Investigations into the Origin of Chloroanisoles Causing Musty Off-Flavor of Raisins

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The formation of chloroanisole in commercially processed and nonprocessed dried-on-the-vine (DOV) raisins under sterile, nonsterile, and low-moisture conditions was determined. The chloroanisoles of raisin samples were steam−solvent extracted using a modified Likens−Nickerson glass distillation apparatus and the concentrated extracts analyzed by gas−liquid chromatography and mass spectrometry. Processed raisins with low water activity (Aw) of 0.48 or 0.80 formed 2,4,6-trichloroanisole (TCA) when incubated during a 3−4 week period. TCA was formed by raisins partially sterilized with propylene oxide or completely sterilized with hydrogen peroxide. Nonprocessed DOV raisins incubated at an Aw of ≤0.80 also formed TCA. Although the amounts of TCA formed in raisins vary greatly from batch to batch, it is demonstrated that sterilized raisins formed TCA under low water activity nonconducive for microbial activity.

Keywords: *Biogenesis; chloroanisole; raisins*

INTRODUCTION

The ubiquitous occurrence of musty off-flavor of stored food products and packaging materials has been recognized (Maarse *et al*., 1985; Nijssen, 1991; Tindale, 1987; Whitfield, 1983). The general tainting of foodstuffs is attributable to polychlorophenol-treated lumber that is widely used as the basic material for the manufacturing of shipping containers, paper, and fiberboard boxes used in the food industry (Crosby *et al*., 1981). Whitfield *et al*. (1985) reported that mustiness in dried fruits was first encountered when van containers were introduced for shipping agricultural products overseas. Changes in temperature and moisture occurring within the cargoes during long transit favored the formation of off-flavor compounds. This problem was exacerbated by the use of lumber, recycled papers, and adhesives containing chlorophenols. These compounds serve as a ready source of substrates for the formation of chloroanisoles by microbial methylation (Tindale *et al*., 1989). Other factors also contribute to the mustiness of dried fruits. Chlorine-based cleaning formulations for washing storage areas, wooden bins, and pallets in the dried fruit industry provided suitable conditions and substrates for generation of chlorophenols (Tindale and Whitfield, 1989). Although the conversion of chlorophenols by microbial methylation to chloroanisoles is a significant source of off-flavor of dried fruit, it is recognized that the development of musty off-flavor can originate from various chemical, physical, and microbial sources (Maarse *et al*., 1988; Reineccius, 1991). The postharvest quality and commercial value of raisins are diminished when musty off-flavor compounds contaminate the product and render it unpalatable and unsalable. It is of interest, therefore, to determine the sources which contribute to the tainting of dried fruits. The purpose of this investigation was to examine whether processed and nonprocessed dried-on-the-vine (DOV) raisins under sterile and nonsterile and relatively low-moisture conditions form chloroanisole compound(s).

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MATERIALS AND METHODS

Processed 1992 and 1993 crop Thompson seedless raisins were obtained from commercial packing companies. The processed raisins have been harvested, tray-dried in the sun, washed, cleaned of debris, and stored under different conditions and have an average moisture content which ranges between 12 and 16%. A nonprocessed 1992 and 1993 crop of DOV raisins (USDA C96−108) selection grown at the ARS Research Farm located at California State University, Fresno, CA, was also used.

Moisture Content and Water Activity (Aw) Determination. The moisture content of raisin samples was determined by a dried fruit moisture meter (Dried Fruit Association of California, Santa Clara, CA). The hydration of raisins was accomplished with the addition of water followed by tumbling for 4 h to provide samples with sufficient moisture content to attain 80% relative humidity within the atmosphere of sealed containers. Water activity of the raisins was determined by measurement of the equilibrium dew point of the atmosphere surrounding the products using a chilled mirror dew point apparatus (General Eastern Hygro M1 Dewpoint Apparatus, Watertown, MA).

Sterilization and Microbiology. Three methods were used to sterilize raisins: (1) autoclaving at 121 °C under 1.1 kg/cm^2 of pressure for 20 min, (2) fumigation with propylene oxide (PO) applied at 0.9 mL/L at 32 °C under vacuum (130 mmHg) for 6 h, and (3) hydrogen peroxide sterilization of a 1 h cycle at ca. 10 ppm H_2O_2 vapor for 1 h using a peroxide generator (VHP 1000, AMSCO, Apex, NC).

To determine microbe populations, four subsamples were taken from 50.0 g samples of raisins and macerated in a high-speed blender for 2 min in 0.2 M phosphate buffer (pH 7.2), diluted in a series and a 0.2 mL aliquot was plated. Four media were used: (1) potato dextrose agar, a nonselective medium for all microbes, incubated at 20 °C for 5 days; (2) total plate count agar, selective for bacteria, incubated at 35 °C for 3 days; (3) a high-osmotic strength medium, Czapek Dox agar with 20% sucrose, selective for osmophilic fungi and bacteria, incubated at 25 °C for 5 days; and (4) DRBC agar base, a medium of choice for enumeration of products with high fungal populations (it limits colony spreading), incubated at 25 °C for 5 days.

Incubation and Chloroanisole Extraction. Raisin samples of 400 g were placed in a 946 mL wide-mouthed Teflon

S0021-8561(96)00241-5

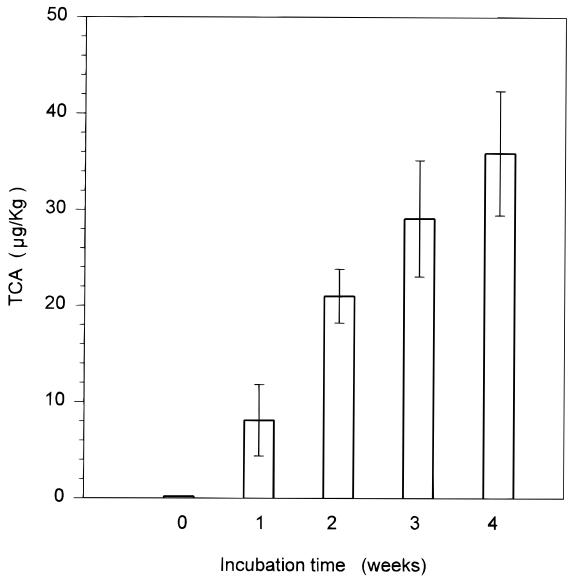

Figure 1. Formation in the levels of 2,4,6-TCA in unsterilized, processed raisins over 4 weeks of incubation at 25 °C (see details in Materials and Methods). Values are means of two experiments. Error bars denote the standard deviation.

Table 1. Sterilization on Chloroanisole Content of Processed Raisins Incubated for 4 Weeks[a]

treatment[b]	chloroanisole content[c] (μg/kg)
expt 1	
control	14.0 ± 9.5
propylene oxide	1.0 ± 0.9
expt 2	
control	72.9 ± 60.2
autoclaving	21.2 ± 10.1

[a] Commercially processed 1992 crop of Thompson seedless raisins; values are means of four samples ± standard deviation. [b] Propylene oxide, 0.9 mL/L at 32 °C under vacuum (130 mmHg) for 6 h; autoclaving at 121 °C under 1.1 kg/cm^2 of pressure for 20 min. [c] Refers to 2,4,6-trichloroanisole; see Identification of Chloroanisoles by GLC/MS in Materials and Methods.

Table 2. Sterilization on Chloroanisole Content of Nonprocessed Dried-on-the-Vine Raisins Incubated for 4 Weeks[a]

	chloroanisole content[c] (μg/kg)	
treatment[b]	expt A	expt B
control (unsterilized)	8.0 ± 2.8	9.5 ± 1.7
propylene oxide	0.3 ± 0.2	1.1 ± 0.4

[a] Nonprocessed 1992 (expt A) and 1993 (expt B) crop of dried-on-the-vine raisins USDA C96-108 selection; values are means of four samples ± standard deviation. [b] See details in Materials and Methods. [c] Refers to 2,4,6-trichloroanisole.

jar having a screw top lid with two sealable openings to allow humidity determinations. After incubation for 24 h at 25 °C, the percent relative humidity in each test sample jar was determined, and the sample jars were stored at 25 °C for 1−4 weeks. Sample jars were taken at appropriate intervals for extraction of chloroanisoles.

Extraction was carried out using a modified Likens−Nickerson glass distillation apparatus (Schultz *et al*., 1977). Samples of 100.0 g of raisins and 1 L of distilled water were placed in a 3 L round-bottom flask. A surrogate standard 2,4,5-trichloroanisole (2,4,5-TCA) of 100 ng/100 μL of toluene was added to monitor recovery. The distillation flask was attached to a simultaneous steam−solvent extraction system. A recommended (R. Flath, personal communication) solvent mixture of 27 mL of pentane, 3 mL of diethyl ether, and 100 μL of toluene was added and the mixture with raisins extracted for 2 h. After extraction, the solvent extract was kept at −18 °C to freeze residual water and then concentrated in a microdistillation system. The small volume (about 100 μL) of concentrates was transferred to glass vials for injection and analysis by gas−liquid chromatography/mass spectrometry (GLC/MS).

Identification of Chloroanisoles by GLC/MS. Chloroanisoles were analyzed and identified using a Hewlett-Packard 5890 gas−liquid chromatograph equipped with a DB-5 MS 30 m × 0.25 mm column and film thickness of 0.5 μm. The carrier gas was helium at 1 mL min^{-1}. The injector temperature was 280 °C, and the oven temperature was programmed from 80 to 280 °C at 4 °C min^{-1}. The gas−liquid chromatograph was directly coupled to a HP-5895 MS detector with the transfer line maintained at 280 °C. The mass spectrometer was operated in SIM (selective ion monitoring) with an electron energy of 70 eV. All GLC/MS data were monitored, stored, and processed using a Hewlett-Packard G1030A ChemStation. The following ions were monitored simultaneously: *m/z* 195, 196, 197, 198, and 210 for 2,4,6-trichloroanisole (2,4,6-TCA); *m/z* 212, 197, and 214 for 2,4,5-trichloroanisole as the surrogate standard; *m/z* 246, 203, and 244 for 2,3,4,6-tetrachloroanisole; and *m/z* 280, 265, and 237 for pentachloroanisole.

RESULTS AND DISCUSSION

Processed unsterilized raisins at an Aw of 0.48 formed TCA in increasing amounts when incubated at 25 °C over a 4 week period (Figure 1). The incubation of processed raisins sterilized by PO or by autoclaving also formed TCA (Table 1). Similarly, nonprocessed DOV raisins incubated at an Aw of 0.80 also formed TCA but in lesser amounts. The PO-sterilized DOV raisins at an Aw of 0.80 formed significantly less TCA compared to nonsterilized DOV (Table 2).

Propylene oxide significantly reduced microbe populations by approximately 85% (Table 3). Hydrogen peroxide sterilized the raisins. Microbe populations did not increase during storage (Table 3).

Table 3. Microbe Populations and TCA Content of Processed Raisins Partially Sterilized by Propylene Oxide or Sterilized by Hydrogen Peroxide Initially and after 3 Weeks of Incubation[a]

		microbe population (colony-forming units/gram raisins)		
treatment	TCA (μg/kg)	TPC	PDA	CZS
initial				
untreated	0.01	14.1 a	12.0 a	5.0 a
propylene oxide	0.01	4.5 b	1.5 b	1.0 b
hydrogen peroxide	0.01	0.0 c	0.0 c	0.0 c
after 3 weeks of incubation				
untreated	1.4 ± 1.3	30.0 a	4.7 a	6.0 a
propylene oxide	9.5 ± 1.0	4.5 b	0.6 b	1.2 b
hydrogen peroxide	4.1 ± 7.0	0.0 c	0.0 c	0.0 c

[a] Commercially processed 1993 crop of Thompson seedless raisins; TCA = 2,4,6-trichloroanisole. Data were analyzed using a one-way ANOVA followed by Fisher's protected LSD ($P = 0.05$) to separate means. Means in columns for each incubation period followed by the same letter are not significantly different. [b] TPC = total plate count agar, PDA = potato dextrose agar, and CZS = Czapek Dox agar with 20% sucrose.

The amount of TCA formed during 3 week incubation of the raisins following treatments exceeded the control (Table 3). On raisins with very low microbe populations (PO-treated) or with zero microbe population (hydrogen peroxide-treated), more TCA was generated than with the untreated controls after 3 weeks incubation. It should be pointed out that the overall results of several incubation tests showed PO sterilization had no effect (control = 5.1 ± 3.4 μg/kg versus PO-treated = 3.8 ± 2.3 μg/kg) on TCA formation. The apparent discrepancy of PO on TCA is due more to the variable amounts of TCA formed by batches of commercial raisins than to PO treatment.

The incubation experiments demonstrated that processed and nonprocessed DOV raisins under the specified laboratory conditions are able to form chloroanisoles. Under sterile conditions and at low water activity nonconducive for microbial activity, the formation of chloroanisoles occurred. These conditions did minimize the microbial contribution in the generation of chloroanisoles. Nonprocessed DOV raisins further precluded the possible interference of other extraneous factors such as washing with chlorinated water and exposure to polyphenol substrates which may have contributed to chloroanisole formation. The use of several methods of sterilization gave an assurance of reducing microbe populations to insignificant levels (Table 3). Tindale *et al.* (1989) observed that the ability of fungi species to methylate chlorophenols to TCA varies and indicated that the quantity of TCA generated cannot be attributed to any single microorganism. In our investigations, no colonies appeared on fungi selective DRBC agar and confirmed that filamentous fungi were absent. The low Aw of raisins is not favorable for growth and metabolism of microorganisms and would exclude the role of microorganisms in the generation of TCA (Scott, 1957).

The development of musty off-flavor in dried fruits can originate from various sources. It can originate from microbial contaminants, chemical reactions within the commodity, and airborne contaminants (Reineccius, 1991). Maarse *et al.* (1988) observed that, even when off-flavor was known to be caused by a halogenated phenol or chloroanisole, it was still difficult to ascertain the origin of these compounds. For example, Buser *et al.* (1982) identified by GC/MS that 2,4,6-trichloroanisole was the compound responsible for the musty cork taint of wines. But how 2,4,6-TCA originated was uncertain. The authors indicated that 2,4,6-TCA and other related compounds may be produced from chlorination of lignin-related substances during chlorine bleaching in the processing of cork. Presently, there is no known biochemical mechanism to explain the production of 2,4,6-TCA of incubated processed or nonprocessed raisins. However, there are some indications that plant organs may have similar capabilities to biotransform native phenols to chloroanisoles as found in animal and microbial systems (Crosby, 1981). It is conceivable that chlorination could occur by enzymatic or nonenzymatic reactions with the phenolic substrates (Jerina *et al.*, 1968). In investigating the biogenesis of 2,4,6-TCA of Rio coffee beans, Liardon *et al.* (1991) suggested that the phenols derived from the Shikimic acid pathway were chlorinated to trichlorophenols by fungal or host chloroperoxidases and finally methylated to chloroanisoles. The existence of both methylating (Axelrod and Daly, 1968) and chlorinating (Jerina *et al.*, 1968) reactions in living systems is known. Thus, it is plausible that the available biochemical information could provide a basis for further investigation into the mechanism of biogenesis of TCA of raisins.

ACKNOWLEDGMENT

We thank Gary Obenauf and Edwin Soderstrom for interest and encouragement of this work, Jane Tebbets and Joel Jenner for technical assistance, and Judy Litster for typing the manuscript.

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Received for review April 7, 1996. Revised manuscript received July 9, 1996. Accepted August 12, 1996.[⊗] This work was partially supported by funds from the California Raisin Advisory Board.

JF960241J

[⊗] Abstract published in *Advance ACS Abstracts,* September 15, 1996.