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Pistachio Skin Phenolics Are Destroyed by Bleaching Resulting in Reduced Antioxidative Capacities

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Pistachio shells split naturally prior to maturity leading to their unique crack-shell form. Within 24 h of harvest, hull-trapped moisture may cause shell staining. The illegal process of bleaching has been used to restore a desirable white color to pistachio shells. It is not known whether bleaching adversely affects phytochemical levels in pistachios. Therefore, we identified for the first time multiple pistachio skin phenolics as quercetin (14.9 μg/g), luteolin (10.0 μg/g), eriodictyol (10.2 μg/g), rutin (1.6 μg/g), naringenin (1.2 μg/g), apigenin (0.2 μg/g), and the anthocyanins, cyanidin-3-galactoside (696 μg/g) and cyanidin-3-glucoside (209 μg/g). We investigated the effects of bleaching (0.1−50% hydrogen peroxide) on phenolic levels and antioxidative capacities in raw and roasted nuts. Because of their flavylium cation structures, anthocyanins were the most sensitive to bleaching. Bleaching decreased total anthocyanin levels [μg/g of skins (% hydrogen peroxide)]: 905 and 549 (0%); 653 and 145 (0.1%); 111 and 18.4 (5%); 6.1 and 3.2 (25%); 0 and 0 (50%) for raw and roasted nuts, respectively. Bleaching also reduced antioxidative capacity [μM/g of Trolox (% hydrogen peroxide)]: 945 and 725 (0%); 940 and 472 (0.1%); 930 and 455 (5%); 433 and 370 (25%); 189 and 173 (50%), for raw and roasted nuts, respectively. Raw nuts preserved phenolic levels and antioxidant capacity better than roasted nuts, suggesting contributing effects of other substances and/or matrix effects that are destroyed by the roasting process. The destruction of bioactive phenolics in pistachio skins may negatively impact the potential health benefits arising from pistachio consumption.

KEYWORDS: ***Pistacia vera*; pistachios; skin phenolics; antioxidant; bleaching; roasting**

INTRODUCTION

A growing body of studies suggests that consumption of nuts might confer beneficial effects on coronary heart disease (*1*, *2*). Although the major research interest on health benefits of nuts has focused on their natural content of monounsaturated fats, emerging evidence indicates that phytochemicals present in their skins (peels) including polyphenols, also may play an important role (*3*). Polyphenols have antioxidant properties, and their presence in outer layers (skins, peels, and hulls) of fruits, vegetables, and tree nuts may offer protection against oxidative stress. In fact, almond and walnut skin phenolics have been shown to inhibit oxidation of LDL, a key step in atherogenesis (*4*, *5*).

Pistachio (*Pistacia vera* L.) nuts are widely consumed and are of significant economic importance with the top major worldwide producers being Iran followed by the United States (*6*). The United States pistachio industry, which is located almost exclusively in California, has experienced phenomenal growth in the past 30 years growing in production from nearly zero in 1976 to $333 million by 2002. Pistachios are unique among tree nuts in that their endocarp (shell) splits naturally prior to maturity. This allows pistachios to be marketed largely in-shell for fresh consumption, because their kernels can be easily extracted without mechanical cracking. However, pistachios have to be processed within 24 h after harvest to avoid hull-trapped moisture, which causes staining of the pistachio shell (*7*, *8*). Therefore, bleaching methods to whiten pistachio shells are sometimes used, although this practice has been made illegal in some countries. Whether bleaching adversely affects the levels of potentially beneficial phytochemicals contained in pistachios is not known.

Phytochemicals previously identified from pistachios include fatty acids, phytosterols, lutein, resveratrol, and anthocyanins (*9*−*13*). The aim of the current study was to identify phenolics in pistachio skins using high performance liquid chromatography with ultraviolet (HPLC-UV) and mass spectrometry (HPLC-MS) detection and to investigate the effects of bleaching in combination with roasting on their levels and antioxidative activities, using Trolox equivalent antioxidant capacities (TEAC) assays. Although identification of some phenolics in other tree nuts, such as almonds (*14*, *15*), macadamia (*16*), walnuts (*5*), and cashews (*17*), as well as peanuts (*12*, *18*), have been reported, these data are not available for pistachio nuts. Given

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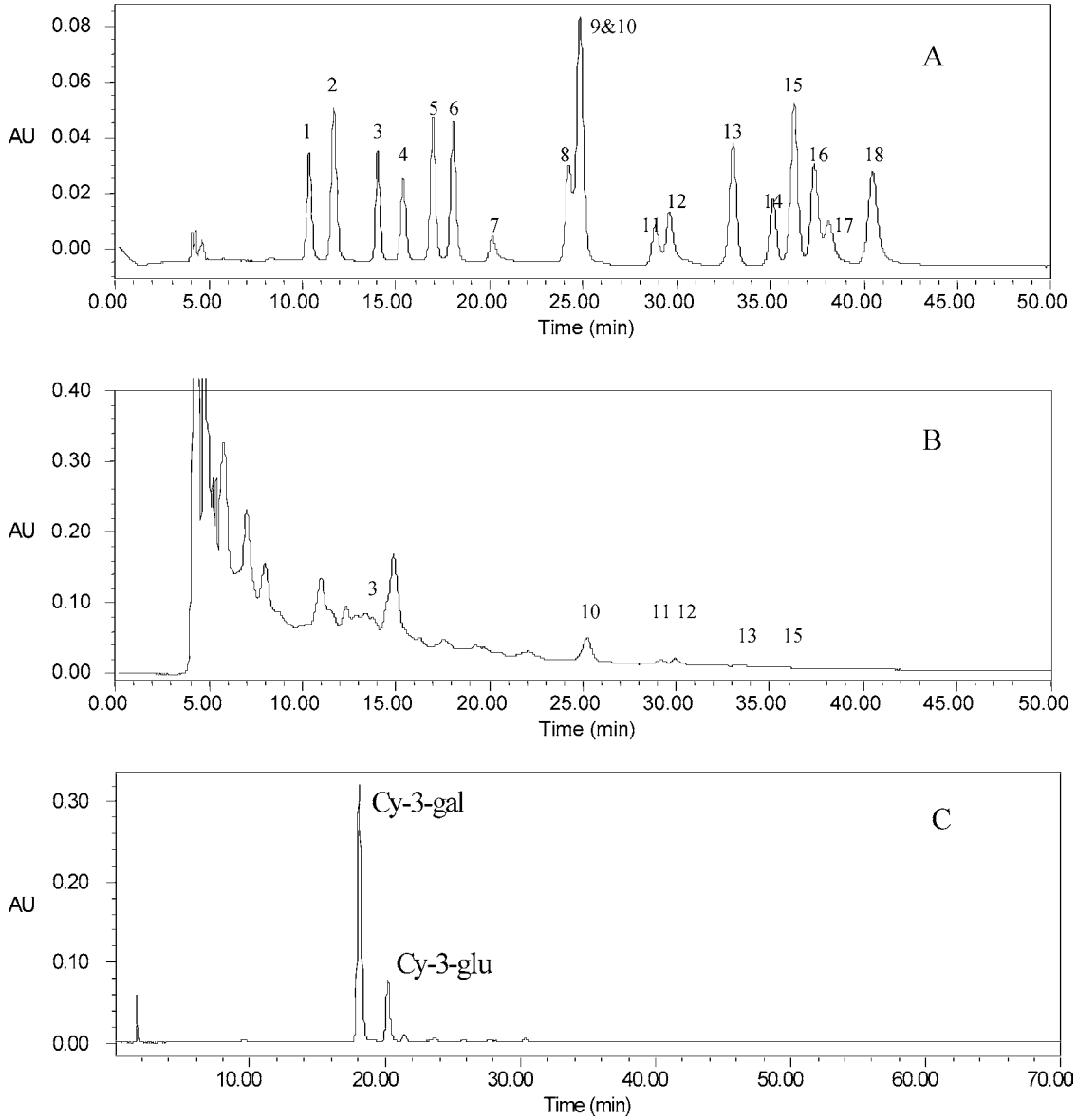

Figure 1. HPLC chromatograms of (A) 18 phenolic reference standards at 292 nm; (B) pistachio skin extract at 292 nm; and (C) pistachio skin extract at 520 nm for anthocyanin detection. 1 = daidzin, 2 = eriocitrin, 3 = rutin, 4 = genistin, 5 = naringin, 6 = hesperidin, 7 = myricetin, 8 = baicalin, 9,10 = daidzein/eriodictyol (coeluting), 11 = quercetin, 12 = luteolin, 13 = naringenin, 14 = genestein, 15 = apigenin, 16 = hesperetin, 17 = kaempferol, 18 = baicalein, Cy-3-gal = cyanidin-3-galactoside, Cy-3-glu = cyanidin-3-glucoside.

the recent report on the effects of pistachio consumption on plasma lipid profile and oxidative status in human volunteers (*19*), this information is needed to evaluate the potential health benefits of pistachios. Furthermore, the effects of bleaching on levels and antioxidative capacities of potentially beneficial phytochemicals found in the pistachio have not been previously examined.

MATERIALS AND METHODS

Chemicals and Materials. Pistachios were provided by Paramount Farms (Lost Hills, CA). All solvents were HPLC grade and purchased from Fisher Scientific Co. (Tustin, CA). Commercially available standards of flavonoids (daidzin, daidzein, eriocitrin, eriodictyol, rutin, quercetin, genestin, genestein, naringin, naringenin, hesperidin, hesperetin, myricetin, baicalin, baicalein, luteolin, apigenin, and kaempferol) were purchased from Indofine Chemical Company (Hillsborough, NJ). Cyanidin-3-glucoside was purchased from Chromadex (Santa Ana, CA).

Preparation of Standards. All phenolic standards (1 mg) were individually dissolved in methanol (1 mL) except for the anthocyanin, cyanidin-3-glucoside, which was dissolved in acidic methanol. All stock solutions were sonicated for 20 min and then further diluted to provide 20, 10, 5, 2.5, and 1.25 μg/mL concentrations that were used to provide standard calibration curves by HPLC analyses.

Preparation of Samples. Skins were separated from the nuts, crushed, and weighed, and aliquots (200 mg) were extracted with acidic methanol (2 mL) by ultrasonication for 30 min. The homogenate was then centrifuged at 10 000 rpm for 10 min, and the supertanant (50 μL) was injected directly into the HPLC or LC-MS for analyses. All analyses were performed in triplicate.

Bleaching of Nuts. Bleached samples of both raw and roasted in-shell pistachios were provided by Paramount Farms (Lost Hills, CA). In-shell pistachios were exposed to different levels of hydrogen peroxide solutions (0.1, 0.5, 1, 3, 5, 15, 20, 25, and 50%) as follows. Whole pistachios (500 g) were introduced and soaked in the appropriate hydrogen peroxide solution (1 L) for 1 min by light stirring to ensure equal exposure. The treated product was then allowed to drip-dry. For raw samples, the treated pistachios were then rinsed in distilled water for 1 min, drip-dried to remove any excess solution, and then dried in a conventional oven at 140 °F for approximately 20 min. For the roasted samples, a conventional oven set at 280 °F temperature was used for

Table 1. Identification and Quantification of Phenolics in Pistachios[a]

phenolics	MW	MS	concentration (μg/g) skin	nuts
rutin	610.5	609.5	1.6	0.55
eriodictyol	288.3	287.3	10.2	1.1
quercetin	302.3	301.3	14.9	0.29
luteolin	286.3	285.3	10.0	1.04
naringenin	272.2	271.2	1.2	0.12
apigenin	270.3	269.3	0.2	0.03
cyanidin-3- galactoside	448.0	449 and MS/MS = 287	696	n.d.
cyanidin-3- glucoside[b]	448.0	449 and MS/MS = 287	209	n.d.

[a] MW= molecular weight; MS = mass spectrometric molecular ion (*m/z*); n.d. = not detected. [b] Confirmed by comparison of its retention time to an authentic reference standard of cyanidin-3-glucoside.

approximately 20 min. The actual time that the samples spent in the oven was determined by their final moisture level, which has a target of 3.5−5.5% moisture for raw and 1.5−2.5% moisture for roasted products.

Identification and Quantification of Phenolics by High Performance Liquid Chromatography with Ultraviolet Detection (HPLC-UV). The HPLC system consisted of a model 600 pump, 717 Autosampler, 996 Photodiode Array detector (PDA) and Millenium[32] chromatography software (Waters, Milford, MA). The conditions for detection of anthocyanins was different from that used for the other phenolics. For anthocyanins, the mobile phase, solvent A (acetonitrile) and solvent B (4% aqueous phosphoric acid) was used under binary linear gradient conditions: 0−60 min, 5−25% A; 60−70 min, 25% A, with a flow rate of 0.75 mL/min, detection wavelength of 520 nm and a 150 × 4.6 mm, 3.5 μ NovaPak C18 column (Waters). For the other phenolics, the mobile phase consisted of solvent A (1% acetic acid in acetonitrile) and solvent B (1% aqueous acetic acid) under gradient conditions as follows: 0−10 min, 15−20% A; 10−30 min, 20−28% A; 30−45 min, 28−30% A; 45−55 min, 30−60% A; 55−60 min, 60% A with a flow rate of 0.8 mL/min, detection wavelength of 292 nm and a 250 × 4.6 mm, 5 μm C18 column (Phenomenex). All sample injection volumes were 50 μL. Phenolics were identified by comparison of their retention times to those of reference standards (**Figure 1**) and were confirmed by LC-MS analyses. Anthocyanins were quantified from their HPLC-UV peak area by using the equation for linear regression obtained from the calibration curve (**Table 1**). The anthocyanins were quantified by using an external standard of cyanidin-3-glucoside and are reported as cyanidin-3-glucoside equivalents (**Table 1**).

Identification and Quantification of Phenolics by Liquid Chromatography Electrospray Ionization Mass Spectrometry (LC-ESI/MS). Analyses were carried out on an LCQ Classic Finnigan LC-MS/MS system (ThermoFinnigan, San Jose, CA), equipped with a HP 1100 series HPLC system consisting of an autosampler/injector, quaternary pump, column heater, and diode array (DAD) detector. Data handling was carried out using Xcalibur 1.2 software (Finnigan Corp). A binary mobile phase system of solvent A (1% acetic acid in methanol) and solvent B (1% aqueous acetic acid) was used as follows: 0−60 min, 10−70% A; 60−70 min, 70−80% A, with a flow rate 0.2 mL/min; injection volume 20 μL; a Zorbax C18, 150 × 2.1 mm, 5 μm column (Agilent) set at a column temperature of 30 °C, DAD range 210−600 and 520 nm as detection wavelength for anthocyanins. MS parameters: Ionization mode, electrospray ionization (ESI) in positive mode for anthocyanins and negative mode for all other phenolics; scan range: 200−600 amu; scan rate: 1 scan/s; cone voltage: 17 eV. Compounds were confirmed by matching their molecular ions (M^+) compared to authentic standards.

Trolox Equivalent Antioxidative Capacity (TEAC). The assay was performed as previously reported (*20*). Briefly, 2′,2′-azinobis(3-thylbenzothiazline-6-sulfonic acid) diammonium salt (ABTS) radical cations were prepared by adding solid manganese dioxide (80 mg) to a 5 mM aqueous stock solution of $ABTS^{\bullet+}$ (20 mL using a 75 mM Na/K buffer of pH 7). Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), a water-soluble analog of vitamin E, was used as an antioxidant standard. A standard calibration curve was constructed for Trolox at 0, 50, 100, 150, 200, 250, 300, and 350 μM concentrations. Skins obtained from raw and roasted nuts exposed to different levels of bleach (0, 0.1, 0.5, 1, 3, 5, 15, 20, 25, 50% hydrogen peroxide) were extracted in methanol/water (1:1, v/v) (10 mg/mL concentrations) by vortexing for 30 min, sonicating for 5 min, and centrifuging for 10 min at 2000*g*. Samples were diluted appropriately according to antioxidant activity in 75 mM Na/K buffer of pH 7. Diluted samples were mixed with 200 μL of $ABTS^{\bullet+}$ radical cation solution in 96-well plates, and absorbance was read at 750 nm after 10 min in a ThermoMax microplate reader (Molecular Devices, Sunnyvale, CA). Samples were assayed in six replicates. TEAC values were calculated from the Trolox standard curve and expressed as Trolox equivalents (in μM Trolox/g).

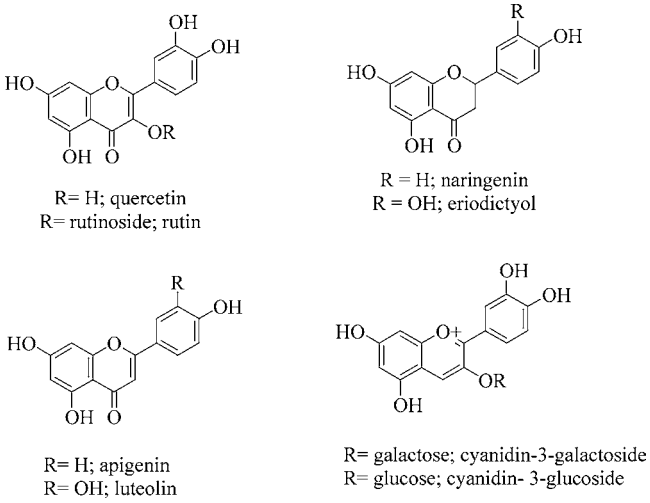

Figure 2. Chemical structure of pistachio skin phenolics (PSPs).

RESULTS AND DISCUSSION

Identification and Quantification of Pistachio Skin Phenolics (PSPs). Figure 1 shows the HPLC-UV chromatograms of 18 phenolic reference standards (**Figure 1A**), pistachio skin extract at 292 nm (**Figure 1B**), and pistachio skin extract at a detection wavelength of 520 nm for anthocyanins (**Figure 1C**). The phenolic standards, in order of elution, are daidzin, eriocitrin, rutin, genistin, naringin, hesperidin, myricetin, baicalin, daidzein/eriodictyol (coeluting), quercetin, luteolin, naringenin, genestein, apigenin, hesperetin, kaempferol and baicalein. Of these compounds, pistachio skins contained rutin, eriodictyol, quercetin, luteolin, naringenin, and apigenin. In addition, the anthocyanins, cyanidin-3-galactoside and cyanidin-3-glucoside, were identified in pistachio skins as reported (*10*, *11*). The chemical structures of the phenolics are shown in **Figure 2**. We also observed broad peaks at a detection wavelength of 254 nm by HPLC-UV, characteristic of proanthocyanidins (polymeric phenolics), which are currently being identified in our laboratory.

Table 1 shows the quantities of the individual phenolics found in pistachio skins and nuts. The major phenolics were anthocyanins (cyanidin-3-galactoside, 696 μg/g; cyanidin-3-glucoside, 209 μg/g), quercetin (14.9 μg/g), eriodictyol (10.2 μg/g), and luteolin (10.2 μg/g). Rutin (1.6 μg/g), naringenin (1.2 μg/g), and apigenin (0.2 μg/g) were present in lower concentrations. The anthocyanins were quantified by HPLC-UV, whereas the other phenolics were quantified by LC-MS methods.

Because of levels of detection and coeluting peaks in the HPLC-UV analyses, we utilized LC-MS/MS methods for

Table 2. Levels of Anthocyanins, Cyanidin-3-galactoside, and Cyanidin-3-glucoside, in Skins Obtained from Raw and Roasted Pistachio Nuts Exposed to Different Levels of Bleaching Agent (Hydrogen Peroxide)[a]

	raw			roasted		
hydrogen peroxide (%)	Cy-3-gal (μg/g)	Cy-3-glu (μg/g)	total (μg/g)	Cy-3-gal (μg/g)	Cy-3-glu (μg/g)	total (μg/g)
0	696 ± 78	209 ± 22	905 ± 100	462 ± 54	87 ± 12	549 ± 66
0.1	554 ± 63	99 ± 14	653 ± 77	105 ± 16	40 ± 5.7	145 ± 22
0.5	457 ± 51	74 ± 10	531 ± 61	87 ± 13	33 ± 4.9	120 ± 18
1.0	341 ± 39	43 ± 6.2	384 ± 45	70 ± 11	26 ± 4.9	96 ± 16
3.0	179 ± 22	12 ± 2.3	191 ± 24	28 ± 5.2	8.2 ± 1.1	36.2 ± 6.3
5.0	103 ± 12	7.4 ± 1.1	110.4 ± 13	15 ± 2.4	3.4 ± 1.0	18.4 ± 3.4
15.0	33 ± 4.7	3.3 ± 0.6	36.3 ± 5.3	9.8 ± 1.6	1.8 ± 0.4	11.6 ± 2.0
20.0	19 ± 2.2	2.4 ± 0.5	21.4 ± 2.7	4.8 ± 1.4	1.1 ± 0.3	5.9 ± 1.7
25.0	6.1 ± 1.4	0	6.1 ± 1.4	3.2 ± 0.7	0	3.2 ± 0.7
50.0	0	0	0	0	0	0

[a] Values are mean ± SD (n = 3). Cy-3-gal = cyanidin-3-galactoside; Cy-3-glu = cyanidin-3-glucoside.

confirmation of phenolic identities as reported(*21*). Therefore, although daidzein and eriodictyol coeluted in their HPLC-UV chromatogram, they yielded different molecular ions in their LC-MS analyses (data not shown). A combination of HPLC-UV and LC-MS analyses using a reference standard of cyanidin-3-glucoside enabled us to differentiate between the galactoside and glucoside forms of cyanidin (data not shown). Our finding that cyanidin-3-galactoside was the major anthocyanin in pistachio skins is in agreement with the previous report (*11*).

The presence of phenolics in pistachio skins is not unusual. Other tree nuts and peanuts have been reported to contain a diverse range of phenolics, including rhamnetin, quercetin, and kaempferol aglycones (*10*−*14*). However, this is the first report of rutin, eriodictyol, quercetin, luteolin, naringenin, and apigenin in pistachios. Furthermore, to the best of our knowledge, pistachios are the only tree nut that contains anthocyanins (*11*), pigments that are responsible for the attractive colors of berries and many other fruits and vegetables.

PSP Levels in Raw and Roasted Nuts Exposed to Bleach. The visual effects of bleach on pistachio shells and nuts are shown in **Figure 3, panels A and B**, respectively. Because pistachios are popularly consumed in both raw and roasted forms, we investigated the effects of bleaching on phenolic levels in both raw and roasted nuts. The levels of all of the phenolics decreased on bleaching and roasting, but the anthocyanins were the most sensitive chemical markers to these processes. This may be explained by the flavylium cationic structure of anthocyanins, which causes these compounds to be more sensitive to pH and temperature changes (*22*) compared to other classes of flavonoids. Total anthocyanin levels for raw and roasted nuts exposed to bleach are shown in **Table 2**. Anthocyanin levels in skins decreased from 905 to 549 μg/g of skins when raw nuts were roasted. Similarly, bleaching starting at levels of 0.1% hydrogen peroxide solution decreased anthocyanin levels from 905 to 653 μg/g for raw nuts and from 549 to 145 μg/g for roasted nuts.

Antioxidative Capacity of Raw and Roasted Pistachio Skin Extracts exposed to Bleach. The oxygen radical absorbing capacity of raw and roasted nuts exposed to different levels of bleach are summarized in **Table 3**. When raw nuts were roasted, their oxygen radical absorbing capacity decreased from 945 to 725 μM Trolox equivalents/g. In addition, raw nuts preserved the antioxidant capacity of the skins better than the roasted nuts when exposed to bleach. For example, on exposure to 0.5% bleach, the antioxidative capacity of skins from raw nuts decreased from 945 to 932 μM Trolox equivalents/g, whereas skins from roasted nuts decreased from 725 to 463 μM Trolox equivalents/g. This suggested that other substances or matrix effects in the raw nuts preserved the antioxidant capacity of the skins and that the roasting process reduced these effects. Therefore, our examination of the qualitative effects of bleaching

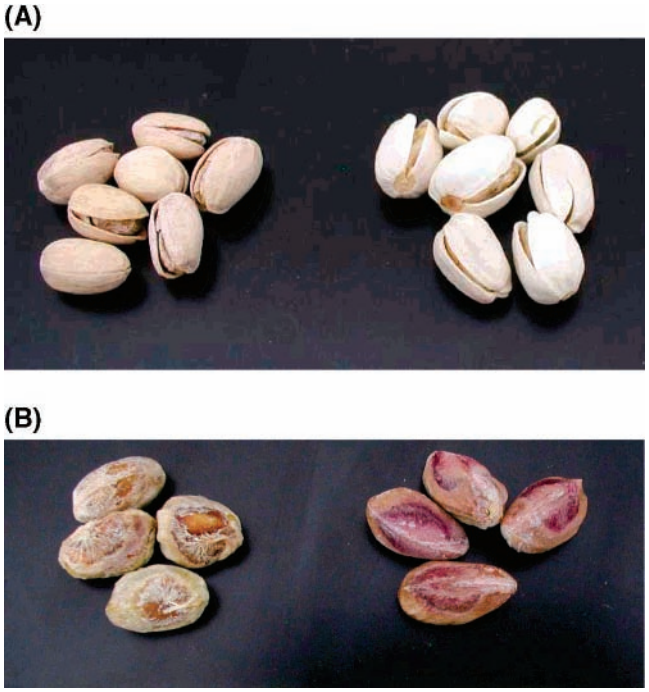

Figure 3. Pictures of (A) raw (left) and bleached (right) "in shell" pistachios and (B) raw (right) and bleached (left) pistachio nuts.

Table 3. Antioxidative Capacities of Raw and Roasted Pistachio Skins Exposed to Different Levels of Bleaching Agent (Hydrogen Peroxide) Determined by the Trolox Equivalent Antioxidant Capacity (TEAC) Assay[a]

	raw	roasted
hydrogen peroxide (%)	TEAC (μM/g of Trolox equivalents)	TEAC (μM/g of Trolox equivalents)
0	945.9 ± 64.7	725.4 ± 9.9
0.1	940.3 ± 61.3	472.8 ± 9.1
0.5	932.4 ± 22.5	463.9 ± 15.5
1.0	934.4 ± 19.3	465.6 ± 5.2
3.0	952.5 ± 34.6	466.0 ± 26.9
5.0	930.9 ± 41.1	455.7 ± 36.1
15.0	453.1 ± 27.2	438.8 ± 15.9
20.0	437.5 ± 14.4	377.9 ± 27.1
25.0	433.8 ± 11.6	370.3 ± 23.3
50.0	189.5 ± 3.9	173.8 ± 16.6

[a] Values represent the mean ± SD (n = 6).

on raw and roasted nuts showed that bleaching of roasted nuts caused a greater decrease in antioxidative capacities of pistachio skins than bleaching of raw nuts.

It can be surmised that the skin plays an important role in protecting the seed from becoming rancid via oxidation after harvest. Bleaching reduced the levels of naturally protective substances in the skins. In addition, the identities and levels of bleaching agents used in this illegal process is currently unknown. Studies on whether residual effects of the bleaching agents in the nuts are deleterious to human health is warranted.

The health value of the pistachio is derived not only from its content of monounsaturated fatty acids and satiety effects present in other tree nuts but also its unique content of lutein, and the anthocyanins, flavonols, and other phenolics that contribute to its total content of antioxidants that protect the nut fatty acids from oxidation. The relative importance of polyphenols found in the skins compared to the effects of the lipids in pistachios on health remain to be determined.

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