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Changes in phenolic profiles of red-colored pellicle walnut and hazelnut kernel during ripening

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ABSTRACT

In studies of secondary metabolites in nuts, many constituents in the kernel remain unidentified due to a high content of phenolic compounds in the pellicle. In the present study, we focused on the investigation of the phenolic and dicarboxylic acid profiles of walnut and hazelnut pellicle-less kernels. High-performance liquid chromatography with diode array and mass spectrometric detection (HPLC-DAD-MSn) was used to carry out the determination of individual phenolics and dicarboxylic acids in brown and red-pellicle walnut and hazelnut. Results show that hexahydroxydiphenic acid (HHDP) di-galloyl hexose isomer, vanillic acid hexoside, quinic acid derivative and catechin are the main constituents of the phenolic profile of walnut, while galloylquinic derivative, caffeoyl hexoside and catechin are the main constituents of the hazelnut kernel. Even though both walnut and hazelnut kernels have a considerably lower content of phenolic compounds and dicarboxylic acids in comparison to the pellicles, when calculated as a percentage of the total mass, the kernel makes a significant contribution to the total phenolic content of the whole nut.

1. Introduction

Walnut (*Juglans regia* L.) and hazelnut (*Corylus avellana* L.) kernels constitute more than 60 percent of tree nuts consumed in Europe. USA and China lead global production of walnuts, while Turkey is the main producer of hazelnuts (INC. Nuts, 2017). In the last ten years, global consumption of tree nuts has increased by 22%, indicating the significant increase of this phenolic source in human diet.

Tree nuts are generally consumed as raw, roasted and/or salted snacks. Even though tree nuts are a high-energy food, they are a valuable source of fat-soluble bioactive compounds and nutrients. Their phytochemical profile also contains a large number of phenolic compounds (Alasalvar & Bolling, 2015). Walnuts have the most diverse phenolic profile and highest content of phenolics among tree nuts. The total phenolic content of walnuts ranges between 1558 and 1625 mg/100 g, while hazelnuts have a significantly lower content of total phenolics (291–875 mg/100 g) (Bolling, Chen, McKay, & Blumberg, 2011). In both walnuts and hazelnuts, phenolic compounds mainly consist of phenolic acids, flavanols, condensed and hydrolysable tannins and, to a lesser extent, tyrosols, dihydrochalcones and flavonols (Chang, Alasalvar, Bolling, & Shahidi, 2016). Alasalvar and Shahidi (2008) single out hydrolysable and condensed tannins as the most abundant phenolic class in walnuts and hazelnuts. The pellicle has been found to be the main source of phenolic compounds in walnut (Colaric, Veberic, Solar, Hudina, & Stampar, 2005), even though the pellicle accounts for only 5–8% of total kernel mass (Martínez, Labuckas, Lamarque, & Maestri, 2010).

During ripening, the content of phenolic compounds in edible fruits changes rapidly and significantly. On reaching ripeness, most edible fruits have a lower content of phenolics in the pulp and a higher content of color-related phenolics (anthocyanins) in the skin (Bizjak, Mikulic-Petkovsek, Stampar, & Veberic, 2013; Castrejón, Eichholz, Rohn, Kroh, & Huyskens-Keil, 2008; Zorenc, Veberic, Stampar, Koron, & Mikulic-Petkovsek, 2016). This is because the biological process of seed ripening is over and protection from herbivores in the form of the unpleasant taste of unripe fruit is no longer needed. The focus of color-related phenolics is subsequently transferred from protection to the attraction of seed carriers.

The structure and ripening of tree nuts is somewhat different from that of other temperate fruits. In the first place, tree nuts are not typical fruit composed of an edible pericarp wrapping inedible seeds. In the case of walnuts, the edible seed is enfolded in a hard, woody shell covered by a green husk, which is rich in phenolics (Stampar, Solar, Hudina, Veberic, & Colaric, 2006; Trandafir, Cosmulescu, & Nour, 2017). Seeds consist of two large cotyledons covered by a thin layer of skin (pellicle), which accounts for less than 10% of the total kernel

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weight (Martínez et al., 2010). The pellicle serves as a phenolic-rich barrier protecting the fatty kernel from oxidation.

A large number of studies to date have dealt with the problem of identifying phenolic compounds in nuts (Anjum et al., 2017; Grace, Warlick, Neff, & Lila, 2014; Gómez-Caravaca, Verardo, Segura-Carretero, Caboni, & Fernández-Gutiérrez, 2008; Jakopic et al., 2011). A major breakthrough in characterizing walnut phytochemicals was the identification of the novel compounds, glansrins (phenolics) and glansreginins (Ito, Okuda, Fukuda, Hatano, & Yoshida, 2007), and their comprehensive identification by Regueiro et al. (2014), who confirmed a total of 120 different phenolics. Following these accomplishments, glansreginins were also identified in hazelnut kernels (Slatnar, Mikulic-Petkovsek, Stampar, Veberic, & Solar, 2014). However, there are scarce data on the phenolic composition of skinless (without pellicle) walnut or hazelnut kernels (Colaric et al., 2005; Cristofori, Bertazza, & Bignami, 2015).

In fresh nuts, the pellicle contributes to a slightly astringent and bitter taste (Schmitzer, Slatnar, Veberic, Stampar, & Solar, 2011). However, astringency is not desirable in roasted nuts. In addition to giving taste properties to raw nuts, the pellicle can contribute to the visual appearance. Uncommon, red cultivars of walnut and hazelnut, such as the red 'Robert Livermore' cultivar, grown and sold in U.S.A., can attract demanding consumers and offer growers a better price over traditional, light-colored cultivars (Souza, 2016).

According to available research information, walnuts and hazelnuts are rich sources of phenolic compounds, but existing studies have mainly focused on the nut kernel and less on the individual parts. Additionally, most analyses have been carried out on fully ripe and often dried nuts, even though walnuts and hazelnuts are often eaten fresh and sometimes not fully ripe (Cristofori et al., 2015). Fruit peel is often presented as the main source of phenolic compounds in fruit (Jakopic, Stampar, & Veberic, 2009) and it is often forgotten that, when calculated as a percentage of the mass, pulp makes a significant contribution to the total phenolic content of the whole fruit (Persic, Mikulic-Petkovsek, Slatnar, & Veberic, 2017).

The aim of our research was to characterize the phenolic profiles of walnut and hazelnut kernels without pellicle and to study the dynamics of phenolic changes in kernels during ripening. Analysis of the pellicle-less kernel will provide more detailed phenolic profiles of walnut and hazelnut, since low-content phytochemicals are often disguised by a large concentration of phenolic compounds in the pellicle. The obtained results could expand the existing knowledge of the phytochemicals of nuts. A further objective was to compare the kernel phenolic profiles of red pellicle cultivars of walnut and hazelnut with common brown pellicle cultivars. These results could be used for determining the pellicle color of walnuts and hazelnuts on the basis of kernel phenolic profile, before the pellicle color is developed.

2. Materials and methods

2.1. Sample

Walnuts and hazelnuts were sampled from the collection orchard of the Biotechnical Faculty located in Maribor (lat. 46° 32′ N, long. 15° 39′ E, elevation 275 m), Slovenia. Both walnuts and hazelnuts were grown in a continental climate.

Samples of whole nuts were collected during ripening, approximately every 30 days (22nd July, 19th August and 15th September). For walnuts, sampling was carried out on a walnut cultivar with a red pellicle (RW) and on cultivars with a brown pellicle ('Lara' and 'Fernor'). In the first sampling in July, the inner part of the walnut kernel was not yet formed in the 'Fernor' and 'Lara' cultivars, so data on the phenolic composition of the RW kernel in July is only given as total analyzed phenolics. Sampling of hazelnuts was carried out on a cultivar with a red pellicle (RH) and brown pellicle cultivars ('Tonda di Giffoni' (TDG) and 'Clark'). Immediately after harvesting, approximately two kilograms of fresh walnuts and hazelnuts were removed from the green husks. The hard shells were shattered and the kernels were peeled from the pellicle. The kernel without pellicle (hereinafter kernel) was then frozen in liquid nitrogen and stored at −80 °C until further analysis.

2.2. Chemicals

The following standards were used for quantification of phenolic compounds: gallic, ellagic, vanillic, caftaric and 3-caffeoylquinic acid from Sigma-Aldrich Chemie GmbH (Steinheim, Germany), phloridizin, apigenin-7-glucoside, quercetin-3-D-galactoside, kaempferol 3-glucoside, epicatechin from Fluka Chemie (Buchs, Switzerland), myrcetin-3-rhamnoside from Apin Chemicals (Abingdon, UK), (+)-catechin and caffeic acid from Roth (Karlsruhe, Germany). Methanol and hexane, both purchased from Sigma-Aldrich Chemie GmbH, were used in the phenolic extraction procedure. The chemicals for the mobile phases were HPLC-MS grade acetonitrile and formic acid from Fluka Chemie. Water for the mobile phase was double distilled and purified with the Milli-Q system (Millipore, Bedford, MA, USA).

2.3. Extraction and quantification of phenolic compounds

The method previously described by Pirisi, Cabras, Cao, Migliorini, and Muggelli (2000) was used for the extraction of phenolic compounds, with some modifications. The kernel was ground in liquid nitrogen. Approximately 1.5 g of ground material was extracted in 3 ml of methanol in a cold ultrasonic bath for 1 h. The temperature was controlled by the addition of ice. After extraction, 1.5 ml of hexane was added to the extract. The samples were stirred on an electric vortex for 10 s and subsequently centrifuged for 10 min at 11,500*g* (Eppendorf centrifuge 5810 R, Hamburg, Germany). The methanolic phase was filtered through polyamide Chromafil AO-20/25 filters (Macherey-Nagel, Düren, Germany) into vials. Analyses were carried out using the Thermo Finnigan Surveyor HPLC system (Thermo Scientific, San Jose, USA) on a Gemini C18 (15 × 4.6 mm, 3 µm; Phenomenex (Torrance, CA, USA)) column operated on 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 a 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.

Phenolic compounds were identified using mass spectra (Thermo Scientific, LCQ Deca XP MAX) with electrospray ionization (ESI) operating in negative (all phenolic groups except anthocyanins) and positive ion mod (anthocyanins). Parameters for the ESI were as follows: the capillary temperature was 250 °C, the source voltage was 4 kV, and the sheath gas and auxiliary gas were 20 and 8 units, respectively. The injection volume of sample was 10 µl and the flow rate was set at 0.6 ml/min. Full scan data-dependent MSn scanning from *m/z* 115 to 1600 was carried out. All phenolic compounds were confirmed based on their mass fragmentation pattern, by comparing their UV–VIS spectra and by adding standard solution to the sample.

The concentration of individual phenolic compounds was calculated from the peak area of an identified individual phenol and corresponding standards. For missing compounds, the concentration was calculated as follows: derivatives of ellagic acid were calculated from the standard curve of ellagic acid, derivatives of quinic acid were calculated from the standard curve of quinic acid and caffeic acid derivatives were calculated from the standard curve of caffeic acid. The content of identified dicarboxylic acids was calculated from the gallic acid standard curve. All analyses were carried out in five repetitions. The content of the identified compounds was expressed in µg/g of fresh weight (FW).