Natural Formation of Styrene by Cinnamon Mold Flora

J.-L. Lafeuille, M.-L. Buniak, M.-C. Vioujas, and S. Lefevre

ABSTRACT: Tests on 106 dried pure cinnamon samples of diverse origins showed that some samples were naturally contaminated with high levels of styrene, up to 524 μg/g. Styrene taint can be associated with high water activity levels and thus with microorganism growth. The mold flora of a Korintji cinnamon sample in which styrene had been found at a 50 μg/g concentration was analyzed and 5 species of mold were isolated. An investigation into the ability of the 5 species of mold to produce styrene showed that 3 of them—namely, *Penicillium citrinum*, *Penicillium oxalicum*, *Aspergillus niger*—produced styrene *in vitro* in buffered peptone water at 25 °C within 5 d in the presence of several natural cinnamon volatile constituents containing the styrene structure. The conversion of these compounds into styrene by these 3 cinnamon fungal species has never been previously reported. A standardized inoculation with the 3 mold species was carried out on 10 g cinnamon samples of various origins followed by a 10-d incubation and highlighting styrene production except for Sri Lanka origin.

Keywords: cinnamon, fungi, gas chromatography-mass spectrometry (GC-MS), spices, styrene

Introduction

Styrene (CAS 100-42-5) was discovered at the beginning of the 19th century via the pyrolitic decarboxylation of organic acids from storax, the resin of the tree *Liquidambar orientalis* Mill. A century later, styrene became commercially essential for the development of synthetic rubber.

As an extremely important commodity chemical used in the manufacture of numerous polymers and copolymers, styrene is obtained from crude oil or liquefied petroleum gas for industrial purposes. Some of these polymers are largely used in packaging applications. Styrene is liquid at room temperature and has a 145.2 °C boiling point. It has a pungent and plastic-like odor. Styrene polymerizes when exposed to light and air but it can also oxidize to form certain aldehydes and ketones giving a sharp, penetrating, disagreeable odor (Boundy and Boyer 1952).

Styrene has a low acute toxicity (WHO 2003). After long-term exposition to styrene in air, irritation of the mucous membranes, respiratory tract, and depression of the central nervous system can be caused (WHO 2003) and hepatotoxicity has been suggested. However, its plastic-like odor and the low threshold concentration at which it is detected by taste (Linssen and others 1993; WHO 2003) make it unlikely that foods contaminated with this component would be ingested in any quantity. The most important exposure is active smoking (WHO 2003).

As styrene was detected in water and foods, a total daily intake (TDI) of 7.7 μg/kg of body weight was established (WHO 2003) as a guideline value assuming that 10% is allocated to drinking water. Based on this data, a guideline value of 20 μg/L in drinking water can be calculated which is also the lowest observed odor threshold for styrene in water (WHO 2003). Concerning styrene limitation in food products, there is no legislation in the European Union. Styrene is listed in the USFDA (2004) regulations as a synthetic flavoring substance or adjuvant, to be used in the minimum amount required to produce a desired effect, and it can be used alone or in combination with other acceptable flavorings.

In many instances, low levels of styrene in food products (a few ng/g) reflect the migration of styrene from food packaging made of styrene polymers or copolymers (Murphy and others 1992). But there is also evidence that styrene occurs naturally at different low concentrations in various types of food products. Therefore the crucial question was to determine the extent to which styrene occurs in raw agricultural commodities that have never been in contact with polystyrene packaging. A study (Steele and others 1994) was realized with different food products taken at the production site to ensure that only endogenous styrene was measured. Styrene was detected in wheat, peanuts, coffee, peaches, oats, strawberries, and beef ranging from 0.0003 to 0.008 μg/g except in cinnamon, which represents a special case among vegetal foods with high values up to 39.2 μg/g. A British survey (EC DG III 1996) showed that styrene could be found in foods at different levels from 0.0001 μg/g in fruits, to 0.2 μg/g in beer, or 0.36 μg/g in coffee. In olive oil, there are increasing levels of styrene during storage up to 1.2 μg/g possibly due to decarboxylation of cinnamic acid naturally present in olive pulp (Ollivier and Guerere 2000). Up to 7.8 μg/L of styrene were detected in vines with possible sources described including microbiological formation by metabolism of cinnamic acid (Hupf and Jahr 1990). The offensively strong off-odors that result can make consumers claim that the product has been contaminated by unnatural ''chemicals.''

Many precursors have been proposed (Steele and others 1994) for the styrene formation by chemical reaction or by biodegradation: carotenoids, long-chain hydrocarbons, fatty and shikimic acids, methyl arachidonate, 2-phenylethanol, glucose and phenylalanine, aldehydes and cinnamic acid, benzoate, and cinnamate.

The conversion of precursors to styrene in food products can be associated with molds and yeast growth. Styrene production has been reported principally for certain species of *Penicillium* molds (Adda and others 1989; Larsen 1999; Pinches and Apps 2007), *Trichoderma* molds (Pinches and Apps 2007), and yeasts (Chen and Peppler 1955; Saxby 1996; Daly and others 1997). Even at the

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beginning of the 20th century, the transformation of cinnamic acid into styrene was proposed as a test for the presence of *Aspergillus* and *Penicillium* in aqueous extracts of molds, the styrene being at once recognized by its odor (Oliviero 1906).

As for cinnamon spice, an attempt to understand the conversion of cinnamaldehyde to styrene by a chemical degradation pathway was realized (Fragniere and others 2003) but the results were not sufficient to formally establish the formation mechanism of styrene in cinnamon suggesting a more complex reaction mechanism. Knowing that the chemical reactions leading to the production of styrene from cinnamaldehyde and cinnamic acid in cinnamon are rather simple but difficult to take place at ambient temperature, we directly focused on a microbiological origin of the phenomenon.

There are no reports for the conversion into styrene of cinnamaldehyde and derivatives in food by naturally occurring cinnamon molds because all these components contain the styrene structure that makes one think of a simple chemical conversion (Fragniere and others 2003).

The current study demonstrates that the styrene occurring in cinnamon may have a biological cause. The capacity of certain *Aspergillus* and *Penicillium* species to produce styrene *in vitro* in the presence of cinnamaldehyde and other cinnamon volatile constituents is shown.

Materials and Methods

Materials

A total of 106 samples of unsulfited cinnamon/cassia in quill (inner bark), stick or ground form were collected from our various suppliers of certified origins.

Styrene (99%), cinnamaldehyde (>98%), cinnamic acid (>99%), deuterated cinnamic-d7 acid (99.8%), cinnamyl alcohol (>97%), cinnamyl cinnamate (>95%), methyl cinnamate (>99%), tridecane, heptane, and Tween 80 were purchased from Sigma-Aldrich Chemical Co. (St. Louis, Mo., U.S.A.). Ethyl cinnamate (98%) was obtained from Acros Organics (Geel, Belgium). Deionized water was used to prepare diluents and agars. Peptone salt diluent (1 g peptone + 8.5 g NaCl in 1000 mL water), yeast extract glucose chloramphenicol agar (YGC), and plate count agar (PCA) media were purchased from Biokar Diagnostics (Beauvais, France) and buffered peptone water (BPW) from AES Chemunex (Bruz, France).

Determination of the styrene content

The samples were finely ground in a laboratory cutting mill. Samples of cinnamon were extracted by hydrodistillation according to ISO 6571 (2008) method. The distillate was collected in 1 mL of heptane containing a known concentration of tridecane used as the internal standard.

The organic phase was injected in split mode on a Agilent 6890 gas chromatograph equipped with both a 30 m, 0.25 mm ID fused silica polar capillary column Grace EC-wax with 0.25 μm film thickness connected to a FID detector and a 30 m, 0.25 mm ID fused silica apolar capillary column Grace EC-1 with 0.25 μm film thickness linked to a mass spectrometer detector (Agilent 5973; Agilent, Santa Clara, Calif., U.S.A.). Helium was the carrier gas at a flow of 1.2 mL/min with a split ratio of 5:1 in each column. Injector temperature was 240 °C. The mass-spectrometer acquisition settings in scan mode were: source temperature, 240 °C; quadrupole temperature, 140 °C; EI mode; electron energy, 70 eV; 33–260 m/z scan. Oven temperature was programmed as follows: held initially at 50 °C for 5 min then from 50 °C (2 °C/min) → 70 °C/min (2.5 °C/min) → 120 °C (20 °C/min) → 240 °C. With an Agilent 6890 injector, sample (1 μL) was injected into the GC and styrene was identified in relation to its mass spectrum on the apolar column. On the polar column, the styrene concentration was determined in relation to the internal standard, tridecane, as μg/g cinnamon. The quantitation limit of this method was 0.5 μg/g, which was considered sufficient given the high levels of styrene found in cinnamon.

Determination of the water activity (a_w)

For cinnamon in quill or stick samples, water activity was measured by the dew point technique with an Aqualab 3T (Decagon, Pullman, Wash., U.S.A.) and, in the case of ground cinnamon by resistivity using a LabMaster-aw (Novasima, Zurich, Switzerland) because the high volatile oil content released by ground cinnamon samples during analysis interferes with water dew point measuring.

Mycological studies

According to ISO 7954 (1987) method, 10 g of sample were collected and homogenized in 90 mL portion of sterile, peptone salt diluent. After a 30–35 min resuscitation phase conducted at room temperature, the suspension was homogenized for 1 min. Decimal serial dilutions were then prepared. One milliliter of each dilution was mixed with YGC in Petri dishes and incubated at 25 °C for 3 d. The different colonies of molds were isolated and purified by subculture on the above medium to obtain isolates for further identification.

The fungus identification down to the species was phylogeny based by the sequence analysis of the ARN_r D_2 LSU gene at the CEERAM (European Centre for Expertise and Research on Microbial Agents, La Chapelle sur Erdre, France).

Inoculation of buffered peptone water containing cinnamic compounds

Buffered peptone water (BPW) was prepared and was fortified by adding one or several cinnamic compounds to 900 mL of sterile BPW to give a total weight of 0.09 g (0.1 mg/mL). Thirty milliliters of this solution were transferred to a wide-mouth, 180 mL, sterile polypropylene container free of styrene. A touch loop inoculation was realized from a 3-d culture grown on YGC at 25 °C to the fortified BPW samples. The incubation was carried out for 5 d at 25 °C. Two types of control samples were systematically prepared: a blank BPW without inoculation but fortified with cinnamic compounds and another blank BPW with no fortification but inoculated. One milliliter of the incubated BPW was then inoculated on the YGC Petri dish to check after incubation by visual inspection that mold species had developed, and on PCA media to confirm the absence of bacterial contamination. The styrene production was monitored using solid phase micro extraction (SPME) followed by gas chromatography-mass spectrometer detector (GC-MS).

Semiquantitative assessment of styrene production by SPME-GC-MS

Wide-mouth container lids were pierced using the heated tip of a needle and were then closed immediately using styrene-free, adhesive tape. Headspace extraction (100 μm polydimethylsiloxane PDMS fiber phase) was carried out at ambient temperature (25 ± 2 °C) for 10 min. Then the SPME needle was removed from container and inserted into a gas chromatograph injector port for 1 min so that the analytes were desorbed from the PDMS fiber phase. Reconditioning of the SPME fiber phase was performed thermally for 10 min at 250 °C. The GC-MS was the same as used for the determination of styrene content in distillates described in a previous

paragraph and with the same instrumental setup. The oven temperature was programmed as follows: held initially at 50 °C for 1 min then from 50 °C (4 °C/min) → 100 °C/min (8 °C/min) → 200 °C (20 °C/min) → 240 °C. Semiquantitation of the styrene levels was performed by determining the styrene peak area in total ion current mode (TIC).

Quantitation of CO_2 by GC-MS

Twenty microliters of gas were collected using a gas syringe in the headspace of the wide-mouth containers and then injected into a GC-MS (as described in the previous paragraph). The oven was isothermally run at 60 °C for 3 min. In the column connected to the mass-spectrometer detector, helium flowed at 0.5 mL/min with a split ratio of 300:1. The areas of all the m/z fragments = 28, 32, 40, and 44 of the 4 main gases present in the Earth atmosphere, namely, dinitrogen, dioxygen, argon, and carbon dioxide, were extracted from the total ion current (TIC). These areas were subsequently corrected by the relative ionization yield of each molecule in the detector on one hand and, on the other hand, by the abundance of the molecular ion compared to the total abundance of all the ions of the same m/z. The percentage of CO_2 could then be calculated by dividing its area corrected by the total sum of the areas corrected.

Standardized inoculation of cinnamon samples

The standardized inoculation used below has been described elsewhere (Guiraud and Galzy 1980). Three Petri dishes, each containing 15 mL of YGC, were inoculated with an isolated mold strain and incubated for 3 d at 25 °C. Five milliliters of a sterile 0.05% Tween 80 solution were then poured over the medium on each of the Petri dishes. Media surfaces were scraped by means of a sterile L-shaped spreader. Supernatants were mixed together and decimal dilutions were prepared with the 0.05% Tween 80 solution until the turbidity completely disappeared. The inoculum is ready as soon as turbidity is no longer present. A mold enumeration was performed on the inoculum according to ISO 7954 (1987) to check the level of inoculation. Under a microbiological safety cabinet, 10 g of pure nonsterilized cinnamon were weighed in a wide-mouth, 60-mL, polypropylene container (3.5 cm in diameter). This open container was placed in another sterilized wide-mouth, 180-mL, polypropylene vessel (5 cm in diameter) containing 20 mL of sterilized deionized water to obtain high water activity inside this dual container system following closure with a polypropylene lid immediately following the inoculation of the cinnamon samples with molds. Ten grams of cinnamon were inoculated with 2 mL of inoculum. A control sample was also prepared by replacing the inoculum with 2 mL of the sterile 0.05% Tween 80 solution. The inoculated cinnamon sample in the dual container system and the control cinnamon sample in the same system were placed in an incubator at 25 °C. The styrene concentration in both systems was compared by SPME-GC-MS after 10-d incubation. Mold activity was assessed by determining the headspace CO_2 concentration by GC-MS after incubation.

Results and Discussion

Determination of the styrene content

Hydrodistillation was carried out with pure cinnamaldehyde and no styrene could be detected by GC. Use of the standard addition method on sample 45 from Table 1 containing initially 15 μg/g of styrene showed that no styrene was produced using the hydrodistillation method (see Figure 1) and the recovery rate for styrene was 72%. Styrene results in this report are uncorrected for recovery rate.

Table 1 — Styrene concentration and water activity of different cinnamon samples from diverse origins.

Sample nr	Cinnamon origin	Cinnamon aspect	a_w	Styrene content μg/g
1	Chine	Broken quills	0.543	5.0
2	Chine	Broken quills	0.796	5.5
3	Chine	Broken quills	0.729	6.0
4	Chine	Broken quills	0.804	6.5
5	Chine	Broken quills	0.792	7.5
6	Chine	Broken quills	0.797	8.0
7	Chine	Quills	0.704	8.4
8	Chine	Broken quills	0.562	9.0
9	Chine	Broken quills	0.695	10.0
10	Chine	Broken quills	0.600	12.0
11	Chine	Broken quills	–	17.0
12	Chine	Broken quills	0.642	17.5
13	Chine	Sticks	0.725	18.5
14	Chine	Sticks	0.708	20.5
15	Chine	Broken quills	0.753	21.0
16	Chine	Broken quills	0.593	32.5
17	Chine	Sticks	0.713	43.0
18	Chine	Broken quills	–	79.0
19	Chine	Broken quills	0.790	100.0
20	Chine	Broken quills	0.775	167.5
21	Chine	Broken quills	0.775	245.5
22	Indonésie	Broken quills	0.650	4.8
23	Indonésie	Broken quills	–	14.5
24	Indonésie	Broken quills	–	17.0
25	indonésie	Broken quills	0.821	19.0
26	Indonésie	Broken quills	0.716	21.0
27	Indonésie	Sticks	0.653	25.0
28	Indonésie	Broken quills	–	54.5
29	Indonésie	Broken quills	–	60.0
30	Indonésie	Sticks	0.705	127.5
31	Indonésie	Broken quills	0.706	8.4
32	Indonésie (Korintji)	Broken quills	0.700	2.8
33	Indonésie (Korintji)	Broken quills	0.658	5.0
34	Indonésie (Korintji)	Broken quills	0.675	8.5
35	Indonésie (Korintji)	Broken quills	0.691	9.5
36	Indonésie (Korintji)	Broken quills	0.780	10.0
37	Indonésie (Korintji)	Broken quills	0.660	11.0
38	Indonésie (Korintji)	Broken quills	0.658	11.0
39	Indonésie (Korintji)	Broken quills	0.695	11.5
40	Indonésie (Korintji)	Broken quills	0.670	12.5
41	Indonésie (Korintji)	Broken quills	0.775	13.0
42	Indonésie (Korintji)	Broken quills	0.590	13.5
43	Indonésie (Korintji)	Broken quills	0.675	14.0
44	Indonésie (Korintji)	Broken quills	0.647	14.0
45	Indonésie (Korintji)	Broken quills	–	15.0
46	Indonésie (Korintji)	Broken quills	–	16.0
47	Indonésie (Korintji)	Broken quills	0.755	16.5
48	Indonésie (Korintji)	Broken quills	0.725	19.5
49	Indonésie (Korintji)	Broken quills	0.685	19.5
50	Indonésie (Korintji)	Broken quills	0.743	22.0
51	Indonésie (Korintji)	Broken quills	–	22.0
52	Indonésie (Korintji)	Broken quills	0.740	24.0
53	Indonésie (Korintji)	Broken quills	–	27.0
54	Indonésie (Korintji)	Broken quills	0.748	28.0
55	Indonésie (Korintji)	Sticks	0.742	29.5
56	Indonésie (Korintji)	Broken quills	0.815	32.5
57	Indonésie (Korintji)	Broken quills	0.747	33.8
58	Indonésie (Korintji)	Broken quills	0.780	35.0
59	Indonésie (Korintji)	Broken quills	0.783	35.0
60	Indonésie (Korintji)	Sticks	0.755	35.5
61	Indonésie (Korintji)	Ground	–	36.5
62	Indonésie (Korintji)	Broken quills	0.785	37.0
63	Indonésie (Korintji)	Broken quills	0.761	38.2
64	Indonésie (Korintji)	Broken quills	0.600	42.0
65	Indonésie (Korintji)	Broken quills	0.561	50.5
66	Indonésie (Korintji)	Broken quills	0.660	52.0
67	Indonésie (Korintji)	Broken quills	0.755	52.4
68	Indonésie (Korintji)	Broken quills	0.792	53.0
69	Indonésie (Korintji)	Broken quills	0.710	55.0

Continued

Table 1—Continued

Sample nr	Cinnamon origin	Cinnamon aspect	a_w	Styrene content μg/g
70	Indonésie (Korintji)	Broken quills	0.732	57.0
71	Indonésie (Korintji)	Broken quills	0.728	59.0
72	Indonésie (Korintji)	Broken quills	0.675	87.0
73	Indonésie (Korintji)	Broken quills	0.726	92.0
74	Indonésie (Korintji)	Broken quills	0.635	101.0
75	Indonésie (Korintji)	Broken quills	—	113.0
76	Indonésie (Korintji)	Broken quills	0.695	115.6
77	Indonésie (Korintji)	Broken quills	0.730	127.0
78	Indonésie (Korintji)	Broken quills	0.759	130.0
79	Indonésie (Korintji)	Broken quills	0.330	140.5
80	Indonésie (Korintji)	Broken quills	0.360	146.0
81	Indonésie (Korintji)	Broken quills	0.773	187.5
82	Indonésie (Korintji)	Broken quills	0.720	189.5
83	Indonésie (Korintji)	Broken quills	0.740	201.0
84	Indonésie (Korintji)	Broken quills	0.773	247.8
85	Indonésie (Korintji)	Broken quills	0.775	403.5
86	Indonésie (Korintji)	Broken quills	0.881	523.8
87	Indonésie (Padang)	Sticks	0.550	1.1
88	Indonésie (Padang)	Sticks	0.685	3.0
89	Indonésie (Padang)	Sticks	0.753	7.2
90	Indonésie (Padang)	Sticks	0.719	8.2
91	Indonésie (Padang)	Sticks	0.690	29.0
92	Indonésie (Padang)	Sticks	0.739	49.0
93	Madagascar	Sticks	0.368	1.1
94	Madagascar	Broken quills	0.851	6.5
95	Madagascar	Broken quills	0.587	9.0
96	Madagascar	Broken quills	0.728	18.5
97	Madagascar	Broken quills	—	33.0
98	Madagascar	Broken quills	—	42.0
99	Madagascar	Broken quills	—	55.0
100	Seychelles	Sticks	0.794	45.0
101	Sri Lanka (Ceylon)	Broken quills	0.795	<0.5
102	Sri Lanka (Ceylon)	Broken quills	0.596	<0.5
103	Sri Lanka (Ceylon)	Broken quills	0.767	0.5
104	Sri Lanka (Ceylon)	Sticks	0.435	0.5
105	Sri Lanka (Ceylon)	Sticks	0.721	0.7
106	Sri Lanka (Ceylon)	Sticks	0.795	0.9

— = analysis not realized.

The styrene content was thus determined in over 100 cinnamon samples from different origins. The results are presented in Table 1 and expressed as micrograms per gram cinnamon. Cinnamon volatile oil contains at least 60% (w/w) of cinnamaldehyde (ISO 3216 (1997c); Ravindran and others 2004; European Pharmacopoeia 2008).

In Table 1, cinnamon samples are listed in order of increasing concentrations of styrene and also according to origin. True cinnamons (Sri Lankan, Seychelles, and Madagascan types) are *Cinnamomum zeylanicum* Blume according to ISO 6539 (1997a). Cassias (Chinese type and Indonesian type) are, respectively, *Cinnamomum cassia* Blume and *cinnamomum burmanii* Blume according to ISO 6538 (1997b).

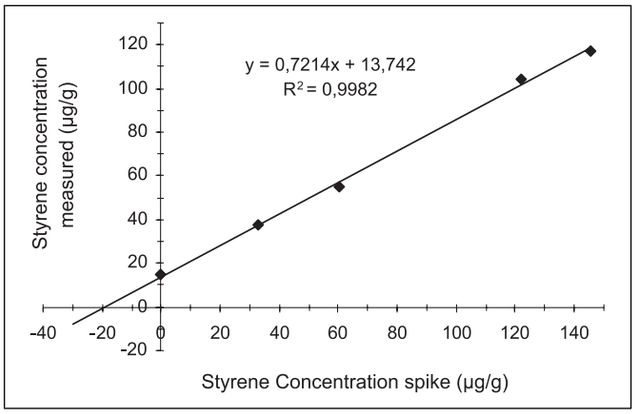

Figure 1—Standard addition plot used to determine recovery rate of styrene in cinnamon by hydrodistillation-GC-FID.

None of the cinnamon samples of Sri Lanka origin (commercially known as Ceylon cinnamon) had a styrene content exceeding 0.9 μg/g—regardless of the water activity level ranging from 0.435 up to 0.795—in line with the values reported so far (Steele and others 1994; Fragniere and others 2003). Although the cinnamons of Sri Lanka, Seychelles and Madagascar origins are theoretically all *Cinnamomum zeylanicum* Blume, only the Sri Lankan cinnamons contain a low styrene content of less than 0.9 μg/g whereas those originating from Madagascar can contain over 40 μg/g of styrene. Conversely, cinnamons of Indonesia origin and especially cinnamon from the Korintji region on the West coast of Sumatra might have very high styrene contents on arrival in Europe or America. Table 1 shows 2 very high values in particular (403.5 and 523.7 μg/g)—values that far exceed those reported in the literature. The 523 μg/g value can be seen to correspond to a very high water activity of 0.881, which suggests that the cinnamon was transported from Indonesia to France with high water content. Among the Indonesian cinnamon samples listed in Table 1, 63% have a styrene content exceeding 20 μg/g, which is a threshold below which we consider that the styrene plastic-like odor is not apparent as it is easily masked by the strong fragrance of cinnamaldehyde with a much higher concentration.

It is difficult to pinpoint a trend between a_w and styrene content since the latter can also be produced during drying, particularly if the drying process is long and poorly controlled. However, if the drying is not sufficient, styrene formation will increase during transportation, fuelled by high temperatures and high relative humidity. Naturally, during the life of the cinnamon tree, only small quantities of up to 0.26 μg/g styrene are produced in the plant tissues through the degradation of cinnamaldehyde and cinnamic acid (Fragniere and others 2003; BfR 2006). Thus, even taking into account any dehydration factor between the fresh and dried cinnamon quills to express this residual value compared to dried cinnamon, and overlooking styrene losses due to the drying process, the cinnamon could, at best, contain only a few μg/g of styrene. These values are recorded in the least contaminated types of cinnamon. Then, under unfavorable transport and storage conditions, styrene may be formed in far higher concentrations. The characteristic odor of styrene was noted many times during transportation of cinnamon quills. However, no studies clearly showing the role of microorganisms in styrene formation in cinnamon have been carried out to date. It was shown (Fragniere and others 2003) that for samples stored at ambient and 35 °C temperatures the formation of styrene was correlated to the oxygen level and humidity and that no variation was observed when stored at 60 °C. It was somehow an indication for microbial activity but the authors did not really consider the microbiological transformation and prioritized the chemical degradation pathway instead.

Fungal contamination

Fungal isolates were investigated. They were isolated from a dried Indonesian Korintji cinnamon, which presented with a 50μg/g styrene content (sample 65, Table 1). The mold flora level was 8.0E6 CFU/g and 5 fungal species were identified: *Syncephalastrum racemosum* (S1), *Penicillium citrinum* (S2), *Penicillium oxalicum* (S3), *Aspergillus niger* (S4), and *Paccilomyces puntonii* (S5). No yeasts were found (<10 CFU/g). Normally, *Aspergillus* spp. are the main components of the mold counts in spices except in

cinnamon and a few other spices in which *Penicillium* spp. predominated (Garrido and others 1992). Spice fungus contamination is probably due to the fact that the majority of spices on sale in international markets have a common origin and come from countries with optimum environmental conditions for the growth and development of molds. The genus *Syncephalastrum* is represented only by a single species, namely, *Syncephalastrum racemosum*. This is a thermophilic mold, commonly found in tropical, subtropical, and temperate regions. As for the *Paccilomyces puntonii* species, these are cosmopolitan molds found in the soil, plants, and food products.

Inoculation of BPW containing cinnamic compounds

The structure of styrene is contained in the several natural flavoring compounds found in cinnamon (Ravindran and others 2004) such as cinnamaldehyde, cinnamyl alcohol, methyl cinnamate, and ethyl cinnamate but also in less volatile compounds such as cinnamic acid. The structures of these compounds are presented in Figure 2. A buffered peptone water (BPW) medium was enriched with these molecules to a concentration of 0.1 mg/mL, and then inoculated with the five S1 to S5 molds isolated from the Indonesian Korintji cinnamon. BPW is a medium that is sufficiently lacking in nutrients to promote the consumption of these cinnamic substrate molecules by the appropriate molds. Low quantities were added to the BPW medium because the aromatic aldehydes are among the most powerful antifungal aromatic molecules found in volatile oils. We have also added cinnamyl cinnamate to the list even though it does not seem to be part of the cinnamon volatile oil (Nijssen and others 1996; Ravindran and others 2004) but contains 2 substructures of styrene.

To confirm that styrene production is definitely due to the biodegradation of cinnamic compounds and not to that of BPW, this medium was inoculated with a mixture of the 5 species of S1 to S5 molds, and enriched with deuterated cinnamic-d7 acid. Cinnamic acid was selected because it forms the basis of styrene production by numerous microorganisms and in many food products. After 5-d incubation, production of styrene-d7 was confirmed by the appearance of a molar mass peak of 111 Da at virtually the same retention time as nondeuterated styrene with a molar mass of 104 Da (Figure 3) and a mass spectrum clearly highlighting the deuterated benzyl fragment $m/z = 83$ (Figure 4). Both blank BPW without inoculation but fortified with deuterated cinnamic-d7 acid and blank BPW with no fortification but inoculated with the mold cocktail, showed no evidence of deuterated or undeuterated styrene production. All of these experimental data showed that the mold mixture clearly used deuterated cinnamic acid as the substrate, implying only a decarboxylation without affecting the styrene structure. A hydrogen atom took the place of the deuterated cinnamic acid carboxyl group via the biological decarboxylation.

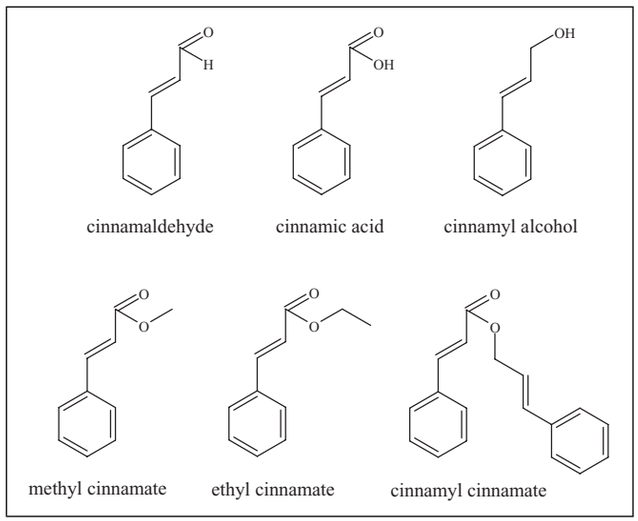

Figure 2—Cinnamic compounds used to enrich BPW medium to confirm styrene production after cinnamon mold flora inoculation.

The 2nd stage involved establishing whether each of the 5 species of mold actually produced styrene in the presence of a mixture of the 6 cinnamic compounds (Figure 2). Each of the molds was, therefore, incubated individually in the presence of this mixture and only 3 species, namely, *Penicillium citrinum* (S2), *Penicillium oxalicum* (S3), *Aspergillus niger* (S4), were proved capable of producing styrene under the conditions given. Finally, each species of styrene-producing mold was inoculated in the presence of just 1 cinnamic compound at a time and, out of these 18 possible combinations, 17 resulted in styrene formation after incubation. Only *Penicillium oxalicum* (S3) in the presence of cinnamaldehyde did not produce styrene. This was undoubtedly due to the acute fungicidal effect of this molecule, which inhibited the development of this mold. Furthermore, this was confirmed by the absence of mold after inoculation on YGC. It should be noted that cinnamyl cinnamate, which is not present in cinnamon volatile oil, was also transformed into styrene by the producing molds. The cinnamic compounds generating greater quantities of styrene, regardless of the species of producing mold, were cinnamic acid and cinnamyl alcohol.

Standardized inoculation of cinnamon samples with S2, S3, and S4

Further, to confirm the *in vitro* production of styrene by 3 species of mold, namely, S2, S3, and S4 obtained from the Indonesian Korintji cinnamon, we performed standardized inoculation of cinnamon samples of varying origin: China, Indonesia (Padang and Korintji), and Sri Lanka with both the whole and ground forms. All of the cinnamon samples used had a styrene content less than or equal to 10 μg/g before inoculation. The tests were carried out in duplicate with noninoculated controls, which were also prepared in duplicate. The level of inoculation was calculated after counting each species of mold in the inoculums. Inoculation with the S2 molds was equivalent to 1.8E3 CFU/g of cinnamon, 1.4E3 CFU/g for S3, and 1.6E4 CFU/g for S4 for whole cinnamon and, respectively, 1.4E3 CFU/g, 1.6E3 CFU/g, and 1.9E2 CFU/g for ground cinnamon.

The results are presented in Table 2. Water activity was measured after 10-d incubation showing that the latter reached a value that was at least greater than 0.913 and therefore much higher than the mold formation a_w threshold. Styrene content was measured semiquantitatively by SPME. Samples of whole cinnamon of Indonesia Korintji origin, whether or not inoculated, naturally developed a large quantity of styrene during incubation compared to cinnamon samples of other origins. It should be noted that, in the case of noninoculated samples of this origin, the low mold formation marked by a small CO_2 content, did not prevent important styrene release. Their styrene content is, however, 10 times higher after inoculation, increasing by the same factor of 10 the percentage of CO_2 in the headspace, thus indicating potent fungal activity. In order of size, samples of Korintji cinnamon 1 and 2 presented styrene contents of 5.0 and 10.0 μg/g, respectively, increasing to 156 and 195 μg/g after inoculation and subsequent incubation. The tendency for Indonesian Korintji cinnamon to release a large quantity of styrene was already evident from numerous samples in Table 1.

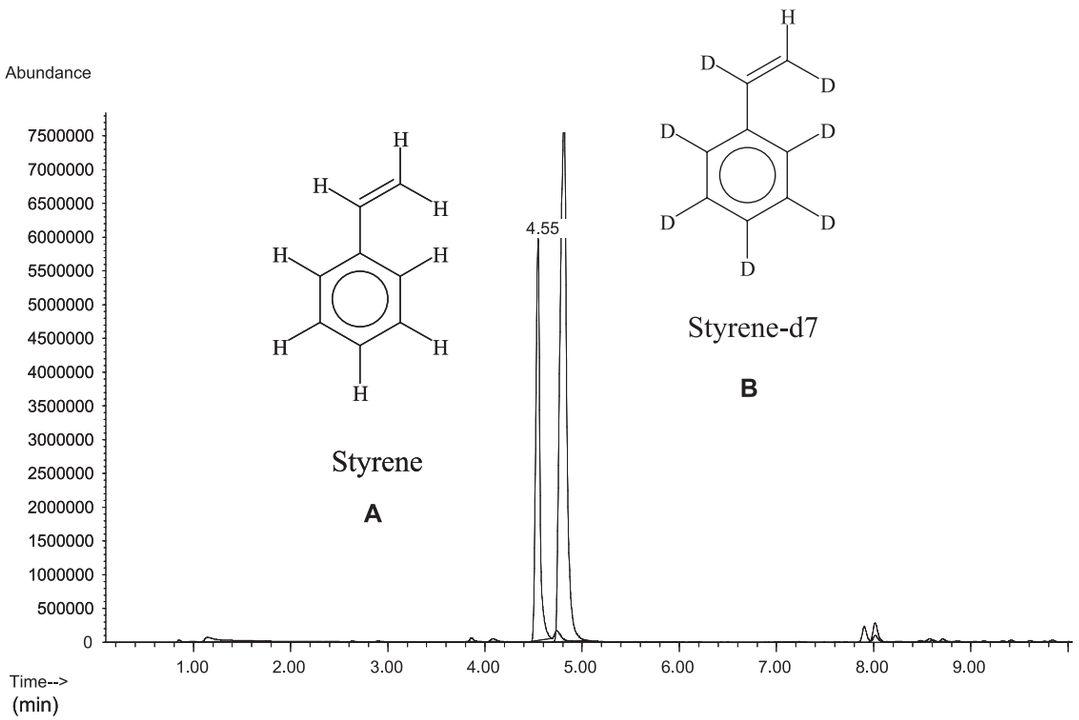

Figure 3 — Superimposed SPME-GC-MS chromatograms of styrene from producing molds on BPW containing cinnamic acid (A) and cinnamic-d7 acid (B).

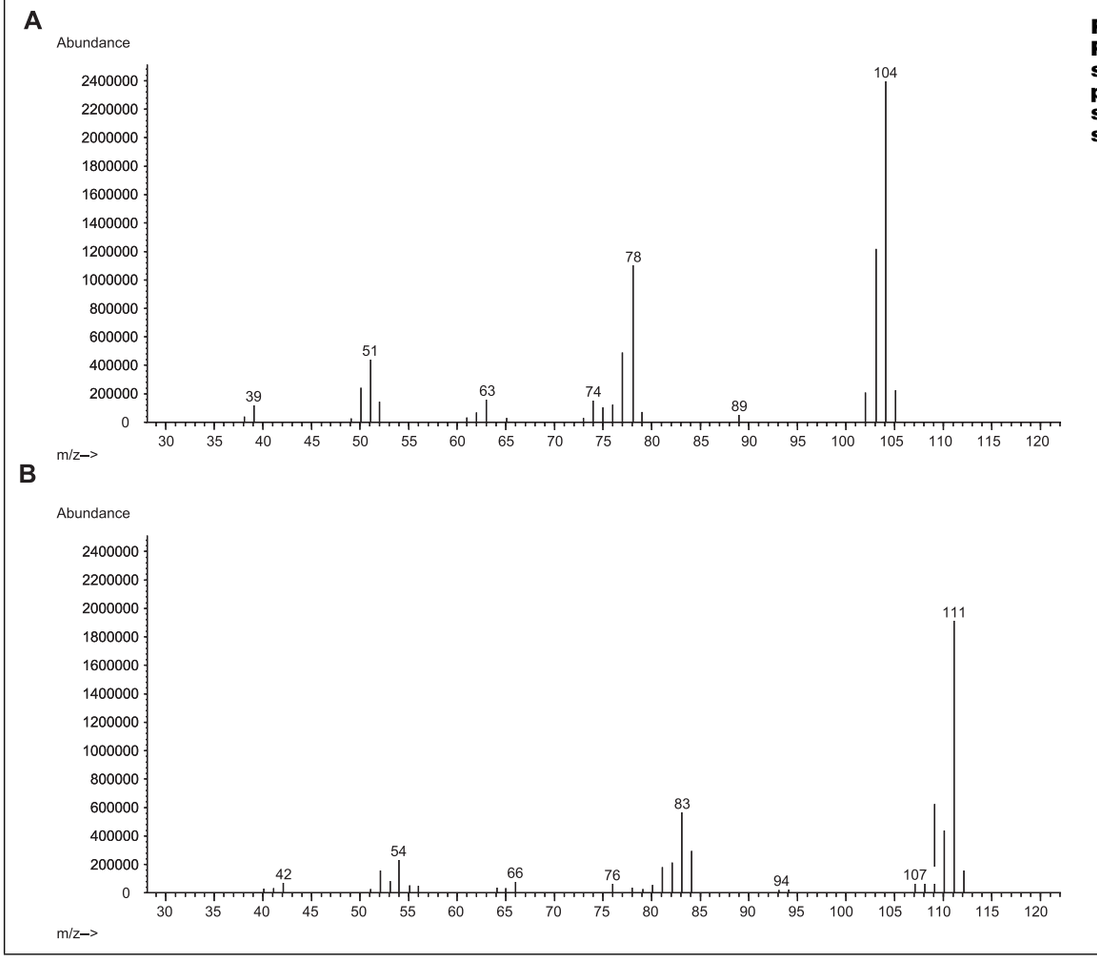

Figure 4 — Recorded mass spectra at the peak apex for styrene (A) and styrene-d7 (B).

Table 2—Effect of a 10-d incubation at 25 °C on inoculated and uninoculated cinnamon samples.

Uninoculated cinnamon samples									
	Before incubation			Whole form after 10-d incubation			Ground form after 10-d incubation		
Cinnamon origin associated sample	Initial a_w	Mold CFU/g	Volatile oil[a] mg/100 mL	Styrene area × 10^3	CO_2 %	Final a_w	Styrene area × 10^3	CO_2 %	Final a_w
Korintji	0.658	<10	1.68	63516	0.5	0.946	838	0.1	0.925
Sample 33, Table 1				78872	0.2	0.943	997	0.1	0.937
Korintji	0.780	100	2.21	67417	0.7	0.973	701	0.2	0.924
Sample 36, Table 1				34143	0.5	0.995	1091	0.1	0.930
Padang	0.685	<10	2.06	2834	0.1	0.970	<300	0.1	0.928
Sample 88, Table 1				1242	0.1	0.938	<300	0.1	0.932
China	0.543	5.2E4	2.10	414	8.9	0.984	2456	0.3	0.927
Sample 1, Table 1				822	15.9	0.986	2752	0.2	0.929
Sri Lanka	0.596	6.1E5	1.12	449	2.2	0.965	<300	0.7	0.943
Sample 102, Table 1				590	6.9	0.984	<300	0.3	0.938
Inoculated cinnamon samples									
	Before incubation			Whole form after 10-d incubation			Ground form after 10-d incubation		
Cinnamon origin associated sample	Initial a_w	Mold CFU/g	Volatile oil[a] mg/100 mL	Styrene area × 10^3	CO_2 %	Final a_w	Styrene area × 10^3	CO_2 %	Final a_w
Korintji	0.658	<10	1.68	547476	4.5	0.940	470	0.1	0.928
Sample 33, Table 1				573118	2.2	0.933	353	0.1	0.932
Korintji	0.780	100	2.21	344631	1.5	0.923	1965	0.1	0.921
Sample 36, Table 1				358779	3.0	0.917	2156	0.1	0.928
Padang	0.685	<10	2.06	168615	1.3	0.931	<300	0.1	0.928
Sample 88, Table 1				103682	0.4	0.913	<300	0.1	0.936
China	0.543	5.2E4	2.10	705	15.7	0.960	5235	0.3	0.931
Sample 1, Table 1				622	15.0	0.933	7648	0.3	0.929
Sri Lanka	0.596	6.1E5	1.12	491	9.6	0.938	<300	0.2	0.941
Sample 102, Table 1				486	7.7	0.936	<300	0.8	0.934

[a]Determined according to ISO 6571 (2008).

The sample of Indonesian Padang cinnamon in its whole, non-inoculated form generated only a small quantity of styrene after incubation but contrastingly larger quantities after inoculation while also releasing more CO_2.

Finally, samples of whole Chinese and Sri Lankan cinnamon behaved in the same way: no effect of inoculation, release of very small quantities of styrene despite marked fungal formation highlighted by substantial CO_2 levels after incubation. This result corroborates the information provided in Table 1 with regard to cinnamon of Sri Lanka origin in which all styrene contents were less than or equal to 0.9 μg/g. Moreover, the Sri Lankan cinnamon sample has the lowest volatile oil content of all the samples, thus ruling out the assumed anti-fungal properties of excessive quantities of cinnamaldehyde. On the other hand, Table 1 showed that cinnamon of Chinese origin contained styrene values of up to 245.5 μg/g whereas the incubated samples had almost no styrene production.

As regards the ground cinnamon samples and regardless of origin, these were virtually devoid of styrene production with or without inoculation. The quantity of CO_2 released was still very low, thus highlighting inhibition of the formation of the mold flora in ground cinnamon. We propose that although cinnamaldehyde is transformed by certain species of *Aspergillus* or *Penicillium* endogenous to cinnamon, grinding of the latter generates such an amount of volatile oil in the gaseous volume that the molds can no longer form. This strong antifungal property of cinnamon volatile oil has been demonstrated on many occasions (Sinha and others 1993; Singh and others 2007). Consequently, regardless of the water activity of cinnamon of any origin, grinding of the bark naturally inhibits mold formation and styrene release.

Conclusions

Styrene is a natural compound in dried cinnamon where it can be found at levels up to 524 μg/g. Therefore it appears prudent to perform controls of the styrene level in cinnamon raw material. Styrene is a by-product of several species of fungus naturally occurring in cinnamon especially from Indonesia-origin cinnamon. Isolated from an Indonesian-Korintji cinnamon, *Penicillium citrinum*, *Penicillium oxalicum*, and *Aspergillus niger* are capable of producing styrene *in vitro* not only from 5 cinnamic compounds present in cinnamon but also from cinnamyl cinnamate which is not mentioned as a cinnamon constituent. If inoculated, these 3 species of molds can also provoke styrene production in cinnamon from diverse origins except from Sri Lanka. The Sri Lankan cinnamon does not seem prone to contain high levels of styrene whatever the conditions of water activity that can take place during drying or transportation. Once ground, cinnamons from all origins show a reduced styrene production that is likely due to the volatile oil emission, which has a high inhibitory effect on mold flora.

Acknowledgments

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