

Ex situ volatile survey of ground almond and pistachio hulls for emission of spiroketals: Analysis of hull fatty acid composition, water content, and water activity

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ABSTRACT

The spiroketal conophthorin has recently been implicated as an important semiochemical of the navel orangeworm moth (*Amyelois transitella*), a major insect pest to California tree nuts. Additionally, new evidence demonstrates that fungal spores in the presence of linoleic acid produce conophthorin. Numerous investigations have analyzed the volatile emissions of almonds and pistachios under varying conditions, yet there are few reports of conophthorin as a volatile component. Previous studies by our laboratories have suggested almond hulls may be a source of conophthorin production. Accordingly, the volatile emissions of ex situ almond and pistachio ground hulls were surveyed at several developmental stages. Each ground sample was analyzed at various intervals to determine if conophthorin was produced. The almond and pistachio samples were presumed to have a natural fungal bouquet present. Additionally, the fatty acid composition, water content, and water activity of the hulls were analyzed for each sample. Conophthorin and the structurally similar compound chalcogran were detected from almond hulls and shells, but not from the pistachio samples. The almond and pistachio hulls were investigated for four fatty acid components – palmitic, oleic, linoleic, and linolenic. The fatty acid composition of almond hulls varied greatly throughout the growing season, whereas the composition of pistachio hulls remained relatively constant. Both water content and activity were constant in early stages of almond growth then dropped in the later stages of hull split. Spiroketal emission along with other associated volatiles is discussed. This is the first report of the fatty acid composition, water content, and water activity of developing almond and pistachio hulls.

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1. Introduction

The structurally simple spiroketal conophthorin (7-methyl-1,6-dioxaspiro[4.5]decane, **1** in Fig. 1) has recently been reported as an active component in a blend of host plant volatiles that attract both male and female navel orangeworm (*Amyelois transitella*) moths (Beck et al., 2012a). *A. transitella* is a major insect pest that inflicts significant economic loss to California almonds, pistachios, and walnuts (Campbell et al., 2003). Moreover, *A. transitella* larvae are purported to vector toxigenic aspergilli to almonds (Palumbo et al., 2008). Yet, conophthorin and the isomeric chalcogran (2-ethyl-1,6-dioxaspiro[4.4]nonane, **2** in Fig. 1) have long histories as semiochemicals of scolytid beetles with insect and plant origins (Francke and Kitching, 2001). Compared to the history of these

spiroketals and scolytid beetles, reports of conophthorin (**1**) and chalcogran (**2**) from almonds are relatively new. As an example, recent investigations reported compound **1** from almonds at hull split (Beck et al., 2012a) and both **1** and **2** from mechanically damaged almonds (Beck et al., 2008). More recent was a report of **1** and **2** from various fungal spores on fatty acids common to almond and pistachio (Beck et al., 2012b).

What was interesting regarding the recent detection of **1** and **2** from almonds (all components present – hull, shell, and kernel) was their lack of detection in a number of investigations that reported on the volatile emissions of almonds or pistachios under varying conditions. For instance, spiroketal **1** or **2** was not detected from the following: the vacuum steam volatiles of almond hulls (Buttery et al., 1980); in situ intact and undamaged almonds (Beck et al., 2009); ambient almond orchard volatiles (Beck et al., 2011a); intact ex situ *Pistacia* spp. (Roitman et al., 2011); and, almond kernels naturally contaminated with orchard fungi (Beck et al., 2011b).

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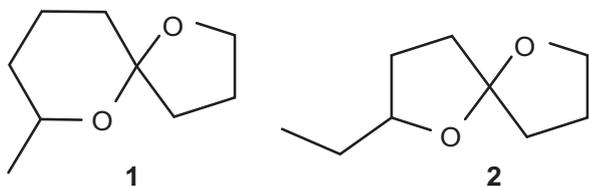


Fig. 1. Conophthorin (7-methyl-1,6-dioxaspiro[4.5]decane) (1) and chalcogran (2-ethyl-1,6-dioxaspiro[4.4]nonane) (2).

The present investigation was initiated to help delineate the true origin of conophthorin from almonds, and to determine if pistachios were a possible source. This is important for a number of reasons: the inconsistent detection of spiroketals in the aforementioned studies; the rich history of spiroketals and scolytid beetles; the recent assessment that conophthorin (1) is a semiochemical of *A. transitella*; and, the recent study that demonstrated spores produce both spiroketals (Beck et al., 2012b). Despite the mounting evidence, the question remains – is conophthorin produced by fungi, the host plant, or both? The fungal spore study demonstrated that spores were capable of producing both conophthorin (1) and chalcogran (2), but did not decisively eliminate the plant as an active participant – not just a carbon source for the spores.

Anecdotal evidence from previous volatile emission studies and preliminary exploratory experiments suggested we turn our attention to the hulls of almonds and pistachios as a possible source of compounds 1 and/or 2. Thus, the objectives of this study were to: (1) monitor the volatiles emitted from ground almond and pistachio components, primarily the hulls, to determine a condition for consistent spiroketal production; (2) determine the corresponding fatty acid profiles of the hulls at progressive stages of hull development; and, (3) determine the water content and water activity of the hulls at the varying stages of hull development.

2. Results and discussion

The objectives of determining the relative fatty acid composition, water content, and water activity of the hulls were successfully met. Furthermore, both spiroketals were observed from ground almond hulls and shells at varying times and amounts (Table 1), yet no spiroketals were observed from the ground almond kernel treatments or any of the pistachio material. The objective of determining specific conditions for consistent spiroketal production was more elusive; however, based on our results various plausible mechanisms/conditions for spiroketal genesis can be considered.

The relative percentage of the four major fatty acids in the almond and pistachio hulls was evaluated at regular intervals throughout the growing season. The fatty acid data for almond hulls (Fig. 2) were surprising when compared to the fatty acid content of developing almond kernels (Soler et al., 1988). Several differences in fatty acid composition were noted. First, for hulls the major component was palmitic, whereas palmitic in kernels is on average the second lowest. In hulls, palmitic acid started at ca. 30% and steadily increased to ca. 46% of the fatty acid composition. This is in contrast to kernels where palmitic acid starts at 19% and decreases to ca. 7%. Second, oleic acid, normally the predominant fatty acid on average in kernels, starts as the lowest fatty acid in hulls at ca. 13% gradually increases to ca. 28% by August then decreases to 16% by mid-September. Third, linoleic acid, the second most predominant fatty acid in kernels, starts as the major component in hulls, decreases at the start of hull split, then increases slightly to about 20%. Finally, linolenic acid, which is a minor to trace component in kernel development shows as a

consistent composition in hulls, starting off at ca. 25% and gradually decreasing to ca. 20% by mid-September.

The fatty acid composition in pistachio hulls also provided a distinct difference when compared to the fatty acid composition in the developing pistachio kernel (Chahed et al., 2006). Pistachio kernel fatty acid composition is similar to that of almond kernels. For example, oleic and linoleic also change relative percent compositions during the early stage of development to end with oleic acid as the highest (ca. 70%) and linoleic approximately the next highest at ca. 12%. Also in pistachio kernels, linolenic starts as a minor component and ends as a trace fatty acid, and palmitic gradually decreases from ca. 20% to ca. 12% of the composition (derived from Chahed et al., 2006 and Soler et al., 1988). The fatty acid composition of developing pistachio hulls (Fig. 3) was consistent in its relative percentages. Linoleic was the predominant fatty acid and showed a gradual decrease from ca. 45% in June to 34% in September. Palmitic was the second highest fatty acid with a slight increase from ca. 27% to ca. 30%. Oleic and linolenic were approximately equal; with oleic slightly favored over linolenic, both starting at ca. 15% and increasing to ca. 18%. Unlike almond hulls and kernels, or pistachio kernels, the pistachio hull fatty acid compositions did not undergo any crossing of relative percentages.

The water content and water activity (a_w) of the developing almond and pistachio hulls (Figs. 4 and 5) were measured to provide an overview of the growth environment for ubiquitous fungi present on the intact nuts. For both matrices there was a high Pearson product-moment correlation coefficient value between water content and a_w . For almond the Pearson's $r = 0.94$ and for pistachio $r = 0.96$. Water content is an important factor for water activity, a vital parameter for fungal growth (Ayerst, 1969). Non-xerophilic fungi prefer a_w values above 0.85 for optimal growth (Hocking, 2001). Interestingly, a surprising number of aspergilli common to tree nut orchards (Bayman et al., 2002) are considered xerophilic and thus can grow at relatively low a_w values (Hocking, 2001). For example, *Aspergillus niger* needs a minimum $a_w = 0.77$ for growth, and *A. flavus* and *A. parasiticus* need $a_w = 0.80$ for growth. Results from the fungal spores on fatty acids study (Beck et al., 2012b) showed spiroketal emission may be from the spores and not necessarily fungal growth; thus, water and fatty acid conditions in the hulls should be optimal in order for spores to undergo transition from resting to germination.

In almond hulls the a_w values remained fairly high (mean $a_w = 0.992$) for the first six samples, which is fairly surprising given the amount of hull split seen in the pictures in Table 1. These values would fully support fungal growth for the vast majority of microbes (Ayerst, 1969; Hocking, 2001). The sample labeled 8/31 ($a_w = 0.799$) showed a significant ($P < 0.0001$; all pairwise comparisons are one-way ANOVA followed by Tukey–Kramer HSD) drop in water activity when compared to the 8/17 sample ($a_w = 0.987$). The 8/31 sample $a_w = 0.799$ is a value likely to support fungal activity of the noted xerophilic aspergilli. The final sample, 9/14 ($a_w = 0.454$), showed another significant drop ($P < 0.0001$) from the 8/31 sample ($a_w = 0.799$). The $a_w = 0.454$ value is low enough that xerophilic fungi common to California tree nut orchards will not develop.

Like almonds, pistachio hull a_w values for the time period between 6/6 and 10/6 also remained high (mean $a_w = 0.989$), but for a longer period. The last pistachio sample analyzed on 10/21 ($a_w = 0.840$) showed a significant drop ($P < 0.0001$) from the previous 10/6 ($a_w = 0.989$). Unfortunately, the a_w values beyond the 10/21 sampling were not performed, but as of that date the a_w value was high enough to accommodate fungal growth of the noted xerophilic fungi.

Analysis of the volatile emission data from the ground almond and pistachio hulls was primarily focused on the presence or

Table 1
Relative amounts^a of volatiles^b of interest from ground almond hulls. Almonds were collected during the 2011 growing season.

Date Days after ground	 Hull/shell			 Hull				 Hull			 Hull – start of hull split				 Hull – hull split				 Hull – hull split			 Hull – hull split			 Hull – wind row				
	6/9			6/23				7/7			7/26				8/4				8/17			8/31			9/13				
	0	4	7	0	4	5	7	0	1	6	0	2	5	7	0	4	6	7	0	1	5	0	2	6	0	2	6	0	2
1-hexanol ^c	+++	+++	++	++	++	+	++	++	+	++	++	++	++	+	++	+	++	+	+	tr	tr	+	+	tr	tr	tr	tr	tr	tr
Hexanal ^c	+			+	+	+		+						+				+		tr	+	+	tr	+	+	+	+	+	
(E)-2-hexenal ^c	++	++		++	++	+		+++	+			++	tr				++		+++	tr		+	tr		tr	tr	tr	tr	
(E)-2-hexen-1-ol ^e	+++	++		++	++			++	++			++	+	tr			++	tr	+										
(Z)-3-hexen-1-ol ^e	++	+	+		+	+			+	tr		+	+	+	+	+	+	+		tr	tr								
(E)-3-hexen-1-ol ^c	++	+	+		+	+	+	+	+			tr	+	+	+	+	tr	+				-							
Hexyl acetate ^c	+++	+	+	+	+	+		+	++			++	++	+			+	+	+	tr	+								
(Z)-3-hexenyl acetate ^e	++	+						+	+			+	+				+	tr											
(E)-2-hexenyl acetate ^e	+++	+		++	+			+	++			++					++				tr								
(E)-2-hexenyl butyrate ^e	++																												
Conophthorin ^f		+	++		+	+				+			tr	tr	tr					tr	tr	+	+	+	+	+			
Chalcogran #1 ^f		tr	+		tr	tr	tr		tr	+	tr	tr	+	+			tr	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr	
Chalcogran #2 ^f		tr	+		tr	tr	tr		tr	+	tr	tr	+	+			tr	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr	
2-pentyl furan ^d	+	++	+		+	+			tr			tr	tr	tr			tr	tr		+	+	+	+	+	+	+	+	+	
Nonanal ^c																			tr			+	+	+	++	+	+	+	
(E)-2-nonenal ^e		++			+	+																tr	+	tr	tr	tr	tr	+	
(E,Z)-2,6-nonadienal ^c		++			+	+																							

^a Relative amounts defined as the average of three GCMS relative abundances: tr=10,000–100,000; +=100,000–1,000,000; ++=1,000,000–10,000,000; and, +++=>10,000,000.

^b All volatiles of interest were verified by comparison of retention times and fragmentation patterns to authentic standards.

^c Aldrich for standard.

^d Alfa-Aesar for standard.

^e Bedoukian for standard.

^f Contech for standard.

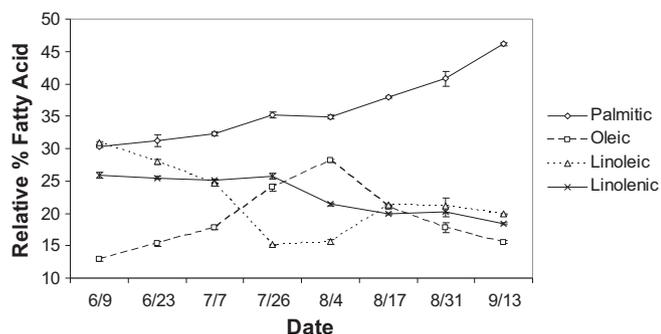


Fig. 2. The relative percentages of the four main fatty acids from almond hulls collected over the 2011 California growing season. Error bars are s.e.m. of triplicates.

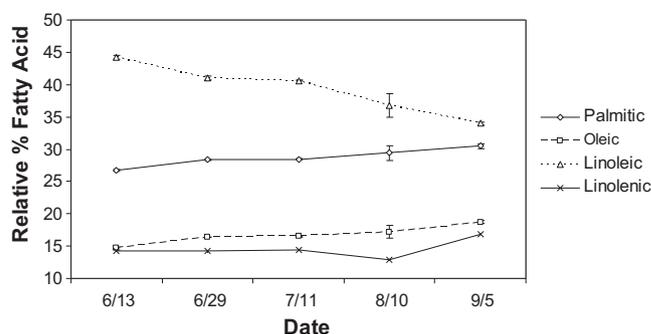


Fig. 3. The relative percentages of the four main fatty acids from pistachio hulls collected over the 2011 California growing season. Error bars are s.e.m. of triplicates.

absence of spiroketals. However, in light of the recent report of spiroketals from fungal spores (Beck et al., 2012b) and the postulated biosynthetic correlations between the detected green leaf volatiles and spiroketals, it was decided to broaden our analysis to include relevant C6 compounds, the spiroketals, 2-pentyl furan, and other C9 compounds (Table 1). The C6 compounds emitted by the spores (Beck et al., 2012b) showed high Pearson's correlation to the spiroketals in some of the fungal strains, thus their inclusion to Table 1 for consideration of trends. Moreover, because the spiroketals contain nine carbons and the proposed ketodiol intermediate is an acyclic C9 compound (Beck et al., 2012b) the choice was made to consider the detected C9 compounds seen in Table 1.

Table 1 provides the survey of volatile emissions from the ground almond hulls at various time points over seven days of volatile monitoring. In terms of spiroketal emission, it was interesting to note that no spiroketals were detected in day 0 while the almonds were still intact (not yet undergone hull split – see pictures in Table 1 corresponding to developmental stage of each intact almond sample, before grinding), but rather were detected at subsequent days of analysis. If the spiroketals are emitted from fungal spores then the spores would require time on the ground internal host material of the hull in order to transform from resting to germination. This time would be dependent upon water activity, temperature, and the individual fungus, thus a delay in spiroketal formation would be expected (Ayerst, 1969). Interestingly, the spiroketals are detected on day 0 of the 7/26 sample, but resume their delay in production in the 8/4 sample. The a_w values of these two sample days are essentially equal; however, there is a drop in linoleic acid between the 7/26 sample and the previous 7/7 sample. Formation of the spiroketals in the

8/17 and 8/31 samples resumes on day 0, with a modest increase in conophthorin production. At this juncture, no explanation is offered for this phenomenon since there is no dramatic change in fatty acid content of the hulls and the a_w value remains within xerophilic growth range. Detection of both spiroketals suddenly drops in the 9/13 sample – perhaps due to the a_w value dropping below that of the noted xerophilic fungi.

The evaluation of volatile emissions from almond shell largely mirrored the results for spiroketal production in Table 1. Contrary to the hull emissions however, was a large drop in emission of C6 and C9 compounds, with only minimal amounts of C6 production throughout the evaluation period. The emissions of the shell did include detection of both conophthorin (1) and chalcogran (2), thus demonstrating that almond shells are also capable of producing these spiroketals, and not just hulls. Unfortunately, the fatty acid content of the shells was not evaluated due to low amounts of material and no reports were found for reference. Almond shells have been reported to contain xylan and phenolics among other components (Esfahlan et al., 2010). A more thorough study of almond shell chemical composition would be required for speculation of the carbon source used for spiroketal production.

A SPME headspace analysis of the containers with the almonds and pistachios was performed prior to their removal and grinding. Table 2 shows the volatile content of the pre-ground headspace for the compounds of interest. The spiroketals were not detected until after hull split was fully in progress. This result is very important when one considers that the almond becomes vulnerable for *A. transitella* infestation at hull split (Gradziel and Martinez-Gomez, 2002) and that conophthorin, in addition to other pertinent volatiles, could be signaling this vulnerability to *A. transitella* (Beck, 2012; Beck et al., 2012a). Also, important to note is the lack of the

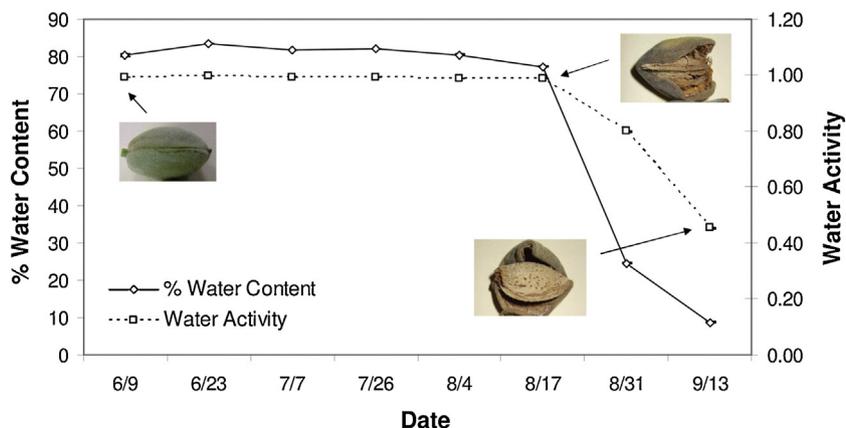


Fig. 4. The water content (left y-axis) and water activity (right y-axis) of almond hulls collected over the 2011 California growing season. Error bars are s.e.m. of triplicates.

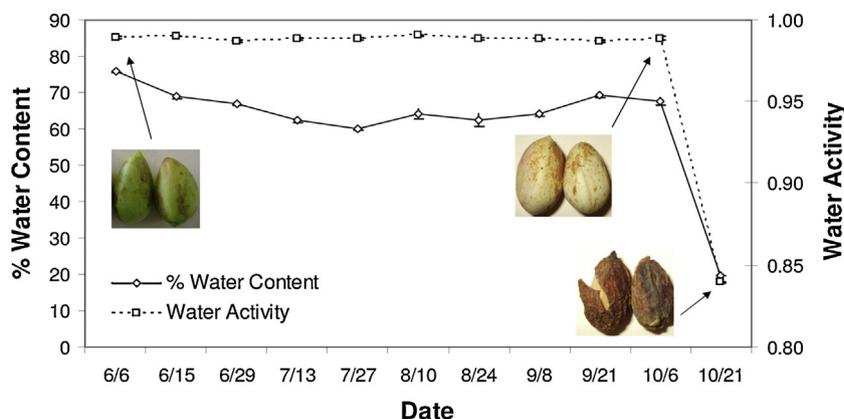


Fig. 5. The water content (left y-axis) and water activity (right y-axis) of pistachio hulls collected over the 2011 California growing season. Error bars are s.e.m. of triplicates.

C6 volatiles in the intact samples (Table 2), but relatively larger production of these volatiles after grinding (Table 1), which assumed to be due to massive tissue injury.

If the carbons at positions 3' or 4' of pentyl furan were to be oxidized in vitro to the corresponding alkene, one can rationalize formation of the spiroketals from hydration of the alkene at either the 3' or 4' carbon followed by subsequent cyclization. However, the presence of pentyl furan on day 0 of all but two of the samples, and its detection in all days of the 9/13 (below xerophilic a_w activity) sample strongly indicate it is plant produced and not fungal produced. Along these same lines, it could be inferred that the C9 compounds noted in Table 1 are also plant produced and not strictly a function of spore production. It should be noted that the C9 compounds were also detected in the ambient almond orchard (Beck et al., 2011a) and the contaminated kernel (Beck et al., 2011b) studies and are most likely primarily a product of fatty acid oxidation.

No tabular data are provided for the pistachio emissions for either intact or ground samples since no spiroketals or other compounds of interest were detected from any of the experiments. The monoterpene composition of the volatiles detected corroborates a recent report for California pistachios (Roitman et al., 2011). The top four monoterpenes consistently detected from the samples were (averages of day 0 samples) limonene (>60%), α -terpinolene (>10%), Δ^3 -carene (>2%), and α -pinene (>1%). It is hypothesized that the large amount of monoterpenes seen in pistachios could be inhibiting spore germination, thus not allowing spiroketal formation. Essential oils containing large percentages of limonene have shown antifungal bioactivity against *Aspergillus* spp. and *Penicillium notatum* (Magwa et al., 2006).

The 9/13 sample proved to be important in demonstrating the relationship between spiroketal production and the presence of moisture. As noted earlier, the a_w value for the hulls for the 9/13

sample was 0.454 and no spiroketals were noted in the ground hulls, ground shells, or pre-ground headspace analyses. Headspace analysis of the ground samples continued for several days and still no spiroketals were detected even after 13 days of monitoring. It was then decided to alter the moisture levels to determine if a change in matrix environment would effect volatile emission. The relative humidity in the container was increased by placing a beaker containing a saturated salt solution (no water in contact with the ground material) into the sample jar. The headspace was monitored for several more days. On day 14 post moisture addition the newly hydrated hulls produced a detectable amount of conophthorin, and the shells produced detectable amounts of both conophthorin and chalcogran. Though the water content and water activity of the newly hydrated material were not determined, it was assumed the sample matrix obtained the necessary moisture conditions for spore development.

The fatty acid composition, water content, and water activity of developing almond and pistachio hulls were determined, and the headspace of the ground material surveyed for production of spiroketals. Both conophthorin (1) and chalcogran (2) were detected from the ground almond hull and shell samples at varying times and treatment conditions. No spiroketals were detected from almond kernels or any of the pistachio samples. The results of this study are important for several reasons. Most importantly, the results demonstrate that ground almond hull and shells, but not kernels produced spiroketals. Second, despite a similar fatty acid composition, pistachio hulls did not produce any detectable amounts of spiroketal under the tested conditions. Third, the apparent relationship between hull water activity and spiroketal production suggests that the spores are the source of compounds 1 and 2. Lastly, the data indicate that some form of tissue damage was required for spiroketal formation. This was demonstrated by the data in Table 1 showing the relatively consistent emission of the spiroketals. Moreover, Table 2 data showed that the spiroketals are not formed until hull split, which is a form of natural damage to the almond tissue.

Table 2

Relative amounts^a of volatiles of interest from whole, intact almonds collected and analyzed in 2011.

	Intact almonds							
	6/9	6/23	7/7	7/16	8/4	8/17	8/31	9/13
Hexanal								+
Conophthorin					tr	+	+	
Chalcogran #1						tr	+	
Chalcogran #2						tr	+	
2-Pentyl furan		tr	tr	tr	tr	+	+	tr
Nonanal		+	+	+				+

^a Relative amounts defined as the average of three GCMS relative abundances: tr = 10,000–100,000; + = 100,000–1,000,000; ++ = 1,000,000–10,000,000; and +++ = >10,000,000.

3. Experimental

3.1. Almond and pistachio collections

In the 2011 growing season, 10 undamaged Nonpareil almonds from three different trees were removed and placed in a 1-quart wide-mouth Mason jar (Ace Hardware, El Cerrito, CA) with a modified lid containing a 1 cm hole and lined with Teflon. Jars were shipped overnight to the laboratory. For pistachios, ca. 40 undamaged Kerman pistachios were removed from three different trees and placed in similar jars. Both almonds and pistachios were

collected from commercial orchards located in the southern Central Valley of California (Kern County).

3.2. Almond and pistachio water and volatile analyses

Upon arrival to the laboratory the headspace volatiles of the intact *ex situ* almonds or pistachios were adsorbed onto 100 μm solid-phase microextraction (SPME), polydimethylsiloxane fibers (Supelco, Bellefonte, PA). For the initial headspace volatile analysis of almonds fibers were exposed for 1–2 min. For pistachios the exposure time was decreased to about 0.5 min due to abundance of volatiles. For all almond and pistachio samples the nuts were removed, sliced in half, and kernels (including seed coat) removed from the hulls/shells. Almond shells could not be separated from the hulls for the collection on 6/9; for all other almond samples the shells were separated from hulls. For pistachios, the hulls did not dehisce from the shells until September; therefore, hulls and shells for all pistachio collection times were combined. All tissues were ground in a 250 ml blender cup (Waring MC3, Torrington, CT). The water content was measured with a moisture analyzer (Mettler HB43-S, Columbus, OH), and water activity measured with a water activity meter (AquaLab 4TE, Pullman, WA). All water analyses were measured in triplicate 2.3 g portions. For volatiles, 15 g portions of the remaining hull or hull/shell tissue were weighed in triplicate and transferred to 125 ml Mason jars with lids modified for volatile sampling.

3.3. Volatile analysis

All adsorbed volatiles were desorbed onto either a DB-1 column (60 m \times 0.32 mm i.d. \times 0.25 μm) or a DB-Wax column (60 m \times 0.32 mm i.d. \times 0.25 μm) (J&W Scientific, Folsom, CA) installed on a 6890 gas chromatographs (GC) coupled to HP-5973 mass selective detectors (MS; Palo Alto, CA). Desorbed volatiles were analyzed with the following methods. For DB-1, injector temperature, 200 °C; splitless mode; inlet temperature, 200 °C; constant flow, 2.0 ml min⁻¹; oven settings, initial temperature, 40 °C; hold time, 0.0 min; ramp 1, 4 °C min⁻¹ to 180 °C; hold time 0.0 min; final temperature, 250 °C; hold time, 3 min. MSD parameters: source temperature, 230 °C; MS source temperature, 150 °C; EI mode, 70 eV; solvent delay, 1 min. For DB-Wax the same GC–MS parameters were followed as previously published (Beck et al., 2012b) Volatiles were included in Table 1 if detected in at least two of the three replicates. Values reported in Table 1 are average of the GC–MS relative areas.

3.4. Almond and pistachio fatty acid analyses

The fatty acid composition was analyzed by extraction and conversion of the triglycerides to fatty acid methyl esters and subsequent detection by GC–FID using a published method (Sathe et al., 2008), but with the following modifications and performed in triplicate: to ground almond or pistachio material (1 g) was added MeOH (5.3 mL) and 10 molar KOH (0.7 ml). Capped sample container was placed in a 55 °C water bath for 1.5 h with occasional shaking after which containers cooled in an ice bath, 12 molar H₂SO₄ (0.6 ml) added, and the container returned to the 55 °C water bath for an additional 1.0 h. The sample was removed, cooled in an ice bath, hexanes (3 ml) added, and the samples centrifuged. The hexane layers were removed, dried and neutralized with K₂CO₃, centrifuged once more, and the hexanes transferred to a GC vial.

The fatty acid methyl esters were analyzed on a DB-Wax column (60 m \times 0.32 mm i.d. \times 0.25 μm) (J&W Scientific, Folsom, CA) installed on a Shimadzu GC-2010 Plus, FID (Pleasanton, CA). Fatty acid methyl ester retention times were verified with purchased standards (VWR International, Wayne, PA).

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