used to monitor the eluted carbohydrates as described by Mikulic-Petkovsek et al. (2012). Organic acids were analyzed on the same HPLC system, equipped with a UV detector set at 210 nm, using a Rezex ROA-organic acid H+ (8%) column (300 mm × 7.8 mm) from Phenomenex, as described by Mikulic-Petkovsek et al. (2012). The column temperature was set at 65 °C. The elution solvent was 4 mM sulfuric acid in double distilled water at a flow rate of 0.6 mL min^{-1}. The duration of the analysis was 30 min. Sugars and organic acids in fruit extracts were identified by their retention time characteristics and by comparison to known standards; the content levels were calculated with the help of the corresponding standard and expressed in mg g^{-1} fresh weight (FW) of bilberries. The content of all analyzed sugars was summed up and presented as total analyzed sugars. In a similar way, total analyzed organic acids were calculated. Both values were used for the determination of sugar/organic acid ratio.

2.4. Extraction of phenolic compounds

The extraction of fruit samples was performed as described by Mikulic-Petkovsek et al. (2015) with some modification. As for sugars and organic acids, ten repetitions (each included several fruits) were carried out (n = 10) for each type. Berries were ground to a fine paste in a mortar chilled with liquid nitrogen, and 2.5 g was extracted with 8 mL methanol containing 3% (v/v) formic acid and 1% (w/v) 2,6-di-*tert*-butyl-4-methylphenol (BHT) in a cooled ultrasonic bath for 1 h. BHT was added to the samples to prevent oxidation of phenolics. After extraction, fruit extracts were centrifuged for 10 min at 12,857*g*. Each supernatant was filtered through a Chromafil AO-20/25 polyamide filter (Macherey-Nagel, Düren, Germany) and transferred to a vial prior to injection into the HPLC system.

2.5. Determination of individual phenolic compounds using HPLC–DAD–MS^n analysis

Phenolic compounds were analyzed on a Thermo Finnigan Surveyor HPLC system (Thermo Fisher Scientific, Waltham, USA) with a diode array detector at 280 nm (flavanols, hydroxycinnamic acid derivatives, iridoids), 350 nm (flavonols) and 530 nm (anthocyanins). Spectra of the compounds were recorded between 200 and 600 nm. The column was a Gemini C18 (150 × 4.6 mm, 3 μm; Phenomenex) operated at 25 °C. The elution solvents were aqueous 0.1% formic acid in double distilled water (A) and 0.1% formic acid in acetonitrile (B). Samples were eluted according to the linear gradient from 5% to 20% B in the first 15 min, followed by a linear gradient from 20% to 30% B for 5 min, then an isocratic mixture for 5 min, followed by a linear gradient from 30% to 90% B for 5 min, and then an isocratic mixture for 15 min, before returning to the initial conditions (Wang, Zheng, & Galletta, 2002). The injection amount was 20 μL and flow rate was maintained at 0.6 mL min^{-1}. All phenolic compounds were identified using a mass spectrometer (Thermo Fisher Scientific) with ESI operating in negative (all phenolic groups except for anthocyanins) and positive (for anthocyanins) ion mode. The analyses were carried out using full-scan data-dependent MS^n scanning from *m*/*z* 115–1500. The injection volume was 10 μL and the flow rate maintained at 0.6 mL min^{-1}. The capillary temperature was 250 °C, the sheath gas and auxiliary gas were 20 and 8 units, respectively, and the source voltage was 4 kV for negative ionization and 0.1 kV for