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Silo-Stored Pistachios at Varying Humidity Levels Produce Distinct Volatile Biomarkers

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Supporting Information

ABSTRACT: Fungal-contaminated tissues are known to produce volatile profiles that are different from uncontaminated tissues. Fungi require certain water activity levels before growth can occur. For nonxerophilic fungi, a water activity of 0.85 is typical for growth, and for extreme xerophilic fungi, the water activity can be as low as 0.64. Recent investigations with stored pistachios (kernels in shell, no hull tissue) at varying relative humidities showed differences among the collected volatile profiles at the tested humidities (ambient, 63, 75, and 84%). Water activities of the kernel and shell were also measured. Results showed significant changes in volatile profiles as a function of water activity of the corresponding pistachio tissue with measured water activity levels at or below that of what is considered extreme xerophilic activities. Because fungal growth, including mycotoxigenic fungi, is dependent upon water activity, the detected volatile profiles could be used for early detection of fungal presence. Multivariate analysis of the volatile data demonstrated significant differences among the volatile profiles at the tested relative humidity levels, and several volatiles were identified as biomarkers of increased humidity and likely fungal development.

KEYWORDS: *detection, fungal spore, percent moisture, water activity, volatile production*

INTRODUCTION

Fungal contamination, particularly mycotoxigenic fungi, of agricultural products represents a major food safety issue[1] with a high associated cost of management.[2] Fungal-contaminated tissues are known to emit volatile profiles different than those of uncontaminated tissues,[3,4] a feature that allows for the detection of fungal-infected food.[3,5] Fungi require certain water activity levels before growth, germination, or transition from dormancy to activity can occur.[6,7] For nonxerophilic fungi, a water activity of about 0.85 is typical for growth or germination.[8,9] For extreme xerophilic fungi, the water activity can be as low as 0.64 to activate the spore from dormant to germ tube growth.[10]

Mycotoxigenic fungi common to tree orchards of California include *Aspergillus flavus* and *A. parasiticus*. Both fungi require a minimum water activity of 0.80 for growth,[8] and mycotoxin production requires higher water activities in almond-based media.[11] Closed-shell pistachio kernels have been shown to be susceptible to aflatoxin growth when contaminated with *A. flavus*.[12] Additionally, stored pistachios have been found to be contaminated with *A. flavus* and *A. parasiticus* with increased fungal counts in the presence of higher than normal moisture levels.[13]

We investigated the emission profiles of typical stored pistachios (kernels in shell, no hull tissue, and obtained from silos of a commercial processor) and placed under varying relative humidities. Additionally, the water activity of the individual kernels and shells was measured. The volatile profiles were monitored over a period of 12 days, and results showed significant changes in volatile profiles as a function of water activity of the corresponding pistachio tissue, with measured water activity levels at or below that of what is considered extreme xerophilic activities. Several volatiles were identified as biomarkers of increased humidity, including 1,8-cineole (**1**), methyl salicylate (**2**), acetoin (**3**), 2-methylpropanal (**4**), (*Z*)-ocimene (**5**), (*E*)-*β*-ocimene (**6**), 1-methylpyrrole (**7**), and *p*-cymene (**8**) (Figure 1, Table 1). This ability to resolve humidity levels suggests that volatile profiles could potentially be used in an early warning detection system. The objectives of

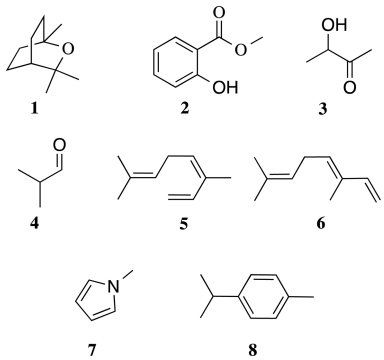

Figure 1. Biomarker volatiles identified in the headspace of pistachios at varying humidity levels: 1,8-cineole (**1**), methyl salicylate (**2**), acetoin (**3**), 2-methylpropanal (**4**), (*Z*)-ocimene (**5**), (*E*)-*β*-ocimene (**6**), 1-methylpyrrole (**7**), and *p*-cymene (**8**).

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Table 1. Compounds Identified through Multilevel Pattern Analysis as Driving Differences in Volatile Profiles Across Time and Humidity Treatments

		group					
compound	RT[a]	day	humidity	specificity[b]	sensitivity[c]	association index[d]	*P*-value[e]
4*E*-nonenal[f]	16.19	0	63	0.72	1.00	0.85	0.003
1,8-cineole	14.28	0	75	0.59	1.00	0.77	0.006
methyl salicylate	19.65	0	84	0.41	1.00	0.64	0.049
trimethyl furanone[f]	12.58	4, 8, 12	0	0.60	1.00	0.77	0.005
acetoin	4.71	4, 8, 12	63	0.92	1.00	0.96	0.001
unknown	8.24	4, 8, 12	63	1.00	0.89	0.94	0.001
unknown	10.26	4, 8, 12	63	0.83	1.00	0.91	0.001
2-methylpropanal	3.45	4, 8, 12	63	0.83	1.00	0.91	0.001
(*Z*)-ocimene	14.57	4, 8, 12	75, 84	1.00	1.00	1.00	0.001
(*E*)-*β*-ocimene	14.97	4, 8, 12	75, 84	1.00	0.94	0.97	0.001
1-methylpyrrole	5.31	4, 8, 12	75, 84	0.90	1.00	0.95	0.001
p-cymene	13.98	4, 8, 12	75, 84	0.68	1.00	0.83	0.001

[a]Retention times (RT) are in minutes. [b]Specificity is the conditional probability that a given sample belongs to a day-humidity treatment given the presence of this compound. [c]Sensitivity is the probability of finding the compound in the day-humidity treatment group. [d]The association index is an average of both specificity and sensitivity. [e]The *P*-value reflects the probability of this association occurring by chance as tested permutationally. [f]Tentatively identified.

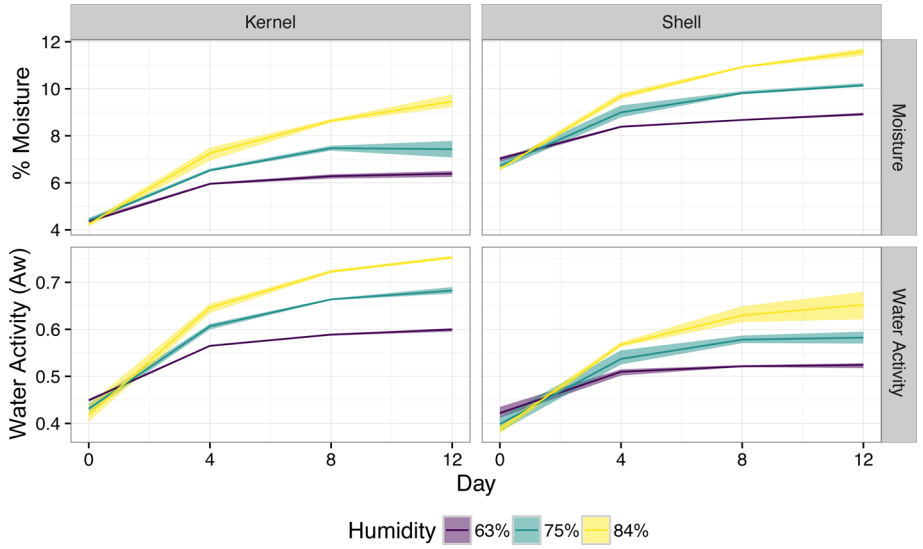

Figure 2. Percent moisture (top) and water activity (bottom) levels from pistachios over time at 63, 75, and 84% ambient relative humidity. Lines and shaded areas denote mean (n = 3) and bootstrapped 95% confidence intervals, respectively.

this research were to determine if the volatile profiles of the stored pistachios at varying relative humidities could be used to distinguish among the different humidity levels and if the profiles included distinctive biomarker signals.

MATERIALS AND METHODS

Pistachios. Pistachios, Kerman variety, were removed from a commercial silo located in Lost Hills, CA, during the month of August 2014 and shipped overnight in paper bags contained within cardboard boxes.

Water Activity and Percent Moisture Analyses. Intact and in-shell, silo pistachios were placed into groups of 19 nuts weighing approximately 24.5 g and placed in 237 mL (8 ounce) Mason jars. A total of 39 groups of pistachios were assembled: triplicates for each treatment (63, 75, and 84%) at each time point (50 min and 4, 8, and 12 d) and one triplicate for the time point 0 min. Relative humidity environments were achieved using 17 mL vials placed within the Mason jars, each containing 6 mL of saturated salt solutions of 63% ($NaNO_2$), 75% (NaCl), and 84% (KCl) at 30 °C.[14,15] For the time zero (d = 0), treatment no salt solutions were added, and pistachios were removed from the bag and separated into shell and kernel, and moisture levels were obtained immediately. All humidity treatments for moisture analyses were analyzed at times identical to the corresponding headspace analyses. After the incubation period at each relative humidity, shell and kernel tissues were again separated by hand and weighed. Each tissue was ground in a small blender cup and measured for water activity using an AquaLab 4TE and percent moisture using a Mettler HB43-S moisture balance.

Collection of Volatiles. In collection chambers identical to those used in the moisture analyses, in-shell silo pistachios weighing approximately 24.5 g were placed into 237 mL (8 ounce) Mason jars with a modified lid for insertion of solid phase microextraction (SPME) fibers. The collection times and intervals were identical to those in previously published protocols.[15] Briefly, after lids were sealed and volatiles were allowed to permeate the headspace for 30 min, PDMS-DVB SPME fibers (Supelco, Bellefonte, PA, United States) were inserted and exposed to the headspace volatiles for 20 min, thus providing the day zero, 50 min collection samples. All volatile collections were performed at 30 °C. After volatile collections, the lids were removed, and the headspace was vented for one min and then resealed for continued permeation. Headspace volatiles were again

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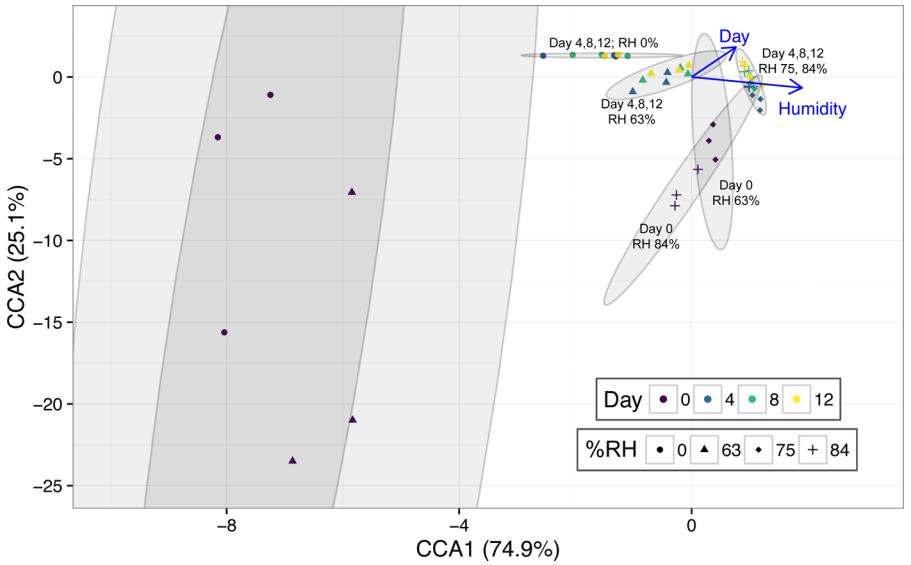

Figure 3. Constrained ordination of volatile profiles as explained by day and humidity (% RH). Points indicate projection of volatile profiles into ordination space, while shaded ellipses denote 95% confidence intervals.

adsorbed at exactly four-day (96 h) intervals (d = 4, 8, and 12), with venting performed for one min after each volatile collection.

Analysis of Volatiles. Volatiles adsorbed onto SPME fibers were thermally desorbed onto an Agilent 7890B GC coupled to a 5977A MSD (Palo Alto, CA) outfitted with an Agilent DB-1 column (60 m × 0.320 mm × 0.25 μm). For further confirmation, identification, and peak resolution, additional injections were occasionally performed on an Agilent 7890B GC coupled to a 5977A MSD and outfitted with an Agilent DB-Wax column (60 m × 0.320 mm × 0.25 μm). Desorbed volatiles were analyzed via protocols identical to those previously published.[15] RI values from both columns were used to assist with initial identification, and identities were further confirmed by comparison to retention times and fragmentation patterns of standards. Compound identities not verified on both instruments with a commercial or other available standard were marked as tentatively identified.

Statistical Analysis. Peak areas (relative abundances) from volatile analysis were used in all statistical comparisons. To investigate the influence of time and humidity on possible fungal volatile production, canonical correspondence analysis (CCA) was applied to volatile profiles collected at the four different humidities and days. CCA is a constrained ordination technique that facilitates visualization and exploration of multivariate differences in two dimensions. Following CCA, multilevel pattern analysis (also known as indicator species analysis) was used to identify biomarkers: those compounds unique to and driving differences between volatile profiles from different humidity and time groups. Finally, correlations between differences in volatile profiles and water activity levels in the kernel and shell were evaluated with a mantel test. All permutational testing was conducted with 10 000 permutations. Data were collated in Microsoft Excel and then analyzed using R version 3.3.1 in the RStudio version 0.99.902 development environment. The following packages facilitated analysis: *dplyr* and *tidyr* for data formatting,[16,17] *ggplot2* and *car* for graphics,[18] *vegan* and *indicspecies* for constrained ordination and indicator species analysis, respectively.

RESULTS AND DISCUSSION

The recommended moisture for stored (Iranian) pistachio kernels has been suggested as 2.5−4.1%.[14] The average initial kernel moisture, prior to addition of increased humidity, of the tested samples was 4.3322 (SEM ± 0.0356), just above the suggested moisture levels (Figure 2). The corresponding water activity of the kernels prior to added humidity was 0.4328 (SEM ± 0.0059) (Figure 2). The ideal storage conditions of Iranian kernels suggest a relative humidity range of 65−70%; thus, the tested relative humidity levels of ambient (denoted 0% in figures) and 63% fall below or at the suggested range, respectively, and the 75 and 84% tested levels provide humidity levels above the suggested range. Acceptable moisture levels for California pistachio processing are 55−65% relative humidity and <6% moisture (private communication to J. Beck). Moisture levels in the shell and kernel met or exceeded this limit after 4 days of treatment (Figure 2). Previous research[12] has shown that the kernel is a better host to aflatoxigenic fungi. Hence, our discussion will focus on the kernel moisture and water activity.

Constrained ordination of the detected volatile profiles revealed that the profiles changed over time and with increasing humidity (Figure 3). Both day (permutation test: pseudo-F = 4.4075; df = 1; P = 0.001) and humidity (permutation test: pseudo-F = 8.8839; df = 1; P = 0.001) significantly explained observed variation in volatile profiles. While there was some variation in volatile profiles in the 0 and 63% humidity treatments at day 0, these profiles nevertheless were distinct from profiles at higher humidities and at later times. The differences between volatile profiles from different humidity treatments at day 0 (volatiles collected 50 min after addition of humidity and start of experiment) suggest that introduction of humidity causes rapid changes in volatile emissions. Additionally, at and after 4 days, volatile profiles from treatments at higher humidities (75 and 85%) separate significantly (t = 33.678; df = 32; P < 0.0001) along ordination axis one from the lower humidities (ambient and 63%).

Volatiles detected over the course of the experiment fell into three distinct categories: constant emissions, low-humidity emissions, and emissions at >70% relative humidity. Volatiles with constant emissions exhibited little or no significant changes across humidity treatments and time (representative selection in Figure 4). These volatiles include 2-ethylfuran, γ-butyrolactone, α-thujene, α-pinene, sabinene, β-pinene, myrcene, Δ-4- and Δ-3-carene, γ-terpinene, p-cymenyl, terpinolene, and 2-(E)-decenal. For discussion purposes, these are being considered as common pistachio kernel volatiles with the vast

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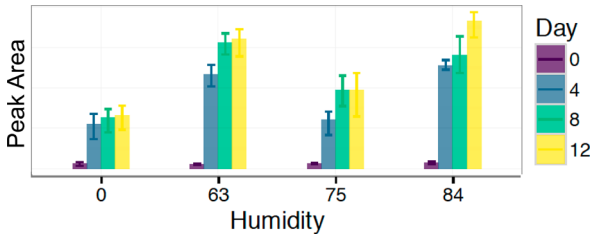

Figure 4. Emission profile of terpinolene, representative of the emission pattern of volatiles that remained constant (little or no significant changes over humidities and days) throughout the experiment.

majority of these compounds having been detected in the headspace of ex situ pistachios.[19]

Volatiles with low humidity emissions produced significantly larger amounts at the 63% relative humidity level. Figure 5

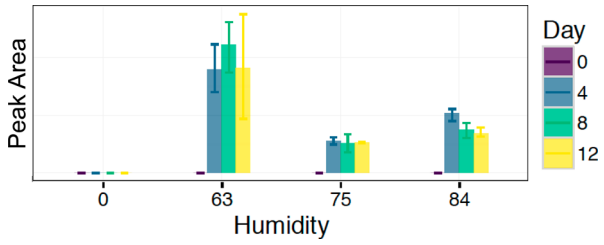

Figure 5. Emission profile of phenylacetaldehyde, representative of the emission pattern of volatiles significant at 63% relative humidity.

provides the emission pattern of phenylacetaldehyde as a representative of the compounds in the category, which includes **3**, 3-methyl-3-buten-1-ol, 3-methyl-1-butanol, furfural, styrene, tricyclene, camphene, benzyl alcohol, phenylacetaldehyde, 2-(*E*)-nonenal, *p*-cymene-8-ol, and bornyl acetate, among others, and a few unidentified compounds. An important aspect of this emission profile is that the individual volatiles are emitted in significantly higher amounts at a humidity level that is considered within an acceptable range for storage, less than 65% (tested at 63%).[14] Moreover, the average water activity for the kernel tissues at 63% relative humidity for days 4, 8, and 12 was 0.5845 (SEM ± 0.0052), a value below that of extreme xerophilic fungi[10] and well below that of xerophilic fungi.[9] This phenomenon is discussed further in proceeding sections but warrants further investigation as to the source of these volatiles on this specific tissue. Many of these compounds have reported emissions from fungi at unreported but presumed higher water activity levels.[20]

Volatiles with emissions greater than 70% relative humidity are compounds emitted in significantly larger amounts by the pistachio tissues at 75 and 84% (Figure 6). The relative humidity value of 70% has been cited as a level that should not be exceeded for both stored pistachios and almonds (private communication to J. Beck).[21,22] Compounds in this category include **1**, **5**, **6**, 7, and **8**. Emission profiles at relative humidity levels above 70% were not statistically different ($t = -1.584$; df = 32; $P = 0.12$) (Figure 3). Such similarities between emission profiles of 75 and 84% may be explained by evaluation of the water activity of the tissues of days 4, 8, and 12, which at the two higher humidity levels are all greater than 0.60. Additionally, the percent moisture of the kernel is greater than the optimal storage value of 6%.

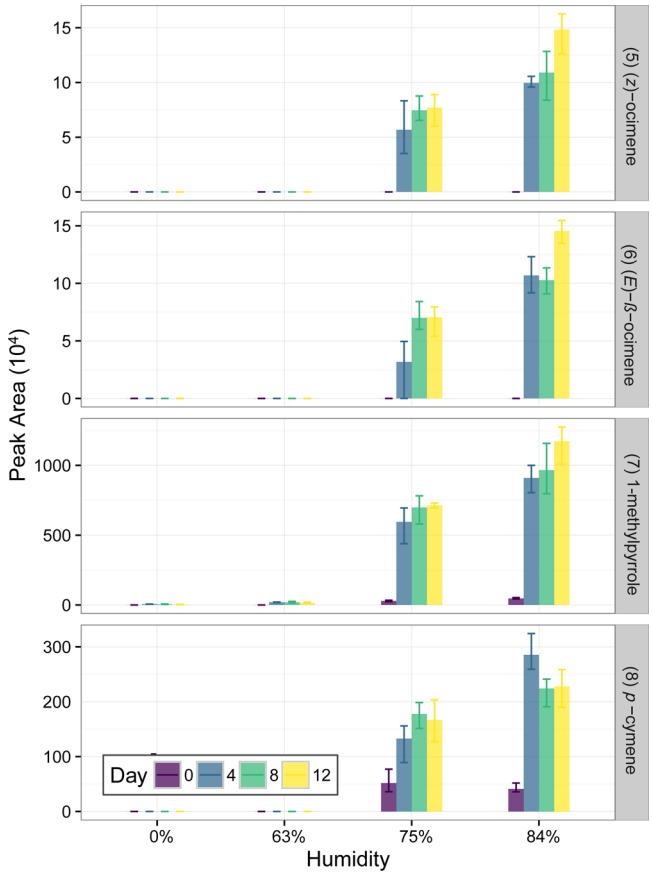

Figure 6. Emission profiles of **5**, **6**, 7, and **8**, representative of the emission pattern of volatiles significant at 75 and 84% relative humidities.

While these changes in volatile profiles could be due to enzymatic activity on the part of the pistachio, fungal-related volatile release could be an alternative explanation for observed differences in volatile profiles. This could be particularly relevant given that many of these changes in volatile profiles were observed at water activity levels below that typically thought to stimulate fungal growth. Indeed, many of the compounds identified through multilevel pattern analysis as driving differences in volatile profiles are known fungal volatiles. Overlaying these biomarkers in ordination space (results of analysis in Table 1, visualization in Figure 7) shows the contributions of given volatile compounds to their respective groups. Table 1 provides an indication of the compounds that drive the differences between groups. For instance, **5** in Table 1 shows a strong (values of 1.00) specificity and sensitivity for the group d = 4, 8, and 12 and high humidity levels of 75 and 84% and is thus a good biomarker for the later days of high humidity levels. The same could be said for the compounds **6**, 7, and **8**. Accordingly, compounds **3** and **4** along with the unknown compounds listed for humidity 63% are biomarkers for the later days of the pistachio tissue at 63% humidity.

Examining these compounds over time and humidity treatments reveals interesting developmental profiles (Figures 4−6). Biomarkers characteristic of high humidity treatments (**5**, **6**, 7, and **8**) are below detectable limits in the lower humidity (ambient and 64%) treatments. This stark contrast is suggestive of an activation threshold and reinforces ideas in the literature that 70% relative humidity is a cutoff for fungal growth.[21,22]

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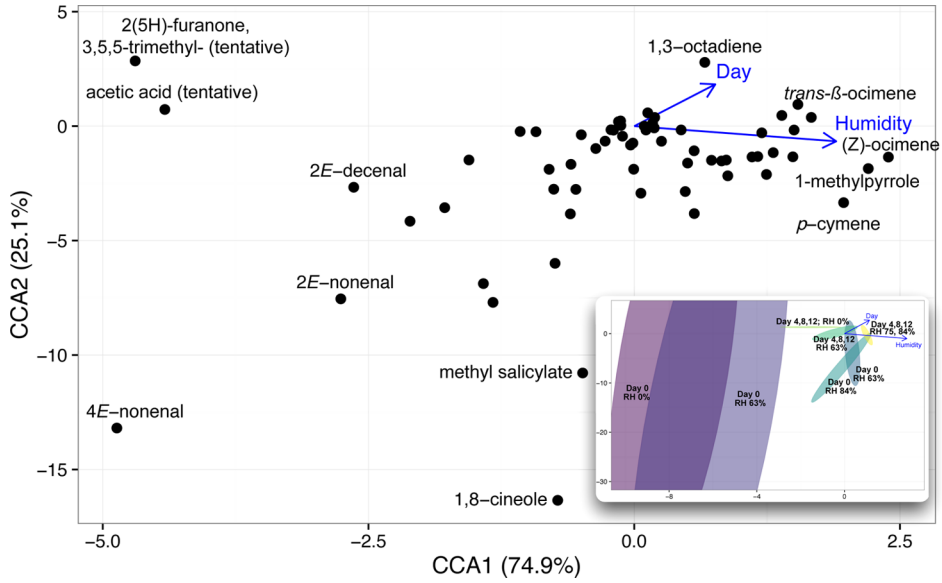

Figure 7. Overlay of biomarkers in ordination space showing the contributions of given volatile compounds to their respective groups.

Results showed significant changes in volatile profiles as a function of relative humidity and water activity of the corresponding pistachio tissue. The experimental conditions used in this study mimicked possible increases in moisture within a pistachio storage silo and the resultant volatile biomarkers at specific moisture levels. An important aspect was that the measured water activity levels were at or below that of what is considered extreme xerophilic activities and that the volatile profiles were suggestive of fungal emissions. Because fungal growth and subsequent mycotoxin production are dependent upon water activity, the detected volatile profiles at the varying humidity levels could be used for early detection of fungal presence by a nondestructive, portable detection system.[15,23]

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jafc.6b04384.

Peaks detected by GC−MS with retention times (min) from DB-1 and DB-Wax columns, corresponding RI values, and authentication sources (Table S1) and differences in select emission levels across tested humidities (Table S2) (PDF)

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Notes

The authors declare no competing financial interest.

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