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HPLC-MS^n identification and quantification of phenolic compounds in hazelnut kernels, oil and bagasse pellets

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ABSTRACT

The paper reports the phenolic content of kernels, bagasse pellets (residue of oil pressing) and oils from five different cultivars and a mix of cultivars. Phenolic compounds were identified by high-performance liquid chromatography coupled with mass spectrometry. Two compounds, glansreginin A and glansreginin B, were detected for the first time in hazelnuts. The main polyphenolic subclass comprised of mono- and oligomeric flavan 3-ols, which accounted between 34.2 and 58.3% in hazelnut kernels and between 36.7 and 48.6% in pellets of the total phenolics detected. In hazelnut oils four compounds have been detected, their content levels ranged from 0.97 to 0.01 μg g^{-1}. Total phenolic content ranged from 491.2 to 1700.4 mg GAE kg^{-1} in kernels, from 848.4 to 1148.5 mg GAE kg^{-1} in pellets and only from 0.14 to 0.25 mg GAE g^{-1} in oils. The percentage of radical scavenging activity ranged from 60.0 to 96.4% for kernels, 63.0 to 73.2% for pellets and from 17.7 to 29.9% for oil. The study provides clear evidence on high phenolic contents and similarly high antioxidant potential of hazelnut kernels and bagasse pellets. The latter could be used as ingredients in cooking and baking or even for production of plant-based pharmaceuticals.

1. Introduction

Hazelnut (*Corylus avellana* L.) belongs to the Betulaceae family and is considered one of the most popular tree nuts consumed worldwide, ranking second in nut production after almond. Hazelnut kernels are typically consumed whole (raw, with skin, or roasted, without skin) or used as ingredients in a variety of processed foods, especially in bakery and confectionery products. Hazelnut skin, hard shell, green leafy cover, as well as leaves are byproducts of roasting, cracking, shelling/hulling, and harvesting processes (Shahidi, Alasalvar, & Liyana-Pathirana, 2007), and are currently investigated for their composition in order to add economic value to waste products emerging from hazelnut industry.

Hazelnut kernels contain dietary fibers and beneficial nutrients such as plant proteins, essential minerals (potassium, calcium, magnesium, selenium), vitamin E, B complex vitamins, as well as unsaturated fatty acids, plant sterols, phytochemicals and micronutrients like tocopherols (Alasalvar, Shahidi, Liyanapathirana, & Ohshima, 2003). Phenolics in hazelnut kernels protect the seed against oxidation and are associated with moderate astringency and characteristic bitter taste of fresh nuts. Among ten nut species analyzed in studies of some authors (Delgado, Malheiro, Pereira, & Ramalhosa, 2010; Kornsteiner, Wagner, & Elmadfa, 2006), hazelnut exhibited an intermediate content of total phenols, which was lower compared to walnuts and pecans but higher compared to pine nuts and macadamias. Various phenolic acids such as gallic, caffeic, *p*-coumaric, ferulic, sinapic, caffeoyltartaric and caffeoylquinic acids have already been quantified in hazelnut samples by several authors (Amaral et al., 2005; Oliveira et al., 2007). Jakopic et al. (2011) identified nine flavan-3-ols, two benzoic acids (gallic and protocatechuic acid), three flavonols and a phloretin glycoside in hazelnut kernels. The composition of hazelnut kernels is relatively well known but a complete profile has yet to be established. Phytochemical studies mostly focus on other nut trees, such as walnut and recently, 16 additional polyphenols, including 3 new hydrolyzable tannins — glansrins A–C, have been reported in walnut kernels (Fukuda, Ito, & Yoshida, 2003). The new tannins were characterized as ellagitannins possessing a tergalloyl group or a related acyl group. The isolated polyphenolic compounds demonstrated a potent antioxidant effect by SOD-like and DPPH radical scavenging assays.

In Slovenia, hazelnut shrubs and trees are naturally widespread all over the country, while the commercial growing is limited to about 1.7% of the Slovene intensive fruit growing area, which accounts for approx. 4000 ha. The average commercial orchard size is 2.2 ha, producing approx. 200 tons of in-shell nuts per year (Avanzato et al., 2009). Growers are striving to produce high quality nuts, which are mostly sold at local markets. In order to increase income from hazelnut production, new products are permanently tested. Oil made of hazelnut kernels is considered a potentially interesting byproduct, which can help to achieve this goal. According to Venkatachalam and Sathe (2006) nut oils are one of the richest plant oils, containing high proportions of unsaturated fatty acids, which are considered important health

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promoting components of the human diet. Furthermore, some studies have shown that microconstituents of tree nut oils exhibited good antioxidant activities (Chandrasekara & Shahidi, 2011; Miraliakbari & Shahidi, 2008; Veldsink et al., 1999), and their source of antioxidants is important for maintaining a healthy lifestyle. Due to many health beneficial effects, which have been highlighted in both scientific press and popular press, hazelnut oil is gaining popularity in Slovenian market in addition to kernels and in-shell nuts. According to Durak et al. (1999) hazelnut based products should frequently be consumed and included in the habitual diet in quantities of approx. 1 g of hazelnut/day/kg of body weight. Based on the monitored total phenolic contents and mean kernel weight of the studied cultivars, reported by Solar and Stampar (2011), a daily portion of 54 kernels or an equivalent amount of pellets (58 g per day) is recommended. Raw kernels with skins have frequently been reported a superior source of bioactive polyphenols compared to peeled kernels (Alasalvar et al., 2009; Arcan & Yemenicioglu, 2009; Locatelli et al., 2010) and could be consumed as healthy snacks.

In order to determine nutraceutical value of selected hazelnut cultivars major phenolic compounds have been identified in kernels and oils. As a residue of oil cold-pressing, bagasse pellets mainly consist of kernel skins, and could thus represent a good source of phenolics. Namely, in walnuts, most of the phenolic compounds are located in the skin and less than 10% are retained in the kernel after the skin is removed. Similarly, a significant portion of phenolics is located in the skin of other nut species (Blomhoff, Carlsen, Andersen, & Jacobs, 2006; Colaric, Veberic, Solar, Hudina, & Stampar, 2005). For this reason the composition of bagasse pellets has also been analyzed with HPLC-MS. To the present day, only less comprehensive chemical methods have been employed to identify phenolic compounds in hazelnut kernels and by-products and a relevant phenolic characterization of hazelnut products is crucial. Moreover, no compositional study on hazelnut oil or pellets has been published to our knowledge. A further objective of the research was to define differences in phenolic contents (individual and total phenolics) and antioxidant activity among hazelnut kernels, pellets and oils of selected cultivars.

2. Materials and methods

2.1. Plant material

Kernels, oils and pellets of 'Clark', 'Fertile de Coutard', 'Istrska dolgoplodna leska', 'Segorbe', and 'Tonda di Giffoni' cultivars were investigated along with a mix of approx. ten different cultivars. Nuts were collected from mature hazelnut trees, growing in a randomized block design with three plants per cultivar. The orchard is located near Maribor (NE Slovenia; 46° 32′ N, 15° 39′ E, elevation 275 m) and is a part of nut cultivar collection field of Biotechnical Faculty. Technologically mature in-shell nuts were dried in a circulated air-flow in a wooden three-level dryer at a constant temperature of 35 °C to approx. 12% moisture content, followed by machine cracking. Five and a half kilograms of kernels additionally dried to 6% humidity was randomly sampled per each cultivar. Sixty raw kernels per cultivar (five replications with 12 kernels) were separated and stored at 7 °C until phenolic analysis. The remaining 5 kg of hazelnut kernels was cold-pressed according to the traditional Slovenian method in a screw-driven oil press with a 12 mm diameter nozzle at the rotation speed of 25 Hz. Press head box temperature was set at 45 °C and the outlet oil temperature did not exceed 31 °C. Oils and bagasse pellets were stored at 7 °C in dark bottles and plastic bags, respectively, until chemical analysis.

2.2. Chemicals

The standards used for the determination of the phenolic compounds in samples were: gallic acid, ellagic acid, *p*-coumaric acid, myricetin-3-*O*-rhamnoside, phloridzin from Sigma-Aldrich, (−)-epicatechin, (+)-catechin, procyanidin B2, phloridzin dihydrate, and quercetin-3-*O*-rhamnoside from Fluka Chemie (Buchs, Switzerland). Methanol and *n*-hexane for extraction of phenolics were acquired from Sigma-Aldrich. The chemicals for the mobile phases were HPLC-MS grade acetonitrile and formic acid from Fluka. Water for mobile phase was double distilled and purified with the Milli-Q system (Millipore, Bedford, MA).

2.3. Extraction and HPLC-MS^n analysis of individual phenolic compounds

Hazelnut kernels were ground with a mechanical grinder. Hazelnuts crumbs or bagasse pellets (2.5 g) were extracted with 5 ml of CH_3OH/H_2O (v/v, 60/40) in a cooled water bath (0 °C) using sonification for 60 min. Hazelnut extracts were centrifuged (Eppendorf centrifuge 5810 R, Hamburg, Germany) at 10,000 rpm for 10 min at 4 °C. The supernatant was filtered through a Chromafil AO-45/25 polyamide filter produced by Macherey-Nagel and transferred to a vial prior to injection into the HPLC system.

10 g of oil was weighted in a centrifuge tube and mixed with 10.0 ml of *n*-hexane and 2.0 ml of CH_3OH/H_2O (v/v, 60/40). The mixture was stirred for 2 min in a vortex apparatus and centrifuged at 3000 rpm. The methanol layer was separated and the extraction repeated twice. The extracts were combined and washed twice with 2 ml of *n*-hexane. The *n*-hexane was discarded. The combined extracts of the hydrophilic layer were brought to dryness in a rotary evaporator under reduced pressure at 40 °C, and the residue was re-dissolved in 500 μl of methanol/water (50:50, v/v) and filtered through a 0.20 μm filter prior to HPLC injection (Pirisi, Cabras, Cao, Migliorini, & Muggelli, 2000). Five replications were performed for each cultivar.

Phenolic compounds were analyzed on a Thermo Scientific Dionex UltiMate 3000 Series UHPLC+ focused (Thermo Scientific, San Jose, USA) with a diode array detector set at 280 nm and 350 nm. Spectra of the compounds were recorded between 200 and 600 nm. The column was a Gemini C_{18} column (150 × 4.6 mm 3 μm; Phenomenex, Torrance, USA) 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 volume was 20 μl and flow rate was maintained at 0.6 ml min^{-1}.

All phenolic compounds were identified by an HPLC-Finnigan MS detector and an LCQ Deca XP MAX (Thermo Finigan, San Jose, CA) instrument with electrospray interface (ESI) operating negative ion mode. The analyses were carried out using full scan data-dependent MS^n scanning from 110 to 1500 *m/z*. Column and chromatographic conditions were identical to those used for the HPLC-DAD analyses. The injection volume was 10 μl and the flow rate was maintained at 0.6 ml min^{-1}. The capillary temperature was 250 °C, the sheath gas and auxiliary gas were 60 and 15 units, respectively; the source voltage was 3 kV for negative ionization and 4 kV for positive ionization and normalized collision energy was between 20 and 35%. Spectral data were elaborated using the Excalibur software (Thermo Scientific). The identification of compounds was confirmed by fragmentation, comparison of retention times and compound spectra as well as by adding the standard solution to the sample.

2.4. Determination of total phenolic content

The extraction of kernels, bagasse pellets and oil samples for the determination of total phenolic content (TPC) was performed according to the same protocol as that of individual phenolics. TPC of the extracts was assessed using the Folin–Ciocalteu phenol reagent method (Singleton & Rossi, 1965). Six milliliters of double distilled water and 500 μl of Folin–Ciocalteu reagent were added to 100 μl of the sample extracts and after waiting for between 8 s and 8 min at