Accepted Manuscript

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Please cite this article as: Čolić, S.D., Fotirić Akšić, M.M., Lazarević, K.B., Zec, G.N., Gašić, U.M., Dabić Zagorac, D.C., Natić, M.M., Fatty acid and phenolic profiles of almond grown in Serbia, *Food Chemistry* (2017), doi: http://dx.doi.org/10.1016/j.foodchem.2017.05.006

Fatty acid and phenolic profiles of almond grown in Serbia

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ABSTRACT

Almond production is not typical for Serbia however the existence of natural populations and unexpectedly suitable agro-climatic conditions initiated this kind of study. Total oil content and concentrations of the fatty acids, total phenolic content and radical-scavenging activity were determined in the kernel oil of 20 local almond selections originating from North Serbia and cultivars 'Marcona', 'Texas' and 'Troito'. Sixteen fatty acids were identified and quantified, with the most abundant being oleic acid and linoleic acid. Nine phenolic acids and nineteen flavonoids were quantified using UHPLC-DAD MS/MS. The predominant polyphenol was catechin, followed by chlorogenic acid and naringenin. Based on oleic acid/linoleic acid ratio, levels of unsaturated fatty acids and specific polyphenols, some selections were chosen for growing and could also be recommended for breeding programs. Our investigation demonstrated that this region could be a suitable for growing almonds with chemical compositions competitive with standard cultivars.

1. Introduction

Almond, Brazil nut, cashew, chestnut, heartnut, hazelnut, macadamia, peanut, pecan, pine nut, pistachio and walnut, one name `nuts`, are nutrient dense foods. In the last 20 years, extensive research has been carried out on the potential bioactive and health-promoting components of nuts (Miraliakbari & Shahidi, 2008, Alasalvar, Shahidi & Amaral, 2009). Nuts are now considered as an important component of a healthy diet because they contain essential micronutrients like tocopherols (Kornsteiner, Wagner & Elmadfa, 2006), minerals (Özcan, Ünver, Erkan & Arslan, 2011), dietary fiber (Salas-Salvadó, Bulló, Pérez-Heras & Ros, 2006), phytosterols (Blomhoff, Carlsen, Andersen & Jacobs, 2006) and other phytochemicals with potential bioactivity. In human supplementation studies nuts have been shown to improve the lipid profile, increase endothelial function and reduce inflammation, all without causing weight gain (Vinson & Cai, 2012). These qualities make nuts a nutritious healthy snack and can be used as ingredient in food formulation.

Almonds (*Prunus dulcis* (Mill.) D.A. Webb) are among the most popular edible nuts, typically used as snack foods or as ingredients in a variety of processed foods, especially in bakery and confectionery products. Although almonds contain high amounts of fat, the lipid fraction does not contribute to cholesterol formation in humans, due to high level of unsaturated (monounsaturated and polyunsaturated) fatty acids (Askin, Balta, Twekinas, Kazankaya & Balta, 2007; Beyhan, Aktas, Yilmaz, Simsek & Gerçekçioğlu, 2011). Generally, the most important unsaturated fatty acids found in almond are oleic acid and linoleic acid (about 90%), while saturated fatty acids are low in content (<10%) (Yada, Lapsley & Huang, 2011). Besides proteins and fats, almonds contain sugars, mainly fructose and sucrose (Balta, Battal, Balta & Yoruk, 2009), vitamins (Segura, Casimiro, Lizarraga, & Ros, 2006) and minerals (Özcan, Ünver, Erkan & Arslan, 2011).

Several investigations on almond seeds and skin extracts revealed the presence of various phenolics compounds, well known to possess antioxidant potential. Vanillic acid, caffeic acid, *p*-coumaric acid, ferulic acid, quercetin, kaempferol, isorhamnetin, delphinidin and procyanidins B2 and B3 were determined in almond seed extract (Amarowicz, Troszynska & Shahidi, 2005). A total of 33 compounds corresponding to flavanols, flavonols, dihydroflavonols and flavanones, and other non-flavonoid compounds were identified by Monagas, Garrido, Lebrón-Aguilar, Bartolome & Gómez-Cordovés (2007) in almond skin. According to Wijeratne, Amarowicz & Shahidi (2006), almond skin, which contains ten times more polyphenols than the kernel, was characterized with isorhamnetin rutinoside, isorhamnetin glucoside, kaempferol rutinoside and kaempferol glucoside. Further, Bolling, Dolnikowski & Blumberg (2009) detected catechins, as well as flavonoids such as naringenin, quercetin and kaempferol, predominantly as glucosides or rutinosides. As for the stilbenes, piceid (a derivative of resveratrol) was also reported in almond skin (Xie & Bolling, 2014).

When incorporated in the diet, almonds positively affect cardiovascular and coronary heart diseases (Blomhoff et al., 2006; Wijeratne et al., 2006). As consumers become more interested in ensuring a healthy life style, the nutritional identification of almond genetic resources is important (Askin et al., 2007). For this reason, CITA (Agrifood Research and Technology Centre of Aragon, Spain) has incorporated chemical quality criteria as an objective in its almond breeding program (Socias i Company, Alonso, Kodad & Gradziel, 2012). Therefore, selection of parents for low linoleic acid and high oil content might be undertaken in a breeding program for increased kernel quality (Kodad, Estopanán, Juan, Alonso, Espiau, & Socias i Company, 2014).

As all these investigations were done in countries with long traditions in almond production, the comparison of Serbian almond selections with cultivars grown worldwide

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would be worthwhile. Therefore, the first objective of the report herein was to monitor fatty acids and phenolic compositions of 20 *Prunus dulcis* selections from North Serbia and their comparison with the cultivars 'Marcona', 'Texas' and 'Troito'. Special attention was given to the composition of polyphenols, as well as to the possibility of establishing chemical compounds helpful for almond characterization and as selection criteria for almond quality evaluation. The final aim was to use multivariate statistics to identify the most promising genotypes based on specific components which could differentiate them. Such investigation enabled the selection of parents for further breeding programs in Serbia which could also be implemented in similar agro-ecological conditions.

2. Materials and methods

2.1. Almond samples

Twenty almond selections were chosen from the large spontaneous population of almonds in North Serbia, called Slankamen Hill, based on phenotypic diversity (Čolić, Rakonjac, Zec, Nikolić & Fotirić Akšić, 2012) and isoenzyme polymorphism (Čolić, Milatović, Nikolić, & Zec, 2010). The orchard was planted at the Experimental Station of Institute PKB Agroekonomik, near Belgrade. It included 20 selections together with three cultivars Marcona (origin - Spain), Troito (origin - Italy) and Texas (origin - USA). Planting distance was 4 × 3 m. The orchard was under a non-irrigated regime. All necessary agro-technical measurements, as well as pest management were done in the orchard during the experimental period. The study location has a continental climate with hot and dry summers (maximum temperature up to 40°C) and cold winters (minimum around -20°C). Average precipitation is 700 mm/y.

Each sample was represented by three trees, trained as an open vase. Selections/cultivars were harvested during two consecutive years, 2014 and 2015, at full maturity (hulls fully

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desiccated and opened along the suture). Harvest time for selections/cultivars was expressed as the number of days before or after Troito full maturity. Kernel color intensity, shell softness, and kernel taste were described on the basis of the International almond descriptor (Gülcan, 1985). Selection/cultivar traits are shown in Table 1.

For biochemical analyses, samples of 30 fruits per selection/cultivar were randomly harvested from cardinally oriented branches with different directions around the canopy. Seeds in endocarps were kept in paper bags in the dark until chemical analysis. Prior to analysis, the endocarp was broken and the seed was pulled out, without extra drying.

2.2. *Chemicals*

Standards of phenolic compounds used for UHPLC MS/MS analysis (protocatechuic acid, *p*-hydroxybenzoic acid, ellagic acid, vanillic acid, aesculin, chlorogenic acid, aesculetin, caffeic acid, *p*-coumaric acid, ferulic acid, sinapic acid, coniferyl aldehyde, phlorizin, phloretin, resveratrol, catechin, rutin, hyperoside, cynaroside, naringin, astragalin, luteolin, apigenin, naringenin, kaempferol, chrysin, pinocembrin, galangin) and Trolox were purchased from Sigma-Aldrich (Steinheim, Germany). Methanol, acetonitrile (both HPLC grade), formic acid, ethyl acetate, and Folin-Ciocalteu reagent were purchased from Merck (Darmstadt, Germany), while 2,2-diphenyl-1-picrylhydrazyl radical (DPPH) was purchased from Fluka AG (Buch, Switzerland). Standard solutions and dilutions were prepared using ultrapure water (TKA Germany MicroPure water purification system, 0.055 µS/cm). All other reagents were of analytical grade. The standard for fatty acid methyl esters determination used for GC-FID analysis was purchased from Restek (37 components Food Industry FAME Mix, RESTEK, lot: #23889).

2.3. Oil extraction and fatty acid methyl esters determination

Forty grams of each sample of almond kernels, with brown skin, was ground until uniform flour (approximately 200 µm particle size) was obtained. Crude oil was extracted from ground almond kernels using the Soxhlet extraction method (AOAC 920.39C). About 5 g of each sample was extracted in a Soxhlet apparatus, using 200 mL of petroleum ether (boiling range 40–60°C) for 8 h. At the end of the extraction period, the residual solvent was removed under a stream of nitrogen, and the extracted oil was stored at -18°C under nitrogen until further analysis.

Fatty acid methyl esters (FAME) were prepared using transmethylation under alkaline conditions, following ISO 12966-2:2012. In a 10 mL screw-top test tube approximately 0.1g of the extracted oil was weighed and dissolved in 2 mL n-hexane. After the addition of 1 mL of 2 mol/l methanolic potassium hydroxide solution, the tube was vortexed for 2 min at room temperature, and centrifuged at 4000 rpm for 5 min. After 2 min, 2 mL of sodium chloride solution (40 g of sodium chloride in 100 mL of water) was added and the tube shaken briefly. The solution was neutralized by adding 1 g of sodium hydrogen sulfate monohydrate. After the salt had settled, 1 mL of the upper phase was transferred to a 2 mL vial for FAME analysis.

Fatty acid methyl esters were analysed by gas chromatography, using a GC DANI 1000 DPC, DANI Instrument SpaA, Italy, with flame ionization detection (FID). A fused-silica capillary column type Rtx-2330, Restec (phase: highly polar phase; biscyanopropyl cyanopropylphenyl polysiloxane, 0.25 mm; column: 60 m x 0.25 mm) was used. The flow rate of nitrogen carrier gas was 1.2 mL/min. Injector and detector temperatures were 250°C and 300°C, respectively. The oven temperature was programmed to maintain a temperature of 100°C for 4 min, then to rise to 240°C at a rate of 3°C/min and to maintain that temperature for 10 min. The sample injection volume was 1 µl. Total run time for one cycle was 60 min.

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Fatty acid identifications were based on retention times by comparing with those of the standard FAME mixture. Quantification of individual fatty acids was based on the peak area obtained, without any corrections (EN ISO 5508:1995). Fatty acid analysis was performed in duplicate for single samples, and average values were reported. The GC method for determining fatty acid methyl esters was validated following recommendations of the EURACHEM guide (Magnusson & Ornemark, 2014) The average relative standard deviation (RSD) of repeatability for minor components (components present at less than 1%) was 5%, while the average RSD for the components present in percentages greater than 1% was 2-3%.

2.4.   *Extraction of polyphenols*

A 10 g of ground almond kernels (particle size approximately 200 µm), in triplicate, was defatted in a 150 mL beaker by adding 50 mL hexane. The solution was stirred in an ultrasonic bath for 30 min at room temperature. After filtration through Whatman No.4 filter paper, the defatted almond residue was then extracted with 50 mL methanol/water (70:30, v/v) in an ultrasonic bath for 30 min at 40°C. The extraction was repeated three times (Arráez-Román, Fu, Sawalha, Segura-Carretero & Fernández-Gutiérrez, 2010). After filtration, all fractions were collected and concentrated to dryness by rotary evaporation under reduced pressure at 40°C. The residue was suspended in 70% methanol to 50 mL and this solution was used for further analysis.

2.5.   *Determination of total phenolic contents (TPC)*

The content of total phenolics in almond extracts was determined colorimetrically by the Folin-Ciocalteu method with some modification (Pavlović et al., 2013). The absorbance of the samples was detected at 765 nm using a GBC Cintra 6 UV–Visible spectrophotometer. The content of total phenolics in the extract was determined using gallic acid as a standard at

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concentrations of 20-100 mg L^{-1}. Total extracted phenolics were expressed as mg gallic acid equivalent (GAE) per kg fresh weight (mg GAE/kg FW).

2.6. Determination of the radical scavenging activity (RSA)

Radical scavenging activity of the almond extracts was measured by 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical according to a slightly modified literature method of Pavlović et al. (2013). The decrease in absorbance was measured at 515 nm using a UV-Vis spectrophotometer to determine the concentration of DPPH˙ remaining. Results were calculated using a Trolox standard curve (100-800 μmol L^{-1}) and expressed in mmol of Trolox equivalents per kg of fresh sample (mmol TE kg^{-1} FW). The reaction was carried out in triplicate and the results were reported as mean values.

2.7. Determination of polyphenolics (UHPLC-DAD MS/MS)

Separation and quantification of phenolic compounds were performed on a Dionex Ultimate 3000 UHPLC system equipped with a diode array detector (DAD) and TSQ Quantum Access Max triple quadrupole mass spectrometer (Thermo Fisher Scientific, Bremen, Germany), according to the slightly modified method already described by Natić et al. (2015). The elution was performed at 40ºC on a Syncronis C18 column (100 × 2.1 mm, 1.7 μm) from ThermoFisher Scientific. The mobile phase consisted of (A) 0.5% aqueous acetic acid solution and (B) acetonitrile MS grade, which were applied in the following gradient program: 5% B in the first minute, 5–95% B from 1.0 to 16.0 min, from 95% to 5% B for 16.0–16.2 min, and 5% B until the 20th min. The flow rate was set to 0.3 mL min^{-1} and the detection wavelengths to 254 and 280 nm. The injection volume was 5 μL.

A TSQ Quantum Access Max Triple-Quadrupole Mass Spectrometer equipped with a heated electrospray ionization (HESI) source was used with the vaporizer temperature kept at

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200°C, and the ion source settings as follows: spray voltage 5 kV, sheet gas (N_2) pressure 40 AU, ion sweep gas (N_2) pressure 1 AU and auxiliary gas (N_2) pressure 8 AU, capillary temperature 300°C, and skimmer offset 0 V. The mass spectrometry data were acquired in negative ion mode, in the m/z range from 100 to 1000. Multiple mass spectrometric scanning modes, including full scanning (FS), and product ion scanning (PIS), were conducted for qualitative analysis of the targeted compounds. The collision-induced fragmentation experiments were performed using argon as the collision gas, and the collision energy was varied depending on the compound. The time-selected reaction monitoring (tSRM) experiments for quantitative analysis were performed using two MS^2 fragments for each compound that were previously defined as dominant in the PIS experiments (Natić et al., 2015). Quantification was done using available phenolic standards. Table S1 presents the list of quantified phenolics in almond samples in negative ionization mode with mean expected retention times (t_R, min), mass of parent ions (*m/z*), masses of product ions (*m/z*) with specified collision energies (eV), correlation coefficients, limits of detection (LOD) and quantification (LOQ), as determined using UHPLC-DAD MS/MS analysis. The limits of detection (LOD) and quantification (LOQ) were calculated using standard deviations (SD) of the responses and the slopes of the calibration curves (S) according to the formulas: LOD = 3(SD/S) and LOQ = 10(SD/S). Standard deviations and slopes were obtained from the calibration curves created in MS Excel.

2.8. *Statistical analysis*

Data for all measurements were expressed as the mean values and Tukey's test was used to detect significant differences ($p \leq 0.05$) between these values. Principal component analysis (PCA) was carried out by PLS ToolBox, v.6.2.1, for MATLAB 7.12.0 (R2011a). All data were auto scaled prior to multivariate analysis. PCA was carried out using a singular

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value decomposition algorithm and a 0.95 confidence level for Q and T2 Hotelling limits for outliers.

3. Results and discussion

3.1.   Oil and fatty acids determination

Oil content in the almond samples and fatty acid composition of the oil are given in Table 2. Generally, our results indicated a high variability among the selections and cultivars in total oil content and fatty acids composition. Considerable variation was established in total oil contents, the highest oil content being found in selection 3/03 (62.86%), while the lowest was obtained in selection 10/03 (36.30%). The range in total oil content in this study was similar to the variability reported in the study of Kodad & Socias i Company (2008). Contents were slightly higher than those obtained by Askin et al. (2007), who found an oil content range from 25.2% to 60.8% for almond genotypes selected from Elazig province in Turkey and Özcan et al. (2011), who established an oil range from 48.8% to 55.7% for five commercial varieties. Oil content in Marcona was slightly lower than those reported by Kodad & Socias i Company (2008), which could be associated with different agroecological growing conditions.

As for fatty acids, sixteen of them were identified in our oil extracts (Table 2). This was fewer than the number of fatty acids found by Beyhan et al. (2011), who studied four commercial and five other almond genotypes from Tokat province and the Aegean region of Turkey. These differences in the numbers of fatty acids could be explained by the different genotypes analyzed, as previously determined by Kodad et al. (2014), and ecological conditions (Kodad & Socias i Company, 2008).

Our study indicated four fatty acids: oleic, linoleic, palmitic and stearic acids, in decreasing order (Table 2) and in the majority of almond samples they represent over 99% of

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the total fatty acid content. Regarding the total fatty acid content in the oil, unsaturated acids - oleic acid and linoleic acid averagely amounted 69.86% and 21.83%, respectively, while saturated acids palmitic acid and stearic acid amounted for 5.48% and 1.90%, respectively. This was in accordance with previous results of Beyhan et al. (2011), Özcan et al. (2011) and Kodad et al. (2014). In a study of the fatty acid composition of almond samples from different regions Zhu, Wilkinson & Wirthensohn (2015) also reported that oleic acid and linoleic acid were found to be the most abundant, Oleic acid in our study ranged from 63.14 (14/03) to 77.37% (16/03), linoleic acid from 15.57 (16/03) to 28.69% (14/03), palmitic acid from 4.68 (1/05) to 6.48 % (13/03) and stearic acid from 1.45 (16/03) to 2.56% (Troito). Selections 16/03, 24/03, 1/05 and ZD1 showed higher contents of oleic acid, varied from 74.61% to 77.37%, compared with Texas, having highest level of oleic acid among standard cultivars (74.59%). The quantity of linoleic acid in nine samples (1/03, 3/03, 10/03, 12/03, 13/03, 14/03, 15/03, 19/03 and 27/03) was above of Troito (22.63%). Palmitic acid was generally higher in the cultivars (except 1/03, 4/03, 13/03 and 19/03), while the level of stearic acid in all genotypes, Marcona and Texas was less than in Troito (2.56%).

Wider variation of oleic acid (50.41-81.2%), palmitic acid (5.46-15.8%) and linoleic acid (6.21-33.1%) in almond genotypes selected from Elazig province (Turkey), reported by Askin et al. (2007), are probably a consequence of a different genotype and agro-ecological conditions (Kornsteiner, Wagner, & Elmadfa, 2006; Kodad & Socias i Company, 2008). Comparing linoleic acid levels in Spanish, Mediterranean, Californian and Australian almonds, Zhu et al. (2015) noticed that the regions producing almonds with lower linoleic acid were not irrigated, whereas Californian and Australian regions routinely apply irrigation to their orchards. Therefore, restriction of irrigation could be a reason for similarity of linoleic acid percentages in this study with Spanish and Mediterranean almonds. According

to Kodad et al. (2014), oil content in almonds, and composition of oil, depends primarily on the genotype, but also on the environmental conditions.

Oleic/linoleic acid ratio (O/L rate) is used in determining the quality of the kernel due to its preventive effect on lipid oxidation (Zacheo, Cappello, Gallo, Santino & Cappello, 2000). Kodad & Socias i Company (2008) showed that it can be used to differentiate genotypes, because it does not change over the years. They also stressed the importance of a high oleic acid percentage to increase the resistance of almond kernels to oxidation during processing, storage and transport. The results presented in Table 2 showed the highest O/L ratio in 16/03 (4.97). Seven selections in total (16/03, ZD1, 24/03, 1/05, 23/03, 25/03 and 28/03) also showed higher value of the O/L ratio compared with 'Marcona' (3.47), which is commonly used as a reference for oil stability. Therefore, these selections are potentially relevant for breeding programs.

Palmitoleic acid, heptadecanoic acid, *cis*-10-heptadecenoic acid, arachidic acid, linolenic acid and eicosenoic acid were present in all samples in very small amounts (<0.5%). Tricosanoic acid was quantified in 20 samples, myristic acid in 18, behenic acid in 17 and pentadecanoic acid in 11 samples. Trace amounts of docosadienoic acid (omega-6), and lignoceric acids were found only in sample 3/03. This could be explained by the softness of the shell, as selection 3/03 was the only almond in this study characterised by a soft shell. Mazinani, Hossein, Rad & Khaneghah (2012) also detected lignoceric acid in an almond sample from Karaj, in ten-fold higher quantity than in our study, but shell softness was not reported in the study.

3.2.   *Determination of TPC, RSA and polyphenol composition*

Generally, most of the antioxidants in nuts are located in the pellicle or outer soft shell (Milbury, Chen, C. Y. & Dolnikowski, 2006). When nuts are peeled or roasted the

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antioxidant activity considerably reduces (Barreira, Ferreira, Oliveira & Pereira, 2008). Therefore, unpeeled raw almond kernels were investigated herein. Our results indicated high variability among the genotypes as each of the 23 almond samples was characterized by a unique phenolic profile. Total phenolic contents, radical-scavenging activity and phenolic profile of 23 almond samples are listed in Table 3. Differences in TPC concentrations were found to be higher amongst selections than amongst the cultivars. The highest TPC concentration was recorded in selection 18/03 (1.39 mg GAE/g), and lowest in Marcona (0.20 mg GAE/g). Those concentrations are much higher when compared with the results of Kiat, Siang, Madhavan, Chin, Ahmad & Akowuah (2014) who obtained TPC concentrations of 0.27 mg GAE/g in aqueous methanol and 0.33 mg GAE/g, in methanol. Our results were lower than those reported by Kornsteiner et al. (2006) who found 1.30-4.56 mg GAE/g and Milbury et al. (2006) who obtained 1.26-2.41 mg GAE/g. The differences in polyphenol content that could be found in the literature largely depended on the type of extraction solvent and standards used (Salcedo, López de Mishima, & Nazareno, 2010).

The results obtained for RSA were similar to those for TPC. Concentrations ranged from 0.81 mmol TE/kg (Marcona) to 24.2 mmol TE/kg (18/03). Kiat et al. (2014) reported higher RSA in methanol extracts compared with 50% aqueous methanol extracts.

A total of 28 polyphenols were determined, nine phenolic acids and 19 flavonoids. Catechin predominated, averaging 46.3% of the total, followed by chlorogenic acid, naringenin, rutin, apigenin and astragalin. Our results are consistent with the findings of Yildirim, San, Koyuncu & Yildirim (2010). On the other hand, Mazinani et al. (2012) found the main phenolic compound to be kampferol (solvent - acetone). Milbury et al. (2006) found no *p*-hydroxybenzoic acid and kaempferol in eight of the most commonly-grown California almonds, while Yildirim et al. (2010) in almond genotypes selected in Isparta province also isolated gallic acid and quercetin. Those differences are probably due to genetic differences,

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environmental conditions and different methods of quantification. Large differences in phenolic profile among genotypes were identified. Generally, fourteen phenolic compounds were present in all almond genotypes though there were differences in relative levels. Galangin was the rarest polyphenol found in six samples. The content of catechin ranged from 2.67 (Marcona) to 80.75 mg/kg (25/03). Ellagic acid was found in 13 samples, ranging from 0.02-1.35 mg/kg. Contrary to Yildirim, Yildirim, Şan, Polat, & Sesli (2016) gallic acid was not found in our samples. Rutin varied from 0.611 (4/03) to 11.33 mg/kg (3/03), and was not found in Troito and 23/03. The lowest content of apigenin (0.1 mg/kg) was found in Marcona, while it was 100-fold higher in sample 23/03 (10.47 mg/kg).

To our best knowledge flavonoids chrysin, pinocembrin, galangin were quantified and reported herein for the first time. This stands also for luteolin and phloretin, as well as for their corresponding glycosides, cynaroside and phlorizin. Significance of flavonoids on human health is well documented so far (Yao et al., 2014).

3.3. *Principal Component Analysis*

PCA was performed to establish which components could be responsible for differentiation of genotypes and to identify the most promising genotypes to be included in breeding programs or to be recommended for production. The initial matrix of 23 (the number of samples) × 47 (TPC, RSA, oil content, fatty acids and phenolics) was processed using the covariance matrix with auto scaling. The PCA resulted in a nine-component model that explained 83.47% of the total variance. The first principal component accounted for 23.67% and the second 12.30% of the total variance. Although no clear clustering on the PCA correlation plot is visible, some conclusions regarding chemical composition could be identified from Fig. 1(A) while the most influential variables were identified using the loading plots Fig. 1(B). The score plot revealed one sample to be an outlier (3/03), lying

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outside the Hotelling T2 ellipse due to the highest oil content, methyl ester of linoleic acid and lignoceric acid, rutin, hyperoside, and naringin, sinapic acid, astragalin and kaempferol compared with the other almond genotypes. We have already stated that selection 3/03 was the only almond sample characterised by a soft shell.

Not an outlier, but fully separated on the Figure 1A is genotype 23/03 (the only one with extremely early harvest time and intermediate intensity of kernel colour) that was distinguished by the highest level of *cis*-10-heptadecenoic acid, *cis*-11-eicosenoic acid, ferullic acid, phlorizin, apigenin, naringenin and pinocembrin.

A small group which was composed of three genotypes 10/03, 25/03 and ZD 2, stored high level of vanillic acid, coniferyl aldehyde, hyperoside and astragalin. Variables for grouping of selections 1/03, 4/03, 13/03, 14/03, 15/03, 18/03, and 27/03 were polyphenols with positive values on PC2, cynaroside, phlorizin and *p*-coumaric acid being the most influential (Table 3).

The biggest group comprised standard cultivars Marcona, Troito, and Texas, and selections 11/03, 12/03, 16/03, 19/03, 24/03, 28/03, 1/05 and ZD1, which were characterized with higher percentages of oleic acid, pentadecanoic acid, and palmitoleic acid (Table 2). Also, those genotypes accumulated very low levels of caffeic acid, phloretin and aesculin, and almost none cynaroside.

In previous PCA applications to almond research Yildirim, Yildirim, Şan, Polat, & Sesli (2016) reported the relevance of catechin, caffeic acid, epicatechin, and *p*-coumaric acid as discriminant parameters to differentiate almond varieties. Our results partly support those findings.

4. Conclusions

This study represents a first analysis of fatty acid and phenolic profile of almond grown in Serbia. This collection revealed a high level of diversity among almond selections and cultivars of different geographical origin growing under the same climatic conditions.

This investigation has shown that almonds from Serbia are a good source of phenolics, mainly catechin followed by rutin, naringenin, astragalin, apigenin, and chlorogenic acid. Taking health promoting compounds into consideration selections 16/03, 1/05 and 12/03 were characterised with the highest levels of unsaturated fatty acids. Finally, selection 18/03 was distinguished by the highest TPC and RSA. Knowing that these assays are non-selective and that could provide some information on synergistic effects and other non-phenolic substances that may contribute to the antioxidant activity, selection 18/03 could be recommended for commercialization or use to introduce new genes or alleles into newly-created cultivars.

The present results demonstrate the importance of maintaining and characterizing the genetic diversity of almond in a gene bank collection for further utilization, mainly for identifying interesting parents to be included in a breeding program as a response to new selection objectives, such as improving the chemical quality of the almond kernel. Our findings may assist breeders to improve the quality of almonds to be grown in Serbian or similar agro-ecological conditions for producing food with a high content of bioactive compounds.

Finally, this study demonstrated that it is possible to grow almond in Northern Serbia with chemical characteristics similar to those of standard cultivars.

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Figure captions

Figure 1. Principal component analysis, scores plot of the first two principal components (A) showing the clustering of samples; loadings plot (B) reflecting the influence of a particular parameter. An ellipse represents the 95% confidence interval using Hotelling's T2 statistics.

Table 1

Almond samples and their characteristics.

Genotype	Pedigree (cultivar/selection)	Harvest time	Kernel color intensity	Softness of shell	Kernel taste
1/03	selection	-11	intermediate	hard	sweet
3/03	selection	-5	dark	soft	sweet
4/03	selection	-7	intermediate	hard	sweet
10/03	selection	-7	intermediate	hard	intermediate
11/03	selection	-11	intermediate	hard	sweet
12/03	selection	-13	intermediate	hard	sweet
13/03	selection	-11	intermediate	hard	sweet
14/03	selection	-13	extremely light	hard	sweet
15/03	selection	-13	intermediate	hard	sweet
16/03	selection	-5	dark	hard	sweet
18/03	selection	-7	light	hard	intermediate
19/03	selection	-5	dark	extremely hard	sweet
23/03	selection	-15	intermediate	hard	sweet
24/03	selection	-15	dark	hard	intermediate
25/03	selection	-13	light	hard	intermediate
27/03	selection	-11	dark	extremely hard	sweet
28/03	selection	-11	intermediate	hard	sweet
1/05	selection	-7	intermediate	hard	intermediate
ZD 1	selection	-11	dark	intermediate	sweet
ZD 2	selection	-3	dark	hard	sweet
Marcona	cultivar	-5	intermediate	hard	sweet
Troito	cultivar	0	intermediate	hard	sweet
Texas	cultivar	+3	dark	intermediate	intermediate

Table 2

Oil content in almond samples and fatty acids composition of oil (average 2014-2015).

Nº*		1/03	3/03	4/03	10/03	11/03	12/03	13/03	14/03	15/03	16/03	18/03	19/03	23/03	24/03	25/03	27/03	28/03	1/05	ZD1	ZD2	Marcona	Troito	Texas
1	Oil content (%)	51.90[f]**	62.86[k]	55.79[h]	36.30[a]	53.74[g]	47.79[d]	43.20[c]	47.45[d]	47.94[d]	39.63[b]	44.57[c]	49.80[e]	52.14[f]	52.87[fg]	50.73[e]	53.64[g]	49.19[e]	54.70[gh]	55.38[h]	46.64[d]	56.74[hi]	57.78[i]	59.14[j]
	Fatty acid composition (%)																							
2	Myristic acid (C14:0)	0.03[ab]	0.03[ab]	<0.01	0.05[ab]	<0.01	0.02[a]	0.22[d]	0.02[a]	0.02[a]	0.05[ab]	0.03[a]	0.05[b]	0.10[c]	0.07[b]	<0.01	<0.01	0.03[a]	0.02[a]	<0.01	0.03[a]	0.03[ab]	0.02[a]	0.03[ab]
3	Pentadecanoic acid (C15:0)	<0.01	0.01[a]	<0.01	0.01[a]	<0.01	<0.01	<0.01	0.03[a]	0.02[a]	<0.01	<0.01	0.02[a]	<0.01	0.06[b]	<0.01	<0.01	0.02[a]	0.02[a]	0.02[a]	<0.01	0.01[a]	0.01[a]	<0.01
4	Palmitic acid (C16:0)	5.84[f]	5.53[i]	5.81[gh]	5.31[a]	5.21[de]	5.14[c]	6.48[de]	5.45[cd]	5.63[d]	4.91[a]	5.41[c]	6.37[g]	4.98[cd]	5.16[d]	4.93[c]	5.37[e]	5.63[d]	4.68[cd]	5.22[e]	5.09[b]	6.43[j]	5.74[h]	5.77[hi]
5	Palmitoleic acid (C16:1)	0.36[cd]	0.29[c]	0.32[c]	0.27[a]	0.31[bc]	0.37[c]	0.37[bc]	0.24[a]	0.27[ab]	0.28[b]	0.34[b]	0.35[c]	0.30[bc]	0.42[de]	0.34[c]	0.26[b]	0.41[d]	0.38[d]	0.30[c]	0.30[b]	0.56[f]	0.38[de]	0.38[de]
6	Heptadecanoic acid (C17:0)	0.06[a]	0.07[b]	0.10[b]	0.06[a]	0.11[b]	0.06[a]	0.07[a]	0.07[a]	0.06[a]	0.06[a]	0.06[a]	0.06[a]	0.10[b]	0.07[ab]	0.09[ab]	0.07[ab]	0.06[a]	0.07[ab]	0.06[ab]	0.06[a]	0.06[a]	0.08[ab]	0.06[ab]
7	cis-10 Heptadecenoic acid (C17:	0.10[b]	0.09[b]	0.11[bc]	0.11[b]	0.09[b]	0.15[c]	0.11[b]	0.08[b]	0.11[b]	0.11[b]	0.12[b]	0.10[b]	0.17[d]	0.12[c]	0.11[bc]	0.03[a]	0.11[b]	0.11[bc]	0.09[b]	0.11[b]	0.10[bc]	0.07[b]	0.10[bc]

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	1)																							
8	Stearic acid (C18:0)	1.73[c]	2.37[f]	2.11[d]	1.99[b]	1.88[cd]	1.51[b]	2.18[c]	1.97[c]	1.52[b]	1.45[a]	1.94[bc]	1.75[bc]	2.17[d]	1.57[bc]	1.96[cd]	2.24[e]	1.72[bc]	1.47[bc]	2.23[e]	1.67[b]	1.58[c]	2.56[g]	2.17[e]
9	Oleic acid (C18:1 n9c)	66.12[d]	66.48[ef]	68.07[e]	67.89[a]	70.94[e]	64.69[bc]	65.56[b]	63.14[b]	65.95[c]	77.37[bc]	70.07[c]	65.08[c]	72.93[e]	75.38[e]	72.52[de]	68.72[de]	71.04[d]	74.61[ef]	75.76[f]	71.10[d]	70.62[ef]	68.22[e]	74.59[g]
10	Linoleic acid (C18:2n6c)	25.48[de]	24.80[f]	22.32[d]	24.08[b]	21.22[cd]	27.72[d]	24.66[c]	28.69[d]	26.17[d]	15.57[a]	21.75[b]	26.04[d]	18.83[b]	16.80[b]	19.63[b]	23.01[d]	19.99[bc]	18.31[c]	16.05[b]	21.34[bc]	20.32[cd]	22.63[de]	16.60[bc]
11	Arachidic acid (C20:0)	0.10[ab]	0.12[c]	0.12[bc]	0.10[a]	0.10[ab]	0.09[a]	0.11[ab]	0.11[ab]	0.09[a]	0.08[a]	0.09[a]	0.08[a]	0.14[c]	0.10[ab]	0.13[bc]	0.11[b]	0.07[a]	0.09[ab]	0.10[ab]	0.09[a]	0.06[a]	0.09[ab]	0.09[ab]
12	cis-11-Eicosenoic acid (C20:1)	0.08[a]	0.10[ab]	0.12[bc]	0.11[a]	0.06[a]	0.10[ab]	0.09[a]	0.10[ab]	0.10[ab]	0.11[a]	0.12[ab]	0.08[a]	0.16[c]	0.12[bc]	0.13[bc]	0.09[ab]	0.09[ab]	0.14[c]	0.09[ab]	0.11[ab]	0.07[a]	0.09[ab]	0.09[ab]
13	α-Linolenic acid (C18:3 n3)	0.04[a]	0.04[ab]	0.72[b]	0.03[a]	0.04[a]	0.09[a]	0.04[a]	0.05[a]	0.03[a]	<0.01	0.04[a]	0.04[a]	0.06[ab]	0.05[ab]	0.03[a]	0.03[a]	0.80[b]	0.03[a]	0.03[a]	0.04[a]	0.03[a]	0.02[a]	0.03[a]
14	Behenic acid (C22:0)	0.02[a]	0.03[ab]	0.10[c]	<0.01	<0.01	<0.01	0.03[a]	0.03[a]	0.02[a]	0.02[a]	0.03[a]	0.02[a]	<0.01	<0.01	<0.01	0.04[ab]	0.02[a]	0.06[b]	0.02[a]	0.01[a]	0.02[a]	0.02[a]	0.02[a]
15	Docosadienoic acid (C22:2cis 13,16)	<0.01	0.01[a]	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
16	Tricosanoic acid (C23:0)	0.04[a]	<0.01	0.10[d]	<0.01	0.05[a]	0.06[ab]	0.09[bc]	0.03[a]	0.01[a]	<0.01	0.02[a]	0.03[a]	0.07[bc]	0.10[cd]	0.13[d]	0.03[a]	0.01[a]	0.03[a]	0.03[a]	0.06[ab]	0.01[d]	0.06[ab]	0.09[cd]
17	Lignoceric	<0.01	0.03	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	0.01	<0.01	<0.01

acid (C24: 0)		1	1			1	1	1		1	1	1	1					1				
Oleic/ Linole ic ratio	2.59^b	2.68^b	3.05^d	2.82^c	3.34^{ef}	2.33^a	2.66^b	2.20^a	2.52^b	4.97^j	3.22^e	2.50^b	3.87^g	4.49^h	3.69^{fg}	2.99^d	3.55^f	4.07^g	4.72^i	3.33^{ef}	3.47^f	3.01^d	4.49^h

*Nº corresponds to PCA variables on loading plot.

**Different letters in the same row denote a significant difference according to Tukey's test, $p < 0.05$.

Table 3

Radical scavenging activity (RSA), total phenolic content (TPC), and content of phenolic

compounds in almond samples. (ND = not detected compound).

Nº*		1/03	3/03	4/03	10/03	11/03	12/03	13/03	14/03	15/03	16/03	18/03	19/03	23/03	24/03	25/03	27/03	28/03	1/05	ZD1	ZD2	Marcona	Troito	Texas
18	RSA (mmol TE/kg FW)	9[cd]*	10[cd]	18[ef]	7[c]	2[a]	10[cd]	12[d]	2[a]	19[ef]	1[a]	24[g]	2[a]	11[d]	4[b]	18[e]	3[ab]	16[e]	2[a]	10[cd]	3[ab]	1[a]	1[a]	20[f]
19	TPC (mg GAE/ kg FW)	593[e]	757[g]	1145[ij]	788[g]	473[d]	390[c]	879[h]	464[d]	1319[k]	382[c]	1392[k]	457[d]	902[h]	683[f]	1106[i]	461[d]	819[g]	448[d]	890[h]	621[e]	204[a]	271[b]	1195[j]
	Hydroxybenzoic acid derivatives (mg/kg)																							
20	Protocatechuic acid	0.75[ef]	ND	0.32[a]	ND	0.60[cd]	0.74[ef]	0.67[de]	0.85[g]	1.07[h]	0.56[c]	1.20[i]	0.58[c]	ND	0.61[cd]	0.43[b]	0.54[c]	0.64[cd]	ND	0.70[ef]	ND	0.56[c]	0.59[cd]	0.77[f]
21	*p*-Hydroxybenzoic acid	0.37[b]	0.32[ab]	0.19[a]	0.45[d]	0.40[cd]	0.36[b]	0.44[d]	0.55[e]	0.58[e]	0.37[b]	0.56[e]	0.40[cd]	0.42[cd]	0.35[b]	0.24[a]	0.55[e]	0.30[ab]	0.32[ab]	0.28[ab]	0.26[ab]	0.37[b]	0.69[f]	0.39[cd]
22	Ellagic acid	0.12[b]	ND	0.09[b]	ND	0.08[b]	0.06[ab]	ND	1.35[e]	0.06[ab]	ND	0.03[a]	ND	0.04[a]	ND	ND	0.02[a]	ND	0.11[b]	ND	ND	0.13[b]	0.35[d]	0.21[c]
23	Vanillic acid	1.55[g]	2.78[n]	0.79[c]	1.44[f]	0.92[d]	1.65[h]	1.62[h]	2.30[k]	2.61[m]	0.38[a]	0.89[d]	0.98[d]	2.84[n]	2.44[l]	1.60[h]	0.48[b]	1.69[h]	1.12[e]	1.82[i]	1.95[j]	0.92[d]	0.92[d]	2.28[k]
	Hydroxycinnamic acid derivatives (mg/kg																							

)																								
24	Aesculin	0.37[d]	0.21[a]	0.23[ab]	ND	0.23[ab]	0.21[a]	0.28[c]	0.25[b]	0.31[c]	0.24[b]	0.28[c]	0.21[a]	0.19[a]	0.20[a]	0.21[a]	0.26[b]	0.21[a]	0.19[a]	0.20[a]	0.22[a]	0.22[a]	0.20[a]	0.22[a]
25	Chlorogenic acid	2.11[e]	4.89[i]	21.03[m]	1.05[a]	5.47[i]	4.38[h]	1.08[a]	1.32[b]	7.99[k]	0.99[a]	13.62[l]	1.87[d]	3.83[g]	1.33[b]	3.05[f]	1.23[b]	1.59[c]	1.20[b]	5.34[i]	2.14[e]	1.35[b]	0.99[a]	6.93[j]
26	Aesculetin	0.27[e]	0.57[i]	0.79[k]	0.55[hi]	0.23[d]	0.08[a]	0.31[f]	0.19[c]	0.26[e]	0.08[a]	0.23[d]	0.13[b]	0.52[g]	0.26[e]	0.81[k]	0.28[ef]	0.30[f]	0.23[d]	0.27[e]	0.68[j]	0.11[b]	0.17[c]	0.19[c]
27	Caffeic acid	0.82[c]	0.83[c]	0.77[ab]	0.79[b]	0.76[ab]	0.75[a]	0.80[b]	0.79[b]	1.48[f]	0.73[a]	0.86[d]	0.75[a]	0.90[e]	0.77[ab]	ND	0.75[a]	ND	0.75[a]	0.73[a]	0.76[ab]	0.78[b]	ND	0.74[a]
28	p-Coumaric acid	0.72[h]	0.21[bc]	0.17[b]	0.25[d]	0.31[e]	0.27[d]	0.39[f]	0.31[e]	0.31[e]	0.18[b]	0.37[f]	0.27[d]	0.28[d]	0.26[d]	0.20[bc]	0.52[g]	0.22[c]	0.20[bc]	0.22[c]	0.26[d]	0.14[a]	0.19[b]	0.18[b]
29	Ferulic acid	0.70[d]	1.71[l]	1.34[j]	1.10[h]	0.83[e]	0.74[d]	1.33[j]	1.72[l]	1.36[j]	0.58[c]	1.64[k]	1.16[i]	2.81[m]	0.84[e]	0.70[d]	0.71[d]	0.41[b]	0.99[g]	0.81[e]	0.89[f]	0.73[d]	0.25[a]	0.59[c]
30	Sinapic acid	1.00[f]	3.50[k]	0.93[e]	0.74[c]	0.56[a]	1.15[h]	0.77[c]	0.57[a]	1.08[g]	ND	1.03[f]	0.65[b]	1.65[i]	1.16[h]	1.96[j]	ND	1.08[g]	0.69[b]	1.03[f]	1.68[i]	ND	ND	0.82[d]
31	Coniferyl aldehyde	0.20[c]	0.61[j]	0.83[l]	0.54[h]	0.24[d]	0.08[a]	0.36[g]	0.21[c]	0.28[e]	0.10[a]	0.27[e]	0.15[b]	0.58[i]	0.29[e]	0.83[l]	0.21[c]	0.33[f]	0.22[c]	0.30[ef]	0.72[k]	0.12[ab]	0.19[c]	0.21[c]
Dihydrochalcones (mg/kg)																								
32	Phlorizin	0.92[g]	1.04[i]	2.09[n]	0.85[f]	1.04[i]	0.64[c]	1.71[k]	0.95[g]	1.82[l]	0.82[e]	2.00[m]	0.74[e]	2.10[n]	0.75[e]	1.06[i]	2.19[o]	1.15[j]	0.97[h]	0.91[g]	0.69[d]	0.41[a]	0.53[b]	1.00[h]
33	Phloretin	0.09[a]	0.18[de]	0.16[cd]	0.13[b]	0.14[bc]	0.09[a]	0.13[b]	0.12[b]	0.15[bc]	0.09[a]	0.24[g]	0.09[a]	0.22[f]	0.12[b]	0.20[e]	0.12[b]	0.12[b]	0.12[b]	0.14[bc]	0.16[cd]	0.08[a]	0.09[a]	0.13[b]
Stilbenoids (mg/kg)																									
34	Resveratrol	0.07[ab]	0.12[d]	0.08[b]	0.09[bc]	0.06[a]	0.10[c]	0.10[c]	0.07[ab]	0.11[cd]	ND	0.06[a]	ND	0.07[ab]	0.06[a]	0.10[c]	0.07[ab]	0.16[e]	0.07[ab]	0.10[c]	0.10[c]	ND	0.08[b]	0.11[cd]
Flavonoids and																									

derivatives (mg/kg)																								
35	Catechin	26.04j	32.91l	49.93p	26.25j	10.85d	21.95h	25.18ij	17.78f	31.0l	4.76b	30.04kl	10.78d	32.15l	19.38g	80.75q	17.06f	29.41k	12.84e	47.99o	35.41m	2.67a	6.85c	41.44n
36	Rutin	0.72a	11.33k	0.61a	3.45i	1.49b	4.10j	1.57bc	0.74a	1.76d	1.62c	2.76h	2.19f	ND	0.61a	2.31g	2.89h	3.01h	1.90e	1.75d	2.13f	0.70a	ND	1.87de
37	Hyperoside	0.50b	5.84m	0.66d	1.28j	0.49b	0.60c	1.00i	0.51b	0.73e	0.33a	0.90h	1.58l	0.93h	0.50b	1.41k	1.01i	0.69d	0.93h	0.79f	0.88gh	0.30a	0.80f	0.82f
38	Cynaroside	4.25l	ND	4.79m	ND	ND	ND	2.21j	0.72c	1.78i	ND	1.77i	ND	ND	ND	1.15d	3.39k	ND	ND	ND	0.52b	0.21a	ND	ND
39	Naringin	ND	0.11h	ND	0.05c	ND	ND	ND	ND	ND	0.003a	ND	ND	ND	0.06d	ND	0.03a	0.05c	ND	0.07e	0.09f	0.04b	ND	0.06d
40	Astragalin	ND	8.66j	ND	6.19h	1.18b	ND	1.44b	ND	1.24b	0.95a	2.60d	0.00	7.77i	ND	4.68g	6.11h	2.82e	2.58d	2.67de	3.20f	ND	ND	2.43c
41	Luteolin	4.27m	3.24k	2.81k	2.31j	0.48b	0.76e	0.82f	0.74de	1.31h	0.59e	6.24n	1.07g	3.78l	0.74de	1.07g	4.23m	0.45b	2.21j	0.69d	1.71i	0.34a	1.09g	0.45b
42	Apigenin	9.94s	6.55p	4.96o	6.08q	0.31c	0.95h	1.05jk	0.46e	1.01ij	0.53f	7.70r	0.57f	10.47t	0.63g	1.09k	4.81n	0.35c	4.94o	0.40d	1.86l	0.10a	2.30m	0.18b
43	Naringenin	14.49t	9.72r	7.29o	9.06q	0.44c	1.39h	1.49jk	0.63e	1.45ij	0.76f	11.43s	0.77f	15.74u	0.88g	1.52k	7.12n	0.48c	7.37p	0.54d	2.68l	0.09a	3.31m	0.22b
44	Kaempferol	1.10h	2.63i	0.82e	2.22k	0.60a	0.76d	0.65ab	0.67bc	0.61a	0.61a	2.04j	0.72cd	1.58i	0.61a	0.82e	1.11h	0.67b	1.04g	0.64ab	0.93f	ND	0.96f	0.62a
45	Chrysin	0.03b	0.07d	0.05c	0.04bc	0.01a	0.02ab	ND	0.08d	0.03b	0.001a	0.04bc	ND	0.05c	0.02ab	ND	0.02ab	0.03b	0.03b	0.02ab	ND	0.01a	0.08d	0.02ab
46	Pinocembrin	0.04b	0.12e	0.11de	0.06c	ND	0.01a	0.02ab	0.03b	0.02ab	ND	0.06c	0.01a	0.13e	0.02ab	0.01a	0.03b	0.01a	0.04b	ND	0.02ab	ND	0.10d	ND
47	Galangin	ND	ND	0.46c	ND	ND	ND	ND	0.47c	ND	0.036a	ND	ND	ND	ND	ND	ND	ND	0.36a	ND	ND	ND	0.40b	0.37a

*Nº corresponds to PCA variables on loading plot.

**Different letters in the same row denote a significant difference according to Tukey's test, $p < 0.05$.

► Fatty acid and phenolics of almonds grown in Serbia were reported for the first time.

► The most abundant fatty acids were oleic acid and linoleic acid.

► The predominant polyphenol was catechin, followed by chlorogenic acid and naringenin.

► Each of the 23 almond samples was characterized by a unique phenolic profile.