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Volatile organic compounds associated with postharvest fungi detected in stored wheat grain

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Abstract Stored wheat grain from across Australia was tested for the presence of postharvest fungi by measuring the production of volatile organic compounds (VOCs). Headspace analysis combined with gas chromatography flame ionization detection (GC/FID) and gas chromatography-mass spectrometry (GC-MS) was used to detect fungi in the grain, based on identification of peaks associated with species such as *Alternaria alternata* and *A. infectoria*. A total of 57 possible compounds were detected by GC/FID. Grain from Queensland, Victoria and New South Wales produced less VOCs compared to grain from South Australia and Western Australia. These compounds appeared at both the early and later stages of fungal growth. For example, *A. alternata* produced volatiles after two days that were only produced by *A. infectoria* after seven days. Gamma-irradiated grain (controls) did not produce these VOCs. A further analysis of the 57 VOCs by GC–MS identified three VOCs: Cyclooctasiloxane, hexadecamethyl-(CAS 556–68-3) produced by *A. alternata*, and Pentadecane (CAS 629–62-9) produced by *A. infectora*. We conclude that fungal VOCs may be useful for early detection of postharvest fungi in grain, prior to visual symptoms occurring.

Keywords *Triticum aestivum* L. · Mould · Chemical detection · Solid phase microextraction · Gas chromatography mass spectrometry

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Introduction

Postharvest fungi in cereal grain destined for human consumption or animal feed can lead to considerable spoilage, manifested through grain weight and nutritional losses, production of mycotoxins, potentially allergenic spores and unpleasant odours (Filtenborg et al. 1996). We recently reported the presence of 15 fungal genera in stored wheat grain in Australia, some of which may pose a risk to human health, such as *Cryptococcus* and *Fusarium* (Barkat et al. 2016). Early detection of postharvest fungi is crucial to avoid contamination of grain.

Analysis of volatile organic compounds (VOCs) by gas chromatography is a particularly promising technique for grain samples as it can reduce the time required to detect the presence of contaminated and mouldy grain, from days to a few hours or even minutes (Girotti et al. 2012). Detection is based on volatile compounds produced by the fungi, such as alcohols, esters, ketones, and mono- and sesquiterpenes and aldehydes.

There are a range of methods that can be used for the early detection of VOCs produced by fungi, as well as other microorganisms or insects in grain. For example, gas chromatography–mass spectrometry (GC-MS) can be used to identify tkey volatile compounds produced by known spoilage fungi (Schnürer et al. 1999). Previous studies have also demonstrated that it is possible to use headspace analyses combined with a gas chromatography to accurately quantify the VOCs produced by fungi in stored wheat, including *Penicillum verrucosum*, *Aspergillus ochraceus* and *A. carbonarius* (Sahgal et al. 2007; Börjesson et al. 1994; Jelen et al. 2003).

The aim of this study was to determine if specific VOCs produced by different fungal species could be used to determine the presence of fungi in wheat grain during storage. The hypothesis was that VOCs produced by storage fungi can be used

for early detection of fungal contamination in stored wheat grain, prior to the development of visible signs and symptoms.

Material and methods

Preparation of farm samples

Twenty-three samples of wheat grain were collected from selected farms from across Australia (previously described by Barkat et al. (2016), plus one additional farm in Queensland) and each was divided into two sub-samples for VOC analysis – comprising the original grain that had its moisture content determined on arrival, and grain that was adjusted to 15% moisture. The moisture content was adjusted by adding a calculated volume of distilled water into a sealed flask (3 L) at 25 °C for 1 week, and confirmed with an electronic moisture meter (Graintec HE 50 electronic moisture meter, Graintec Pty Ltd., Toowoomba, Australia). The control was wheat grain obtained from Cooperative Bulk Handling, Western Australia, and adjusted to either 11% or 15% moisture content, before being gamma-irradiated for one week at 30, 000 GY and then a small sample placed on PDA for 5–7 days to confirm sterility. There were two replicate flasks per farm subsample to give a total of 46 flasks each of the original grain and moisture-adjusted grain, and four control flasks (two at each moisture content).

Preparation of spiked wheat samples

Ten fungal species previously isolated from stored wheat, including four isolates of *Alternaria*, three isolates of *Cladosporium*, two isolates of *Penicillium*, one isolate of *Aureobasidium,* plus an isolate of *Fusarium graminearum* (WAC 11387) known to produce mycotoxins (Tan et al. 2011) obtained from the Department of Agriculture and Food Western Australia were used to inoculate wheat grain (Table 1). The 11 isolates were grown on PDA at 25 °C for 7 days in the dark before 5 plugs of 5 mm^2 in size were taken from the edge of the colonies and used to inoculate sterile, gamma-irradiated grain (adjusted to 15% moisture content) and the flasks were incubated at 25 °C in the dark for 1, 2, 3, 7 or 10 days prior to the VOCs being analysed. The control was gamma-irradiated grain at 15% moisture content inoculated with plugs of sterile PDA. There were two replicates for each isolate to give a total of 22 flasks, with 10 flasks as controls.

Extraction of volatile organic compounds

Each grain sample (50 g) was placed into a 100 mL Erlenmeyer flask (Fisher Scientific, Quickfit, UK; Cat. NoFE 100/3) equipped with a cone/screw-thread adapter (Crown Scientific, Code ST 5313) with a 7/16″ blue septum (Grace Davison Discovery Sciences, Cat. No. 6518). The flasks were placed in a water bath at 45 °C for 3 h incubation, prior to extracting of the volatiles. Two fibres (SPME fibre 50/30 μm polydimethylsiloxane (PDMS; Cat. No. 57348-U from Analytical Sigma-Aldrich), selected according to Qiu et al. (2014a) and conditioned prior to use in accordance with the manufacturer's recommendations were then inserted into the headspace of the flasks containing the samples for three hours. The fibre holder was then removed from the extraction flask and inserted into the injection port of the GC-FID. The fibre was extended into a GC-FID inlet where sample components were desorbed.

Table 1 Fungal isolates used to inoculate sterile, gamma-irradiated grain for volatile production

Species	WAC Code	Region
Alternaria alternata	13842	NSW
A. infectoria	13850	WA
A. infectoria	13852	NSW
A. infectoria	13851	WA
Auerobasidium sp.	13824	WA
Cladosporium herbarum	13833	WA
C. cladosporioides	13838	WA
C. cladosporioides	13841	NSW
Fusarium graminearum	11387	WA
Penicillium cordubense	13831	SA
P. dipodomyicola	13832	NSW

Sampling regions: Western Australia (WA), South Australia (SA), New South Wales (NSW), Victoria (VIC), and Queensland (QLD)

Optimisation of measurement conditions

Optimal headspace solid-phase microextraction gas chromatography conditions following SPME parameters were used according to Qiu et al. (2014a). Briefly, for the optimisation of the standards (C15 and custom retention time index standard), the appropriate volumes of each standard were added into a sealed 250 mL bottle. After 5 min extraction with the fibre 50/30 μm DVB/CAR/PDMS at room temperature (24 ± 1 °C), the fibre was injected into the gas chromatograph at a temperature of 250 °C for desorption of >3 min.

Gas chromatography-flame ionization detector (GC-FID) analysis

VOC analysis was performed on an Agilent 6890 Gas Chromatograph manufactured by Agilent Technology (Palo Alto, CA, USA) and a Flame Ionization Detector (FID; Hewlett Packard 6890 series) was used to analyse the volatile

Table 2 Volatile organic compounds (VOCs) detected by GC-FID in the headspace of samples from farm grain at original moisture content collected from five states across Australia

RT	WA1 10%	WA2 11%	WA3 11%	WA4 9%	WA5 10%	WA6 10%	WA7 10%	WA8 9%	SA1 11%	SA2 10%	SA3 9%	SA4 13%	SA5 9%	SA6 9%	NSW1 11%	NSW2 11%	NSW3 12%	NSW4 14%	NSW5 11%	VIC2 12%	VIC2 12%	VIC3 12%	QLD 10%	Control
13.24	−	−	−	+	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	+	−
15.84	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−
29.85	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
40.89	−	−	−	−	−	−	−	+	−	+	+	+	+	+	−	−	−	−	−	−	−	−	−	−
40.96	−	−	−	−	−	−	+	+	−	−	−	−	−	−	−	−	+	−	+	+	−	−	−	−

RT, retention time; VOCs produced by original grain (+) or not detected (−).Sampling regions: Western Australia (WA), South Australia (SA), New South Wales (NSW), Victoria (VIC), and Queensland (QLD)

Table 3 Volatile organic compounds (VOCs) detected by GC-FID in the headspace of samples from original farm grain adjusted to 15% moisture content from five states across Australia

RT	WA1	WA2	WA3	WA4	WA5	WA6	WA7	WA8	SA1	SA2	SA3	SA4	SA5	SA6	NSW1	NSW2	NSW3	NSW4	NSW5	VIC1	VIC2	VIC3	QLD	Control
10.05	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−
13.24	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
15.84	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
26.4	−	−	−	−	+	+	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−
40.89	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	+	−
40.96	−	−	−	−	−	−	−	−	−	+	+	−	−	+	−	−	−	−	−	−	−	+	−	−

RT, retention time; VOCs produced by grain at 15% (+) or not detected (−)

Sampling regions: Western Australia (WA), South Australia (SA), New South Wales (NSW), Victoria (VIC), and Queensland (QLD)

Table 4 Chemicals putatively detected by GC-FID in the headspace of samples from original farm grain and farm grain adjusted to 15% moisture content

RT (min)	Possible chemicals	CAS No	Formula
10.05			
	(E,E)-2,4- Hexadienal	142–83-6	C_6H_8
	2,4-Dimethyloctane	4032–94-4	$C_{10}H_{22}$
	2-Ethylpyridine	100–71-0	C_7H_9N
	4,5-Dimethylthiazole	3581–91-7	C_5H_7NS
	Heptanal	111–71-7	$C_7H_{14}O$
13.24			
	(4-Fluorophenyl)-methanamine	659–41-6	C_7 H_8 F N. Cl H
	1,3,5-trimethylbenzene	108–67-8	$C_6H_3(CH_3)3$
	(E,Z)-2,4-Heptadienal	4/02/4313	$C_7H_{10}O$
	1-Isopropenyl-4-methylenecyclohexane	499–97-8	$C_{10}H_{18}$
	3-Methylbutyl 2-methylpropionate	3/01/2050	$C_9H_{18}O_2$
	Benzaldehyde	100–52-7	C_7H_6O
	Butyric acid	107–92-6	$C_4H_8O_2$
	Ethyl 2-(methylthio)-acetate	4455–13-4	$C_5H_{10}O_2S$
	Ethyl 2-bromobutyrate	66,025–42-1	$C_6H_{11}BrO_2$
	Ethyl hexanoate	123–66-0	$C_8H_{16}O_2$
	Ethyl propyl disulfide	30,453–31-7	$C_5H_{12}S_2$
	Isopropyl trichloroacetate	3974–99-0	$C_5H_7C_{l3}O_2$
	Methyl 4-methylhexanoate	2177–82-4	$C_8H_{16}O_2$
	tert-Butyl 3-chloropropanoate	55710–80-0	$C_7H_{13}ClO_2$
15.84			
	1,2,4-Trithiapentane	-	$C_2H_6S_3$
	2,3,4-Trimethylfuran	10599–57-2	$C_7H_{10}O$
	2-Ethyl-3-methylpyrazine	15707–23-0	$C_7H_{10}N_2$
	4-Methylphenol	106–44-5	C_7H_8O
	4-Penten-2-yl bromoacetate	-	$C_7H_{11}BrO_2$
	4-tert-Butyltoluene	98–51-1	$C_{11}H_{16}$
	6-Ethyl-2-vinylpyrazine	32736–90-6	$C_8H_{10}N_2$
	6-Ethyl-3-hydroxy-2-methyl-4H-pyran-4-one	8/11/4940	$C_8H_{10}O_3$
	6-Methyl-3,5-heptadien-2-one	1604–28-0	$C_8H_{12}O$
	Benzyl formate	104–57-4	$C_8H_8O_2$
	tert-Pentylbenzene	2049–95-8	$C_{11}H_{16}$
26.4			
	(Z)-3-Dodecen-1-ol	32451–95-9	$C_{12}H_{24}O$
	2,3,6,7,8,8alpha-Hexahydro-1,4,9,9-tetraethyl-1H-3alpha,7-methanoazulene	560–32-7	$C_{15}H_{24}$
	2beta-Hydro-6alpha-methoxy-trans-decalin	-	$C_{11}H_{20}O_2$
26.4			
	3,4,5-Trichloroanisole	54135–82-9	$C_7H_5Cl_3O$
	6-Methyl-1,2,3,4,5,6-hexahydro-(7H)- cyclopentapyridine-7-one	-	$C_9H_{15}NO$
	Acora-4,10-diene	-	$C_{15}H_{24}$
	Cadina-3,5-diene	-	$C_{15}H_{24}$
	Dimethyl 2,5-dichloro-4-bromo-3-oxopentanoate	-	C7H8BrCl2O_3-
	Furan-2-carbaldehyde	98–01-1	$C_5H_4O_2$
	Muurola-4,11-diene	-	$C_{15}H_{24}$
	p-Chlorobenzylidene-propyl-amine	-	$C_{10}H_{12}$ CIN
	p-Ethyl-dimethyl hydrocinnamic aldehyde	-	$C_{13}H_{18}O$

Table 4 (continued)

RT (min)	Possible chemicals	CAS No	Formula
29.85			
	(Z)-2,6-Dimethoxy-4-(prop-1-enyl)-phenol	-	$C_{11}H_{14}O_3$
	(Z)-5-Tridecen-2-yl acetate	-	$C_{15}H_{28}O_2$
	11-Dodecenyl acetate	35153–10-7	$C_{14}H_{26}O_2$
	2beta-Hydroxy-6alpha-cyanide-trans-decalin	-	$C_{11}H_{17}NO$
	Benzophenone	9133–55-5	$C_{13}H_{10}O$
	Cedrenol	28231–03-0	$C_{15}H_{24}O$
	Globulol	51371–47-2	$C_{15}H_{26}O$
40.89			
	(E)-14-Hexadecen-1-ol	-	$C_{16}H_{32}O$
	(E)-2,5-Dimethyl-3-styrylpyrazine	-	$C_{14}H_{14}N_2$
	1-Methyl-4-(1-hydroxy-1-methylethyl)-benzene	1197–01-9	$C_{10}H_{14}O$
	7,12-Dimethylhexadecane	-	$C_{18}H_{38}$
	7,12-Dimethyloctadecane	-	$C_{20}H_{42}$
	N,N-Dimethyl-N-octyl-benzamidine	-	$C_{17}H_{28}N_2$
40.96			
	(Z)-9-Methylhexadecenoate	-	$C_{17}H_{32}O_2$
	9-Tigloylretronecine	-	$C_{13}H_{19}NO_3$

RT, retention time

profiles extracted by HS-SPME. The columns used in this experiment were a Stabilwax® polar column (Dimensions: 30 m × 0.25 mm × 0.25 μm film thickness, ZB-WAX, Cat. No. #10623) and an Rxi®-5 ms non-polar column (Dimensions: 30 m × 0.25 mm × 0.25 μm film thickness, RESTEK, Cat. No. #13423). Helium was used as the carrier gas at a constant speed of 40 mL min^{-1} in the split-less mode. The column temperature program was set at 45 °C for 5 min, then increased by 5 °C min^{-1} to 250 °C and held for 5 min and the GC-FID instrument was operated under the split-less mode. The helium inlet pressure was controlled at a constant flow of 1 mL min^{-1}. The ionization potential was set at 70 eV, and scanning was performed from 35 to 500 amu at a rate of 3.15 scans sec^{-1}. The retention Kovates-index (Kovats 1958) was calculated by using Pherobase (i.e., the relative retention values based on a scale defined by the elution of a series of n-alkanes) obtained freely online from the website www.pherobase.com/database/kovats/kovats-index.

Table 5 Volatile organic compounds detected by GC-FID from 15% moisture content grain spiked with *Alternaria alternata* WAC13842 or one of three isolates of *A. infectoria* (WAC11850, 113851 or 113852) from 1 to 7 days post-inoculation. There were only three retention times detected in the spiked grain samples, and their time of detection (post-inoculation) varied among isolates

RT	Kovats index	*A. alternata*	*A. infectoria*			Control
		WAC 13842	WAC 13850	WAC 13851	WAC 11852	
13.24	996	1,2,7	7	-	-	-
15.84	1076	7, 10	1,2,3,7,10	-	1,2	-
26.4	1076	1,2,3	-	-	7,10	-

RT, retention time; (−) not detected; Numbers indicate days on which detected

Gas chromatography-mass spectrometry (GC-MS) analysis

In addition to the VOC analysis using GC-FID, GC-MS was used to identify specific compounds produced by two species of *Alternaria* (*A. infectoria* WAC13850 and *A. alternata* WAC13842) and *Cladosporium herbarum* (WAC13833). These three isolates were selected because they were previously recovered frequently from wheat samples (Barkat et al. 2016). An Agilent 6890 gas chromatograph equipped with the 30 m × 0.25 mm × 0.25 μm Stabilwax® polar column that was coupled to an Agilent 5973 Network mass selective detector with an Agilent ChemStation was used to identify the separated VOCs. The column temperature program was set the same as GC-FID. The injection port (splitless mode), interface and MS source temperature were 250 °C and 230 °C, respectively. The volatiles were identified by comparison of the mass spectrum (peaks) with the NIST08 mass spectra library together with retention indices (NIST08 mass spectral search program for the NIST/EPA/NIH mass spectral library version 2.0F), built in 2008).

Table 6 GC-FID volatile organic compounds detected by GC-FID from 15% moisture content grain spiked with *Aureobasidium* sp., *C. herbarum*, *C. cladosporioides*, *F. graminearum*, *P. cordubense* and *P. dipodomyicola* colonized grain at 15% moisture content. There were only five retention times detected in the spiked grain samples, and their time of detection (post-inoculation) varied among isolates

RT (min)	Kovats index	*C. herbarum*	*C. cladosporioides*		*P. cordubense*	*P. dipodomyicola*	*Aureobasidium* sp.	*F. graminearum*
		WAC 13833	WAC 13838	WAC 13841	WAC 13831	WAC 13832	WAC13824	WAC11387
10.05	906	-	1	10	-	-	-	-
20.1	1217	-	-	-	1,2,3,7,10	1,2,3,7	-	-
29.85	1604	2,3,7	-	2	-	-	-	-
40.89	1887	-	-	-	1,3,7,10	1,2	-	-
40.96	1888	2	-	2	-	10	1,2,3,7	1,2,3,7,10

RT, retention time; (−) not detected; Numbers indicate days on which detected

Statistical analysis

Differences between farm sample and spike sample VOCs were identified using GCALIGNER 1.0 and GCKOVATS according to Dellicour and Lecocq (2013). The alignment algorithm was based on the comparison between the retention times of each detected compound in a sample. Specific volatiles were identified by comparison of the mass spectrum with the NIST08 mass spectra library together with retention indices.

Results

Volatile organic compounds detected from farm samples

The 23 original farm samples varied in the production of volatiles, between and within each state (Table 2). Individual farm samples from Western Australia (WA), Queensland (QLD) and South Australia (SA) produced peaks at 13.24 min. Grain from one SA farm produced a specific peak at 15.84 min and similarly grain from one WA farm produced a peak at 29.85 min. One VOC at 40.89 min was identified in one farm from WA and five farms from SA, and at 40.96 min there was a peak found in two farm samples from WA, two from New South Wales (NSW) and one from Victoria (Vic). There were no volatiles found on gamma-irradiated grain.

When the farm samples were adjusted to 15% moisture content and re-measured, unique peaks specific to individual farms were found from grain from QLD (at 10.05 min) and one WA farm at 13.24 and 15.84 min (Table 3). At 26.4 min two farms from WA and one from NSW shared a peak. Another shared VOC was produced in the headspace of one SA farm and the QLD farm sample at 40.89 min. Three samples of grain from SA and one from Victoria produced a specific peak at 40.96 min. Gamma-irradiated wheat did not produce any unique peaks.

A total of 57 possible chemicals were identified in original grain and grain adjusted to 15% moisture content based on the peaks (retention time) (Table 4).

Volatile organic compounds detected from spiked wheat samples

Three specific peaks were produced in the samples spiked with the two *Alternaria* species, which varied in the time of detection (Table 5). *A. alternata* produced a peak at 13.24 min at 1, 2 and 7 days post inoculation (dpi) and *A. infectoria* (WAC13850) produced the same peak after 7 dpi. A second specific peak was produced at 15.84 min after 7 dpi by *A. alternata* and the same peak was identified in two of the *A. infectoria* isolates (WAC13850 and WAC13852) from 1 dpi. A third peak at 26.4 min was detected in the headspace

Table 7 Volatile organic compounds identified using gas chromatography-mass spectrometry (GC-MS) analysis from irradiated grain spiked with *Alternaria alternata, A. infectoria* and *Cladosporium herbarum*

Peak area %	*Alternaria alternata*	*Alternaria infectoria*	*Cladosporium herbarum*	Likely Compound	Match quality (%)
	WAC13842	WAC13850	WAC13833		
1.16	+	−	−	Cyclooctasiloxane, hexadecamethyl-	93.3
2.03	−	+	−	Pentadecane	48.1
0.10	−	−	+	Naphthalene	45.5

Volatiles detected (+) or not detected (−)

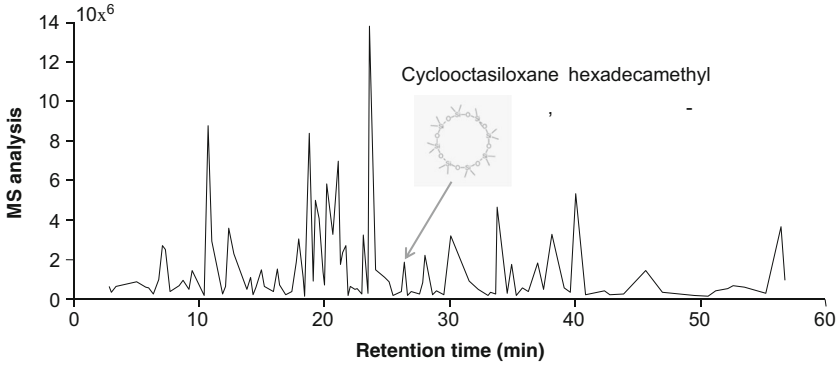

Fig. 1 Chromatogram of GC-MS of the specific VOC Cyclooctasiloxane, hexadecamethyl- from gamma-irradicated grain samples colonised by *Alternaria alternata* (WAC13842)

of *A. alternata* after only 1 dpi and also in *A. infectoria* WAC 13852 at 7 dpi. *A. infectoria* isolate WAC13851 did not produce any unique peaks and neither did the controls.

Five specific peaks were detected across the three isolates of *Cladosporium*, two isolates of *Penicillium* and one isolate each of *Aureobasidium* and *Fusarium* (Table 6). At 10.05 min, a peak unique to *C. cladosporioides* was detected in both isolates, but nine days apart. The two *Penicillium* isolates both had a peak at 20.1 min that was present from 1 dpi until at least 7 dpi, and both also had a second peak at 40.89 min at 1 dpi. Similarly two of the three *Cladosporium* isolates produced a specific peak at 29.85 and also 40.96 at 2 dpi. The peak at 40.96 min was also found to be common to *Penicillium dipodomyicola, Aureobasidium* sp. and *Fusarium graminearum* but varied in the time of detection from 1 dpi to 10 dpi. (Table 6).

Correlation between farm samples and spiked samples

Using GC Aligner it was determined that some peaks were common to the spiked samples and those in the farm samples. For example, samples spiked with *Cladosporium cladosporioides* produced a specific peak at 10.05 min and this peak was also observed in the original farm sample from QLD. A specific peak in grain spiked with *A. alternata* and *A. infectoria* (WAC13850) at 13.24 min, was observed in the original farm samples from WA and the grain adjusted to 15% from WA, SA and QLD farms, and peaks commonly produced by all *Alternaria* species at 15.84 min and 26.4 respectively were observed in original grain from WA farms and grain from SA farms adjusted to 15%. *Penicillum* species produced a specific peak at 40.89 min, and this was observed in grain from WA and SA farms on arrival and in grain adjusted to 15% from SA and QLD farms. A common peak at 40.96 min in grain spiked with *Cladosporium* spp., *Aureobasidium* sp., *F. graminearum* and *P. dipodomyicola* was observed in original grain from SA, QLD and VIC farms, and grain adjusted to 15% from WA, NSW and VIC farms.

GC-MS identification of compounds from *Alternaria alternata*, *A. infectoria* and *Cladosporium herbarum*

Based on GC/FID results, the volatiles produced by three fungal species (*A. alternata* WAC13842, *A. infectoria* WAC13850 and *C. herbarum* WAC13833) were further characterised by comparing mass spectra with library spectra and determining chromatographic retention indices (Table 7). The largest peak in the headspace of *A. alternata* was idenitifed as Cyclooctasiloxane, hexadecamethyl- (Fig. 1). Pentadecane was produced by *A. infectoria* (Fig. 2) and was unique to this fungus. Naphthalene was only identified in the headspace of *C. herbarum* (Fig. 3).

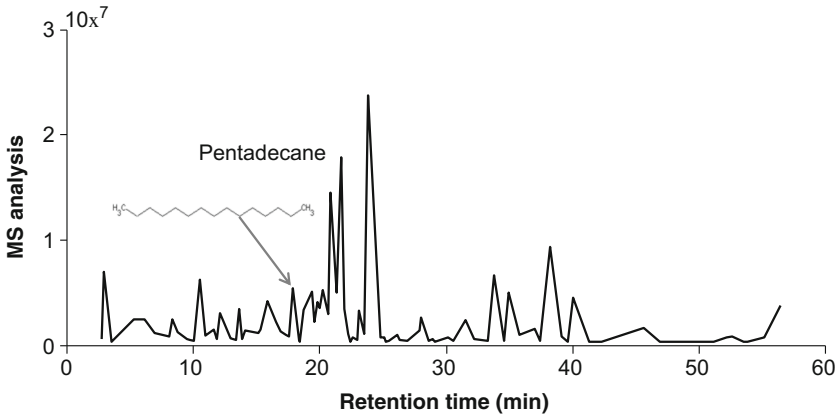

Fig. 2 Chromatogram of GC-MS of the specific VOC Pentadecane from gamma-irradicated grain samples colonised by *Alternaria infectoria* (WAC13850)

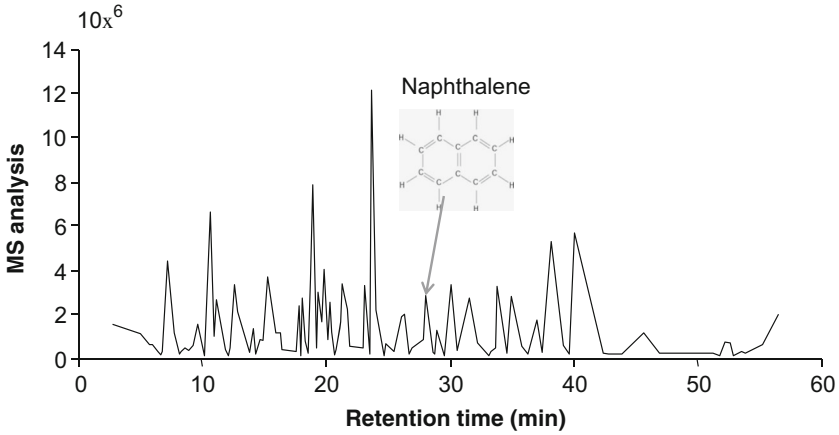

Fig. 3 Chromatogram of GC-MS of the specific VOC Naphthalene(c) from gamma-irradicated grain samples colonised by *Cladosporium herbarum* (WAC 13833)

Discussion

The results of this study confirm the hypothesis that VOCs can be used to determine the presence of fungi in stored wheat grain at an early stage of development. This study found that volatiles could be detected in wheat sampled from farms across Australia, however grain from QLD, VIC and NSW produced less VOCs compared to SA and WA. This result is contrary to expectations, given the diversity of fungi we previously detected in the different states of Australia (Barkat et al. 2016).

The difference in VOCs between states was possibly due to different climate conditions between regions (e.g. temperature or rainfall patterns), which would impact on growth of fungi and in turn on the production of VOCs. In general, it would be expected that the temperature and rainfall would affect the production of metabolites by storage fungi (e.g. as reported by Paolesse et al. 2006 and Magan and Evans 2000). Future studies should focus on the identification of mould species and their associated VOCs on grain at the pre-harvest stage. When combined with climatic information from the different growing regions, this information could be used to develop predictive models of which fungi are likely to be present in stored grain, and their risk to human and animal health.

Another possible reason for the observed variation in VOC production between the states, could be due to different farm practices. Different grain storage and hygiene methods (e.g. drying, aeration and chemical controls of pests) used on farms could influence the growth of fungi (Darby and Caddick 2007) which would in turn affect the production of VOCs. Similarly, Paolesse et al. (2006) indicated that water activity is a critical factor in the pattern of a chemical profile, and Magan and Evans (2000) suggested that this could have a considerable impact on, and modify the significance of, individual volatile compounds produced by a specific fungal species. In the present study we found that fewer volatiles were produced on grain samples adjusted to 15% moisture content compared to those produced on original farm samples which suggested that the variation in number of volatiles produced me be due to the different moisture content of the farm samples. Another explanation could be due to different VOCs being produced in the headspaces, most likely due to the different fungal species present on the grain. De Lucca et al. (2012) reported that variation in the production of metabolites on corn can also be due to sampling variability, due to the distribution of contamination among the individual kernels. Wider sampling of Australian stored grain is required to confirm this.

There were some correlations between VOC peaks in grain samples from farms and the spiked grain samples. This finding suggests that there is relationship between the VOCs produced and the fungi colonising the grain, which might be useful to determine the presence of storage fungi in farm samples. Magan and Evans (2000) demonstrated that there are a range of VOCs produced by fungi when colonising cereal grain, including 3-methyl-1-butanol, 1-octen-3-ol and other 8-carbon ketones and alcohols. However, none of these volatiles were detected in the present study. Likewise, Börjesson et al. (1989) identified a number of volatiles produced in the headspace of wheat spiked with *Aspergillus amstelodami, Asp. flavus, Penicillium cyclopium, and Fusarium culmorum,* including 2-methylfuran, 2-methyl-I-propanol, and 3-methyl-I-butanol, which were not produced in the current study. The reason for the difference in the metabolites between this study and others could be due to the difference in methods used for collection of samples, extraction and analysis (Girotti et al. 2012). Although not included in the present study, in future studies it will also be useful to determine if there is a correlation between samples with VOCs and the climate where the sample is collected. However, to do this meaningfully will require more samples from each region, from different times of the year, and from different storage facilities.

There is significant interest in being able to detect fungal spoilage at an early stage in stored grain. In this study some

compounds appeared predominantly at the early stages of fungal growth, whilst others were only observed later. For example, *A. alternata* produced volatiles two days after inoculation that were only produced by *A. infectoria* seven days after inoculation. These results are consistent with Girotti et al. (2012) who reported the early detection of volatiles of *F. graminarum* in wheat after 48 h. Compounds that are produced after two days are potentially strong indicators of the presence of spoilage fungi on grain (Magan and Evans 2000). A study by de Lacy Costello et al. (2003) confirmed that sensor systems can be used to assess the early detection of *A. flavus*-inoculated wheat grain after 3 days. The present study indicated that it was possible to differentiate between the species tested, based on the volatile production patterns detected after two days. However, more detailed study is required on volatiles produced by more fungal species, and how VOCs compare to conventional methods for the rapid and early detection of fungal spoilage in grain.

Three fungal species were able to produce specific VOCs on sterile grain and they were Cyclooctasiloxane, hexadecamethyl- produced by *A. alternata*, Pentadecane produced by *A. infectora* and Naphthalene produced by *C. herbarum*. Pentadecane has also been reported in cultures of *Asp. flavus* on cracked corn (De Lucca et al. 2012) whilst Naphthalene was also shown to be produced by *Asp. niger* on wheat (Harris et al. 1986. Cyclooctasiloxane, hexadecamethyl- was also produced by *Aspergillus terreus* isolated from soil (Rajalakshmi and Mahesh 2014). Further studies are required to develop knowledge of the types and amounts of VOCs present during postharvest storage in Australia, and their significance. Further, more isolates of each species are required to confirm whether the VOCs are unique, and to confirm that they can be used to detect and quantify fungal growth in wheat stored on a larger scale.

It is clear that there are a range of volatile odours postharvest fungi can produce when colonising wheat grain that might be useful for the early detection of grain spoilage. Indeed, previous reports demonstrate that monitoring for volatiles might be a good early indicator of quality loss and mycotoxin formation in grain (De Lucca et al. 2012; Lippolis et al. 2014). As a consequence, there is an absolute need for early and efficient methods to detect infected grain and to distinguish between relevant and harmless species. The priority for the grain industry should be evaluating the occurrence of moulds in Australian grains produced under different conditions, to determine when various fungal species are present. Developing modern, fast and easy tools for identification of spoilage at early stages will allow significant losses and grain downgrading to be avoided.

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