Journal of the Science of Food and Agriculture *J Sci Food Agric* **88**:1363–1368 (2008)

Comparison of volatile emissions from undamaged and mechanically damaged almonds†

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Abstract

BACKGROUND: The navel orangeworm (NOW) *Amyelois transitella* (Walker) is a major insect pest of almonds causing considerable monetary setbacks for both growers and processors, and thus control of NOW is one of the top priorities for the almond industry. Field observations purport that NOW is attracted to previously injured almonds. Accordingly, in this study the volatile output of damaged almonds was investigated in an effort to identify potential attractants for further studies into the control and/or monitoring of NOW. Mature almonds from the Monterey variety were evaluated for their volatile composition after mechanical damage and compared with the volatile composition of undamaged almonds.

RESULTS: Volatile organic compounds (VOCs) were collected on Tenax, desorbed and identified via gas chromatography/mass spectrometry analysis. VOCs unique to the damaged tree nuts included trace amounts of 3-pentanol and isomers of the spiroketal chalcogran. VOCs that increased in relative amounts after damage include the spiroketal conophthorin and numerous four-carbon ester and ketone as well as alcohol derivatives, in addition to two eight-carbon chain compounds.

CONCLUSION: Several VOCs, both unique and in increased amounts, were identified from damaged almonds. Their presence in damaged almonds warrants further investigation into their role in NOW response to damaged almonds, which may lead to insights into the control and/or monitoring of NOW.
Published in 2008 by John Wiley & Sons, Ltd.

Keywords: almond; damaged; spiroketal; Tenax; volatile

INTRODUCTION

The navel orangeworm (NOW) *Amyelois transitella* (Walker) is a major insect pest of almonds grown in California and causes considerable monetary setbacks for both growers and processors. Control of NOW has been stated as one of the top priorities for the almond industry, with another priority being the development of new pest management tools.[1] There is twofold interest in controlling NOW, namely its direct damage to tree nuts and the associated contamination of toxin-producing fungi (mycotoxins) resulting from NOW feeding damage, which provides avenues for infection by mycotoxigenic fungi. The point of damage into the tree nut from the pest insect exposes the protective layers (hull, shell, seed coat) surrounding the kernel. This point of entry allows for ambient spores of aspergilli to enter and thus contaminate the nut.[2] Contamination of tree nuts by mycotoxins is a chief concern for both human food and animal feed safety, with both areas experiencing major export issues as a result of the contamination.[3] The aflatoxin-producing (aflatoxigenic) fungi most relevant to agriculture include *Aspergillus flavus* and *Aspergillus parasiticus*. Aflatoxin is presently a significant food safety problem owing to its carcinogenic and teratogenic attributes. The current total aflatoxin action threshold for international export of tree nuts is set at 4 ppb compared with the domestic level of 20 ppb set by the Food and Drug Administration (FDA).[2,3] California is the top producer of almonds, supplying 75% of the world's needs.[1] Approximately 5% of California's cropland is dedicated to almond production.[4] The

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† Segments of this report were presented at the 48th Annual Meeting of the American Society of Pharmacognosy, Portland, ME 04101, USA, 14–18 July 2007 and at the 17th Annual Multi-Crop Aflatoxin Elimination Workshop, Sacramento, CA 95814, USA, 25–28 October 2004

‡ Retired

Contract/grant sponsor: USDA-ARS; contract/grant number: CRIS 5325-42000-036-00
Contract/grant sponsor: Paramount Farming Company; contract/grant number: CRADA #58-3K95-7-1198
(*Received 18 September 2007; revised version received 10 December 2007; accepted 28 December 2007*)
Published online 14 April 2008; DOI: 10.1002/jsfa.3224

 J Sci Food Agric 0022–5142/2008/$30.00

California almond industry generates approximately \$2 billion annually, with the total California tree nut industry reporting over \$3.5 billion. About 50–70% of California tree nuts are exported overseas annually, with 80% of almond production alone being exported.[1] The strict export action levels for aflatoxin have resulted in mycotoxin management issues for producers as well as state and federal governments. Actual costs of crop loss due to aflatoxin contamination in California were estimated to have been \$23–47 million over the period 1995–2001.[3] Moreover, the economic and health impacts of mycotoxins have been stated to be severe for developing nations.[5]

In a recent investigation, researchers reported the observation that female NOW moths were attracted to injured almonds.[6] Current attractants used in the field and/or lab for NOW include the female sex pheromone of NOW, (*Z*,*Z*)-11,13-hexadecadienal,[7] a pheromone blend of (*Z*,*Z*,*Z*,*Z*,*Z*)-3,6,9,12,15-tricosapentaene, (*Z*,*Z*,*Z*,*Z*,*Z*)-3,6,9,12,15-pentacosapentaene, ethyl palmitate and ethyl-(*Z*,*Z*)-11,13-hexadecadien-1-yl acetate[8] and the almond oil fatty acids myristic, palmitic, stearic, oleic and linoleic.[9] Investigations on VOCs from almonds report the detection of 2-hexyl-3-methylmaleic anhydride[10] and various alkane, alkene, alkanol, aromatic and furan VOCs.[11] However, a search of the literature does not provide examples of VOC emission as a result of injury to the almond. As part of our ongoing efforts to address the concerns regarding NOW, our labs investigated the VOC output of mechanically damaged (DMG) almonds from the Monterey variety and compared the VOC fingerprint with that of undamaged (CTRL) almonds to ascertain what VOCs, if any, were unique to DMG almonds. The major VOCs from the CTRL and DMG experiments were compared and contrasted.

MATERIALS AND METHODS

Plant material

Fruits of *Prunis dulcis* (P. Mill.) D.A. Webb, variety Monterey, common name sweet almond, were collected in two batches during mid to late June 2006 from the groves of Paramount Farming Company, Bakersfield, CA, USA. Each batch was replicated in triplicate over different days. Batch 1 consisted of almonds that had been injured while intact on the tree, allowed to remain on the tree for approximately 14 days, then removed and placed in glass jars with a Teflon paper seal between the cap and jar. The injury/damage consisted of hull penetration with an 8 penny nail (3 mm diameter). Batch 2 consisted of control almonds that were not injured, removed from the tree and placed in glass jars with a Teflon paper seal between the cap and jar. Batches 1 and 2 were collected during concurrent time frames. Batches were sent via overnight delivery to the USDA-ARS facility in Albany, CA, USA for volatile evaluation.

Collection of VOCs[6]

Almonds (*ca* 500 per experiment) were transferred to a 12 L round-bottomed flask fitted with an inlet for purified airflow at 1 L min^{-1} and a Tenax (25 g) collection system. VOCs were collected for 18 h and desorbed with freshly distilled diethyl ether (100 mL), then the ether was concentrated to a volume of *ca* 1 mL with a warm water bath and a Vigreux distillation column.

Gas chromatography/mass spectrometry (GC/MS) analysis

Separation of the collected VOC mixture was achieved with a DB-Wax column (60 m × 0.32 mm i.d. × 0.25 µm; J&W Scientific, Folsom, CA, USA) installed on an HP 6890 gas chromatograph (GC) coupled to an HP 5973 mass selective detector (MSD) (Hewlett Packard, Palo Alto, CA, USA). Extracts were analysed with the following method: 1 µL injections; injector temperature, 150 °C; splitless mode; inlet temperature, 150 °C; inlet pressure, 7.7 psi; total flow, 11.9 mL min^{-1}; He carrier gas at 7.7 psi; flow, 1.5 mL min^{-1}; velocity, 31 cm min^{-1}; constant flow; oven settings: initial temperature, 30 °C; hold time, 4 min; ramp, 2 °C min^{-1}; final temperature, 200 °C; hold time, 30 min. The MSD parameters were as follows: source temperature, 230 °C; MS quadrupole temperature, 150 °C; electron impact (EI) mode, 70 eV; solvent delay, 1 min; scan group 1, 40–300 amu; scan group 2 at 20 min, 40–450 amu. National Institute of Standards and Technology (NIST), Wiley and internally generated databases were used for fragmentation pattern identification. Retention indices (RIs) were calculated using a homologous series of *n*-alkanes on a DB-Wax column. Compounds that did not match the RIs of known VOCs from our database and/or did not provide sufficient mass fragmentation pattern matches were assigned as unknown in Table 1.

Statistical analysis

GC/MS analysis was performed on each of the three separate samples for both the CTRL and DMG batches of almonds. The relative areas for each of the compounds from the GC/MS runs were normalised to the internal standard cyclodecanone (15 µg) and the means, standard deviations and confidence limits (95%) in Table 1 and Fig. 3 were calculated with Microsoft Excel software (Redmond, WA, USA).

RESULTS AND DISCUSSION

Analysis of the major VOCs emitted by both the CTRL and DMG almonds provides a wide range of compounds, which corroborated other reports and added to the volatile fingerprint of almonds in the literature. Table 1 provides a list of the major VOCs detected from both experiments. Examination of Table 1 showed a number of monoterpenes common to citrus and other plants,[12] namely α-pinene, camphene, β-pinene, β-myrcene, limonene

J Sci Food Agric **88**:1363–1368 (2008)
DOI: 10.1002/jsfa

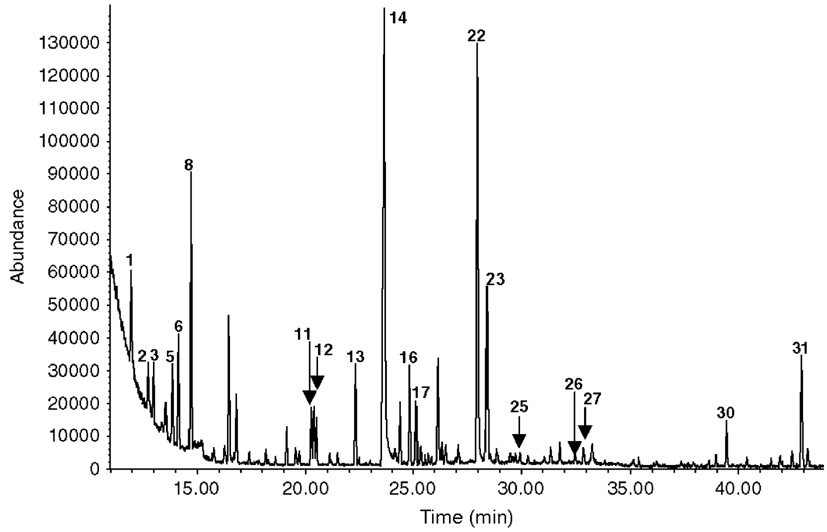

Figure 1. Total ion chromatogram (relative abundance *versus* time) illustrating a typical elution pattern of DMG almond VOCs. Unique, increased and/or notable compounds are labelled with numbers corresponding to compounds listed in Table 1.

and cymene. The compounds α-pinene and camphene were noted to be in relatively large amounts in the CTRL almonds and underwent a small decrease in volatile output for the DMG almonds (Fig. 1 illustrates a typical GC elution pattern for DMG VOCs). Camphene and α-pinene are both common, non-specific plant VOCs that have a wide range of semiochemical activity,[13–15] but neither has been reported for activity against NOW. The remaining monoterpenes are ubiquitous as plant VOCs, and several have been noted as semiochemicals.[14]

The spiroketal conophthorin (7-methyl-1,6-dioxaspiro[4.5]decane), in unknown configuration, was also observed to undergo a small increase in relative amounts in several of the DMG almond volatile analyses. Conophthorin is present in several insects and plants and in varying concentrations of isomers (Fig. 2).[16]

The sesquiterpenes bourbonene (as a mixture with benzaldehyde), β-copaene and aromadendrene also increased in relative amounts in the DMG almonds. These particular sesquiterpenes have been noted to occur together in potato leaf VOCs.[17] Bourbonene and β-copaene are pheromones for the European birch aphid,[18] and aromadendrene has been reported to be an attractant for the Brazilian eucalyptus brown looper.[15] However, none of the noted sesquiterpenes has been implicated as possessing activity against NOW.

The only compounds to demonstrate corroboration of previous reports of almond VOCs were 2-pentylfuran, nonanal, 1-octen-3-ol, benzaldehyde and 2-phenylethyl alcohol.[11,19] Notable differences between the work performed by Buttery *et al.*,[11] which evaluated VOCs from almond hulls, and the VOCs collected in the present study were the detection here of numerous four-carbon ester and ketone as well as alcohol derivatives. Specific examples were the compounds that also showed a general increase in amounts between the CTRL and DMG almond VOCs, namely 2-butanol, ethyl 2-methylbutyrate, ethyl isovalerate, ethyl 2-butenoate, ethyl 3-methylbut-2-enoate, ethyl tiglate and 3-hydroxy-2-butanone. Several of these VOCs have been attributed to fruity, wine aroma and smoky odours[20,21] and are known semiochemicals,[22–24] yet are not associated with NOW semiochemicals. The compounds that demonstrated statistically valid increases were ethyl 2-methylbutyrate, 2-methyl- and 3-methyl-1-butanol, ethyl tiglate and β-copaene (Fig. 3), in addition to one unknown compound.

Several compounds in Table 1 were noted to be indicative of fungal growth. Of particular interest were 2-methyl- and 3-methyl-1-butanol and 2-pentyfuran owing to their relatively large amounts. The butanol

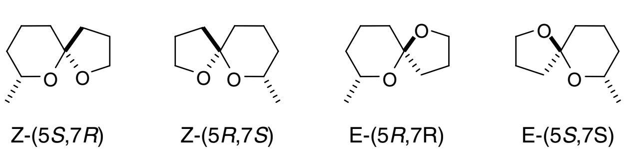

Figure 2. Stereoisomers of conophthorin (7-methyl-1,6-dioxaspiro [4.5]decane).

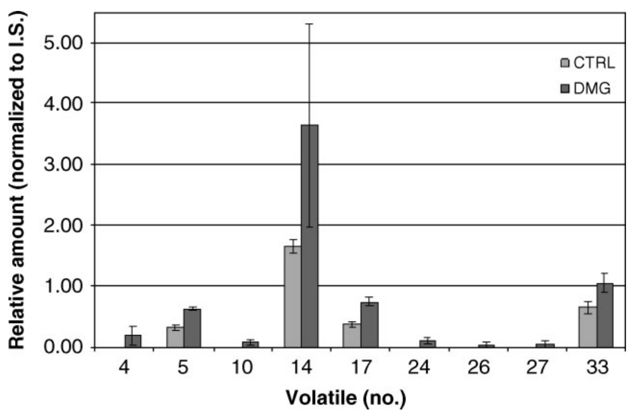

Figure 3. VOCs showing a statistically significant increase (95% confidence limit) in DMG almonds.

Table 1. Major volatile components of Monterey (MO) damaged (DMG) and control (CTRL) almonds[a]

No.	Compound	RI[c]	RI (PMR)[d]	RI (lit.)	Relative amount[b] MO CTRL	Relative amount[b] MO DMG
1	α-Pinene	1016	1020	1014	2.21 (1.42)	1.78 (0.74)
2	2-Butanol	1032	1027	1019, 1025	0.24 (0.12)	0.32 (0.20)
3	Ethyl butyrate	1037	1029		0.51 (0.37)	0.59 (0.35)
4	Unknown	1044			ND	0.19 (0.26)
5	Ethyl 2-methylbutyrate	1053	1046		0.32 (0.09)	0.62 (0.04)
6	Camphene	1058	1063	1048	2.27 (1.24)	1.98 (0.92)
7	Ethyl isovalerate	1067	1062		2.57 (0.25)	3.34 (2.10)
8	β-Pinene	1092	1106	1088, 1094	0.36 (0.14)	0.32 (0.12)
9	Diethyl carbonate	1100	1101		0.53 (0.67)	ND
10	3-Pentanol	1111	1103	1108	ND	0.08 (0.08)
11	Ethyl 2-butenoate	1158	1158		0.14 (0.25)	0.20 (0.27)
12	β-Myrcene	1160	1157	1154, 1159	0.77 (0.04)	0.56 (0.13)
13	Limonene	1188	1197	1182, 1195	1.06 (0.36)	0.80 (0.18)
14	3-Methyl- and 2-methyl-1-butanol	1207	1205	1190	1.66 (0.19)	3.63 (2.95)
15	Ethyl 3-methylbut-2-enoate	1219			0.16 (0.28)	0.23 (0.30)
16	2-Pentylfuran	1227	1226	1220, 1224	1.81 (0.04)	0.93 (0.38)
17	Ethyl tiglate, ethyl hexanoate	1231	1232		0.38 (0.07)	0.74 (0.14)
18	1-Dodecene	1243	1242		0.69 (0.75)	ND
19	Styrene	1247	1252		0.30 (0.02)	0.52 (0.56)
20	3-Octanone	1249	1251		Tr	0.12 (0.11)
21	Cymene isomer (*para*- 1264)	1261	1250		0.16 (0.04)	0.11 (0.10)
22	3-Hydroxy-2-butanone	1274	1278		1.83 (0.76)	2.55 (1.85)
23	Conophthorin	1280			0.34 (0.26)	0.79 (0.88)
24	Unknown	1286			ND	Tr
25	*E*-4,8-Dimethyl-1,3,7-nonatriene	1301	1302		0.11 (0.10)	0.15 (0.04)
26	Chalcogran isomer #1	1343			ND	Tr
27	Chalcogran isomer #2	1348			ND	Tr
28	Nonanal	1387	1389	1390, 1400	0.30 (0.36)	ND
29	Tetradec-1-ene	1444	1446		1.02 (1.77)	ND
30	1-Octen-3-ol	1451	1448	1428, 1446	0.28 (0.06)	0.42 (0.12)
31	Bourbonene/benzaldehyde mix	1505	1516		0.21 (0.07)	0.47 (0.66)
32	*trans*-α-Bergamotene	1577	1582		0.19 (0.19)	0.13 (0.02)
33	β-Copaene	1582	1589		0.64 (0.20)	1.04 (0.27)
34	Aromadendrene	1606		1605	0.11 (0.18)	0.24 (0.12)
35	1-Hexadecene	1645	1647		0.63 (1.09)	ND
36	Ethyl benzoate	1654	1661		0.51 (0.60)	0.18 (0.20)
37	1-Methyl-2-pyrrolidinone isomer	1662			0.60 (0.09)	0.55 (0.22)
38	Cyclodecanone[e]	1726	1744		15.00 (0.00)	15.00 (0.00)
39	Unknown	1870			0.58 (0.28)	0.53 (0.21)
40	2-Phenylethyl alcohol	1899	1910	1848	0.16 (0.02)	0.42 (0.62)
41	1-Dodecanol	1968			0.37 (0.13)	0.18 (0.17)
42	2-Phenoxyethanol	2126	2142		0.21 (0.06)	0.14 (0.12)
43	Docosane	2187	2200		0.52 (0.12)	0.30 (0.29)
44	Unknown	2264			0.17 (0.03)	Tr
45	Unknown	2279			0.56 (0.28)	0.53 (0.33)
46	Unknown	2471			0.18 (0.04)	0.21 (0.21)
47	Vanillin	2541	2585		1.88 (0.57)	0.72 (0.68)

[a] Almonds collected on three different days.
[b] Volatile amounts reported as mean, normalised to 15 µg of internal standard, with standard deviation in parentheses; ND, not detected; Tr, trace amount (<0.10 µg).
[c] Retention index relative to *n*-alkanes on DB-Wax column.
[d] RI of volatile compounds based on in-house database.
[e] Internal standard.

VOCs increased in amounts from CTRL to DMG, while 2-pentylfuran decreased between these two experiments. The VOCs noted to occur during fungal growth, particularly *Aspergillus* species, are 2-methyl- and 3-methyl-1-butanol, 2-pentylfuran, 1-octen-3-ol and 3-octanone.[25,26] In addition to its previously reported occurrence in almonds,[11,19] it should be noted that 1-octen-3-ol is also a plant volatile of numerous plants, including genera of the Orchidaceae, as well as a semiochemical for several different

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DOI: 10.1002/jsfa

insects. 2-Pentylfuran is also an Orchidaceae plant volatile, but to a much lesser extent (The Pherobase, www.pherobase.com, accessed 22 August 2007). The eight-carbon VOCs, in addition to the sesquiterpenes myrcene, limonene and copaene, have been reported to be produced by *Penicillium* species.[27] Moreover, sesquiterpene VOCs unique to *A. flavus*[28] were not found in this study, thus indicating the possible presence of *Penicillium* more so than *Aspergillus*, yet this information did not provide enough evidence to exclusively implicate one particular microbe. Both *Aspergillus* and *Penicillium* are known to be present on almonds.[29]

The compounds unique, albeit only present in trace amounts, to the DMG almond VOCs were 3-pentanol, two chalcogran isomers (Fig. 4) and one unknown compound (No. 24, Table 1). 3-Pentanol is relatively new as a semiochemical, with only one study that demonstrated its ability to provoke a response in the male sugarcane weevil.[30] Interestingly, the same study reported ethyl butyrate, among other esters, as eliciting an antennal response in the female sugarcane weevil. The chalcogran isomers, however, have a long history of semiochemical activity, primarily with the European spruce bark beetle *Pityogenes chalcographus*.[31,32] The (2*S*,5*R*) and (2*S*,5*S*) configurations of chalcogran are found in *P. chalcographus*, and as two isomers with unknown configurations in the bark beetle *Pityogenes quadridens*.[33] It is interesting to note that Byers *et al*.[34] used combinations of chalcogran, camphene and α- and β-pinene, all VOCs detected in DMG almond VOCs, along with the compound methyl-*E*,*Z*-2,4-decadienoate to enable host recognition of the bark beetle. Other correlations between DMG almond VOCs and semiochemicals from bark beetles are similar VOCs, among others, emitted from *Ips typographus* males under stress, namely α- and β-pinene, camphene, myrcene, limonene and cymene, and similar VOCs from *Pityogenes* species, namely limonene, chalcogran, 1-octen-3-ol and 2-phenylethyl alcohol.[35] The occurrence of the chalcogran isomers in this and the one associated previous study[6] does not conclusively determine whether the spiroketals are emitted as a result of damage to the almonds or formed by fungal growth. The detection of several VOCs indicative of fungal growth brings into question whether or not the method of removing the DMG almonds after several days on the tree and subsequent transportation to the laboratory allows ambient fungi to initiate growth on the almonds. Investigations into this matter are ongoing.

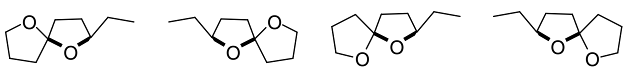

Figure 4. Stereoisomers of chalcogran (2-ethyl-1,6-dioxaspiro [4.4]nonane).

CONCLUSION

The VOC emissions of control and damaged almonds were investigated. VOCs unique to damaged almonds include 3-pentanol and two isomers of the spiroketal chalcogran (unknown configuration) in trace amounts. Other VOCs that increased in relative quantity include the spiroketal conophthorin (unknown configuration), numerous four-carbon ester and ketone as well as alcohol derivatives, in addition to two eight-carbon chain compounds. VOCs suggestive of fungal growth were noted and brought to question whether the chalcogran isomers are damage-induced or a result of fungal growth. Also notable was the apparent correlation between several bark beetle semiochemicals and VOCs from the CTRL and DMG almonds. The detection of the VOCs noted above provides evidence that further investigation into their role in NOW response to damaged almonds is required.

ACKNOWLEDGEMENTS

This research was conducted under USDA-ARS CRIS project 5325-42000-036-00 and a cooperative research and development agreement with Paramount Farming Company (CRADA #58-3K95-7-1198).

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J Sci Food Agric **88**:1363–1368 (2008)
DOI: 10.1002/jsfa