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The role of fungi in the production of chloroanisoles in general purpose freight containers

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Samples of timber from the flooring of six general-purpose freight containers allegedly involved in the contamination of foodstuffs by 2,4,6-trichloroanisole were analysed for chlorophenol, chloroanisole and fungal content. All containers were found to be contaminated with 2,4,6-trichlorophenol, 2,3,4,6-tetrachlorophenol, pentachlorophenol and in all but one container their corresponding chloroanisoles. The concentrations of these compounds varied over a range of five orders of magnitude. In addition, 38 species of fungi were isolated from the timber. The species identified consisted of a variety of *Alternaria* (1), *Aspergillus* (7), *Cladosporium* (2), *Eurotium* (4), *Mucor* (1), *Paecilomyces* (1), *Penicillium* (16), *Phoma* (1), *Trichoderma* (4) and *Ulocladium* spp. No one species was found in all containers; however, *Penicillium chrysogenum* was isolated from five containers and six other species, *Eurotium amstelodami, E. repens, E. rubrum, Penicillium corylophilum, P. aurantiogriseum* and *Ulocladium* sp. were each found in three containers. Of the 38 species isolated, 19 are known to biomethylate chlorophenols in wood and pulped wood products. At least three species of fungi with this ability were found in each container. An attempt has been made to relate the presence of individual fungi in a container to the observed concentrations of the chloroanisoles.

INTRODUCTION

General-purpose freight containers fitted with timber floors are occasionally implicated in the chemical contamination of non-hermetically sealed food, food raw materials and unused packaging components (Whitfield, 1994). Chloroanisoles, and in particular 2,4,6-trichloroanisole (TCA), are a major cause of such contamination (Tindale, 1987; Whitfield *et al.*, 1994). These compounds produce an intense musty taint in food and beverages that renders them unfit for human consumption (Curtis *et al.*, 1974; Buser *et al.*, 1982; Whitfield *et al.*, 1985; Lambert *et al.*, 1993). The precursors of the chloroanisoles in general-purpose freight containers are chlorophenols that have been introduced into the timber floor by accidental spillage, purposeful addition as a preservative (Whitfield *et al.*, 1989) or during cleaning with sanitising agents (Tindale & Whitfield, 1989). By analogy with other materials found to be contaminated with chloroanisoles, e.g. broiler house litter (Gee & Peel, 1974) and fibreboard (Tindale *et al.*, 1989), it has been assumed that the chlorophenols present in the container floors were biomethylated into the corresponding chloroanisoles by fungi (Tindale, 1987). However, until now no attempt has been made to demonstrate that fungi with the ability to biomethylate chlorophenols are part of the microflora present in the timber floors of freight containers.

Over the past 10 years the CSIRO has investigated numerous cases where freight containers have been implicated in the contamination of foodstuffs and packaging components with chloroanisoles. As a result, in recent years the floors of containers involved in such problems have been routinely analysed for chlorophenol, chloroanisole and fungal content. This paper reports the analysis of samples of timber removed from the floors of six containers involved in outbreaks of mustiness in foodstuffs. In addition, an attempt has been made to relate the concentrations of chloro-anisoles in a container floor to the presence of individual fungi known to biomethylate chlorophenols in timber and fibreboard.

MATERIALS AND METHODS

Materials

2,4,6-Trichlorophenol (TCP), 2,3,4,6-tetrachlorophenol (TeCP) and pentachlorophenol (PCP) were purchased

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from Fluka Chemie AG (Buchs, Switzerland); and 2,4,6-trichloroanisole (TCA), 2,3,4,6-tetrachloroanisole (TeCA) and pentachloroanisole (PCA) were obtained from Oxford Chemicals Ltd (Brackley, UK). The internal standards, 3,5-dimethyl-2,4,6-trichlorophenol (DMTCP) and 3,5-dimethyl-2,4,6-trichloroanisole (DMTCA) were synthesised in the laboratory and 2,4-dichlorophenol-d_3 (DCP-d_3) was purchased from Cambridge Isotope Laboratories (Woburn, MA, USA). The purity of these compounds was confirmed by analysis using gas chromatography–mass spectrometry (GC–MS) (Whitfield *et al.*, 1986, 1994). The microbiological agars, Dichloran Rose Bengal Chloramphenicol, Malt Extract Agar and Czapek Yeast Extract Agar were purchased from Unipath Ltd (Basingstoke, UK) and Dichloran Glycerol 18%, Czapek Yeast Extract plus 20% sucrose and 25% Glycerol Nitrate Agars were prepared in the laboratory (Pitt & Hocking, 1985). Distilled water was purified by filtration through a Milli-Q Purification System (Millipore Corporation, Bedford, MA, USA).

Woodshavings were planed from the surfaces of the timber floors of allegedly contaminated freight containers and then stored in glass screw-top jars at 0°C until required for analysis. The samples were taken either at the time the complaint was made or as soon as the container was located by the shipping line.

Isolation of chlorophenols

Woodshavings (1–10 g) were added to filtered distilled water (1·5 litre), acidified to pH 1 with 2M sulphuric acid and allowed to stand at room temperature overnight. The sample was then steam-distilled and the distillate (500 ml) collected in a flask containing 0·2M sodium hydroxide (15 ml). The alkalinity of the distillate was stabilised at pH 11–12 and DCP-d_3, the recovery internal standard (1 μg) was added. The distillate was washed with chloroform (75 ml) and hexane (80 ml), and the aqueous residue was concentrated to about 50 ml on a rotary evaporator (40°C). The concentrate was acidified to pH 2–3 and passed through a Sep-Pak C18 cartridge (Waters Millipore Corporation, Milford, MA, USA). The cartridge was washed with water (20 ml) and the chlorophenols eluted with methanol (3 ml). The extract was made alkaline and after addition of the quantitation internal standard, DMTCP (1 μg), the solution was evaporated just to dryness on a rotary evaporator. Sodium hydrogen carbonate (20 mg) and acetic anhydride (200 μl) were added and the mixture heated to 80°C for 30 min. After the reaction mixture had cooled, iso-octane (200 μl) and water (500 μl) were added and the solution was transferred to a 2 ml screw-cap vial and shaken vigorously. The vial was then centrifuged and the iso-octane layer transferred to another 2 ml screw-cap vial which contained a small quantity of dry sodium hydrogen carbonate. The sample was analysed within hours of acetylation. Reference chlorophenols for the calibration of the GC–MS were derivatised using the same acetylation procedure.

Isolation of chloroanisoles

A sample of woodshavings (1–10 g) was added to filtered distilled water (1 litre). The volatile components were then isolated by combined steam-distillation and solvent extraction with pentane/diethyl ether (9 : 1) as solvent. The extraction was continued for 2 h after boiling commenced. The internal standard, DMTCA (100 ng), was added and the extract dried. Excess solvent was removed by distillation using a microfractionating column (Whitfield *et al.*, 1986). The concentrated extract was stored in a glass vial at −15°C until required for analysis.

Gas chromatography – mass spectrometry

A Varian 1440 gas chromatograph, fitted with a fused silica column (25 m, 0·32 mm i.d.) coated with a methylphenylsilicone BP5 phase of thickness 0·5 μm, was used for all analyses. The gas chromatograph was directly coupled to a Varian-MAT 311A mass spectrometer and the quantitative analyses performed by multiple-ion-detection under software control by a Finnigan-MAT INCOS 2200 data system. The instrument was calibrated for chlorophenol analyses by analysing an acetylated solution that contained measured quantities (1 μg/ml) of TCP, TeCP, PCP and the internal standards, DMTCP and DCP-d_3. For the chloroanisole analyses the calibration solution contained 1 μg/ml each of TCA, TeCA, PCA and the internal standard, DMTCA (Whitfield *et al.*, 1986). Each analysis was repeated until all peaks were within the linear range of the mass spectrometer detector system.

Extraction efficiency

The extraction efficiencies of the steam-distillation technique for the recovery of the chlorophenols were determined by the extraction of the target compounds from the aqueous solutions. The average recoveries for the chlorophenols were as follows: TCP 91%, TeCP 95% and PCP 96%. For the chloroanisoles the recoveries were as follows: TCA 90%, TeCA 94% and PCA 99%. These recovery figures were used to correct the raw data obtained from the analyses of the different samples.

Isolation and identification of fungi

Small samples of woodshavings were direct-plated, in duplicate, on to both Dichloran Rose Bengal Chloramphenicol and Dichloran Glycerol 18% agars (Pitt & Hocking, 1985). These plates were incubated at 25°C for 1 week. Colonies from these plates were subcultured on to different agars, depending on the genera; *Trichoderma* spp. were plated on to Malt Extract Agar, *Eurotium* spp. on Czapek Yeast Extract Agar plus 20% sucrose and the remainder on Czapek Yeast Extract Agar. All cultures were incubated at 25°C for 1 week, with the exception of the *Eurotium* spp. which were incubated for 2 weeks. Most species were identified

from these sub-cultures but *Penicillium* spp. required further sub-culturing for identification.

The pure sub-cultures of the *Penicillium* spp. were inoculated on to Czapek Yeast Extract Agar and incubated at 5, 25 and 37°C for 1 week. The same cultures were also inoculated on to Malt Extract Agar and 25% Glycerol Nitrate Agar and incubated at 25°C for 1 week (Pitt & Hocking, 1985). Microscopic examination was used to identify all isolates to genus level and the majority to species level.

RESULTS AND DISCUSSION

The concentrations of chlorophenols and chloroanisoles found in six freight containers are given in Table 1; the containers are listed in the order that they were received for analysis. In all cases the levels of the chlorophenols were greater than those of their corresponding chloroanisoles, with the exception of Container E. In this container one value, that of PCP, was less than that of the corresponding chloroanisole. The data in Table 1 also show that the degree of conversion of an individual chlorophenol to its corresponding chloroanisole varied between compounds and between containers; the lowest conversion was observed for TCP in Container B (0·06%) and the highest for PCP in Container E (90%). Percent conversion was calculated by dividing the concentration of the chloroanisoles by the total concentration of both the chlorophenols and the chloroanisoles found. Admittedly, these data do not take into account possible losses of the more volatile chloroanisoles into the atmosphere, or into food products. Even so the results generally support the commonly held view that chlorophenols are the principal precursors of chloroanisoles under these conditions (Tindale, 1987). The data in Table 1 also show that the concentrations of the three chloroanisoles in the six containers varied greatly. Containers A, D and F had relatively high concentrations of TCA, Containers B and C had relatively low concentrations and in Container E the concentration of TCA was intermediate between the two extremes. By comparison the concentrations of TeCA and PCA were quite low in Containers B, C, D and E, and only moderately higher in Containers A and F.

Additional support for the biomethylation theory was provided by the detection in all six containers of fungi capable of the methylation reaction (Table 2). Most of these fungi were xerophilic and consequently capable of growth below 0·85 a_w (Pitt & Hocking, 1985). However, some species of *Aspergillus* and *Eurotium* can grow at water activities as low as 0·70 (Pitt & Hocking, 1985). The a_w range of 0·70–0·85 corresponds to moisture contents in timber of between 13 and 18% at 20°C. This level of moisture may occur in the floors of freight containers as a result of climatic conditions (rain), cleaning procedures (steam or water) or as a result of the transportation of moist or wet cargoes such as hides.

A total of 38 species of fungi were isolated from the six containers (Tables 2 and 3). Growth of these fungi occurred under optimal conditions in the laboratory, confirming the presence of spores or hyphae in the wood from the containers; however, as conditions in the containers were not recorded it is not known whether all of the fungi were active. Nineteen of these are known to be capable of biomethylating chlorophenols (Table 2). Fifteen of these methylators have been confirmed as being xerophilic. Sixteen of these species are known to have wide temperature ranges for growth, from −2 up to 48°C (Table 2). Temperatures during transport would fall within this range. The temperature ranges delimiting the growth of *Aspergillus wentii*, *Trichoderma koningii* and *T. pseudokoningii* have not been determined but these species would grow strongly between 15 and 30°C.

Of those species known to be capable of biomethylating the chlorophenols, six different *Penicillium* spp. were identified, five *Aspergillus*, four *Eurotium* and three *Trichoderma*. In addition to these, *Paecilomyces variotii* was isolated from Containers B and F. *Penicillium chrysogenum*, isolated from five of the six containers, was the most frequently found of these 19 fungi. Seven other species were isolated from only one container each and the remaining 11 species of fungi were from two or three different containers.

The methylating ability of the remaining 19 species

Table 1. Concentrations (μg/kg) of chlorophenols and chloroanisoles present in the timber floors of freight containers

Freight container	Concentrations found						% Conversion of chlorophenols to chloroanisoles[c]		
	TCP	TeCP	PCP[a]	TCA	TeCA	PCA[b]	TCP	TeCP	PCP
A	120 000	7100	13 000	1600	260	350	1·3	3·5	2·6
B	12 000	34	51	7·2	1·4	16	0·06	4·0	24
C	4800	5700	68 000	30	0·5	11	0·62	0·09	0·02
D	2900	370	18	1500	4·5	ND[d]	34	1·2	0
E	77 000	2·8	1·6	860	2·4	15	1·1	46	90
F	12 000	620	6900	4700	120	120	37	16	1·7

[a]TCP: 2,4,6-trichlorophenol, TeCP: 2,3,4,6-tetrachlorophenol; and PCP: pentachlorophenol.
[b]TCA: 2,4,6-trichloroanisole; TeCA: 2,3,4,6-tetrachloroanisole; and PCA: pentachloroanisole.
[c]Calculated on total concentrations found.
[d]ND: not detected at a detection limit of 0·1 μg/kg.

Table 2. Fungi that are known to methylate chlorophenols and were isolated from the timber floor of contaminated freight containers

Species	Containers in which found	Percent methylation[a]			Minimum a_w for growth at 25°C[c]	Temperature for growth (°C)[c]	
		TCP	TeCP	PCP[b]		Optimal	Range
Aspergillus flavus	A,B	35	Tr[d]	ND[e]	0·78(33°C)	25–42	12–48
A. niger v. *niger*	B,C	ND	2–8	ND	0·77(35°C)	35–37	7–46
A. sydowii	F	19	10–74	ND	0·78	NA[f]	9–37
A. versicolor	B	ND	6–80	ND	0·78	27	9–39
A. wentii	A	ND	Tr	ND	0·74	NA	NA
Eurotium amstelodami	A,E,F	ND	2–39	ND	0·70[g]	33–35	<45
E. chevalieri	E	ND	Tr-3	ND	0·74	30–35	<43
E. repens	A,C,D	12	25–74	ND	0·72	25–27	4–40
E. rubrum	A,D,E	ND	3–31	ND	0·70	25–27	5–40
Paecilomyces variotii	B,F	59	11–81	ND	0·79	35–40	5–48
Penicillium brevicompactum	A	27	Tr	ND	0·78	23	−2–30
P. chrysogenum	A,B,D,E,F	ND	Tr	ND	0·79	23	4–37
P. corylophilum	B,C,F	5	62–83	ND	0·80	NA	5–37
P. crustosum	A,C	31	2–16	ND	NA	25	−2–30
P. glabrum	A,E	ND	Tr-54	ND	0·80(22–25°C)	23[h]	0–30
P. roqueforti	C	ND	7–65	ND	0·83[h]	23–25[h]	<35
Trichoderma harzianum	C,E	ND	ND	19	0·91	30	5–36
T. koningii	A,C	ND	ND	4	NA	NA	NA
T. pseudokoningii	E	ND	ND	49	NA	NA	NA

[a]Percent methylation of TCP from Tindale *et al.* (1989); percent methylation of TeCP from Gee and Peel (1974); percent methylation of PCP from Cserjesi (1972).
[b]TCP: 2,4,6-trichlorophenol; TeCP: 2,3,4,6-tetrachlorophenol; PCP: pentachlorophenol.
[c]Pitt and Hocking (1985)
[d]Tr, trace.
[e]ND, not determined.
[f]NA, not available.
[g]Wheeler and Hocking (1988).
[h]Magan and Lacey (1984).

of fungi identified in the six containers has not been determined (Table 3). *Penicillium* spp. were again the most common; 10 of the 19 species in this group were from this genus. Two each of *Aspergillus* and *Cladosporium* spp. were isolated, one *Trichoderma* and one *Phoma*. *Ulocladium* spp. were isolated from Containers A, B and C and *Alternaria* spp. from Containers B and D. Although a greater variety of genera were identified in this group not all are known to be xerophilic. Of the eight whose minimum water activity for growth has been determined, six were xerophilic; one, *Cladosporium herbarum*, could be considered to be only just xerophilic and *Mucor plumbeus*, which germinates at a minimum water activity of 0·93, is not xerophilic (Pitt & Hocking, 1985). Temperature ranges for growth of all these species would be suited to those most likely encountered during long-distance transportation.

Examination of the microflora isolated from Container A indicated the presence of four known moderate to strong methylators of TCP; these were *Aspergillus flavus, Eurotium repens, Penicillium brevicompactum* and *P. crustosum*. By comparison, Container D had only one species, *E. repens*, and Container F two species, *Aspergillus sydowii* and *Paec. variotii*. Based on these microbiological findings it is a little surprising that the percentage conversion of TCP to TCA was greater in Containers D and F than in Container A. A possible explanation was the very high concentration of TCP in Container A (120 000 μg/kg) compared with the total concentrations of the three chlorophenols in the other two containers (<20 000 μg/kg). At this high concentration, TCP would exert strong fungicidal activity (Anon., 1989). No fungi known to be moderate or strong methylators of TCP were isolated from Container E. However, five species known to be strong methylators of TeCP and PCP were found in this container. These fungi have not been tested against TCP; hence their ability to methylate this compound is unknown. Two fungi known to be strong methylators of TCP were isolated from Container B (*A. flavus* and *Paec. variotii*) and two from Container C (*E. repens* and *P. crustosum*). The yield of TCA in both of these containers was low, as was the percent conversion of TCP to TCA (Table 1). The low yield of TCA in Container C may be explained by the presence in the floor of relatively high concentrations of PCP (68 000 μg/kg). At this concentration PCP would be an efficient fungicide and could be expected to inhibit the growth of most fungi (Anon., 1987). However, there is no obvious explanation for the low yield of TCA in Container B.

The concentrations of TeCA and PCA found in Containers B, C, D and E were low. However, the percent conversion of TeCP to TeCA was 46% in Container E and the conversion of PCP to PCA was 24% in Container B and 90% in Container E. The

percent conversions of these two compounds in Containers C and D and the conversion of TeCP to TeCA in Container B were all below 5%. Three fungi known to be strong methylators of TeCP were isolated from Container E (*Eurotium amstelodami, E. rubrum* and *Penicillium glabrum*) and two species that were strong methylators of PCP were also isolated from this container (*Trichoderma harzianum* and *T. pseudokoningii*). No species known to be strong methylators of PCP were isolated from Container B. In Containers A and F moderate concentrations of TeCA and PCA were found, although the percent conversion of precursor chlorophenol to chloroanisole was low in both cases. Five species known to be strong methylators of TeCP were found in Containers A (*E. amstelodami, E. repens, E. rubrum, P. crustosum* and *P. glabrum*) and four species in Container F (*A. sydowii, E. amstelodami, Paec. variotii* and *Penicillium corylophilum*). No fungi with a known ability to methylate PCP in high yield were isolated from either container. However, *T. koningii*, a weak methylator of PCP was isolated from Container A.

Table 3 lists those species of fungi isolated in the contaminated containers whose methylating abilities of TCP, TeCP and PCP have not been determined; their role is unknown. The majority of these fungi were isolated from Container A (seven species) and Container B (nine species). Unfortunately, it is not possible to speculate on the ability of these fungi to methylate chlorophenols based on their presence in these two containers. Container A already had seven species of known methylation ability and the low yields in Container B suggest the presence of an unknown factor that inhibits the growth of fungi. Clearly a series of trials to assess the ability of these fungi to methylate TCP, TeCP and PCP is required.

CONCLUSION

At least three species of fungi with some ability to methylate chlorophenols were isolated from each of the six containers examined. Relatively high concentrations of chloroanisoles (> 1500 μg/kg) were found in three of these containers while in a fourth the concentration of TCA was 860 μg/kg. In the two containers with low concentrations of chloroanisoles, the presence of a high concentration of the fungicide PCP (68 000 μg/kg) probably explains the poor conversion of chlorophenols to chloroanisoles in one of these, whilst in the other there is no obvious explanation for the observed results. It is possible that the moisture content of the floor in this container was below that required for fungal growth. The absence of physical data on the condition of the floor leaves this result open to speculation. The degree of biomethylation that occurred in all containers was in most cases inconsistent with the reported methylation abilities of the species identified. However, studies by Gee and Peel (1974) have demonstrated the wide range of methylating abilities that occur amongst different strains of the same species, including the inability of some strains to produce chloroanisoles. Furthermore, there may also have been fungi present which were capable of metabolising the chlorophenols via a separate pathway (Cserjesi, 1972). Importantly, no fungi known to be incapable of producing

Table 3. Fungi isolated from the timber floors of freight containers contaminated with chlorophenols and chloroanisoles that have not been identified as capable of methylating chlorophenols

Species	Containers in which found	Minimum a_w for growth at 25°C[a]	Temperature for growth (°C)[a]	
			Optimal	Range
Alternaria spp.	B,D	NA[b]	NA	NA
Aspergillus fumigatus	B	0·82(40°C)	40–42	12–55
A. nidulans	B	0·80(37°C)	35–37	7–47
Cladosporium cladosporioides	B	0·82[c]	20–25[d]	-5–32
C. herbarum	D	0·85[d]	20–25[d]	-10–32
Mucor plumbeus	D	0·93	20–25	4–35
Penicillium aurantiogriseum	A,B,C	0·81	23	-2–30
P. commune	A,B	NA	NA	NA
P. decumbens	E	NA	NA	NA
P. griseofulvum	B	0·81(23°C)	23	4–35
P. implicatum	F	0·78	NA	NA
P. montanense	A	NA	NA	NA
P. pinophilum	F	NA	NA	NA
P. verrucosum	A	NA	NA	NA
P. viridicatum	A,B	0·80–0·81(23–25°C)	23	-2–36
P. waksmanii	B	NA	NA	5–37
Phoma sp.	A	NA	NA	NA
Trichoderma aureoviride	E	NA	NA	NA
Ulocladium spp.	A,B,C	NA	NA	NA

[a]Pitt and Hocking, 1985.
[b]NA, not available.
[c]Hocking *et al.* (1994)
[d]Magan and Lacey (1984)

chloroanisoles were found in the container floors. Thus in future studies of fungi and the metabolism of chlorophenols in timber, the ability of individual isolates to methylate chlorophenols should be assessed. There is also a need to obtain information on the moisture content of timber samples at the time of collection to verify that conditions are conducive for fungal growth. Even so information obtained in the current study can be interpreted as supporting the opinion that fungi present in the floors of freight containers are responsible for the conversion of chlorophenols to chloroanisoles. However, a number of factors including the concentration of the chlorophenols, the species and strain of fungi present in the timber and the moisture content in the timber can all affect the level of methylation that occurs during the lifetime of the container.

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