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Volatile Analysis of Ground Almonds Contaminated with Naturally Occurring Fungi

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Supporting Information

ABSTRACT: Aflatoxigenic aspergilli inflict major economic damage to the tree nut industry of California, with the highest negative impact to almonds. Aspergilli and fungi in general are known to emit volatiles in varying quantity and composition dependent upon their growth media. The goal of the study was to determine the volatile emission of whole and blanched almonds that had been picked out and labeled as inedible by processors. The aflatoxin content and number of colony forming units of each sample were also determined. A total of 23 compounds were consistently detected and identified. Several volatiles from the blanched almonds demonstrated significant increases when compared to the emissions of whole almonds. Several of these volatiles are considered fatty acid decomposition products and included hexanal, heptanal, octanal, nonanal, 3-octen-2-one, tetramethylpyrazine, and decanal. The almond samples investigated were characteristic of a typical postharvest environment and illustrative of potential contamination within a stockpile or transport container. Volatiles indicative of fatty acid decomposition were predominant in the samples that underwent some form of blanching. The emission amounts of hexanal, heptanal, octanal, and hexanoic acid increased 3-fold in samples contaminated with aflatoxin; however, due to variability between samples they could not be considered as indicator volatiles for aflatoxin content. The emission profile of volatiles from almond kernels contaminated with naturally occurring aspergilli and associated fungi is heretofore unreported.

KEYWORDS: almond, aflatoxigenic aspergilli, fatty acid decomposition, fungal contamination, volatile

INTRODUCTION

In 2009/2010 California orchards generated 1.41 billion pounds (641 million kg) of almonds accounting for 80% of global production.[1] Rigorous import limits set by the European Union for aflatoxin contamination in almonds have resulted in an increase in rejected almond shipments.[2] Aflatoxins are toxic metabolites produced by *Aspergillus flavus* and *A. parasiticus*, ubiquitous fungi of tree nut orchards, and represent a grave food safety problem due to their carcinogenic and teratogenic attributes.[3] *A. parasiticus* is capable of producing aflatoxins B_1, B_2, G_1, and G_2 (AFB1, AFB2, AFG1, and AFG2, respectively) (Figure 1), while aflatoxigenic strains of *A. flavus* produce primarily AFB1 and AFB2. AFB1 is the most predominant aflatoxin and considered to be the most toxic.[4] It should be noted that AFB2, AFG1, and AFG2 are typically not present if AFB1 is absent, and AFG1 production has been correlated to the production of AFB1.[5]

The volatile emission of clean, raw ground almond kernels is known,[6] and there are reported methods for volatile analyses of aflatoxigenic and atoxigenic aspergilli in culture media.[7−9] However, because of the differences of volatile emissions between culture media and growth on host plant materials, results of these investigations do not necessarily translate to field conditions. Studies have demonstrated distinct differences in fungal volatile output as a function of medium. For instance, the production of volatile metabolites from the five fungal species cultured on two media "was highly dependent on both medium and species".[10]

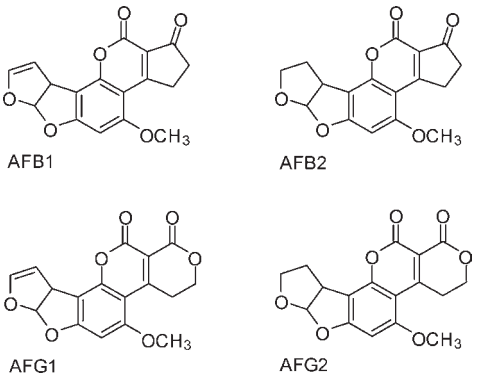

Figure 1. Aflatoxins B_1, B_2, G_1, and G_2 (AFB1, AFB2, AFG1, and AFG2, respectively).

There are limited reports on the volatile output of agricultural products specifically contaminated with microbial bouquets containing *A. flavus* and *A. parasiticus*. In two separate reports Abramson et al.[11,12] demonstrated the existence of three indicator volatiles, 1-octanol, 3-methyl-1-butanol, and 3-octanone for *A. flavus, A. versicolor* and *A. glaucus*, and an additional volatile,

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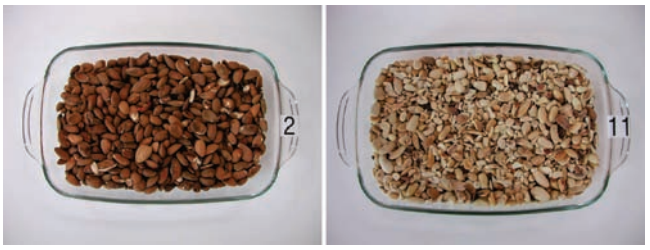

Figure 2. Pictures representative of whole (left) and blanched (right) almond kernels prior to grinding for volatile emissions analysis and aflatoxin content determination.

1-octen-3-ol, for *A. versicolor* on stored barley. Similarly, the indicator volatiles 3-methyl-1-butanol, 3-octanone, and 1-octen-3-ol were noted for wheat contaminated with *A. glaucus*, *A. flavus*, and *A. repens* in addition to *Penicillium*, *Alternaria*, and *Fusarium* genera.[13,14]

Almonds, as well as most agricultural commodities, are known to have ambient microbes associated with them. The following is a sampling of genera found during a study of the mycoflora of almonds: *Cladosporium, Penicillium, Aspergillus, Fusarium,* and *Trichoderma*.[15] Examples of volatiles from these microbes grown individually on agar are relatively general and include small branched alkanols, alkanones, and alkenes, among others.

The limited information of fungal volatiles from whole and blanched almond kernels, versus the reports of volatiles from agar media, prompted this report of the analysis of volatile emission from almonds from their typical environment (e.g., storage before and/or after hulling/shelling) and contaminated with native fungi.

EXPERIMENTAL PROCEDURES

Almond Material. Almonds, 21 batches, were provided by the Almond Board of California from processors throughout the California Central Valley and chosen as pick-outs: almonds with an increased probability of aflatoxin contamination and considered inedible. It should be noted that the material for sampling was unique in that several processors from different locations provided almond pick-outs with the intent there would be a good chance for aflatoxin content for study. Having an adequate number of almond samples with natural aflatoxin contamination is considered an uncommon instance. The collected almonds underwent commercial processing and mimicked that of stored whole almonds ready for transit (Figure 2, left) (samples 1, 2, 4, 5, 8, 9, 12, 13, 15, 17−21). A smaller set of almond samples (samples 3, 6, 7, 10, 11, 14, and 16) consisted of >90% almonds that had undergone blanching (Figure 2, right; see also Supporting Information). Commercial blanching typically involves exposure of the almond kernel to water at 90−100 °C for two or more minutes.[16] Each sample of almond kernels (1 kg) was ground to a fine consistency using a food processor with the nut grater attachment (Electrolux) as per homogeneity regulations for almond aflatoxin analyses.[17]

Almond Fungal Volatile Collections. Ground samples (6 g) were removed from random locations from within storage containers, placed in a 25 mL Erlenmeyer flask, and sealed with a screw cap containing Teflon. Once sealed, the volatiles were adsorbed onto an SPME (Supelco, Bellefonte, PA; 100 μm, polydimethylsiloxane fiber) using the following parameters: *P*, permeation of volatiles = 5 min; *E*, exposure of fiber to volatiles = 1 h; *S*, storage of volatiles on fiber = 1 min; *T*, thermal desorption = 5 min.[18]

Volatile Analyses. All experiments utilized transfer of absorbed volatiles onto either a J&W Scientific (Folsom, CA) DB-Wax column (60 m × 0.32 mm i.d. × 0.25 μm) or a J&W Scientific DB-1 column (60 m × 0.32 mm i.d. × 0.25 μm) installed on one of two HP-6890 gas chromatographs (GC) coupled to HP-5973 mass selective detectors (MS, Palo Alto, CA). Desorbed volatiles were analyzed with methods previously reported,[18] but with the following change in program: ramp one, 4 °C/min; final temp, 180 °C; hold time, 0.0 min; postrun 210 °C; hold time, 5.0 min. NIST, Wiley, and internally generated databases were used for fragmentation pattern identification. The retention indices (RIs) were calculated using a homologous series of *n*-alkanes on the DB-Wax and DB-1 columns. Volatile identifications (Table 1) were verified by injection of authentic samples and comparison to retention times of an internally generated list of volatiles on identical columns. Volatile amounts shown in Tables 2 and 3 are the peak areas from the GC−MS total ion chromatograms (TIC). Strict adherence to sample size and volatile collection parameters allowed for comparison of volatile abundances.

Table 1. Volatiles Collected and Identified from Ground Whole and Blanched Almonds

			DB-Wax (RI)		DB-1 (RI)	
peak	library/ID[a]	source[b]	calcd	lit.	calcd	lit.
1	hexanal	AA	1077	1077		772
2	undecane	Poly	1098	1100	1097	1100
3	2-butylfuran	AA	1126	1126	878	877
4	2-heptanone	AD	1178	1178	867	865
5	heptanal	AD	1181	1180	875	876
6	limonene	AD	1195	1197	1020	1020
7	dodecane	Poly	1198	1200	1197	1200
8	2-pentylfuran	AA	1228	1226	977	977
9	2-octanone	AD	1282	1281	966	967
10	octanal	AA	1285	1284	977	979
11	1-hexanol	AD	1354	1350	848	848
12	nonanal	AD	1390	1389	1079	1082
13	3-octen-2-one	BD	1404	1404	1009	1013
14	acetic acid	AD	1455	1475		580
15	tetramethylpyrazine	PS	1474	1476	1059	1061
16	2-decanone	AD	1491	1491	1171	1172
17	decanal	AD	1495	1495	1181	1184
18	butyrolactone	AD	1618	1623	857	855
19	γ-hexanolactone[c]	PMR	1692	1699	999	1003
20	hexanoic acid	AA	1855	1825/1874	972	890
21	γ-octanolactone[c]	PMR	1907	1916	1206	1210
22	phenol	AD	2002	2000	957	957
23	γ-nonanolactone[c]	PMR	2022	2030	1311	1315

[a] Compound identification by RI relative to *n*-alkanes on DB-Wax column, retention times, mass fragment libraries, and comparison to authentic samples. [b] Source codes: AA, Alfa Aesar; AD, Aldrich; BD, Bedoukian; EM, Eastman; Poly, Polyscience Corp.; PMR, isolated by Plant Mycotoxin researchers. [c] Tentatively identified.

Colony-Forming Unit (CFU) Counts of *A. flavus* and *A. parasiticus*. *A. flavus* and *A. parasiticus* agar (AFPA) were prepared per literature protocol[19] and from the following components: Bacto yeast extract, 20 g/L; Bacto peptone, 10 g/L; ferric ammonium citrate (Sigma), 0.5 g/L; dichloran (Sigma), 1 mL of a 0.2% solution in ethanol; Bacto agar (BD), 15 g/L; chloramphenicol (Sigma) 0.1 g/L. Fungal counts were measured in triplicate for each almond sample. Ground almond (40 g) was added to maximum recovery diluent (200 mL, Oxoid), stirred for 30 min, followed by aliquot dilutions of 1:10, 1:20, and 1:40 (v/v).

Table 2. Volatile and Aflatoxin Amounts from Ground Whole Almonds

		sample no.[a]													
peak	library/ID	1	2	4	5	8	9	12	13	15	17	18	19	20	21
1	hexanal[b,c]	811,054	1,159,907	3,267,829	754,557	313,919	1,835,592	3,471,251	9,269,078	5,446,882	299,246	490,744	369,121	187,406	210,239
2	undecane	322,627	407,638	283,347	148,931	249,916	230,520	342,421	404,490	188,324	172,184	138,230	194,392	112,759	107,607
3	2-butylfuran	61,605	127,043	180,052	129,136	159,239	235,663	343,935	476,221	292,809	114,081	131,792	62,724	29,738	99,144
4	2-heptanone	278,144	285,584	404,423	177,418	179,845	345,599	541,790	648,189	424,192	205,270	290,462	129,423	106,677	129,732
5	heptanal[b,c]	57,285	60,676	259,746	49,525	15,395	257,548	332,549	611,940	471,324	71,660	33,451	18,008	0	18,120
6	limonene	827,149	976,305	479,537	582,875	1,005,972	932,891	731,967	712,103	781,239	1,328,882	1,266,306	1,507,867	1,128,215	1,251,780
7	dodecane	352,237	646,140	1,654,019	251,341	374,029	566,905	1,917,906	2,014,461	523,363	401,035	393,999	332,870	294,984	366,554
8	2-pentylfuran	374,679	446,218	422,546	357,525	394,786	846,772	1,125,061	1,525,031	1,137,986	481,203	503,001	454,596	307,570	354,065
9	2-octanone	47,633	101,828	150,570	63,668	22,692	182,167	226,805	268,995	230,084	39,726	159,685	34,243	13,767	0
10	octanal[b,c]	219,090	307,233	984,936	300,141	156,662	781,474	1,096,122	2,015,029	1,570,152	181,126	338,392	155,137	66,402	73,219
11	1-hexanol	710,149	871,509	2,565,440	335,349	454,212	266,822	1,065,285	660,991	545,101	360,844	571,164	308,200	250,283	163,569
12	nonanal[b]	598,651	722,296	2,104,546	703,150	386,226	1,625,505	1,998,220	3,342,750	2,656,009	695,120	968,235	586,650	456,824	701,108
13	3-octen-2-one[b]	339,724	449,052	837,040	358,009	340,986	602,881	835,380	1,051,237	647,279	292,498	472,235	241,040	300,126	309,556
14	acetic acid[c]	429,630	345,333	243,333	221,050	403,333	727,697	1,332,715	2,300,000	4,760,000	418,187	642,333	1,736,333	411,181	198,640
15	tetramethylpyrazine[b]	12,268	224,077	34,161	39,148	30,885	0	173,487	95,749	108,047	0	0	34,670	229,741	17,296
16	2-decanone	122,310	90,987	394,702	78,587	60,927	257,042	349,759	379,928	372,294	107,243	186,032	114,032	68,102	76,009
17	decanal[b]	268,061	278,043	869,864	256,120	0	430,684	593,316	871,195	715,003	242,902	332,682	241,750	162,151	232,691
18	butyrolactone	518,935	3,422,824	671,618	772,958	594,709	644,070	723,881	757,368	1,802,802	956,660	732,665	3,924,549	454,647	491,632
19	γ-hexanolactone	595,922	699,567	1,634,804	558,990	1,040,502	1,008,969	1,286,512	2,137,488	1,510,795	708,125	1,067,279	816,695	498,751	518,114
20	hexanoic acid	166,819	265,788	1,132,763	85,059	52,953	329,762	1,485,790	3,020,344	1,623,059	62,737	695,405	792,934	196,483	265,365
21	γ-octanolactone	452,786	478,999	1,260,314	396,965	745,088	639,442	1,084,882	1,691,540	981,269	156,735	984,648	845,134	588,377	341,651
22	phenol	95,132	1,419,291	0	56,877	54,768	152,541	48,773	306,794	134,704	84,634	153,854	140,402	0	28,634
23	γ-nonanolactone	483,020	373,486	933,790	282,665	539,295	271,385	1,278,931	1,462,423	888,393	190,657	3,308,495	1,024,995	490,365	279,219
aflatoxin amounts (ppb)															
	B_1 sum	174.2	1.1	123.0	577.5	48.6	264.9	390.8	372.0	27.6	1.4	1.5	130.2	5.7	0.8
	B_2 sum	28.8	0.0	17.0	87.2	10.0	18.4	53.0	41.6	4.6	0.3	0.2	19.2	0.8	0.0
	G_1 sum	0.1	0.4	1.3	0.0	0.2	332.4	0.8	399.0	1.3	0.3	7.6	0.6	0.6	0.1
	G_2 sum	0.0	0.0	0.0	0.0	0.0	19.2	0.0	32.6	0.0	0.0	1.4	0.0	0.0	0.0
	total aflatoxin sum	203.1	1.5	141.3	664.7	58.8	634.8	444.6	845.1	33.5	2.1	10.7	150.0	7.2	1.0

[a] Sample numbers as provided and analyzed blindly; relative amounts are peak surface areas generated by ChemStation software; values are means of triplicates. [b] Volatiles that displayed significant increases in blanched samples. [c] Volatiles that demonstrated >3× increase in emission when AFB1 content >20 ppb, but was not significant at $P < 0.002$ ($n = 5$ <20 ppb; $n = 9$ >20 ppb).

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Table 3. Volatile and Aflatoxin Amounts from Ground Blanched Almonds

		sample no.[a]						
PK	library/ID	3	6	7	10	11	14	16
1	hexanal[b,c]	5,860,857	9,138,521	767,950	17,441,248	21,725,538	13,885,560	5,244,908
2	undecane	890,400	170,905	250,187	328,579	404,004	229,453	210,080
3	2-butylfuran[c]	255,824	191,755	32,624	559,189	702,686	561,063	263,914
4	2-heptanone	463,122	342,854	181,296	842,627	841,549	766,374	418,061
5	heptanal[b,c]	424,713	527,804	48,062	1,023,027	1,186,084	1,072,037	393,257
6	limonene	725,460	635,428	1,167,129	810,997	979,258	739,733	650,396
7	dodecane	1,266,127	283,713	345,169	2,039,813	862,165	2,665,906	2,976,420
8	2-pentylfuran	1,084,131	573,196	261,589	1,791,176	1,540,374	1,524,770	814,319
9	2-octanone	271,959	137,090	0	320,064	355,611	383,649	160,662
10	octanal[b,c]	1,496,340	1,701,341	238,929	3,970,322	3,958,359	4,084,788	1,308,246
11	1-hexanol	418,057	232,681	3,364,826	754,940	811,721	581,341	918,101
12	nonanal[b,c]	2,658,172	2,941,740	481,356	6,030,191	5,757,940	6,445,689	2,726,110
13	3-octen-2-one[b,c]	826,894	1,025,248	153,103	2,054,605	2,374,791	1,954,546	942,731
14	acetic acid	1,755,000	793,333	676,667	1,950,000	3,351,049	3,027,329	2,233,333
15	tetramethylpyrazine[b]	550,478	283,850	116,132	406,135	605,097	301,118	300,084
16	2-decanone[c]	228,796	272,718	0	585,455	726,516	967,717	512,613
17	decanal[b,c]	840,266	764,029	224,412	2,044,545	2,028,489	2,598,349	685,513
18	butyrolactone	2,690,029	329,657	2,010,119	1,231,611	1,397,753	1,015,098	703,013
19	γ-hexanolactone	952,362	970,053	353,652	2,274,579	2,100,953	2,826,657	1,137,917
20	hexanoic acid	1,012,379	1,270,026	125,623	6,939,618	9,909,605	16,080,756	1,598,019
21	γ-octanolactone	677,789	523,408	431,777	1,458,020	1,285,430	2,248,371	935,269
22	phenol	207,050	113,533	89,140	119,453	156,471	198,001	41,745
23	γ-nonanolactone	798,285	296,827	341,666	3,232,200	911,765	1,017,516	879,219
aflatoxin amounts (ppb)								
	B_1 sum	10.0	146.0	2.9	26.0	146.8	37.8	66.6
	B_2 sum	2.1	19.5	0.3	3.8	19.8	6.4	9.6
	G_1 sum	3.5	1.8	0.3	1.1	131.7	1.5	18.0
	G_2 sum	0.9	0.3	0.1	0.1	19.8	0.0	3.5
	total aflatoxin sum	16.5	167.5	3.6	30.9	318.1	45.7	97.6

[a] Sample numbers as provided and analyzed blindly; relative amounts are peak surface areas generated by ChemStation software; values are means of triplicates. [b] Volatiles that displayed significant increases in blanched samples. [c] Volatiles that demonstrated >3× increase in emission when AFB1 content >20 ppb, but was not significant at $P < 0.002$ ($n = 2$ <20 ppb; $n = 5$ >20 ppb).

An aliquot from each dilution (0.1 mL) was spread on an AFPA Petri dish and incubated at 30 °C. Total fungal colonies and colonies of *A. flavus* and *A. parasiticus* showing orange pigmentation on the reverse were counted after 42−48 h.

Aflatoxin Standards and Analyses. Aflatoxin standards were prepared as per AOAC 971.22 (18th edition, 2005) and previously published methods.[19] Upon completion of volatile collection, each sample was subjected to aflatoxin analysis using a method similar to previously published protocols.[20] Ground almond kernels (6 g) were blended in an MC3 minicontainer (Waring) with methanol/water (60:40, 25 mL) and NaCl (1 g) for 1 min. The mixture was gravity filtered through fluted filter paper (Whatman 2V) followed by syringe filtration (Pall 0.45 μm nylon, 13 mm diameter) of a 2.5 mL portion. An aliquot (1.0 mL) of the extract was diluted with an equal volume of water and passed through an Aflatest P affinity column (Vicam) followed by a water wash (2 mL). Aflatoxins were eluted from the column with acetonitrile (2 mL), and the eluate evaporated to dryness under a stream of nitrogen at 40 °C. The dried sample was dissolved in methanol (1.0 mL) and analyzed for aflatoxins by reversed-phase HPLC (Agilent 1100, Santa Clara, CA). Conditions for HPLC analyses (Inertsil ODS-3, 4.6 × 250 mm): mobile phase water/acetonitrile/methanol (45:25:30); flow, 1.0 mL/min; temperature, 25 °C; detector, fluorescence, 365 nm excitation, 455 nm emission; derivatization, photochemical reactor (PHRED, Aura Industries), 25 m × 0.25 mm i.d. coil; injection volume, 20 μL; retention times, AFG2, 7.8 min; AFG1, 8.7 min; AFB2, 9.4 min; AFB1, 10.6 min. All volatile and aflatoxin experiments were performed in triplicate with mean of the triplicate being reported (Tables 2 and 3) and used for all analyses; tables were generated in Excel (Microsoft Inc.). Paired *t* tests (whole vs blanched) (>20 ppb AFB1 vs <20 ppb AFB1) (>10 ppb AFG1 vs <10 ppb AFG1) were performed using Microsoft Excel. A very conservative approach was taken for identifying volatiles whose amounts were different between comparisons by using the Bonferroni *P* value at $\alpha = 0.05/23 = 0.002$. Discriminant analysis of the two assigned groups (whole and blanched) was performed as a pairwise comparison by using Bionumerics 4.6 (Applies Maths, Inc.).

RESULTS AND DISCUSSION

The volatile analysis of ground almonds provided a total of 23 compounds (Table 1) from the 21 almond samples. A number of these compounds have been shown to be commonly associated with *Aspergillus* contamination on various commodities and include hexanal, 2-pentylfuran, 1-hexanol, nonanal, 1-octen-3-ol, and 1-octanol;[7,8,21] however, none of these reports were for aspergilli

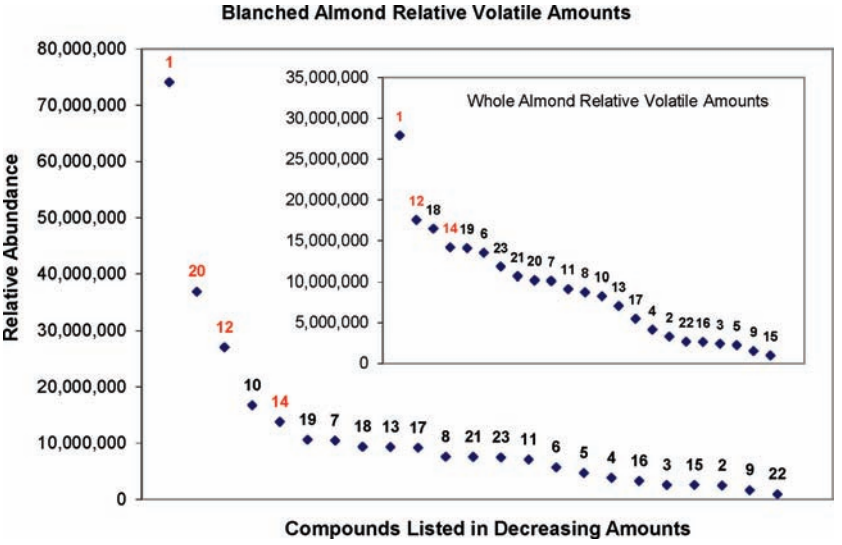

Figure 3. Comparison of volatile amounts for both whole (inset) and blanched almonds, plotted in decreasing order. Compound numbers in red denote high emissions in both series and/or noteworthy presence as a major emission.

on almonds. There were no detectable sesquiterpenes in the present analysis though an earlier study demonstrating sesquiterpene emission being related to aflatoxigenic aspergilli grown on agar media.[9]

Most notable was the relatively large amount of hexanal emitted in both whole and blanched samples (Figure 3). Hexanal is well-known as an off-flavor volatile from the auto-oxidation of linoleic acid,[22] as well as the thermal oxidation of linoleates.[23] It should be noted that hexanal was *ca.* two times higher in emission than the next highest volatile amount. When plotted in descending orders the volatile amounts for the whole and blanched series declined in a near-exponential fashion (Figure 3). Another volatile with significant emission in both samples, but more prominent in the blanched series, was hexanoic acid. Hexanoic acid was noted in an almond oil oxidation investigation,[22] but not in a steamed almond hull investigation[24] despite the large presence of octanoic and nonanoic acid. Another major volatile was nonanal, which is known as a decomposition product of oleic acid.[23] Oleic acid is the major fatty acid found in almonds, followed by linoleic acid. In general, the C_6-C_9 alkanals from the present study showed high parity with emissions of steamed almond hulls,[24] raw almonds,[6] and oxidized almond oil.[22] This high incidence of alkanal emissions between these GC−MS investigations raises questions as to the specific conditions of the almond kernels during analysis and the specific origin/cause of the associated volatiles. It should be noted that C_8-C_{10} (2*E*)-alkenals, similar to what Buttery et al.[24] and Beltran et al.[22] reported, were detected in the present study; however their emission was inconsistent (<50% of all samples) and relatively low, and thus not included in Tables 1−3. It could be conjectured that (2*E*)-alkenal emission may be influenced by stressors to lipid enzyme activation such as oxidation, heat, drought conditions, or microbial presence as has been indicated by investigations of other systems.[6,22,25]

The volatile γ-butyrolactone, common in wines, Orchidaceae plants, and to a lesser extent roasted coffee beans, was also found in relatively high amount. For wines, it was suggested that γ-butyrolactone may originate from glutamic acid;[26] conversely, γ-lactones in general have been reported to be formed from free oleic acid.[23,27] An interesting feature noted in Figure 3 was the relatively modest amounts of the branched γ-lactones (compounds **19**, **23**, and **21**). These compounds are common flavor odors and are thought to be degradation products of fatty acids that may involve lipoxygenase from aspergilli.[28] What is important about the presence, as well as the relatively high amounts, of γ-butyrolactone detected in the present study is its unique emission relative to the other parallel studies.[6,22,24] The presence of these γ-lactones also highlights the varying composition and quantities of volatile emission as a function of kernel conditions and/or stressors.

Another notable emission difference of the present study and the investigation of steamed almond hulls[24] was the composition of terpenoids and aromatic compounds. This could be attributed to different collection techniques used for each study. Interestingly, the present study did not detect any alkadienals, which were found to be present in the investigation by Buttery et al.[24]

Other volatile analyses of almonds have been performed: *in situ* volatile emission of Nonpareil almonds[18] and *ex situ* damaged and undamaged whole almond volatiles.[29] Interestingly, very few volatiles were common between these investigations and the present study. For example the *ex situ* method detected the volatiles limonene, 2-pentylfuran, nonanal, and 1-octen-3-ol, and the latter was detected as a transient in the present study. The *in situ* method detected the volatiles γ-butyrolactone and nonanal. These large disparities are most likely due to the volatile extraction method used, but could also be due to differences in nut phenology, level of fungal contamination, and/or state of the almond (sliced, whole, in-shell).

Fourteen of the 21 samples (1, 2, 4, 5, 8, 9, 12, 13, 15, and 17−21) underwent typical processing without blanching and were analyzed as whole almond kernels (Table 2 and Figure 2, left). The remaining seven samples (3, 6, 7, 10, 11, 14, and 16) contained >90% of almonds that underwent blanching (Table 3 and Figure 2, right; see also Supporting Information). Discriminant analysis of the blanched and whole almonds provided moderate separation of the two groups (Figure 4). The three top discriminants responsible for the observed separation were tetramethylpyrazine, hexanal, and nonanal. Additionally a paired *t* test of each volatile was used to identify volatiles whose amounts

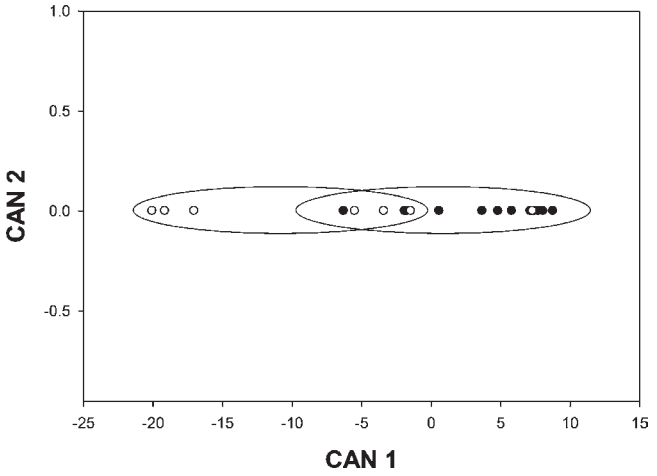

Figure 4. Plot of the first canonical variable resulting from the discriminant analysis of the GC−MS volatile data, whole vs blanched volatile differences: ● = whole kernels; ○ = blanched kernels.

differ between blanched and whole almonds. A significant increase in the quantities of a number of volatiles from the blanched series was observed and included: hexanal, heptanal, octanal, nonanal, 3-octen-2-one, tetramethylpyrazine, and decanal. All of these volatiles, excluding tetramethylpyrazine, have origins from fatty acid decomposition. The volatile emission increase of tetramethylpyrazine (ca. 2.5 times greater in the blanched series) may perhaps be explained by the heat involved during the blanching process. Pyrazine formation typically involves reaction of an amino acid and reducing sugar under thermal conditions.[30] Though the emission increases for a number of volatiles between whole and blanched almonds were statistically different, no volatile could be used as an indicator volatile for either whole or blanched almonds due to the overlap in ranges of the amount of any particular volatile.

Almond samples were evaluated to determine if any volatiles were indicative of aflatoxin content. Comparisons were made within the whole and blanched series where samples with differing aflatoxin content (>20 ppb AFB1 vs <20 ppb AFB1; and >10 ppb AFG1 vs <10 ppb AFG1) were delineated. Statistical analysis of data from Tables 2 and 3 revealed no difference in emission of any volatiles. Additionally, correlation analysis was performed to determine if aflatoxin content was correlated to the emission amount of a particular volatile; again, no noteworthy correlations were observed (data not shown). Nevertheless, there were some noteworthy increases in volatile emissions in samples containing more AFB1 and AFG1 that may warrant future investigation (Tables 2 and 3). For the whole almond data set (Table 2), the volatiles hexanal, heptanal, octanal, acetic acid, and hexanoic acid increased greater than 3-fold when AFB1 content in the corresponding samples was >20 ppb. Also in the whole almond data set, the volatiles hexanal, heptanal, octanal, and hexanoic acid increased greater than 3-fold when AFG1 content was >10 ppb. Similarly, in the blanched almond data set (Table 3), the volatiles hexanal, 2-butylfuran, heptanal, octanal, nonanal, 3-octen-2-one, 2-decanone, decanal, and hexanoic acid increased greater than 3-fold when AFB1 content was >20 ppb, while no volatiles changed greater than 3-fold in blanched almonds containing >10 ppb AFG1. The parity of volatiles (hexanal, heptanal, octanal, and hexanoic acid) between the two sample sets that showed an increase in emission during AFB1 presence is noteworthy; though it should be stressed that they

Table 4. *Aspergillus* and non-*Aspergillus* Fungal Colony Forming Unit (CFU) Counts for Samples 1−21 and Percent Aspergilli per Sample

	total[a]		*A. flavus* and *A. parasiticus*[a]		
sample	av	std dev	av	std dev	% aspergilli
1	51900	20600	722	255	1.4
2	42400	15100	0	0	0.0
3[b]	91400	17700	11800	2770	12.9
4	2780	839	111	96	4.0
5	101000	41400	19700	4840	19.5
6[b]	68800	2300	4330	601	6.3
7[b]	0	0	0	0	no CFUs
8	13800	3920	611	96	4.4
9	29400	2780	4720	1250	16.1
10[b]	1060	1420	111	192	10.5
11[b]	278	481	0	0	0.0
12	17600	3280	9440	1690	53.6
13	18000	2740	278	255	1.5
14[b]	0	0	0	0	no CFUs
15	48500	2850	15400	2500	31.8
16[b]	3440	1710	0	0	0.0
17	76200	16200	1940	1230	2.5
18	69400	2360	778	694	1.1
19	44500	9080	17400	2990	39.1
20	35400	9060	13200	3750	37.3
21	120000	24600	333	577	0.3

[a] Colony forming unit (CFU) counts . [b] Blanched almond samples.

are not being defined as reliable indicators to diagnose the presence of aflatoxin content in an almond sample. It should be noted that the three alkanals (C_6−C_8) were also associated with the increase in volatile emission between blanched and whole almonds.

Finally, material from each sample was evaluated for fungal CFU counts (Table 4) to provide information regarding the percentage of aspergilli versus nonaspergilli. Conventional wisdom would suggest that, due to heating, the blanched samples would be void of CFU counts for microbes; yet, evaluation of the CFU data provided in Table 4 showed disparity involving the CFU counts for all fungi and the almond samples that had undergone some form of the blanching process. Four of the seven blanched samples exhibited anticipated CFU counts: two samples (7 and 14) showed no CFUs present, and two samples (11 and 16) showed no *Aspergillus* CFUs, but did show non-*Aspergillus* CFUs. An unusual result for these four samples was the comparison of CFU data and aflatoxin content. Though samples 7 and 14 did not provide any CFU counts, aflatoxins were detected in each sample (Table 3), albeit a small amount for sample 7. Likewise, samples 11 and 16 showed no *Aspergillus* CFUs yet both samples displayed relatively high amounts of aflatoxins. It should be noted that data of CFU counts and aflatoxin content did not produce any viable relationships.

Though the blanched almonds exhibited significantly higher volatile amounts, on average they contained less aflatoxin content (whole almonds, 151, 20, 53, 4, and blanched almonds, 62, 9, 23, 4 ppb AFB1, ABF2, AFG1, and AFG2, respectively). This suggests that the blanching process does eliminate and/or diminish fungal contamination, but does *not* eliminate aflatoxin presence previously established by the aflatoxigenic aspergilli. Little is known

regarding the blanching process and its effect on aflatoxin content. A review of peanut characteristics and blanching[31] implied that blanching/color sorting reduces aflatoxin by 99%.

The data from Table 4 and the possible explanation regarding fungal absence highlight an important point. It has been reported that certain aspergilli are able to produce extracellular lipase for deposit onto the host-plant tissue.[32] Lipase activity from fungal infection releases fatty acid from host-plant tissue (e.g., almond kernel) and activates the lipoxygenase and hydroperoxide lyase pathways. The lipoxygenase and hydroperoxide lyase pathways convert linoleic and linolenic acid to hexanal and (*E*)-2-hexenal.[33] Additionally, the lipoxygenase pathway is known to produce the fatty acid decay products C_6-C_{10} alkanals and alkenals.[25,33] If the extracellular lipase activity is able to continue almond fatty acid breakdown in the absence of the fungi, it may explain the lower associations between volatile emissions, aflatoxin content, and aspergilli presence. In support of this theory was the reported detection of C_5-C_9 alkanals and alkenals when exogenous lipase is applied to cotton bolls.[33]

In addition to the alkanals and alkenals noted above, it is known that the minor components of triolein decomposition also include methyl ketones and γ-lactones;[23] thus, providing a further explanation, and origins from almond fatty acids, for the presence of 2-octanone, 3-octen-2-one, 2-decanone, γ-hexalactone, γ-octalactone, and γ-nonalactone.

Twenty-one samples of pick-out almonds chosen from random processors throughout the California Central Valley were evaluated for their volatile emission, aflatoxin content, and CFU counts. The samples were segregated into whole and blanched subsets. The blanched almonds exhibited a significant increase in the amounts of the volatiles hexanal, heptanal, octanal, nonanal, 3-octen-2-one, tetramethylpyrazine, and decanal when compared to the whole almonds, this despite their origins from different processors. This result corroborates a report that almond skins provide antioxidative protection.[34] Moreover this difference in volatile emission between whole and blanched almond implies the almond skin may also inhibit fatty acid autoxidation. The volatiles hexanal, heptanal, octanal, and hexanoic acid demonstrated a greater than 3-fold increase in emission when AFB1 content was >20 ppb, but were not significant at $P < 0.002$. Thus, the use of HS-SPME GC−MS at this juncture does not appear to be an effective tool for detection of aflatoxins and/or aspergilli. This said, the 3-fold emission increase in some volatiles does raise the question regarding AFB1 content, but the results do not provide an answer to aflatoxin presence.

■ ASSOCIATED CONTENT

Supporting Information. Thumbnail photographs of all almond samples prior to homogenization and sampling. This material is available free of charge via the Internet at http://pubs.acs.org.

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