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Abstract: The fatty acid, tocopherol, sterol, phospholipid and phenolic compositions of Polish hazelnuts (Kataloński and Webba Cenny) were examined. Particularly, free + esterified and bound tocopherol, sterol and phenolic compounds were determined.
The major fatty acids found in hazelnuts were oleic and linoleic acids. α-tocopherol was the most abundant tocopherol accounting for 90-92% of the total content. Bound tocopherols represented 45.5 and 21.7% of total tocopherols in Kataloński and Webba Cenny cultivar, respectively. Total free + esterified sterols were between 62.0 and 75.7% of total sterols and β-sitosterol was the first sterol in the two samples. Phosphatidylcholine was the most common phospholipid, accounting for 72.2% for Kataloński and 67.5% Webba Cenny, respectively. The most abundant fatty acids in the phospholipid fraction were oleic equally with palmitic acids.
Twelve free and six bound phenolic compounds were identified and quantified in hazelnut kernel, instead nine free and six bound phenolic compounds were determined in hard shell.

Highlights

- free and bound tocopherol, sterol and phenolic compounds were determined
- Twelve free and six bound phenolic compounds were identified in kernel
- nine free and six bound phenolic compounds were identified in hard shell
- Bound tocopherols represented 45.5 and 21.7% of total tocopherols

Determination of lipid and phenolic fraction in two hazelnut (*Corylus avellana* L.) cultivars grown in Poland

Running title: ***Determination of lipid and phenolic compounds in Polish hazelnuts***

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Abstract

The fatty acid, tocopherol, sterol, phospholipid and phenolic compositions of Polish hazelnuts (*Kataloński* and *Webba Cenny*) were examined. Particularly, free + esterified and bound tocopherol, sterol and phenolic compounds were determined.

The major fatty acids found in hazelnuts were oleic and linoleic acids. α-tocopherol was the most abundant tocopherol accounting for 90-92% of the total content. Bound tocopherols represented 45.5 and 21.7% of total tocopherols in *Kataloński* and *Webba Cenny* cultivar, respectively. Total free + esterified sterols were between 62.0 and 75.7% of total sterols and β-sitosterol was the first sterol in the two samples. Phosphatidylcholine was the most common phospholipid, accounting for 72.2% for *Kataloński* and 67.5% *Webba Cenny*, respectively. The most abundant fatty acids in the phospholipid fraction were oleic equally with palmitic acids.

Twelve free and six bound phenolic compounds were identified and quantified in hazelnut kernel, instead nine free and six bound phenolic compounds were determined in hard shell.

Keywords: hazelnuts, tocopherols, sterols, phenolics, phospholipids

1. Introduction

Hazelnuts (*Corylus avellana* L.) are the second most popular nuts worldwide just after almonds with a global production average at nearly one million tonnes (MT) annually (Contini, Baccelloni, Massantini & Anelli, 2008; Schmitzer, Slatnar, Veberic, Stampar & Solar, 2011). The world's hazelnut production is mainly covered by two main market players; Turkey produces about 430 000 MT/year and Italy about 130 000 MT/year in unshelled basis (FAOSTAT, 2011). However, there are also smaller but significant producers, such as, USA, Azerbaijan, Georgia, China, Iran, Spain, France, Kirgizstan, Poland and Croatia, listed here in descending order of production abundance.

Poland has no long standing tradition of producing large-fruited hazelnuts, but there has been a constant increase in output since beginning production in the 1980s. In accordance with the FAO database, production output came very recently to over 3000 MT/year in unshelled basis (FAOSTAT, 2011). The cultivation area is mainly composed of scattered and small orchards, located mostly in Lublin region (south-eastern part of Poland) and also in south and central Poland.

The lipid fraction forming the major part of hazelnuts (~60%), is composed of non polar (98.8%) and polar (1.2%) constituents (Alasalvar, Shahidi, Liyanapathirana & Ohshima. 2003a; Alasalvar, Amaral, Satir & Shahidi, 2009a). Triacylglycerols are the major nonpolar lipid class, representing nearly 100% of the total nonpolar lipids in hazelnut oil (Alasalvar, Shahidi, Liyanapathirana & Ohshima, 2003b). Minor lipidic compounds are the sterols and tocopherols: total sterol content varies from about 120 to 250 mg/100 g of hazelnut oil (Alasalvar et al. 2009a; Amaral, Casal, Citova, Santos, Seabra & Oliveira, 2006a; Crews et al. 2005). The differences occur among cultivars, though, sitosterol is the major sterol. Hazelnuts are an excellent source of tocols ranging from 11 to 45 mg/100 g of oil (Alasalvar et al. 2009a; Amaral, Casal, Seabra & Oliveira, 2006b; Crews et al. 2005). The major tocopherol is

68 α-tocopherol, accompanied by γ- and β-tocopherols. Hazelnut oil has been reported to have
69 the highest α-tocopherol level among tree nut oils (Kornsteriner, Wagner & Elmadfa, et al.
70 2006).
71 Hazelnuts are an abundant source of several vitamins and minerals. They are a source of fibre,
72 which has a nutritional function for humans, but also contain an array of phytochemicals
73 including phenolics.
74 The content of phenolics in hazelnuts serves as a significant criterion in evaluating hazelnut
75 quality (Alasalvar, Karamać, Kosińska, Rybarczyk, Shahidi & Amarowicz, 2009b; Contini et
76 al., 2008). Five phenolic acids have been identified and quantified (both, free and esterified
77 forms) by Alasalvar, Karamać, Amarowicz & Shahidi (2006) and Shahidi, Alasalvar &
78 Liyanapathirana (2007) in the hazelnut kernel and by-products. Recently, Jakopic, Mikulic
79 Petkovsek, Likozar, Solar, Stampar & Veberic (2011) detected nine flavan-3-ols, two benzoic
80 acids derivatives, three flavonols and phloretin glycoside, as free phenolic compounds in
81 hazelnuts.
82 The phenolics in fruits have been quantified as a freely extractable and the nonextractable
83 polyphenols (Arranz, Saura-Calixto, Shaha & Kroon, 2009). At present, only the extractable
84 phenolics in free and esterified forms of hazelnuts and its by-products have been thoroughly
85 studied (Shahidi, Alasalvar & Liyana-Pathirana, 2007), whereas significant amounts of
86 bioactive bound polyphenols that remain in the residues from free phenolic extraction have
87 not been studied qualitatively yet. However, Yang (2009) took into account the concentration
88 of total, free and bound phenolics in kernels of nine types of tree nuts, including hazelnuts.
89 Nevertheless, the identification of the single bound phenolic compounds have not been
90 studied so far.
91 The principal aim of this research was to investigate the content of lipidic (including fatty
92 acid composition, tocopherol, sterol and phospholipid analysis), and phenolic compounds of

93 Polish hazelnut varieties *Kataloński* and *Webba Cenny* commonly cultivated in Poland.
94 Analytical data on total, free and bound tocopherols, as well as sterols and phenolics collected
95 with up-to-date instrumental methods were compared and related to available literature data.
96
97 **2. Materials and Methods**
98
99 *2.1. Chemicals*
100 All the solvents and reagents were purchased from Merck (Darmstadt, Germany). The
101 standard compounds were supplied by Sigma-Aldrich (Saint Louis, MO, USA).
102
103 *2.2. Samples*
104 The investigated hazelnuts (*Corylus avellana* L.), *Kataloński* and *Webba Cenny* varieties were
105 procured at the orchard located in the south of Poland (Jankowice, Pszczyna 50°0′5″N 18°59′
106 18″E) in 2012. The plants (both varieties) were grown in open field with the same
107 agronomical conditions. Preliminary sun-dried nuts (3 days at 20–25 °C) were stored
108 unshelled at 10°C for one month until they were analysed (final moisture content <7% water
109 in d.m.). The hazelnuts *Kataloński* and *Webba Cenny* listed in *The Polish National List of*
110 *Fruit Plant Varieties 2013* are the varieties of which seed material is eligible for production
111 and marketing in Poland and is allowed to be distributed on the European Union territory.
112 Hazelnuts were manually cracked and shelled before chopping. The fibrous skin, particularly
113 distinctive for *Kataloński* variety, was removed by hand and a brown skin (pellicle) was left
114 for analysis.
115 *2.3. Moisture content*
116 Approximately 5 g of the sample were dried in a drying oven for 8 hours in 105°C until
117 constant weight according to AOAC (1995), following with moisture calculations.

118 *2.4. Total lipid extraction (TL)*

119 The hazelnut oil was extracted using the procedure described by Boselli, Velazco, Caboni &
120 Lercker (2001). Approximately 10 g of the sample was homogenised with 100 mL of a
121 chloroform/methanol solution (1/1 *v/v*) in a glass bottle with a screw-cap. The bottle was kept
122 at 60°C for 20 min before adding an additional 100 mL of chloroform. After 3 min of
123 homogenisation, the content of the mixture was filtered through the filter paper. The filtrate
124 was mixed thoroughly with 70 mL of 1 M KCl solution and left overnight at 4°C in order to
125 phase separation. The organic phase was collected and dried with a rotary evaporator at 40°C,
126 dissolved in 5 mL *n*-hexane/isopropanol solution (4/1, *v/v*) and stored at -18°C until it was
127 analysed.

128

129 *2.5. Isolation of total unsaponificable compounds by hot saponification (HS)*

130 Hot saponification was performed as described by Caligiani, Bonzanini, Palla, Cirlini & Bruni
131 (2010) with minor modifications. Briefly, approximately 10 g of hazelnuts were finely
132 chopped using the manual chopper (Kitchen Mate, UK), the 0.5 mL of dihydrocholesterol (c
133 = 2.0 mg/mL) was added and saponification was carried out by boiling and stirring for 1 h
134 with 100 mL of 1.0 N potassium hydroxide in ethanol-water solution (4/1 *v/v*). After cooling,
135 100 mL of distilled water was added, and the sample transferred to a separating funnel and
136 extracted four times with 50 mL of diethyl ether. The ether extracts were pooled into a
137 separating funnel and washed four times with 50 mL of distilled water. The organic phase was
138 dried with anhydrous sodium sulphate, filtered, dried and the residue was weighed.

139

140 *2.6. Total fatty acid analysis*

141 The fatty acid composition of hazelnut oil samples was determined from TL extract as
142 FAMEs by capillary gas chromatography analysis after alkaline treatment as described by

Christie (1982). The chromatographic conditions were the same as reported by Verardo, Gómez-Caravaca, Gori, Losi, & Caboni (2013).

2.7. Tocopherol analysis

Due to free and total tocopherols determination, approximately 300 mg of fat from TL and 5 mg of fat from HS extract were dissolved in 1 mL and 7 mL of *n*-hexane, respectively. The solutions were filtered through a 0.45 µm nylon filter. The tocopherols were determined by HPLC (Agilent 1200 series, Palo Alto, CA, USA) equipped with a fluorimeter detector (Agilent, Palo Alto, CA, USA). The excitation wavelength was 290 nm and the emission one was 325 nm. The column used was a Luna Hilic Phenomenex column (250 mm x 4.6 mm i.d., 5 µm particle size) in isocratic conditions as reported by Gómez-Caravaca, Verardo & Caboni (2010). Calibration curve was constructed with α-tocopherol standard solution and it was used for quantification.

2.8. Free + esterified and bound phytosterol (PS) analysis

To determine the free + esterified phytosterols, approximately 0.5 mL of dihydrocholesterol (c = 2.0 mg/mL) was added to 250 mg of fat from TL extract and saponification was conducted at room temperature. After saponification, the organic fraction was washed with 10 mL diethyl ether/water (1/1 *v/v*), and further the unsaponifiable matter was extracted in sequence; two times with 10 mL diethyl ether, and washed two times with 10 mL 0.5 N aqueous KOH and again two times with 10 mL of distilled water. The diethyl ether solvent was removed under vacuum and the unsaponificable matter was used for the free + esterified sterol determination.

To determine the total sterols, 5 mg of fat from HS extract was used for analysis.

167 The unsaponificable matter from TL and HS extracts were sylilated according to Sweeley,
168 Bentley, Makita & Wells (2002), and they were analysed using a GC/MS (GCMS-QP2010
169 Plus, Shimadzu, Tokyo, Japan) in the chromatographic conditions reported by Cardenia,
170 Rodriguez-Estrada, Baldacci, Savioli & Lercker (2012).
171 Data were filed and processed by the software GCMSsolution ver. 2.50 SU1 from Shimadzu.
172 PS identification was achieved by comparing peak mass spectra with peaks of standard
173 mixture and with GC-MS data reported by Pelillo, Iafelice, Marconi & Caboni (2003).
174 Quantification of identified phytosterols was performed in relation to dihydrocholesterol used
175 as internal standard.
176
177 *2.9. Phospholipid determination*
178 To determine the phospholipids in hazelnut, approximately 100 mg of fat from TL extract
179 were weighed and dissolved in 1 mL of 88/12 (*v/v*) chloroform/methanol system and used for
180 the HPLC analysis.
181 The quantitation of the phospholipid classes was performed using HPLC-ELSD. The
182 chromatographic method used for the separation of the polar lipids extracted from hazelnut oil
183 by Verardo, Gomez-Caravaca, Gori, Losi & Caboni (2013) was carried out. Phospholipid
184 separation was performed on an Agilent liquid chromatography HP 1200 Series (Agilent
185 Technologies, Palo Alto, California, USA). The detector was an evaporative light scattering
186 detector (ELSD; PL-ELS1000, Polymer Laboratories, Church Stretton, Shropshire, UK). The
187 control of the HPLC system was accomplished by the software Agilent ChemStation (Agilent
188 Technologies, Santa Clara, CA, USA) whilst chromatograms registration and data processing
189 were assessed by ClarityLite (ver. 2.4.0.190, DataApex, Praha, The Czech Republic). The
190 separation was achieved using a silica column, 150 mm × 3 mm with 3 μm particle diameter

(Phenomenex, Torrance, CA, USA). The calibration curves were prepared separately for each phospholipid identified.

2.10. Determination of phospholipid fatty acids

About 20 mg of fat from TL extract were dried under nitrogen, dissolved in 0.5 mL of chloroform and loaded on a Silica gel 60 TLC plate 20 x 20 cm (Merck KGaA, Darmstadt, Germany)**.** The mobile phase was 100 mL of a mixture *n*-hexane/diethyl ether 3/2 (*v/v*). Phospholipid band was visualised under UV light (254 nm) by spraying the TLC plate with a 0.02% (*m/v*) ethanolic solution of 2,7-dichlorofluorescein (sodium salt) and then scraped off and collected. Phospholipids were extracted three times with chloroform (3 x 1 mL). Organic extracts were pooled, dried under nitrogen and, to convert fatty acids to the corresponding methyl esters (FAMEs), the method of Christie (1982) was carried out. FAMEs were determined by GC-FID according to Verardo et al. (2013).

2.11. Extraction and determination of free phenolic compounds

The free phenolic compounds were extracted using the optimised protocol described by Verardo, Bendini, Cerretani, Malaguti, Cozzolino & Caboni (2009). Briefly, 4 g of the chopped hazelnuts kernel and shell were extracted in an ultrasonicator using 40 mL of ethanol/water solution (4/1 *v/v*) at 40°C for 10 min. After centrifugation at 3,500 rpm for 15 min, the supernatant was collected and the residue was re-extracted under the same conditions. The free phenolic composition of the extracts was determined with RP-HPLC–DAD–MS, as previously described by Bocalandro et al. (2012).

2.12. Extraction and determination of bound phenolic compounds

Both, the kernel and shell residues of free phenolics ethanol extraction were taken under alkaline hydrolysis as reported by Bonoli, Verardo, Marconi & Caboni (2004). Briefly, the residue from free phenolic extraction was digested with 100 mL of 2 N NaOH in distilled water at room temperature for 20 h with no light access and stirring under nitrogen gas. The mixture was then brought to pH 2-3 by adding 37% hydrochloric acid in a cooling ice bath and extracted with 100 mL of *n*-hexane to remove the lipid fraction. The final solution was extracted three times with 100 mL of ethyl acetate/diethyl ether (1/1 *v/v*). The organic fractions were pooled and evaporated to dryness. The phenolic compounds were reconstituted with 4 mL of 1/1 methanol/water (*v/v*) and stored at -18°C until it was analysed. The polyphenol composition of the extracts was determined with RP-HPLC–DAD–MS, as previously described by Bocalandro et al (2012).

2.13. Statistical analysis

Relative standard deviation was obtained, where appropriate, for all data collected. One-way analysis of variance, ANOVA (Tukey's honest significant difference multiple comparison) was evaluated using Statistica 8 software (2006, StatSoft, Tulsa, OK, USA). *p*-values lower than 0.05 were considered statistically significant. All chemical analyses were carried out in triplicate (n=3) for each sample, and the analytical data were used for statistical comparisons.

3. Results and Discussion

3.1. Fatty acid composition

The fatty acid composition of studied hazelnuts is given in Table 1. The predominant fatty acid was oleic acid (C18:1 ω9), followed by linoleic acid (C18:2 ω6), with the mean values being in close agreement with the ranges reported elsewhere (Amaral et al., 2006a; Alasalvar et al., 2003b; Bacchetta et al., 2013). Based on the analysis conducted, statistically significant

differences (p<0.05) concerning content of major fatty acids in *Kataloński* and *Webba Cenny* were found.

Kataloński reported higher percentages of linoleic acid and, consequently, PUFA content as compared to *Webba Cenny*. *Webba Cenny* however showed the higher content of oleic acid, MUFA and SFA than *Kataloński.* Several authors (Alasalvar et al., 2003a) demonstrated that the content of oleic acid in various hazelnut cultivars is inversely correlated with the content of linoleic acid. This is reasonably consistent with the results herein reported for *Kataloński* and *Webba Cenny* cultivars.

Other major fatty acids were palmitic and stearic acids that represented more than 4 and 1% of total fatty acids, respectively.

SFA content for *Kataloński* and *Webba Cenny* varieties in present study were less than 6 and 7%, respectively. The average SFA content of 75 cultivars from Spain, Italy, Greece, Slovenia, France and Portugal studied by Bacchetta et al. (2013) accounted for 8.43%, total SFA value in the Portuguese cultivars ranged from 7.5 to 10% (Amaral et al., 2006a), and Turkish cultivar made up with 7.85% SFA of the total fatty acids of hazelnut oil (Alasalvar et al., 2003b). The data obtained in this work can be due to the different weather conditions in Poland; that are colder and rainy if compared with the Mediterranean climate of Turkey, Italy or Spain (Gantner, 2005). However, according to Piskornik & Korfel (1993), nuts obtained in these conditions contain considerably less saturated fatty acids (SFA).

3.2. Free (+ esterified), bound and total tocopherol and sterol content

Firstly, all tocopherol and sterol values presented in literature on hazelnuts so far have been reported on the oil basis and expressed in mg/kg, μg/g or mg/100g of oil (Matthäus & Özcan, 2012; Kornsteriner et al., 2006). However, the total values of tocopherols and sterols

264 expressed on whole-nut basis have not been published yet, excepting one report on total α-
265 tocopherol content presented by Alasalvar et al. (2003a).
266 In the present study, the hot saponification was preliminary tested using 2.2 N KOH solution,
267 as reported by Caligiani et al. (2010), however, the tocopherols, especially β- and γ-
268 homologues were degraded during the saponification process. The similar KOH concentration
269 for direct saponification (11% *w/v* solution ≈ 2 N) was previously used by Katsanidis & Addis
270 (1999) on animal origin matrix and they concluded that the investigated method is only useful
271 for the analysis of the α-homologues. Because of that, lower KOH concentrations were tested
272 (data not showed) and the data underlined that a solution of KOH 1 N reported better results
273 for this matrix.
274 After establishing the procedure, free, bound and total tocopherols were determined. Results
275 are shown in Table 2 and were expressed as dry base weight (moisture was 3.96 and 3.34 %
276 *w/w* for *Kataloński* and *Webba Cenny* cultivars, respectively). Among the tocopherols
277 identified, α-tocopherol was most abundant accounting for 90-92% of the total tocopherol
278 content, followed by γ- (4.3-7.0%) and β-tocopherol (2.4-4.1%).
279 The extraction method (TL and HS) influenced significantly (p<0.05) all tocopherol recovery
280 and total amounts of α-, γ- and β-homologues. Herein, total tocopherol content was 236.2 and
281 205.8 mg/kg of d.w. nut for *Kataloński* and *Webba Cenny* cultivars, respectively. The α, γ and
282 β tocopherols obtained directly from hot saponification of the matrix were on average 1.5, 1.3
283 and 2.0 fold higher, respectively, than free forms obtained on the oil basis. These data
284 confirmed that bound tocopherols are contained in hazelnuts and the direct saponification of
285 the sample is needed to determine their content.
286 The free α-tocopherol concentration also expressed as mg/kg d.w. nut amounted to 204.3 and
287 186.5 for *Kataloński* and *Webba Cenny* cultivars, respectively. These results were in the same
288 order of magnitude as the data reported by Alasalvar et al. (2003a) where α-tocopherol was

289 determined with concentration of 24 mg/100 g of nut. In order to compare the hazelnuts
290 cultivated in Poland with wider range of literature, the results of free tocopherols were
291 recalculated and expressed on the oil basis. As can be seen in Supplemental Figure S1,
292 *Kataloński* cultivar had significantly (p<0.05) higher concentration of α- (298.6 μg/g oil), γ-
293 (20.7 μg/g oil) and total tocopherols (327.1 μg/g oil) than *Webba Cenny* (283.6, 18.6, 309.6
294 μg/g oil, respectively). In case of β-tocopherol (*Kataloński* 7.8 μg/g oil, *Webba Cenny* 8.4
295 μg/g oil), there was no statistically significant differences between cultivars (p<0.05). In
296 general, results of free tocopherol contents are in agreement with available data (Kornsteriner
297 et al., 2006; Alasalvar et al., 2003b; Savage, McNeil & Dutta, 1997).
298 Bound tocopherols represented the 45.5 and 21.7% of total tocopherols in *Kataloński* and
299 *Webba Cenny* cultivar, respectively. As reported for the free form, α-tocopherol was the first
300 bound tocopherol and accounted for the 81.0 and 88.2% of total bound tocopherols in
301 *Kataloński* and *Webba Cenny* samples, respectively. Bound γ-tocopherol was the second
302 bound tocopherol and its content was 15.4 and 3.1 mg/kg of nut for *Kataloński* and *Webba*
303 *Cenny* samples representing the 14.3 and 6.9% of total bound tocopherols, respectively.
304 Bound β-tocopherol accounted for the 4.7 and 4.9% of total bound tocopherol content.
305
306 Seven sterols and three stanols were identified. Table 3 presents their free + esterified, bound
307 and total form content.
308 Total content (free + esterified + bound) of phytosterols found in *Kataloński* and *Webba*
309 *Cenny* samples was 1522.2 and 1303.2 mg/kg d.w. nut, respectively. These results are in the
310 same order of magnitude as those of Nyström, Schär & Lampi (2012).
311 No statistical differences were reported in free + esterified phytosterols content in both
312 samples; total free + esterified sterol compounds were the 62.0 and 75.7% of total sterols in
313 *Kataloński* and *Webba Cenny* samples, respectively (p<0.05). Sitosterol was the first free

sterol in the two samples tested and accounted for the 84.0% of total free + esterified sterols. The other free + esterified sterols, in decreasing order of abundance, were campesterol (53.5 and 50.0 mg/kg d.w. for *Kataloński* and *Webba Cenny* samples), and Δ^5-avenasterol (36.7 and 39.5 mg/kg d.w. for *Kataloński* and *Webba Cenny* samples). Other major compounds were the saturated sterols, namely sitostanol and campestanol.

Other minor free + esterified sterol compounds that were determined were Δ^7-avenasterol, stigmasterol, chlerosterol, fucosterol and cholesterol.

Bound sterols in *Kataloński* and *Webba Cenny* samples were the 38 and 24% of total sterols, respectively. As reported for the free sterols, sitosterol, campesterol and Δ^5-avenasterol were the first, second and third bound sterols in all samples. The sum of sitosterol, campesterol and Δ^5-avenasterol was 92 and 85.5% of total bound sterols in *Kataloński* and *Webba Cenny*, respectively.

In order to provide comparable results, the concentration of free + esterified sterols was recalculated and expressed on the oil basis (Supplemental Figure S2). The most abundant phytosterols, namely, sitosterol, campesterol, and Δ^5-avenasterol found in *Kataloński* and *Webba Cenny* oils accounted for 1894.5 and 1689.7 mg/kg, 128.9 and 101.5 mg/kg, 88.4 and 80.2 mg/kg, respectively. Obtained sterol results for both cultivars are in the wide agreement with those presented in literature. As an example, Matthä us & Özcan (2012) determined that hazelnut cultivars oil grown in Turkey contained 1222.2–4947.3 mg/g sitosterol, 81.1–445.9 mg/g campesterol, and 60.1–143.1 mg/g Δ^5-avenasterol, whereas Savage et al. (1997) reported 1416–1693 mg/g sitosterol, 78–114 mg/g campesterol, and 33–170 mg/g Δ5-avenasterol.

3.3. Phospholipid (PL) analysis

The phospholipid content and the distribution of individual phospholipid species were determined in the two hazelnut cultivars (Table 4). Three phospholipids (PC – phosphatidylcholine, PE – phosphatidylethanolamine; PI – phosphadidylinositol) were fully separated and identified according to Parcerisa, Codony, Boatella & Rafecas (1999) and Alasalvar et al. (2003b). The *Kataloński* cultivar was significantly higher in total phospholipid content than the *Webba Cenny* ($p<0.05$). The PC fraction was the most common phospholipid, accounting for 72.2% for *Kataloński* and 67.5% *Webba Cenny*, and followed by PE and PI, respectively. These results are in the close agreement with the data of Mirliakbari & Shahidi (2008), that reported the contents of PC and PI in the amount of 4.8 and 0.8 mg/g of oil, respectively. Different results were reported by Parcerisa et al. (1999), where the total PL content were accounted as ten fold lower (1.09 mg/g of oil). The occurred discrepancy could be coupled with the applied response factor for the PL calibration curve; herein the calibration curve was prepared separately for PC, PE and PI, whereas Parcerisa et al. (1999) assumed the PC response factors for PE and PI calculation. Additionally, the same authors (Parcerisa et al., 1999) analysed different cultivars that were obtained in different harvest time, agronomical management, soil, and climate conditions.

Nevertheless, limited information regarding phospholipids in hazelnuts is available in the literature.

To determine the phospholipid fatty acids and to obtain the purified phospholipid fraction from the total lipid extract, a TLC separation was carried out. This technique permits the isolation of the different lipid classes (Montealegre, Verardo, Gómez-Caravaca, García-Ruiz, Marina & Caboni, 2012).

The most abundant fatty acids in the PL fraction were oleic equally with palmitic acid, followed by stearic, palmitoleic and linoleic acids, whereas *Kataloński* cultivar was approximately three fold richer in the linoleic acid than *Webba Cenny* (Table 5). Fatty acid

363 profiles detected for PL fraction of both cultivars were consistently different and richer in
364 SFA than that observed for total lipids (triglycerides + polar fats) according to Parcerisa et al.
365 (1999) that reported the considerable higher concentration of SFA in PC that in TAG class.
366
367 *3.4. Determination of free and bound phenolic compounds in kernel and shell*
368 Phenolic compounds in raw shelled hazelnuts were analysed. Two different extraction
369 methods were carried out to obtain free and bound phenolics from *Kataloński* and *Webba*
370 *Cenny* cultivars grown in Poland.
371 The extracted compounds were identified by analysing UV and MS data and quantified by
372 DAD detection. The quantification was performed by comparison with calibration curves of
373 proanthocyanidin B-2 (0.03–1.00 g/L), taxifolin (0.06–1.00 g/L), (+)-catechin (0.10–1.00
374 g/L), gallic acid (0.12–1.20 g/L), quercetin (0.10–1.0 g/L), phloretin (0.05-1.00 g/L) and
375 syringic acid (0.10–1.0 g/L) dissolved in methanol and filtered with a 0.20 µm filter.
376 Twelve free phenolic compounds were identified and quantified in hazelnut kernel. Briefly,
377 two phenolic acids (gallic and protocatechuic acids) and ten flavonoids (catechin and
378 epicatechin, 2 procyanidin dimmers, 2 procyanidin trimers, 3 flavonols and 1 chalcone) were
379 determined. These compounds were previously identified in hazelnut kernel by several
380 authors (Shahidi et al., 2007; Jakopic et al., 2011; Schmitzer et al., 2011).
381 Table 6 shows all the identified compounds determined in the hazelnut samples. *Kataloński*
382 cultivar showed the highest content of total free phenolic compounds (31.7 µg/g d.w. nut) and
383 its content was about two times higher than for *Webba Cenny*.
384 Phenolic acids accounted for the 38 and 32% of total free phenolic compounds in *Kataloński*
385 and *Webba Cenny* kernel, respectively. Gallic acid was the first phenolic acid in the two
386 samples and its concentration ranged from 4.1 µg/g in *Webba Cenny* to 11.1 µg/g in
387 *Kataloński*, where the highest concentration of this phenolic compound was reported. These

388 results accorded to other authors (Alasalvar, Pelvan & Amarowicz, 2010; Pelvan, Alasalvar &
389 Uzman, 2012).
390 Flavan-3-ols were the first free phenolic compounds in the both samples; in fact catechin and
391 their oligomers represented the 48 and 89% of total free phenolics, respectively. Procyanidin
392 dimmers were contained in higher quantities and ranged from 9.7 to 11.8 µg/g d.w. nut in the
393 analysed samples. These results were in the same order of magnitude as those reported in
394 literature (Jakopic et al., 2011; Schmitzer et al. 2011).
395 Three flavonols, namely myricetin-3-O-rhamnoside, quercetin-pentoside and quercetin-3-O-
396 rhamnoside were identified and quantified. According to Jackopic et al. (2011) quercetin-
397 rhamnoside was the first flavonol, and herein it is two times higher in *Kataloński* than in
398 *Webba Cenny*.
399 Six bound phenolic compounds were identified in the hazelnut kernels (Table 6). All of them
400 are phenolic acids.
401 *Kataloński* and *Webba Cenny* samples showed a similar total content of phenolic compounds
402 ranged between 17.4 and 19.7 µg/g d.w.
403 Compound with principal fragment ions at m/z 179 and 139 m/z was identified as caffeic acid
404 derivative and it was the first bound phenolic compound in the two studied cultivars. It
405 accounted for 40.3 and 71.9% of total bound phenolics in *Kataloński* and *Webba Cenny*
406 samples, respectively.
407 The other compounds that were identified in both samples, according to the following
408 characteristic ions, were: $[M-H]^-$ 153, corresponding to protocatechuic acid; $[M-H]^-$ 167,
409 vanillic acid; $[M-H]^-$ 223, sinapic acid; $[M-H]^-$ 163, *p*-coumaric acid; $[M-H]^-$ 191, quinic
410 acid. Their content in two cultivar tested was reported in Table 6.
411 HPLC-DAD-ESI-MS was used to determine the free and phenolic component present in hard
412 shell hazelnuts. Up-to-date, there are no sufficient data available on free phenolics contained

413 in hazelnut hard shell. Shahidi et al. (2007) reported free phenolic compounds in hazelnut
414 hard shell by HPLC-DAD method for the first time, but there were tentatively identified and
415 quantified merely five phenolic acids. The other authors determined the free phenolic fraction
416 in the hazelnut shell by spectrophotometrical methods (Stévigny, Rolle, Valentini & Zeppa,
417 2007; Contini et al., 2008).
418 Nine free phenolic compounds were identified in *Kataloński* and *Webba Cenny* samples used
419 MS detector. Briefly, five phenolic acids, three flavonoids and a chalcone compound were
420 determined in the hazelnut samples (Table 6). Total free phenolic content in *Kataloński*
421 samples was two fold higher than in *Webba Cenny*.
422 According to Shahidi and co-workers (2007), gallic acid was the first phenolic acid in hard
423 shell; its content was 2.7 and 0.8 µg/g d.w. nut in *Kataloński* and *Webba Cenny*, respectively.
424 The second and third phenolic acids were protochatechuic and vanillic acids respectively.
425 Moreover, sinapic and *p*-coumaric acids were, also, identified, but their content was lower
426 than LOQ. Quercetin-pentoside and catechin were the principal flavonoids in the hard shell
427 but myricetin-3-O-rhamnoside and phloretin-2-O-glucoside were also identified and
428 quantified in both tested cultivars.
429 As far as we know, six bound phenolic compounds were identified and quantified in hazelnut
430 shell for the first time. Total bound phenolic content in hard shell was about 7-8 times higher
431 than free phenolic content.
432 Four phenolic acids and two flavonoids were identified (Table 6). Phenolic acids were the
433 73.1 and 71.3% of total bound phenolic compounds in *Kataloński* and *Webba Cenny*,
434 respectively. Protocatechuic and vanillic acids were the first phenolic acids and their content
435 was higher than 30 µg/g d.w. nut. Gallic acid and its quinic derivative were also quantified.

436 The two representative flavonoids were taxifolin and catechin. Taxifolin was not previously
437 described in hazelnut but it is a component of other woody structure, such as pine bark
438 (Bocalandro et al. 2012).
439
440 **4. Conclusions**
441 To sum up, different extraction methods were used for examining lipids and phenolic
442 antioxidants of two hazelnut cultivars grown in Poland. The results confirmed that direct
443 saponification of hazelnut is necessary to determine the total sterol and tocopherol content,
444 because these components in hazelnuts are present in both free and bound forms.
445 HPLC-DAD-ESI-MS was used to identify and quantify the free and bound phenolic
446 compounds in hazelnut kernels and in the hard shells. Hazelnut shells represent a rich source
447 of natural phenolic antioxidants.
448
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576

Table 1. Fatty acid composition (g/100 g of total fatty acids) of hazelnut varieties grown in Poland.

Fatty acids	Variety	
	Kataloński	*Webba Cenny*
C14:0	0.02 ± 0.00 a	0.02 ± 0.00 a
C16:0	4.33 ± 0.02 a	4.61 ± 0.05 b
C16:1	0.18 ± 0.00 a	0.17 ± 0.00 b
C17:0	0.03 ± 0.00 a	0.03 ± 0.00 a
C17:1	0.08 ±0.00 a	0.07 ± 0.00 b
C18:0	1.47 ± 0.02 a	2.13 ± 0.08 b
C18:1 ω9	80.25 ± 0.50 a	81.42 ± 0.14 b
C18:2 ω6	13.16 ± 0.51 b	11.06 ± 0.17 a
C18:3 ω6	0.03 ± 0.00 a	0.02 ± 0.01 a
C18:3 ω3	0.12 ± 0.00 a	0.11 ± 0.00 b
C20:0	0.09 ± 0.00 a	0.11 ± 0.00 b
C20:1	0.14 ± 0.00 a	0.14 ± 0.01 a
C22:0	0.02 ± 0.00 a	0.02 ± 0.00 a
C22:2	0.05 ± 0.01 a	0.05 ± 0.01 a
C24:0	0.03 ± 0.00 a	0.02 ± 0.00 a
Σ SFA	5.99 ± 0.02 a	6.95 ± 0.04 b
Σ MUFA	80.65 ± 0.50 a	81.80 ± 0.13 b
Σ PUFA	13.36 ± 0.52 b	11.25 ± 0.17 a

Data expressed as means ± standard deviation (n=3). The different lower case letters (a-b) in the same row indicate significantly different values ($p < 0.05$)

Table 2. Tocopherol content in the two hazelnut varieties cultivated in Poland (mg/kg d.w. nut)

Tocopherols		Variety	
		Kataloński	*Webba Cenny*
α-tocopherol	**free**	117.3 ± 2.4 a	147.1 ± 4.9 b
	bound	87.0 ± 2.9 a	39.4 ± 1.4 b
	total	204.3 ± 23.3 a	186.5 ± 16.2 a
γ-tocopherol	**free**	7.9 ± 0.4 a	10.2 ± 0.00 b
	bound	15.4 ± 0.5 a	3.1 ± 0.5 a
	total	23.3 ± 0.2 a	13.3 ± 0.5 b
β-tocopherol	**free**	3.5 ± 0.0 a	3.8 ± 0.2 a
	bound	5.1 ± 0.2 a	2.2 ± 0.5 b
	total	8.6 ± 0.2 a	6.0 ± 0.3 b
Total	**free**	128.7 ± 2.8 a	161.1 ± 5.1 b
	bound	107.5 ± 6.9 a	44.7 ± 5.4 b
	total	236.2 ± 23.7 a	205.8 ± 15.4 a

Data expressed as means ± standard deviation (n=3). The different lower case letters (a-b) in the same row indicate significantly different values ($p < 0.05$)

Table 3. Sterols content in two hazelnut varieties cultivated in Poland (mg/kg d.w. nut)

Sterols		Variety	
		Kataloński	*Webba Cenny*
Cholesterol	**free + esterified**	2.4 ± 0.2 a	1.6 ± 0.6 a
	bound	6.7 ± 0.4 b	1.8 ± 0.2 a
	total	9.1± 0.5 b	3.4± 0.3 a
Campesterol	**free + esterified**	53.5 ± 1.0 b	50.0 ± 0.6 a
	bound	35.8 ± 2.1 b	17.3 ± 1.5 b
	total	89.3 ± 1.3 b	67.3 ± 1.0 a
Campestanol	**free + esterified**	11.0 ± 1.0 b	8.0 ± 0.7 a
	bound	2.7 ± 0.6 a	6.1 ± 1.5 b
	total	13.7± 0.8 a	14.1± 1.0 a
Stigmasterol	**free + esterified**	6.1 ± 0.5 a	6.0 ± 0.1 a
	bound	3.2 ± 0.3 b	1.2 ± 0.0 a
	total	9.3 ± 0.4 b	7.2 ± 0.5 a
Stigmastanol	**free + esterified**	1.2 ± 0.3 a	0.9 ± 0.1 a
	bound	2.3 ± 0.5 b	1.2 ± 0.1 a
	total	3.5 ± 0.3 b	2.1 ± 0.1 a
Chlerosterol	**free + esterified**	5.7 ± 0.3 b	4.3 ± 0.7 a
	bound	7.1 ± 1.2 b	2.8 ± 0.6 a
	total	12.8 ± 0.5 b	7.1 ± 0.3 a
Sitosterol	**free + esterified**	789.6 ± 31.2 a	832.7 ± 36.2 a
	bound	472.1 ± 23.4 b	242.1 ± 18.4 a
	total	1261.7 ± 48.5 b	1074.8 ± 29.2 a
Sitostanol	**free + esterified**	28.2 ± 1.7 a	32.7 ± 0.2 b
	bound	15.9 ± 0.8 a	29.1 ± 0.9 b
	total	44.1 ± 0.9 a	61.8 ± 1.2 b
Δ5-avenasterol	**free + esterified**	36.7 ± 2.6 a	39.5 ± 1.7 a
	bound	24.7 ± 2.5 b	11.3 ± 2.0 a
	total	61.4± 2.7 b	50.8± 2.5 a
Fucosterol	**free + esterified**	3.4 ± 0.5 a	4.2 ± 0.6 a
	bound	1.1 ± 0.1	1.3 ± 0.3 a
	total	4.5± 0.4 a	5.5± 0.5 a
Δ7-avenasterol	**free + esterified**	6.1 ± 0.1 a	6.8 ± 0.4 a
	bound	6.7 ± 0.3 b	3.8 ± 0.5 a
	total	12.8± 0.6 b	9.0± 0.4 a
Total	**free + esterified**	943.9 ± 47.0 a	986.7 ± 31.6 a
	bound	578.3 ± 33.4 b	316.5 ± 30.8 a
	total	1522.2 ± 41.6 b	1303.2 ± 36.3 a

Data expressed as means ± standard deviation (n=3). The different lower case letters (a-b) in the same row indicate significantly different values ($p < 0.05$)

Table 4. Phospholipid content (mg/g of oil) and percentage of each phospholipid (on total phospholipid content) in Polish hazelnut varieties.

Phospholipids	Variety			
	Kataloński		*Webba Cenny*	
	mg/g of oil	%	mg/g of oil	%
PC	7.9 ± 0.0 a	72.2	5.3 ± 0.1 b	67.5
PE	2.1 ± 0.0 a	19.6	1.8 ± 0.0 b	23.2
PI	0.9 ± 0.0 a	8.2	0.7 ± 0.0 b	9.3
Total	10.9 ± 0.1 a		7.9 ± 0.1 b	

PC - Phosphatidylcholine, PE - Phosphatidylethanolamine; PI - Phosphadidylinositol.

Data expressed as means ± standard deviation (n=3). The different lower case letters (a-b) in the same row indicate significantly different values ($p < 0.05$)

Table 5. Phospholipid fatty acid composition (% area of total methyl esters) of hazelnut varieties grown in Poland.

Fatty acids	Variety	
	Kataloński	*Webba Cenny*
C16:0	32.3 ± 0.1 a	34.7 ± 0.6 b
C16:1	11.0 ± 1.4 a	12.5 ± 0.8 a
C18:0	14.6 ± 1.9 a	14.9 ± 0.5 a
C18:1 ω9	32.4 ± 0.4 a	34.5 ± 0.8 b
C18:2 ω6	9.7 ± 0.2 a	3.4 ± 0.5 b
Σ SFA	46.8 ± 1.9 a	49.6 ± 1.0 a
Σ MUFA	43.4 ± 1.7 a	47.0 ± 1.5 a
Σ PUFA	9.7 ± 0.2 a	3.4 ± 0.5 b

Data expressed as means ± standard deviation (n=3). The different lower case letters (a-b) in the same row indicate significantly different values ($p < 0.05$)

Table 6. Free and bound phenolic compounds (µg/g d.w. nut or shell) isolated in kernel and hard shell of analysed hazelnuts

Phenolic compounds	[M-H]$^-$ (m/z)	Fragments (m/z)	Free phenolics				Bound phenolics			
			Kernel		Hard shell		Kernel		Hard shell	
			Kataloński	*Webba Cenny*	*Kataloński*	*Webba Cenny*	*Kataloński*	*Webba Cenny*	*Kataloński*	*Webba Cenny*
Gallic acid [1]	169	125	11.1 ± 0.1 b	4.1 ± 0.0 c	2.7 ± 0.0 d	0.8 ± 0.0 e	nd	nd	16.9 ± 0.1 a	13.9 ± 0.0 b
Caffeic acid derivative [2]	179	135	nd	nd	nd	nd	7.9 ± 0.0 b	12.5 ± 0.0 a	nd	nd
Protocatechuic acid [1]	153	109	1.1 ± 0.1 d	1.1 ± 0.1 d	0.5 ± 0.0 e	1.1 ± 0.0 d	2.5 ± 0.0 c	4.8 ± 0.0 b	33.0 ± 0.2 a	33.6 ± 0.1 a
Procyanidin dimmer [3]	577	451 289	10.5 ± 0.1 a	5.3 ± 0.0 b	nd	nd	nd	nd	nd	nd
Procyanidin trimer [3]	865	577	0.8 ± 0.0 a	1.2 ± 0.1 a	nd	nd	nd	nd	nd	nd
Catechin [4]	289	245	0.5 ± 0.0 d	1.0 ± 0.0 c	0.8 ± 0.0 c	0.3 ± 0.0 d	nd	nd	16.0 ± 0.1 b	20.5 ± 0.0 a
Vanillic acid [1]	167	123	nd	nd	0.4 ± 0.0 e	0.2 ± 0.0 e	2.9 ± 0.0 d	3.5 ± 0.0 c	34.8 ± 0.1 b	36.0 ± 0.0 a
Procyanidin trimer [3]	865	577	2.2 ± 0.1 b	3.0 ± 0.5 a	nd	nd	nd	nd	nd	nd
Taxifolin [5]	303	-	nd	nd	nd	nd	nd	nd	21.5 ± 0.1 a	22.1 ± 0.0 a
Procyanidin B2 [3]	577	451 289	1.3 ± 0.0 b	4.5 ± 0.2 a	nd	nd	nd	nd	nd	nd
Galloylquinic acid [1]	343	191	nd	nd	nd	nd	nd	nd	17.4 ± 0.0 b	22.3 ± 0.0 a
Sinapic acid [2]	223	-	nd	nd	< LOQ	< LOQ	4.0 ± 0.1 b	6.9 ± 0.0 a	nd	nd
***p*-coumaric acid** [2]	163	119	nd	nd	< LOQ	< LOQ	5.1 ± 0.0 a	2.1 ± 0.2 b	nd	nd
Quinic acid [1]	191	-	nd	nd	nd	nd	5.1 ± 0.0	< LOQ	nd	nd
Epicatechin [4]	289	245	0.1 ± 0.0 a	0.1 ± 0.0 a	nd	nd	nd	nd	nd	nd

Myricetin-3-O-rhamnoside [6]	463	317	0.8 ± 0.1 a	0.5 ± 0.1 b	0.2 ± 0.0 c	< LOQ	nd	nd	nd	nd
Quercetin-pentoside [6]	433	301	1.0 ± 0.0 b	2.9 ± 0.0 a	1.0 ± 0.0 b	0.2 ± 0.0 c	nd	nd	nd	nd
Quercetin-3-O-rhamnoside [6]	447	301	2.8 ± 0.0 b	4.9 ± 0.1 a	nd	nd	nd	nd	nd	nd
Phloretin-2-O-glucoside [7]	435	273	0.3 ± 0.0 b	0.9 ± 0.0 a	0.1 ± 0.0 c	0.1 ± 0.0 c	nd	nd	nd	nd
Total			31.7 ± 0.2 c	16.7 ± 0.4 d	5.6 ± 0.2 e	2.5 ± 0.1 f	19.7 ± 0.2 d	17.4 ± 0.2 d	139.6 ± 0.6 b	148.5 ± 0.2 a

Data expressed as means ± standard deviation (n=3). nd: not detected. The different lower case letters (a-b) in the same row indicate significantly different values ($p < 0.05$)

LOQ: limit of quantification

1= quantified with gallic acid at 280 nm; 2 = quantified with syringic acid at 280 nm; 3 = quantified with procyanidin B2 at 280 nm; 4 = quantified with catechin at 280 nm; 5 = quantified with taxifolin at 280 nm; 6 = quantified with quercetin at 350 nm; 7 = quantified with phloretin at 280 nm

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Supplementary Material

Click here to download Supplementary Material: Fig1.pdf

Supplementary Material

Click here to download Supplementary Material: Fig2.pdf