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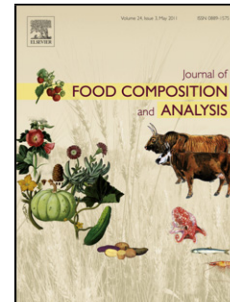

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Bioactive phytochemicals of tree nuts. Determination of the melatonin and sphingolipid content in almonds and pistachios

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Highlights

- Pistachios are richer in melatonin than almonds (2.6±3.0 vs 1.2±0.5 ng/g)
- One pistachio cultivar showed a melatonin content higher than 12000 pg/g
- Pistachios are richer in sphingolipids than almonds (302±77 vs 165±21 pmol/g)
- Among screened ceramide fatty acyl homologs the most represented is the C16:0

Abstract

Tree nuts are healthy foods rich in bioactive phytochemicals. Their regular, moderate consumption has been associated with a reduced risk of chronic-degenerative diseases, in the context of a healthy diet and lifestyle. This study aimed to investigate the phytochemical profile of almonds and pistachios, in order to add new elements in the complex scenario of nut chemistry. A LC-MS/MS method was developed to quantify melatonin and ceramides in almonds and pistachios. In general, pistachios were richer in melatonin (2609±3096 *vs* 1222±500 pg/g) and total ceramides (302±77 *vs* 165±21 pmol/g) than almonds. Among total ceramides fatty acyl homologs, the most represented was the C16:0 species, both for ceramides and dihydroceramides, and both in almonds (37-40%) and pistachios (51-74%). Overall, these results add a piece of information to elucidate the chemical composition of almonds and pistachios and provide a rationale for the nutraceutical potential of nuts in the Mediterranean diet.

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Keywords: functional foods; nutraceuticals; indoleamines; lipids; ceramides; Mediterranean diet

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1. Introduction

Tree nuts are dry fruits or kernels enclosed in woody shells or hard husks, which in turn are generally covered by a thick, fleshy/fibrous outer husk that is removed during harvest. According to FAO, almonds (*Prunus dulcis* Mill.) and pistachios (*Pistacia vera* L.) are included among the ten primary nut crops (http://www.fao.org/waicent/faoinfo/economic/faodef/fdef05e.htm). These nut products, *i.e*. shelled nuts, contain macro- and micronutrients of high biological value, as well as an array of bioactive phytochemicals including polyphenols, carotenoids and phytosterols (Alasalvar & Bolling, 2015). Because of their content of healthy lipids, low glycaemic potential and antioxidant profile, regular nut consumption has been associated with a reduced risk of cardiovascular disease, metabolic syndrome, type 2 diabetes mellitus, certain types of cancer and neurodegenerative disorders (Rusu, Gheldiu, Mocan, Vlase, & Popa, 2018). Noteworthy, nuts represent a relevant component of the healthy Mediterranean diet, characterized by consumption of nuts that is almost two-fold higher than American diet (Aranceta, Pérez Rodrigo, Naska, Vadillo, & Trichopoulou, 2006).

Melatonin is a pleiotropic molecule, widespread among living organisms. From prokaryotes to higher eukaryotes, this indoleamine is involved in a plethora of biological, hormonal, physiological and pathophysiological processes at cell, tissue and organ levels. Melatonin is an amphipathic molecule able to cross barriers as cell membranes and the blood-brain barrier, and, also, it possesses an intrinsic, powerful antioxidant capacity, scavenging the harmful reactive oxygen and nitrogen species (ROS and RNS). In the plant kingdom, melatonin has been detected in a number of medicinal and food plants, where it exerts an auxin-like activity, besides protecting cells and organelles, particularly chloroplasts and mitochondria, from oxidative stress arising from photosynthesis and respiration as well as from other biotic and abiotic stresses. Melatonin concentration in plants varies considerably not only from species to species but also among varieties within the same species and in different organs of a given plant, usually ranging from pg g^{-1} to μg

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g^{-1} of tissue, depending on the environmental and ecological conditions (Kocadağli, Yilmaz, & Gökmen, 2014). In addition, circadian and circannual melatonin fluctuations have been observed in some plant species. In general, seeds and other reproductive organs, rich in lipids and highly vulnerable to oxidative stress caused by environmental pollution or UV irradiation, present the highest levels of melatonin, with concentrations several orders of magnitude higher than those typically measured in vertebrate serum (pg mL^{-1}) (Iriti & Varoni, 2015)

Sphingolipids are an ubiquitous class of nitrogen-containing lipids found in all living organisms as essential structural components of biomembranes, as well as in lipoproteins and other lipid-rich structures. They play a role as intracellular messengers that modulate cell growth, differentiation and apoptosis, also involved, in mammals, in inflammation and immunity. Sphingolipids are amphipathic molecules structurally consisting of three moieties: a polar head group and two nonpolar tails, the sphingoid base and an amide linked long-chain fatty acid (these two-part constitute ceramides). The former is an aliphatic amino alcohol that varies in alkyl chain length (from 12 to 22 carbon atoms, with C18 compounds being the most common), degree of saturation and position of the double bonds, presence of a hydroxyl group and branching of the alkyl chain. As previously introduced, the amino group of the sphingoid base is usually substituted with a long-chain fatty acid via an amide group. In turn, the fatty acids vary in chain length (14-30 carbon atoms), degree of unsaturation, and presence or absence of a hydroxyl group on the carbon atom (Canela et al., 2016). Unlike mammalians, plant sphingolipids are mainly cerebrosides (mono- and oligohexosylceramides) containing glucose, galactose, mannose and inositol as head groups (Markham, Lynch, Napier, Dunn, & Cahoon, 2013, Dean, 2018). Health-promoting effects of dietary sphingolipids have been demonstrated *in vivo*; in particular, their anticancer activity has been documented in a number of animal models (Canela et al., 2016).

In this report, two LC-MS/MS methods have been applied to measure melatonin and ceramides in pistachios and almonds of different origin.

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2. Materials and Methods

2.1 Plant material

Pistachio products (n = 15) were purchased from local market (Milan, Italy) and kindly supplied by Professor Ali Mostafavi; almonds (n = 8) were purchased from local market (Bari, Italy). The origin and the characteristics of the nut products are reported in Table 1.

2.2 Reagents, chemicals and standards

Sphingolipid standards were purchased from Avanti Polar Lipids (Alabaster, USA). Melatonin (Mel) standard was purchased from Sigma-Aldrich. The isotopomer Melatonin-OCD3 (Mel-OCD3) was synthesized by Prof. Andrea Penoni (Dipartimento di Scienza e Alta Tecnologia, Università dell'Insubria, Varese). Methanol, ethanol, acetonitrile, ammonium formate and formic acid (all analytical grade) were supplied from Merck (Darmstadt, Germany). Water was MilliQ-grade (Millipore, Milford, MA, USA).

2.3 Melatonin extraction procedure

The extraction procedure is a modification of the method described by Kocadağli et al. (Kocadağli et al., 2014). The main differences are the addition of Mel-OCD3 as internal standard and the introduction of a purification step of the samples by solid phase extraction. The fine nut powder previously obtained (125 mg) was added with 50 μL of IS (Mel-OCD3, 1 ng added) and extracted with ethanol in triplicate. The samples were vortexed for 3 min and, after centrifugation for 5 min at 10000 rpm, the organic supernatants were pooled (about 3 mL) and evaporated under a gentle stream of nitrogen. After reconstitution with 1 mL of water/methanol (95:5 v/v), the sample was loaded on Strata X 100 mg cartridges (Phenomenex, CA, USA) connected to Supelco Visiprep Dil SPE apparatus. Cartridges were previously conditioned with 1 mL methanol and 1 mL distilled water. The cartridges were rinsed with 1 mL water, then with 1 mL 5% MeOH in water and vacuum-dried for 5 min to remove excess water. Finally, the retained compounds were eluted with

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1 mL of methanol. The eluate was evaporated until dryness by a gentle nitrogen stream. Finally, the residue was re-dissolved with 150 µL acetonitrile/water (1:1) and 5 µL were injected for LC-MS/MS analysis.

2.4 Sphingolipid extraction procedure

Plant materials (1-2 g) were finely ground to powder with an electric blender, keeping the samples in ice. After the addition of 10 µL of IS (Cer C12, 20 mM), the powder (250 mg) was extracted in a microtubes with O-ring seal screw with 500 µL of methanol, 100 µL water and 250 µL chloroform (Dalmau, Jaumot, Tauler, & Bedia, 2015). The samples were sonicated for 30 min and incubated overnight in an oscillator bath at 48 °C. After centrifuging, the supernatant was evaporated under a stream of nitrogen. The residues were dissolved in 150 µL of methanol and then centrifuged for 10 min at 13000 rpm. The clean supernatant (130 µL) was transferred into the autosampler vials and 10 µL directly injected in LC-MS/MS.

2.5 LC-MS/MS instrumentation

The mass spectrometry measurements were performed with a computerized integrated system consisting of a liquid chromatography coupled to a tandem mass spectrometer. The liquid chromatography system was an UltiMate® 3000 LC Systems (Dionex™, Sunnyvale, CA, USA), with autosampler, binary pump, and column oven (Thermo Fisher Scientific, USA). The tandem mass spectrometer was an AB Sciex 3200 QTRAP LC-MS/MS instrument with electrospray ionization (ESI) TurboIonSpray™ source (AB Sciex Framingham, MA, USA). Instruments were managed with manufacturers' software and according to manufacturers' instructions. The analytical data were processed by Analyst software (version 1.6.2).

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2.6 Targeted LC-MS/MS analysis of melatonin in almonds and pistachios

Chromatographic separation was attained on a reversed-phase Zorbax Bonus-RP (3.0x100 mm, 3.5μm) analytical column preceded by a security guard cartridge. Linear gradient was obtained between eluent A (5 mM ammonium formate and 0.1% formic acid in water) and eluent B (0.1% formic acid in methanol). The elution gradient was set as below: 0-1 min (20% B), 1-5 min (20-60% B), 5-7 min (60% B), 7-7.2 min (60-95% B), 7.2-8.2 min (95% B), 8.2-8.5 min (95-20% B) and 8.5-10 min (20% B). The flow rate was 0.4 mL/min, the autosampler and the column oven were kept at 10°C and 40°C, respectively. In the ion spray source, Nitrogen was used as a nebulizing gas (GS 1, 25 psi), turbo spray gas (GS 2, 25 psi) and curtain gas (30 psi). The ion spray voltage in the positive ion mode (ESI+) was set at 5.5 kV and the source temperature was set at 300 °C. Quantitative analysis was performed by Multiple reaction monitoring (MRM). Collision-activated dissociation (CAD) with Nitrogen was performed at the Low gas pressure setting. The dwell time was set at 0.25 s. Quantitative analysis was performed calculating the area/area ratio (Mel/Mel-OCD_3) and interpolating with the calibration curve (Table S1).

2.7 Targeted LC-MS-MS analysis of sphingolipids in almonds and pistachios

LC-MS/MS analysis was developed based on the method already described with some modifications (Merrill, Sullards, Allegood, Kelly, & Wang, 2005). Separation was accomplished in a ACQUITY UPLC BEH C-8 Column, 130 Å, 1.7 μm, 2.1 mm × 100 mm (Waters, Millford, MA) preceded by a security guard cartridge. The two mobile phases were: phase A, 2 mM ammonium formate in water and phase B 1 mM ammonium formate in methanol, both containing 0.2% formic acid (v/v). A linear gradient was programmed: the column was equilibrated with 80% (B), increased to 90% (B) in 3 min, held for 3 min, increased to 99% (B) in 9 min, held for 3 min, back to the initial conditions in 2 min and kept for 2 min at 80% (B). The flow rate was 0.3 mL/min; the

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autosampler and the column oven were kept at 15°C and 30°C, respectively, the operating pressure was 450 Psi. In the ion spray source, Nitrogen was used as a nebulizing gas (GS 1, 45 psi), turbo spray gas (GS 2, 50 psi) and curtain gas (25 psi). The ion spray voltage in the positive ion mode (ESI+) was set at 5.5 kV and the source temperature was set at 300 °C. Quantitative analysis was performed by Multiple reaction monitoring (MRM), with the parameters reported in Table S2 that were optimized before by direct infusion of standard mixtures. Collision-activated dissociation (CAD) with Nitrogen was performed at the Low gas pressure setting. The dwell time was set at 0.10 s. Quantitative analysis was performed by calculating the area/area ratio (ceramide/IS) for each species and interpolating with the calibration curve.

2.8 Analytical procedure and performance of melatonin analysis

Stock solutions of Mel and Mel-OCD3 were prepared at 1 mg/mL in methanol. An intermediate solution 10 μg/mL in methanol was prepared before the final working solution 100 ng/mL from which the ten-point calibration curve was obtained by serial dilutions of this solution with water in the range 500-1.25 ng/mL. The stock solution of Mel-OCD_3 was diluted 1:500 (v/v) and then 1:100 with water at a final working concentration of 20 ng/mL. For each calibration point 10 μL of the standard curve solution were added with 50 μL of IS (1 ng) and extracted by SPE as already described (melatonin in the range 0,025-5 ng). Calibration curves were calculated by plotting the area ratio (Mel/IS) vs ng Mel. Linearity was assessed by un-weighted least squares regression ($y=0.7596x-0.0082$, $R^2=0.9998$). Validation parameters for precision and accuracy were calculated using different replicates of samples on the same day (intra-day) and in different working days (inter-days). Accuracy was expressed as the relative error (RE%), while precision was measured as coefficient of variation (CV%): they were determined at 0.25, 1, 4 ng in sets of three replicates per day. CV% and RE% resulted below 15% and thus considered suitable. LOD was considered as the

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concentration that generates a signal-to-noise (S/N) ratio > 3 whereas LLOQ was that yields at S/N >10. LLOQ was found at 25 pg and LOD at 5 pg.

2.9 Analytical procedure and performance of sphingolipid analysis

All samples were dissolved in methanol at a concentration of 0.6 mM (stock solution) heating at 55-60°C if necessary. Ceramide (Cer)-C12 was used as internal standard (IS) diluting 1:30 the stock solution with methanol. The working solution mixture 40 μM was prepared by mixing 150 μL of each standard and bringing to a final volume of 2.25 mL with methanol. The six-point calibration curve was obtained by serial dilutions of this solution in the 40-2.5 μM range. For each calibration point, 10 μL were mixed with 10 μL of IS and 130 μL of methanol directly into the autosampler vial (range 25-400 pmol added in vial). Calibration curves were calculated by plotting the area ratio (ceramide/IS) *vs* pmol added. Linearity was observed for each analyte in the whole range ($R^2>0.99$). Precision and accuracy, expressed as CV% and RE%, resulted <15% and the results were accordingly reported in Table S3.

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3. Results and Discussion

3.1 Melatonin content in almonds and pistachios

The results obtained from analysis of melatonin in almonds and pistachios evidenced a high variability depending on the different cultivars and/or suppliers. Pistachios were generally richer in melatonin than almonds (2609±3026 vs 1222±500 pg/g) (Figure 1A). In particular, one pistachio sample (P2) showed a melatonin content higher than 12000 pg/g, and in another one (P3) it was around 6000 pg/g (Figure 1B). Melatonin content in different almond cultivars was somewhat more homogeneous ranging from 643 (A5) to 2110 pg/g (A1) (Figure 1C).

In a previous study, 39 ng/g of melatonin were measured in almonds by HPLC-ECD (Manchester et al., 2000). In different pistachio varieties, exceptionally high melatonin concentrations (227-233 μg/g, the highest levels reported for any plant organ to date) were detected by spectrofluorometric detection (Oladi, Mohamadi, Shamspur, & Mostafavi, 2014). To the best of our knowledge, these are the only reports available on the presence of melatonin in these nuts, and differences may arise from diverse extraction methods and analytical instruments used to determine the indoleamine. A possible confounding factor to explain the significant difference is the co-measurement of melatonin and its isomers, including tryptophan ethyl ester, a compound with the same molecular weight of melatonin and previously considered as a melatonin isomer (Gardana, Iriti, Stuknyte, De Noni, & Simonetti, 2014; Vigentini et al., 2015; Vitalini, Gardana, Simonetti, Fico, & Iriti, 2013). To note, in this paper melatonin levels were assessed by using the isotopomer analogue 5-OCD_3-melatonin, which shows a specific LC-MS/MS fragmentation and an improved performance for quantification.

3.2 Sphingolipid levels in almonds and pistachios

The results of targeted analyses of ceramides (standard panel of seven main molecular species) and dihydroceramides (standard panel of five main chemical species) in three almond and nine pistachio

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samples are summarized in Tables S5 and S6. Since the intended purpose of the study was nutritional, rather than technological, compositional data were not normalized to water or total lipid content of the different seeds. A summary of the results indicates that, in the considered samples, total ceramides (ceramides + dihydroceramides, all measured fatty acyl homologs) were more abundant in pistachios than in almonds (302±77 vs 165±21 pmol/g) (Figure 2A). Among total ceramide fatty acyl homologs, the most represented was the C16:0 species, both for Cer and DHCer species, and both in almonds (37-40%) and pistachios (51-74%), followed by 18:1, 18:0 and 14:0. Fatty acid composition of ceramides and dihydro-ceramides was more homogeneous in the three almond cultivars than in the seven pistachio samples. In particular, the higher and more variable content of ceramides in pistachios was mostly due to the larger variability of the content in C16:0 fatty acyl ceramide (35-38% in almonds, 50-73% in pistachios; Figure 2B). Furthermore, results can be plotted as two superimposed piled histograms (Figure 2C,D) to compare sphingolipid levels among the different samples of almonds and pistachios. Not all pairs of Cer/DHCer homologs were measured in the targeted analysis, and the Cer/DHCer ratio could be determined only for pairs in which both components yield measurable amounts, *i.e.*, only for the C16:0 and C18:1 fatty acyl homologs. The mean Cer/DHCer ratio was 3.75, with a higher and more homogeneous ratio in almonds (mean 4.72; 4.58-4.92) than in pistachios (mean 3.42; 2.81-4.40) (Figure 3). The Cer/DHCer ratio of the C18:1 homolog was consistently lower than that of the C16:0 homolog (mean 1.35 vs. 3.15 among all almond and pistachio samples). This observation suggests that the ceramide homologs have in part a different origin, and that the more abundant acyl-sphingosine homologs may derive both from the corresponding dihydro-sphingosines (*de novo* pathway) and from the degradative pathway of sphingomyelins, thus indicating that a more systematic characterization of the complete ceramidome may highlight quali-quantitative nutritional differences also during seeds processing and shelf life.

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Previous studies on plant sphingolipids show that sphingosine d18:1Δ4(E) is only a minor component of the long chain base pool of plants. Among screened plants are 1) *Arabidopsis thaliana* leaves (Markham, Li, Cahoon, & Jaworski, 2006) where the major sphingosine base is t18:1(E) approx. 10%, 2) sunflower seeds (Salas, Markham, Martínez-Force, & Garcés, 2011) where the major sphingosine bases are d18:1 Δ8(E) and t18:1 Δ8(E), and 3) pulp and skin of Moro Blood Orange (*Citrus sinensis*), where at least 11 differents sphingosine bases were identified (Valsecchi et al., 2012). Ceramide composition also changes among different organs of the same plant, such as between pollen and leaves in *Arabidopsis* (Luttgeharm et al., 2012). There is scanty information on occurrence and levels of ceramides in nuts. To the best of our knowledge, only one article (Miraliakbari & Shahidi, 2008) reports the total sphingolipid content of almond and pistachio oils, as 240 and 330 mg/100 g of oil, respectively, measured utilising TLC-FID as a non-specific method of quantification. Moreover, in almonds, a monoglucocerebroside, 1-*O*-*β*-D-glucopyranosyl-(2*S*,3*R*,4*E*,8*Z*)-2-[(2*R*)-2-hydroxyhexadecanoylamino]-4,8-octadecadiene-1,3-diol, containing the major sphingoid base sphinga-4,8-dienine (d18:2, a dihydroxy base) in the hydrophobic moiety was identified on the basis of high-resolution 1D and 2D NMR data (Sang et al., 2002). The major sphingoid bases of ceramides identified in almonds (Fang, Ho, Sang, & Rosen, 2005) were t18:1 and d18:2, associated with very long chain fatty acids (saturated C22, C23, C24, C25), and the corresponding *α*-hydroxy acids. They also identified and measured the corresponding cerebrosides at 0.068 mg/g (sphinga-4,8-dienine, *α*-hydroxy-palmitic acid (C16:0h) and glucose as the head group (Fang et al., 2005).

The aim of this study is nutritional, rather than phytochemical. Thus, this work measures a standard series of d18:1 Δ4(E) ceramides since they are the fraction of sphingoid pool which is common to human and plant metabolism. With a targetted analysis for the identification of 13 selected species, we found in almonds and pistachios a prevalence of C16:0 ceramide. A recent article (Neeland et al., 2018), reports that circulating shorter-chain saturated fatty acid ceramides (C16:0, C18:0)

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positively correlate with insulin resistance, while longer-chain polyunsaturated ceramides (C24:2, C30:10 and C32:11) are inversely associated. Wang et al., 2017 showed a positive association between plasma ceramide concentrations and incident of cardiovascular disease (CVD), while Mediterranean dietary intervention (with nuts or olive oil) may mitigate this deleterious effect. Despite the fact that bioavailability in human of dietary administered sphingolipids still needs further elucidation, we are convinced that the composition of sphingoid components in nuts is much more complex than the target analysis here reported. For this reason here we used a brode extraction procedure (escaping alkaline methanolysis typical of targeted sphingolipid analysis) useful for un-targeted and discovery lipidomic analysis. A cognate article will present our un-targetted investigation of the occurrence of other related compounds in these cultivars.

4. Conclusions

Plants are complex systems, rich in hundreds of bioactive phytochemicals that belong to multiple chemical families. The health benefits of plant foods can thus be ascribed to the likely additive and/or synergistic effects of their constituents. As for all food with high nutritional value and a high commercial value, quality check and enforcement of marketed goods is of great importance. Chemometric techniques applied to constituents such as bulk and minor fatty acids are a viable approach to ascertain food authenticity and geographic source. One such case is possible adulteration of ground almond with much less prized apricot kernel. The relative proportions of five main fatty acids: palmitic (C16:0), palmitoleic (C16:1), stearic (C18:0), oleic (C18:1) and linoleic (C18:2) acid, measured as FAME, were studied with the use of several techniques of Principal Component Analysis (PCA), and allowed to pinpoint and measure the proportion of the extraneous component (Esteki et al., 2017). The occurrence of a specific minor isomer of eicosenoic fatty acids, gondoic acid (C20D11) in pistachios allowed discriminating the origin of Iranian pistachios of five different cultivars (Esteki et al., 2019).

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Ceramides are distinctive components of plants, and with this work, we have started adding new facets to the complex scenario of nut chemistry, thus contributing to further elucidate the pivotal role of nuts as a component of the Mediterranean diet. Forthcoming developments stem from understanding that the ceramide composition of pistachios and almonds contains a large proportion of as yet poorly investigated compounds that are the object of our pending studies.

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Declarations of interest: none

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Figures captions

Figure 1. (A) Average of melatonin concentration in almonds (n=8) and pistachios (n=15). **(B)** Melatonin levels in the different pistachios and **(C)** almonds cultivars. Each nut cultivar has been extracted and analysed in triplicate. Results are presented as mean±SD.

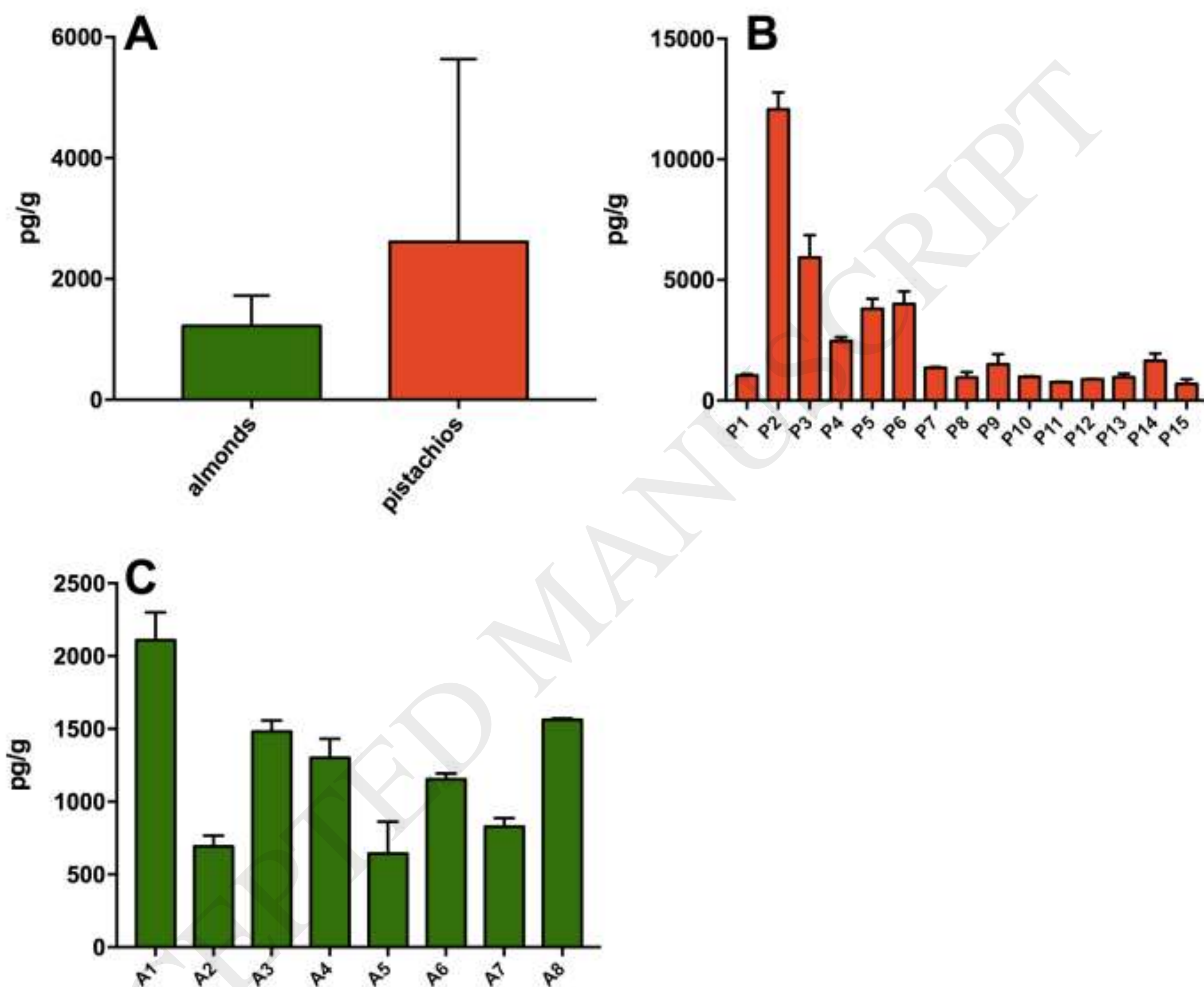

Figure 2. (A) Mean±SD of total ceramides and dihydroceramides amount found in almonds (n=3) and pistachios (n=9). **(B)** Mean concentration (±SD) of single ceramides and dihydroceramides species of in almonds (green, n=3) and pistachios (orange, n=9) cultivars. **(C)** Composition in ceramides and dihydroceramides species of the almond cultivars (n=3) **(D)** Composition in ceramides and dihydroceramides species of pistachios samples (n=9).

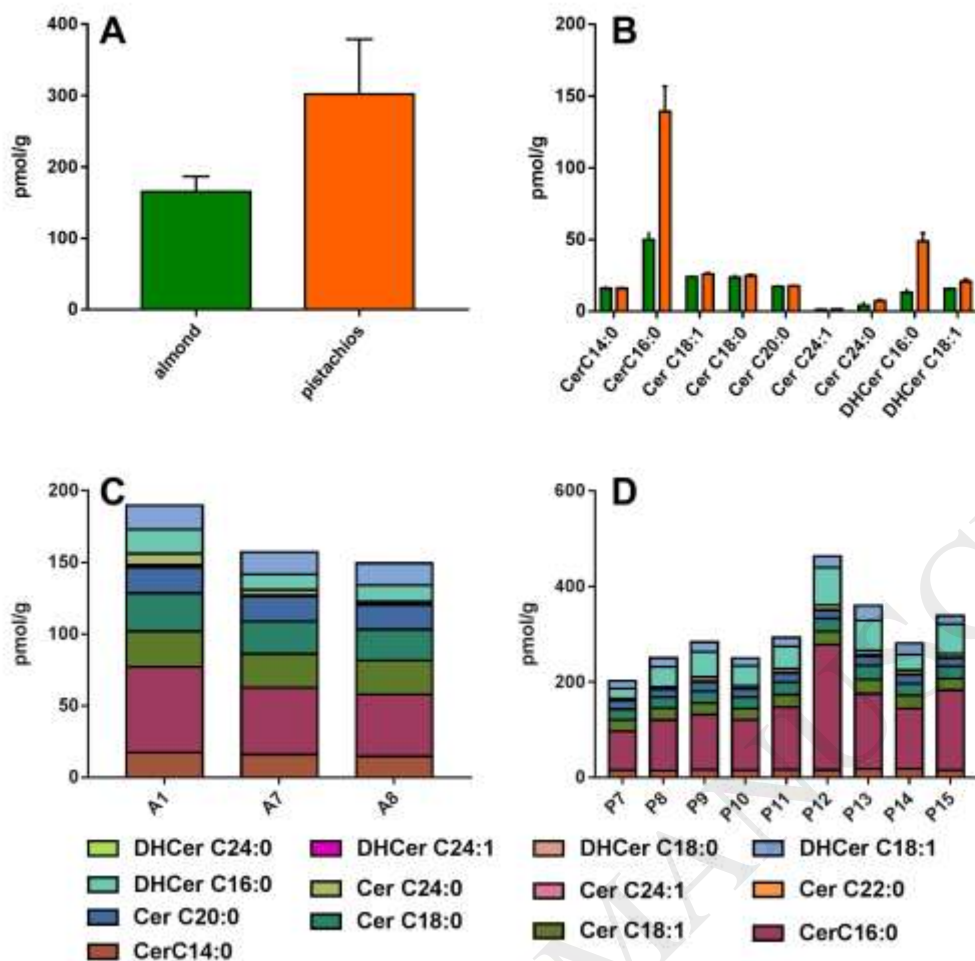

Figure 3. Mean±SD ratio Cer C16:0/DHCer C16:0 and Cer C18:1/DHCer C18:1 in almonds (n=3) and pistachios (n=9).

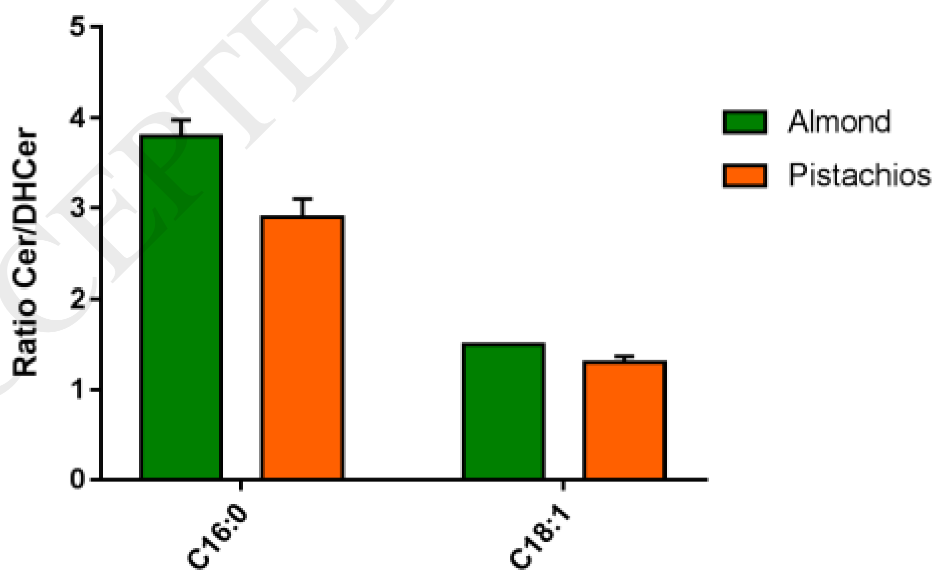

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Table 1. Origin and characteristics of the nut product samples.

Species	Sample	Nut product	Origin	Characteristics
Pistachio (*Pistacia vera* L.)	P1	Shelled	Gut & Gunstig , EDEKA (Hamburg)	Roasted, salted
	P2	Shelled	Seeberger, Wertvolle Snacks (Ulm)	Roasted, salted
	P3	Shelled	Iran	Not roasted, not salted
	P4	Shelled	Iran	Not roasted, not salted
	P5	Shelled	Iran	Not roasted, not salted
	P6	Shelled	Iran	Not roasted. not salted
	P7	Shelled	Bronte DOP (Sicily, Italy)	Not roasted, not salted
	P8	Shell	USA	Roasted, salted
	P9	Shell	Non-EU	Organic, roasted, not salted
	P10	Shell	Iran	Not roasted, not salted
	P11	Shell	USA	Roasted, not salted
	P12	Shelled	Noberasco	Not roasted, not salted
	P13	Flour	Iran	-
	P14	Flour	Italy	-
	P15	Flour	Italy	-
Almond (*Prunus dulcis* Mill.)	A1	Shelled	cv. Palo (Puglia, Italy)	Dried
	A2	Shelled	cv. Collepasso (Puglia, Italy)	Dried
	A3	Shelled	cv. Barletta (Puglia, Italy)	Dried
	A4	Shelled	cv. Cassano (Puglia, Italy)	Dried
	A5	Shelled	cv. Minervino (Puglia, Italy)	Dried
	A6	Shelled	California (USA)	Died
	A7	Shelled	California (USA)	Dried
	A8	Shelled	California (USA)	Dried