Evaluation of Fatty Acid Composition, Antioxidant and Antimicrobial Activity, Mineral Composition and Calorie Values of Some Nuts and Seeds from Turkey

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Abstract: The samples of the hazelnut, peanut, pistachio, almond, walnut, chestnut, pumpkin seed and sunflower seed were collected from Turkey. The fatty acid compositions of Turkish nut and seed oils were analyzed by Gas Chromatography (GC) were determined. The antioxidant activity of the samples was assessed by 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging capacity assay toward BHT and Vitamin C. Retinol and α-tocopherol were analyzed using High-Pressure Liquid Chromatography with UV Detector (HPLC-UV). The antimicrobial and antifungal activities of Turkish nut and seeds were evaluated using the disk diffusion method toward 9 bacteria and 5 yeasts. The nut and seeds showed strong antimicrobial activity against the test organisms. Spectroscopic determination of minerals (Calcium, magnesium, potassium, sodium, iron, copper, manganese, selenium, zinc, chromium, aluminum) of nuts and seeds was performed with inductively coupled plasma-atomic emission spectrometer (ICP-AES). The calorie values of samples were measured using a Bomb Calorimeter.

Keywords: Nut and seed; fatty acids; antioxidant; antimicrobial activities; calorie values; mineral content; hazelnut.

1. Introduction

Nuts are considered to be one of the most nutritious human foods, due to their high contents of proteins, carbohydrates, unsaturated fatty acid, vitamins and essential minerals [1]. Nut consumption
lowers the risk of cardiovascular heart disease (CHD), which may be partly explained by the cholesterol-lowering effect [2,3]. The favorable fatty acid composition and lipid lowering effect of nuts have been demonstrated in experimental studies with almonds, peanuts, pistachios and walnuts [4-7]. Recently oilseeds have been thoroughly investigated taking into account especially the phytochemicals representing the minor components (like tocopherols, squalene, chlorophylls, and phenolic compounds) [8]. This interest is connected with the activity of such compounds against CHD [9], lipid oxidation, protein cross-linking and DNA mutations [10-12]. Most of these beneficial effects are due to antioxidant activity; especially when the presence of phenolic compounds and tocopherols is involved in the stability of oils [13].

Natural antioxidants function as free radical scavengers are widely used in the food industry to enhance the sensory, health-promoting, or keeping quality of foods [13]. Consumption of foods rich in natural antioxidants has been reported as being protective against certain types of cancer and may also reduce the risk of cardiovascular and cerebrovascular events. These actions of antioxidants have been attributed to their ability to scavenge free radicals, thereby reducing oxidative damage of cellular biomolecules such as lipids, proteins, and nucleic acids [14]. Therefore, dietary supplements, consisting of antioxidants such as flavonoids and vitamins, could be used to effectively protect body cells from the attack by oxidative stress and to preserve human body health in general [15]. Several nuts such as walnuts and peanuts are among these dietary plants known to have significant antioxidant contents [16-19]. The stable radical species 1,1-diphenyl-2-picrylhydrazyl (DPPH), has been widely used for antioxidant capacity screening and estimation due to its clear reaction mechanism, solvent compatibility and the technical simplicity of its assays which requires no special equipment [20].

In recent years, multiple resistances in human pathogenic microorganisms have developed due to the indiscriminate use of commercial antimicrobial drugs commonly employed in the treatment of infectious diseases. This situation, the undesirable side effects of certain antibiotics and the emergence of previously uncommon infections [21] have forced scientists into looking for new antimicrobial substances from various sources like medicinal plants. The screening of plant extracts and plant products for antimicrobial activity has shown that higher plants represent a potential source of new anti-infective agents [22].

Essential elements, including the main elements and a number of trace elements, fulfill various functions: as electrolytes, in enzymes, vitamin, hormone constituents [23,24]. Nuts are an important source by the essential minerals for the health [2,25-28]. Moreover, there has been growing interest in evaluation of the macro and microelements in a variety of food samples. The importance of mineral composition is due to their nutritional properties and beneficial health effects, as well as their meeting of dietary guidelines required for a healthy diet [1].

In this study we have investigated fatty acid composition, total ω-3 and ω-6 fatty acids, α-tocopherol, retinol, radical scavenging capacity (DPPH), antimicrobial activity, calorie values and mineral content of hazelnut, peanut, pistachio, almond, walnut, pumpkin seed and sunflower seed grown in Turkey.

2. Materials and Methods

2.1. Plant Material

Materials were collected from hazelnut samples (Corylus maxima Mill.), in Trabzon on Blacksea Coast; peanut samples (Arachis hypogaea L.), in Osmaniye; pistachio samples (Pistacia vera L.), in Gaziantep; almond samples (Prunus amygdalus Batsch.), in Muğla; walnut samples (Junglas regia L.), in Balıkesir; chestnut samples (Castanea sativa Mill.), in Bursa; pumpkin seeds (Cucurbita pepo L.) and sunflower seeds (Helianthus annus L.) in Edirne province in 2008 harvesting time.

2.2 Fatty Acid Composition

Fatty acid methyl esters (FAMEs) were prepared from oil samples and determined by gas chromatography (GC) according to the method described by Slover and Lanza [29]. FAMEs were
preparing using $\text{BF}_3$ in methanol (20% of $\text{BF}_3$ in methanol) and extracted with $\text{n-hexane}$ and then analyzed by GC.

2.3. Radical-Scavenging Activity (Antioxidant Activity)

The free-radical-scavenging activity of the extracts was determined by the DPPH assay as described by Bloiss [30]. Briefly, each sample was diluted in methanol prior to the analysis (1 mg/mL). An aliquot (0.1 mL) of the solution was added to 3.9 mL of DPPH solution (6x$10^{-5}$ M in methanol), thoroughly mixed, and the absorbance of the sample at 515 nm was recorded after the time necessary for the reaction to reach a plateau [31]. The absorbance of DPPH solution in methanol, without any antioxidant (control), was also measured. The percentage of remaining DPPH was calculated as follows:

$$\text{% DPPH scavenging} = \left[ \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \right] \times 100$$

Where $A_{\text{sample}}$ is the absorbance of sample after the time necessary to reach the plateau (30 min) and $A_{\text{control}}$ is the absorbance of DPPH.

2.4. Vitamin A (Retinol) and Vitamin E ($\alpha$-Tocopherol) Content

Retinol and $\alpha$-tocopherol exhibit their characteristic maxima of UV absorption at 324 and 292 nm, respectively. The best separation within a reasonable time as well as good peak shape were achieved with a mobile phase consisting of methanol and n-hexane in proportion of 72:28 at a flow rate of 1 mL/min. The retention times were: 2.24±0.01 and 2.95±0.03 min for retinol and $\alpha$-tocopherol respectively. To determine the amounts of retinol and $\alpha$-tocopherol samples calibration curves were constructed by plotting the peak areas versus the concentration. Linearity was achieved in the concentration range 0.35–70 and 0.23–46 µM for all-trans-retinol and $\alpha$-tocopherol respectively. The detection limits calculated as signal-to-noise ratio equal to 2 were: 1.10 µM for retinol and 0.78 µM for $\alpha$-tocopherol [32-34].

2.5. The Antimicrobial Activity

The antimicrobial activities are evaluated against Gram positive (Staphylococcus aureus ATCC 6538, Bacillus cereus ATCC 7064, Mycobacterium smegmatis CCM 2067, Listeria monocytogenes ATCC 15313, Micrococcus luteus La 2971) and Gram negative (Escherichia coli ATCC 11230, Klebsiella pneumoniae UC57, Pseudomonas aeruginosa ATCC 27853, Proteus vulgaris ATCC 8427) bacteria and the yeast cultures (Candida albicans ATCC 10231, Kluyveromyces fragilis NRRL 2415, Rhodotorula rubra DSM 70403, Debaryomyces hansenii DSM 70238 and Hanseniaspora guilliermondii DSM 3432) using disk diffusion method. Antimicrobial screening: Disk diffusion method: Sterilized antibiotic discs (6 mm) were used following the literature procedure [35-38].

2.6. Mineral Content

Spectroscopic determination of minerals (Calcium, magnesium, potassium, sodium, iron, copper, manganese, selenium, zinc, chromium, aluminum) of samples was performed with a Varian Liberty Series II AX sequential model inductively coupled plasma-atomic emission spectrometer (ICP-AES) with a glass nebulizer and SPS 5.

2.7. Calorie Values

The calorie values of samples were measured using a LECO-AC (350) Bomb Calorimeter. The samples were placed in the bomb chamber, pressurized to 425 psi with pure oxygen, combusted and the amount of heat liberated recorded on a plotter. The calorimeter was calibrated against benzoic acid standard before the analysis of samples [39].
3. Results and Discussion

Oil yields of nuts and seeds are given in Table 1. The highest fat content was detected in walnut (76.0%) and the lowest in chestnut (3.54%). The fat content in descending order was walnut, pistachio, hazelnut, pumpkin seed, almond, peanut, sunflower seed and chestnut. For almond hazelnut peanut pistachio and walnut were in agreement with the values found by Miraliakbari and Shahidi [18], Kornsteiner et al. [40]. Chestnut was in agreement with the values found by Borges et al. [41], Pugliese et al [42].

As can be seen form Table 1, almond, hazelnut, pistachio, walnut, pumpkin seed, peanut and sunflower seed oils contain high amounts of unsaturated fatty acids (93.36, 89.44, 89.73, 89.71, 89.59, 86.97 and 82.75%, respectively), while chestnut oil contains them in the lowest amount (69.85%). The highest amount of monounsaturated fatty acids (MUFA) was found in almond, pistachio and hazelnut oils (72.75, 68.45 and 63.52%, respectively); while peanut, chestnut and sunflower seed oils (56.69, 36.00 and 33.05%, respectively) contained lower amount of MUFAs. Pumpkin seed and walnut oils had the highest amount of MUFAs (18.19 and 13.85%, respectively). The most abundant MUFAs was oleic acid (18:1) found in the range of 13.55-71.98%, followed by palmitoleic (16:1), eicosenoic (20:1) and heptadecenoic acids (17:1). The highest oleic acid content was found in almond, pistachio and hazelnut oils (71.98, 67.18 and 63.21%, respectively); while peanut, chestnut and sunflower seed oils contained relatively lower amount of oleic acid (55.41, 33.88 and 32.69%, respectively). The lowest quantities were found in pumpkin seed and walnut oils (17.62 and 13.55%, respectively). Polyunsaturated fatty acids (PUFA) content of walnut and pumpkin seed oils were 75.86 and 71.40%, respectively; which were the highest values found in this study. Sunflower seed, chestnut, peanut, hazelnut, pistachio and almond oils contained lower amounts of PUFAs (49.70, 33.85, 30.28, 25.92, 21.28 and 20.61%, respectively). The linoleic acid (18:2) content in the samples studied in this research was found to be in the range of 13.64-70.17%. The highest linoleic acid content was determined in the pumpkin seed and walnut oils as 70.17 and 63.42%, respectively. The linoleic content of sunflower seed, peanut, pistachio and almond oils was found to be 48.58, 26.51, 20.53 and 20.37%, respectively; while hazelnut oil had the lowest linoleic acid content (13.64%). Eicosadienoic acid (20:2 n-6) and docosadienoic acid (22:2 n-6) were found to be in the ranges of 0.04-0.38% and 0.03-0.40%, respectively. The composition of α-linolenic acid (18:3 n-3) (ALA), γ-linolenic acid (18:3 n-6) and eicosatrienoic acid (20:3 n-6) in the samples were in the ranges of 0.04-12.22%; 0.03-1.46%, 0-2.00%, respectively. The highest ALA content was found in walnut (12.22%) and hazelnut oils (12.14%), while almond oil (0.04%) was the poorest in ALA acid. Peanut (1.46%) and chestnut oils (0.57%) were the nuts richest in γ-linolenic acid, while hazelnut oil was the lowest in γ-linolenic acid (0.03%). The highest amount of eicosatrienoic acid was found in peanut oil 2.00%, followed by pumpkin seed oil 0.75%, while there was no eicosatrienoic acid in almond oil. The highest eicosadienoic acid content was found in chestnut oil (0.38%), while hazelnut and sunflower seed oils were the poorest in eicosadienoic acid (0.04%). Chestnut oil was the richest in terms of docosadienoic acid (0.40%), while hazelnut and walnut oils were the poorest (0.03%). The other PUFAs such as the arachidonic acid (20:4 n-6) was found only in chestnut oil and the eicosapentaenoic acid (20:5 n-3) (EPA) was found in chestnut and sunflower seed oils (Table 1).

Oleic and linoleic acid content of Pistachio oil was in agreement with the values found by Satil et al. [43], Seferoglu et al. [44]; for hazelnut oil was in agreement with the values found by Ozdemir et al. [27], Alasalvar et al. [28], Garcia et al. [45], Parcerisa et al. [46], Kirbaslar and Erkmen [47]; for peanut oil was in agreement with the values found by Tuberoso et al. [8] and Chiou et al. [48]. Oleic acid content of pumpkin seed oil was found to be lower than that of literature while linoleic acid content was higher [8,49,50]. For sunflower seed oil, the data were in agreement with the data found by Tuberoso et al. [8]; for walnut and almond oils were in agreement with the value found by Arranz et al. [51]; for chestnut oil was in agreement with the value found by Pugliese et al. [42]. Oleic acid can reduce the risk of heart attack by lowering the level of serum triacylglycerides and LDL cholesterol and increasing the HDL cholesterol [52], and recently was found to influence the breast cancer risk by dramatically cutting the levels of gene involved in the disease development [53].
In the samples used in this study, oleic acid and linoleic acid were found to be the main MUFA and PUFA, respectively. This shows that the sum of the oleic and linoleic acids comprises the highest amount of the unsaturated fatty acids. In our study, moreover the sum of the oleic and linoleic acid amounts of the sampled nuts and seeds was found in the range of 92.35-62.75. According to Yıldız et al. [54], this is of particular interest, considering that mainly of the unsaturated fatty acids can be attributed to oleic and linoleic acids, both of which are important from a nutritional point of view besides oil stability.

The highest amount of saturated fatty acids was found in chestnut oil (30.14%), followed by sunflower seed, peanut, hazelnut, pumpkin seed, walnut and pistachio oils (17.10, 13.03, 10.56, 10.41, 10.29 and 10.27%, respectively); while almond oil contained the lowest saturated fatty acid amount (6.64%). The main fatty acids found in the samples were palmitic acid (16:0) 5.39-15.16%, stearic acid (18:0), 1.20-4.89%, and lower amount myristic acid (14:0) 0.03-2.00%. The highest palmitic acid content was found in chestnut and sunflower seed oils (15.16 and 11.83%, respectively), while peanut, pistachio, hazelnut, walnut, pumpkin seed and almond oils had lower amounts of palmitic acid (9.48, 8.20, 7.37, 7.18, 5.62 and 5.39%, respectively). The richest in stearic acid were sunflower seed and pumpkin seed oils (4.89 and 4.49%, respectively), while hazelnut, walnut, peanut, pistachio, chestnut and almond oils had considerably lower amounts of this acid (3.08, 3.07, 2.98, 1.93, 1.90 and 1.20%, respectively). Chestnut oil was the richest in myristic acid (2.00%), while pistachio, sunflower seed, pumpkin seed, almond, hazelnut, walnut and peanut oils (0.11, 0.09, 0.06, 0.05, 0.04, 0.03 and 0.02%, respectively) contained relatively low amounts of myristic acid. The other saturated fatty acids were found in the samples investigated in this study were hexanoic acid (6:0) found only in sunflower seed oil, 0.03%; octanoic acid (8:0) found in chestnut, 1.78% and sunflower seed oils, 0.03%; decanoic acid (10:0) 1.69%, undecanoic acid (11:0) 1.85%, dodecanoic acid (12:0) 1.84%, tridecanoic acid (13:0) 1.37%, pentadecanoic acid (15:0) 1.25% and hexadecanoic acid (16:0) 0.24% found only in chestnut oil. Arachidic acid (20:0) 0.07% was found only in hazelnut oil. Beheic acid (22:0) was found in chestnut oil 0.58%, peanut 0.54%, pumpkin seed oil 0.24% and pistachio oils 0.03%. No beheic acid was found in hazelnut, almond, walnut and sunflower seed oils. Tricosanoic acid (23:0) was found only in chestnut (0.48%) and sunflower seed oils (0.23%) (Table 1).

The unsaturated/saturated ratio (ratio of the sum of unsaturated fatty acids to the sum of saturated fatty acids) previously was used by Pershern et al. [26] for hazelnuts. This ratio showed a significant difference between nuts and oil seeds in this study: almond (14.51), pistachio (8.74), walnut (8.72), pumpkin seed (8.61), hazelnut (8.47) and peanut (6.67) had significant higher unsaturated/saturated ratio than sunflower seed (4.84) and chestnut (2.32) (Table 1). The unsaturated/saturated fatty acids ratio of hazelnut (8.47) was in agreement with the value (8.6) found by Ozdemir et al. [27] and was lower than the value (13.1) found by Koksal et al. [55]. The lower the ratio, the longer was the shelf life. The rates of oxidation of fatty acids are approximately 1:10:100:200 for stearic, oleic, linoleic and linolenic acid, respectively [56]. Acids containing unsaturated bonds like linoleic and linolenic were strongly oxidized at higher temperature [57]. In the study of roasting operation of hazelnut, linoleic acid in hazelnut oil was found to start degradation after 20 minutes at 135 degrees [47].

ALA and EPA were found to be the main ω-3 fatty acids. The highest amount of ALA was found in walnut and hazelnut oils (12.22 and 12.14%, respectively), the other oils contained low amounts of ALA. EPA also was found in chestnut (1.15%) and sunflower seed oils (0.43%); while EPA was not found in the other oils. Linoleic acid (18:2), γ-linolenic acid (18:3), docosadienoic acid (22:2) and eicosatrienoic acid (20:3), lower amount arachidonic acid (21:4) were found to be the main ω-6 fatty acids. The highest amount of linoleic acid were found in pumpkin seed, walnut and sunflower seed oils (70.17, 63.42 and 48.58%, respectively), chestnut, peanut, pistachio almond and hazelnut oils (28.86, 26.51, 20.53, 2037 and 13.64%, respectively) contained lower amounts of linoleic acid. The highest amount of γ-linolenic acid was in peanut oil (1.46%). The highest amount of docosadienoic acid was in chestnut oil (0.40%).
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<th>Pistachio</th>
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<th>Walnut</th>
<th>Chestnut</th>
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The highest amount of eicosatrienoic acid was in peanut oil (2.00%), the other oils contained low amounts of eicosatrienoic acid and there was no eicosatrienoic acid in almond oil. Arachidonic acid only was found in chestnut oil (0.22%). Linoleic acid is essential for humans and is a precursor of many substances that regulate blood clotting, blood pressure, temperature, inflammation, immunological activity, regulation of cell differentiation, repair of DNA damage and many other functions [52,58].

According to Yang et al. [3] walnuts are good sources of both antioxidants and ω-3 fatty acids, in particular high amounts of ALA (6.3 g/100 g), whereas other nuts such as almonds and pistachios possess much lower amounts (0.4–0.7 g/100 g). In this study, the highest amount of ALA and total ω-3 fatty acid were found in walnut. Although almond and pistachio values were lower than the value found by Yang et al. [3] walnut value (12.22%) was higher than. EPA was found in only chestnut and sunflower seed. The highest amount of total ω-6 fatty acid was found in pumpkin seed and walnut. Walnut was found only nut which contained both in very high rates the highest amount of total ω-3 and ω-6 fatty acids.

In this study, α-tocopherol and retinol contents were investigated. The highest amount of α-tocopherol was found in pumpkin seed (530 mg/kg), followed by sunflower seed, peanut, hazelnut, almond, walnut and pistachio (170, 160, 150, 150 and 140 mg/kg, respectively). α-Tocopherol was not detected in chestnut. Retinol content was in almond and pumpkin seed 280; walnut and sunflower seed 270 mg/kg, and it was not detected in the others. The pharmacological properties of α-tocopherol, widely used as a natural antioxidant, are well known [59].

The DPPH radical scavenging abilities of nuts and seeds along with the reference standards BHT and ascorbic acid were expressed as IC\(_{50}\) values. The IC\(_{50}\) value of BHT was 9.64 mg/mL and of ascorbic acid was 2.51 mg/mL. All of nuts and seeds showed DPPH scavenging activities, but DPPH scavenging activity of chestnut was the greater than BHT and ascorbic acid with the lowest IC\(_{50}\) value (1.26 mg/mL). Pistachio 46.44, hazelnut 47.21, pumpkin seed 47.53, walnut 48.24, almond 48.30, peanut 53.13 and sunflower seed 57.11 mg/mL also demonstrated lower activity toward BHT and ascorbic acid. Pistachio, hazelnut, pumpkin seed, walnut, almond, peanut and sunflower seed (46.44, 47.21, 47.53, 48.24, 48.30, 53.13 and 57.11 mg/mL) respectively also demonstrated lower activity toward BHT and ascorbic acid.

Traditionally, the scientific literature has suggested that tocopherols were the main antioxidant component of nuts [3]. In our study, chestnut showed the greatest DPPH scavenging activity toward the others although α-tocopherol and retinol were not found in chestnut. This great radical scavenging effect may be originated from some other phenolic molecules rather than α-tocopherol.

Most antibacterial medicinal plants attack Gram-positive strains while few are active against Gram-negative bacteria [60]. The Turkish nuts and seeds showed strong antimicrobial activity against the Gram (+) and Gram (-) bacteria and the fungi cultures studied, chestnut and pistachio showed higher antimicrobial activity according to the other nuts and seeds.

Hazelnut inhibited more effective against Proteus vulgaris; peanut against Debaryomyces hansenii; pistachio against Escherichia coli, Staphylococcus aureus, Micrococcus luteus, Listeria monocytogenes, Rhodotorula rubra; almond against Proteus vulgaris, Pseudomonas aeruginosa; walnut Proteus vulgaris, chestnut against Escherichia coli, Bacillus cereus, Mycobacterium smegmatis, Candida albicans, Pseudomonas aeruginosa; pumpkin seed against Staphylococcus aureus, Micrococcus luteus, Kluyveromyces fragilis; sunflower seed against Proteus vulgaris, Listeria monocytogenes, Kluyveromyces fragilis.

Calorie values of nut and seed samples were investigated. The highest calorie value was found in walnut and the lowest calorie value was found in chestnut. The values found were for walnut 7730 cal/g, hazelnut 7406 cal/g, pistachio 7384 cal/g, almond 7027 cal/g, sunflower seed 6991 cal/g, pumpkin seed 6907 cal/g, peanut 6856 cal/g, chestnut 4986 cal/g.

Calcium, magnesium, potassium, sodium, iron, copper, manganese, selenium, zinc, chromium and aluminum contents of nut and seed samples investigated as mineral content. Calcium content of samples was in the range of 476.5-1221.74 mg/kg. The highest calcium contents were found in pistachio 1221.74, almond 1075.25, walnut 977.5 and hazelnut 806.23 mg/kg. Magnesium content of samples was in the range of 420.90-427.83 mg/kg and contents of all samples were close values.
Potassium content of samples was in the range of 2166.83-4739.15 mg/kg, the highest potassium contents were in chestnut 4739.15, pumpkin seed 4050.61 and sunflower seed 3094.09 mg/kg. Sodium content of samples was in the range of 403.91-1417.94 mg/kg and of chestnut 403.91 mg/kg which was the lowest content. Sodium content of other samples was close values. Iron content of samples was in the range of 8.79-40.98 mg/kg, contents of pumpkin seed 40.98, sunflower seed 28.30, walnut 24.90 and almond 23.95 mg/kg were the highest. Copper content of samples was in the range of 1.87-10.13 mg/kg, the highest contents were in sunflower seed 10.13 and walnut 8.14 mg/kg. Manganese content of samples was in the range of 5.82-35.30 mg/kg, the highest contents were obtained for pumpkin seed as 35.30, walnut 35.13 and chestnut 20.81 mg/kg. Selenium content of samples was in the range of 0-3.08 mg/kg, the highest contents were in almond 3.08 and walnut 3.05 mg/kg, and there was no in chestnut. Zinc content of samples was in the range of 8.02-53.14 mg/kg, content of pumpkin seed 53.14 and sunflower seed 38.65 mg/kg being highest. Chromium content of samples was in the range of 0-0.35 mg/kg, the highest contents were in pumpkin seed 0.35 and pistachio 0.34 mg/kg and chromium was not found in chestnut. Aluminum content of samples was in the range of 1.43-11.03 mg/kg and its have at toxic concentrations. Similar values were found in the literature, although variability within each nuts and seeds variety can be observed and explained by geographical origin, harvest year, climate and methods of cultivation [25,26].

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Supporting Information

Supporting Information accompanies this paper on http://www.acgpubs.org/RNP

References


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