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Spoilage fungi and their mycotoxins in commercially marketed chestnuts

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Abstract

A nationwide survey was carried out to assess mould spoilage of *Castanea sativa* nuts sold in Canadian grocery stores in 1998–99. Morphological and cultural characters, along with secondary metabolite profiles derived from thin-layer chromatography, were used to sort and identify fungi cultured from nut tissue. Three mycotoxigenic fungi dominated (*Penicillium crustosum*, *Penicillium glabrum*/*spinulosum* and *Penicillium discolor*) and were isolated at frequencies of 67.1%, 18.6% and 17.7%, respectively, from a total sample size of 350 nuts. Another mycotoxin producer, *Aspergillus ochraceus* was also isolated, but at a much lower frequency. HPLC and diode array detection were used to confirm the suspected presence of the mycotoxins penitrem A, chaetoglobosin A and C, emodin and ochratoxin A in extracts prepared from naturally infected nut tissue. To the best of our knowledge, this is the first time emodin has been found in a naturally contaminated food source.

Keywords: *Castanea sativa*; *Penicillium*; *Aspergillus*; Penitrem A; Ochratoxin A; Emodin; Chaetoglobosins

1. Introduction

Casual observations of visible mould growth, insect damage and malleability of imported chestnuts sold in Canadian grocery stores during the late 1990s suggested a high frequency of contamination by spoilage fungi. Two chestnut species dominate the chestnut market in Canada, the Chinese chestnut (*Castanea mollissima*), grown primarily in Asia, as well as in discrete populations in the southern US and the European chestnut (*Castanea sativa*), grown in temperate regions of continental Europe. The most recent surveys of fungal spoilage of chestnuts were published in 1960 and 1975 for *C. mollissima* (Wright, 1960; Wells and Payne, 1975) and earlier for *C. sativa* (Lanza, 1950; Riccardo, 1963). These studies implicated several species from a range of mycotoxigenic fungal genera, including *Alternaria*, *Fusarium*, *Penicillium* and *Aspergillus*. Since that time, species concepts in these genera have undergone considerable revision (especially those of *Penicillium* and *Aspergillus*; taxonomic review in

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Samson and Gams, 1984), resulting in more precise concepts with a high correlation with mycotoxin profiles.

In the mould genera *Penicillium* and *Aspergillus*, secondary metabolite profiles have become critical components of species concepts, and have utility as identification tools and as taxonomic characters (Frisvad, 1981; Filtenborg et al., 1983; Frisvad and Filtenborg, 1983; Abramson and Clear, 1996). Grouping strains by secondary metabolite profiles simplifies the task of sorting them for identification. Because secondary metabolites are critical aspects of interactions between these moulds and their substrates, they allow strains to be identified on the basis of characters that reflect their biological significance. Furthermore, mycotoxin profiling of moulds is particularly germane when studying strains isolated from substrates intended for consumption by humans or animals.

Chestnuts have a higher water activity, starch and moisture content and lower protein levels than most other nuts (Beuchat, 1978). Thus, they have the potential to support the growth of a different spectrum of mycotoxigenic fungi than do other nuts or grains. Spoilage organisms generally infect nut tissue through a breach in the outer shell. These breaches can be caused by weevils (Wells et al., 1975; Wells and Cole, 1977) such as *Currulio sayi* (Payne et al., 1972), which produce exit tunnels 1–2 mm in diameter. Fungi recovered from weevil damaged *C. mollissima* nuts are capable of producing toxic compounds when grown in vitro (Wells and Payne, 1975). This indicates that there is a clear potential for mycotoxin production in *C. sativa* nuts infected by spoilage fungi.

Contaminated nuts or grains are potential sources of mycotoxin exposure for the average consumer who may not realize the significance of mould or insect damage, and may not carefully examine these foods before they are cooked. The survey reported in this paper was undertaken to identify and quantify spoilage fungi found in *C. sativa* nuts sold commercially in Canada, sorted using secondary metabolite profiles and identified using modern species concepts. We also made preliminary attempts to identify mycotoxins isolated directly from spoiled *C. sativa* nuts using qualitative HPLC.

2. Materials and methods

2.1. Sample collection

Chestnut samples (*C. sativa*) of about 1 kg were purchased by correspondents (see acknowledgements) from grocery stores at the following locations across Canada: Charlottetown (PEI), Kentville (Nova Scotia), Lethbridge (Alberta), Montreal (Québec), Oakville (Ontario), Saskatoon (Saskatchewan) and Winnipeg (Manitoba) and sent to Ottawa. A sample of *C. mollissima* nuts received from Summerland (British Columbia) was excluded. The samples were random, with no attempt made to deliberately choose or reject nuts with spoilage symptoms, such as visible mould growth, cracks or insect tunnels. Samples were sent by overnight courier to Ottawa and processed within 1–2 days. Fifty nuts from each 1-kg sample were randomly selected, scored for visible signs of damage or infection (using the following categories: damage of outer shell, i.e., cracks or holes, discoloration of tissue, insect holes in seed tissue and tissue having visible mould growth) and then subsequently used for culture isolations. The total sample size was 350 *C. sativa* nuts. Additional nonrandom subsets of 25 nuts displaying internal mould colonization (apparent to the unaided eye) were selected from the Nova Scotia sample, and from an additional sample purchased in Ottawa, Ontario, and used for mycotoxin analysis of nut tissue.

2.2. Isolation of spoilage moulds

Each nut was surface sterilized by immersing it in 95% ethyl alcohol for 30 s, removed from the ethanol and then cleaved in half with a sterilized blade. Explants 1–5-mm diameter were removed from the exposed center of the seed to 2-ml screw cap vials containing 1–1.5 ml of Czapek Yeast Extract agar with micronutrients (CYA, Samson and Pitt, 1985) emended with 5 mg/l chloramphenicol to retard bacterial growth. For nuts lacking obvious infection symptoms, tissue explants were taken from the burr crown region (containing a portion of the radicle and cotyledon) and from the calyx region of the nut (containing only a portion of

the cotyledon) and placed in separate vials. For nuts having tissue discoloration or insect holes, explants were taken from the symptomatic region and from a region free of visible symptoms. If sporulation was visible in the nut tissue or between the tissue surface and the shell, only the sporulating region was sampled. Sample vials were incubated in the dark for two weeks at room temperature, sealed with Parafilm and stored at 4 °C until processed for identification.

Axenic isolates were derived from the CYA vials with visible mould colonies. Spores were removed from the vial with a disposable sterile plastic loop, suspended in semi-solid agar and then streaked across CYA in 9-cm polystyrene Petri dishes. Growth was monitored for 4–7 days in the dark at room temperature. Mixed colonies were separated by restreaking spores until pure isolates of each colony type were obtained. Representative cultures of the following species have been deposited in the CCFC under the following accession numbers: *Aspergillus japonicus* var. *japonicus* 231271; *Aspergillus ochraceus* 231270; *Penicillium bilaii* 230242; *Penicillium crustosum* 230238; *Penicillium discolor* 230237; *Penicillium spinulosum* 230097, *Penicillium glabrum* 230098, 230099, 230100; *Penicillium glandicola* 230240; *Penicillium miczynskii* 230239; and *Penicillium thomii* 230241.

2.3. Use of thin layer chromatography (TLC) for chemotaxonomic characterization

Purified strains to be grown for TLC analysis were transferred to CYA in Petri plates by single-point inoculation and placed in a dark incubator for 9–10 days at 25 °C. After incubation, the isolates were sorted into groups based on similarities in colony color, texture, growth rate, production of soluble pigments and liquid exudates. Secondary metabolite chromatograms of all strains were prepared using a TLC technique developed by Filtenborg et al. (1983; illustrated in Singh et al., 1991) in which an agar plug from each strain was extracted with chloroform/methanol (2:1; v:v) and spotted on Whatman LKDF60 channeled silica gel TLC plates on duplicate plates. Each plate included griseofulvin (5 mg griseofulvin: 2 ml methanol) as an internal standard for the calculation of relative Rf values (=Rfg). TLC plates were developed in tanks lined with solvent-saturated filter paper, one duplicate in toluene/ethyl acetate/90% formic acid (TEF; 5:4:1, v/v/v) and the other in chloroform/acetone/2-propanol (CAP; 85:15:20, v/v/v).

Plates were air-dried and examined under a long-wave UV lamp (366 nm), then sprayed with diluted sulfuric acid (18 M diluted to 50% with methanol) and reexamined under long-wave UV light. The plates were then heated on a hot plate until coloured bands appeared. Long-wave UV light profiles were photographed using Kodak Elite chrome film (ISO 100, exposure 45 s, 1 or 2 min, with combinations of Kodak 2E and Sky 1A filters) and images of post-heating profiles were captured for comparison by digital scanning using a Hewlett Packard ScanJet ADF color scanner.

2.4. Identification of mould species using micromorphology

Strains with similar secondary metabolite profiles and colony characters were sorted into groups for morphological species identification. Selected cultures from each group were transferred to CYA and 2% Malt Extract Agar (MEA, Samson and Pitt, 1985) using a three-point inoculation technique (Pitt, 1979). Cultures were incubated in the dark for 7 days at 25 °C. The micromorphological taxonomic systems and keys of Pitt (1979, 1988), Samson (1979) and Tzean et al. (1990) were used for species identification.

2.5. Chemotaxonomic comparison of species grown on various media

To determine whether secondary metabolites produced on CYA could be produced on different media, representative strains of the predominant species were grown on CYA, MEA and chestnut agar [500 g Valade™ chestnut purée (composed of chestnuts in salt and water), 20.0 g agar and 1 l water]. Metabolites were extracted and TLC plates prepared as above. Metabolite profiles were compared based on patterns before spraying, after spraying and after heating between media for individual species and between species on the basis of

Rfg values and colour reactions of the metabolite spots.

2.6. Isolation of mycotoxins from naturally contaminated chestnut tissue

Two samples of 25 nuts each, one from Nova Scotia and one from Ottawa, displaying internal infection symptoms were selected to assay for mycotoxins produced in naturally colonized nuts. Nuts were removed from their shells, macerated in 600 ml of distilled water/acetonitrile (1:1 v:v) and transferred to a 2-l round-bottom flask. The tissue was extracted for 1 h in 300 ml of *n*-hexane (final mixture of 1:1:1 distilled water/acetonitrile/hexane) with mechanical rotation. The flask contents were vacuum filtered through Whatman #1 filter paper and the filtrate transferred to a 2-l separatory funnel. The filtrate was vigorously shaken, left to stand for 5 min and the resulting phases were collected. The hexane phase was concentrated using roto-evaporation, then evaporated to dryness under a stream of nitrogen (fraction B). The acetonitrile/water phase was then extracted with 400 ml chloroform, and the two resulting phases were collected in two 1-l round-bottom flasks. The chloroform phase was roto-evaporated to dryness (fraction C), as was the water/acetonitrile phase until the acetonitrile was evaporated. A 50-ml aliquot from the water phase was loaded onto three 900-mg C18 Sep-Pak cartridges linked in series. The cartridges were flushed twice with 10 ml of distilled water (fractions D and E), once with 10 ml of ethyl acetate (fraction F) and finally three times with 10 ml of methanol (fractions G, H, I). The organic solvent phases of all fractions were evaporated under nitrogen and then lyophilized to remove residual water.

The second sample of 25 nuts displaying internal mould symptoms, purchased in Ottawa, Ontario, was treated as above, with an additional chloroform extraction of the macerated chestnuts to facilitate the isolation of chaetoglobosins. The chestnut material remaining after the hexane/water/acetonitrile extraction was placed in a 2-l beaker, resuspended in 500 ml of chloroform and stirred for 1 h, filtered under vacuum using a Buchner funnel and roto-evaporated to dryness (fraction A).

2.7. HPLC analyses of the nut tissue fractions

In Lyngby, dried fractions were resuspended in 1 ml of methanol and a 10-μl aliquot was injected into an HP 1090M HPLC with a built-in photodiode array detector. HPLC analyses were performed with a Agilent Hypersil BDS C-18 column (4 mm i.d., 100 mm length, particle size: 3 μm). Metabolites were separated with the following solvent gradient system (solvent A = water and solvent B = 50 ppm trifluoroacetic acid in acetonitrile): initial solvent gradient conditions were set at 15% of solvent B and raised to 100% in 40 min, held at 100% for 3 min and finally returned to 15% in 7 min at a flow rate of 1.0 ml/min (column temperature maintained constant at 40 °C). Mycotoxin identifications were made using retention times, retention indices and UV absorbance spectra. The identifications of chaetoglobosin C, emodin, penitrem A and ochratoxin A were confirmed using standards purchased from Sigma and identification of chaetoglobosin A was confirmed using a standard from the Mycology Group, BioCentrum-DTU standard collection.

The presence of ochratoxin A in fraction H was reconfirmed in Ottawa with a commercial standard using a Varian Vista 5500 HPLC and Bio-Rad Bio-Dimension UV-Vis Scanning detector with a CSC (Chromatography Sciences) Hypersil 120A/ODS column (4.6 mm i.d., 100 mm length, particle size: 3 μm) using the following solvent gradient: 15% acetonitrile (0.1% TFA)/85% water (0.1% TFA) to 100% acetonitrile (0.1% TFA) at a flow rate of 1 ml/min for 40 min.

3. Results

3.1. Spoilage moulds identified from the chestnuts

Chestnut samples were obtained from seven provinces in Canada. The spoilage symptoms observed from the *C. sativa* nuts are summarized in Table 1. A large majority of the total 350 *C. sativa* nuts exhibited visible damage, including visible mould growth, insect tunnels, tissue discoloration or cracks in the outer shell. Virtually all damaged nuts (98%) yielded culturable fungi. Discoloration of nut tissue, varying in appearance from light to dark brown, was an important symptom that was correlated with the

Table 1
Correlation of combinations of spoilage symptoms observed in seven samples of chestnuts purchased in Canada in November–December 1998 (total sample size, $n=350$ nuts)

	Damaged outer shell	Discolored tissue	Tissue with insect holes	Tissue with visible mould
Damaged outer shell	265	151	72	70
Discolored tissue	147	174	67	70
Tissue with insect holes	68	67	68	45
Tissue with visible mould	70	72	45	74
Total sample ($n=350$)	265	174	68	74

isolation of moulds. Visible mould was observed in 20.6% of the nuts, usually associated with tissue discoloration and/or outer shell damage. Insect holes were noted in 19.4% of the nuts, and were strongly associated with dark tissue discolorations and the presence of visible mould.

Twelve hyphomycete species were isolated and identified, primarily species of *Penicillium* and *Aspergillus*, and enumerated based on colony characters, TLC profiles and micromorphology (Table 2). Three species of *Penicillium* were routinely isolated from each of the seven samples: *P. crustosum* Thom (67.1% frequency), *P. glabrum*/*spinulosum* (18.6 %, see below) and *P. discolor* Frisvad et al. (17.7%). All three species have been reported to produce mycotoxins in agar culture. *A. ochraceus* Wilhelm, isolated at a relatively low frequency of 2.0%, and occurring in only two of the seven samples, was considered noteworthy because of its ability to produce ochratoxin A. The following fungi were isolated at frequencies of less than 2.0%: *Penicillium chermesinum* Biourge, *P. glandicola* (Oud.) Seifert and Samson, *Penicillium echinulatum* Fassatiová, *P. miczynskii* Zaleski, *P. thomii* Maire, *P. bilaii* Chalabuda, *A. japonicus* K. Saito var. *japonicus* and a *Trichoderma* spp. Microfungi that are not normally considered mycotoxin producers, such as zygomycetes and yeasts, were not counted or identified, but occurred at low frequencies.

3.2. Chemotaxonomic characterization

Secondary metabolite profiles on CYA were an effective means of sorting cultures into groups and differentiating between species. Minor variations among metabolite profiles were observed for all species grown on CYA, MEA and chestnut agar. All

Table 2
Spoilage fungi isolated from chestnut samples purchased in seven Canadian provinces in November–December 1998 (total sample size, $n=350$)

Species	Numbers of isolates							
	Que. ($n=50$)	N.S. ($n=50$)	Man. ($n=50$)	P.E.I. ($n=50$)	Ont. ($n=50$)	Sask. ($n=50$)	Al. ($n=50$)	Isolation frequency percentage ($n=350$)
P. crustosum	35	38	37	35	36	28	26	67.14
P. glabrum/*spinulosum*[a]	23	2	3	7	10	15	5	18.57
P. discolor	4	5	17	8	3	5	20	17.71
P. chermesinum	6	0	0	0	0	0	0	1.71
P. glandicola	0	1	0	3	0	0	0	1.14
P. echinulatum	0	0	0	0	0	0	2	0.57
P. miczynskii	0	1	0	0	0	0	0	0.29
P. thomii	2	0	0	0	0	0	0	0.57
P. bilaii	1	0	0	0	0	0	0	0.29
A. ochraceus	1	6	0	0	0	0	0	2.00
A. japonicus	0	0	0	1	0	0	0	0.29
Trichoderma spp.	0	1	0	0	0	0	0	0.29

[a] We did not attempt to distinguish between *P. spinulosum* and *P. glabrum* during the survey. See text for explanation.

species displayed relatively consistent TLC profiles, except strains of the *P. glabrum*/*spinulosum* complex. Strains of *P. glabrum* and *P. spinulosum* are difficult to separate using micromorphological characteristics (Pitt et al., 1990), and we did not attempt to distinguish between them in this survey. Variation in TLC profiles and in the pigmentation and reverse colouration observed on CYA, MEA and chestnut agar were observed among strains belonging to this complex. Strains within this complex could be grouped into at least three different metabolite profiles differing from each other by more than one metabolite spot.

3.3. Identification of mycotoxins from naturally infected nut tissue using HPLC

Five mycotoxins were identified from several of the collected fractions by comparison of their HPLC retention times and the UV spectra of their peaks with those of standards (see Table 3). From the Nova Scotia sample, penitrem A was isolated from fraction C with a retention time of 23.38 min, emodin was isolated from fraction G with a retention time of 17.75 min and ochratoxin A was isolated from fraction H with a retention time of 16.08 min. Emodin and ochratoxin A were obtained in a pure form. The mycotoxins chaetoglobosin A and C were isolated from fraction A with retention times of 16.87 and 18.47, respectively, along with penitrem A from fraction C of the Ontario sample. Ochratoxin A production was confirmed by independent HPLC analysis of the same fraction (Nova Scotia fraction H) in Ottawa with a retention time of 18.39 (standard retention time 18.22) and matching UV spectrum.

Table 3
Mycotoxins extracted from chestnut tissue summarized by extraction fraction, retention times and associated fungal species

Mycotoxin	Fraction	Retention time (min)	Suspected producing species
Chaetoglobosin A	A	16.87	*Penicillium discolor*
Chaetoglobosin C	A	18.47	*Penicillium discolor*
Penitrem A	C	23.38	*Penicillium crustosum*
Emodin	G	17.75	*Aspergillus ochraceus*
Ochratoxin A	H	16.08	*Aspergillus ochraceus*

Metabolites were separated with the following solvent gradient system (solvent A = water and solvent B = 50 ppm trifluoroacetic acid in acetonitrile): initial solvent gradient conditions were set at 15% of solvent B and raised to 100% in 40 min, held at 100% for 3 min and finally returned to 15% in 7 min at a flow rate of 1.0 ml/min (column temperature maintained constant at 40 °C).

4. Discussion

Fungal spoilage of commercially purchased chestnuts is clearly a cause for concern. Fungal colonization of the nuts may have occurred at any stage from flowering, harvest, storage, sorting or transport via damaged portions of the outer shell. The results show that virtually all *C. sativa* nuts with visible damage yield culturable moulds. Moreover, a number of fungi isolated in this survey are well-known mycotoxin-producing, spoilage organisms specializing in proliferation within storage environments. The relative frequencies of *P. crustosum*, *P. discolor* and *P. glabrum*/*spinulosum* may be higher because of their ability to grow under the refrigeration conditions that are commonly used for chestnut storage. The fungi isolated at frequencies below 2.0% are less tolerant to cold and will not grow at refrigeration temperatures (Domsch et al., 1980; Payne et al., 1983; Pitt, 1988; Frisvad et al., 1997).

As previously suggested by Frisvad (1989a) and implemented by Mills et al. (1995), we found that colony morphology used in conjunction with thin layer chromatograms of secondary metabolites was an effective means of sorting large numbers of micromorphologically similar isolates into taxonomically meaningful groups. A high level of interspecies consistency was observed when metabolite profiles produced on a single medium were compared. The only exception was with strains identified morphologically as belonging to the *P. glabrum*/*spinulosum* species complex, whose variations in metabolite profiles indicated the presence of two or more species. Variations were also noted in metabolite profiles of isolates grown on CYA, MEA or chestnut agar, suggesting that secondary metabolism may be induced at different intensities when a strain is exposed to nutritional variations. These observations reinforce the idea that media composition and brands of media ingredients should be standardized when applying metabolite profiles to sort and identify species (Filtenborg et al., 1990; Okuda et al., 2000).

The fungi derived from *C. sativa* nut tissue were relatively well-known *Penicillium* and *Aspergillus* species. *P. crustosum* predominated in this survey, confirming the reputation of this species as a ubiquitous spoilage organism (Domsch et al., 1980). Wells and Payne (1975) also isolated this species (as *Penicillium terrestre sensu* Raper and Thom) at a high frequency from *C. mollissima* nuts. *P. discolor*, which did not appear in the Wells and Payne (1975) survey, is a recently described species not previously reported in Canada, although known from the southern USA (initially identified as *Penicillium aurantiovirens* Biourge , cf. Springer et al., 1976; Frisvad et al., 1997). Both *P. crustosum* and *P. discolor* have previously been reported from other nut species, specifically walnuts, black walnuts and pecans (Huang and Hanlin, 1975; Frisvad et al., 1997; Frisvad, unpublished), and *P. crustosum* has also been isolated from coconut and pistachio nuts (El-Banna and Leistner, 1988; Hatton and Kinderlerer, 1991). Wells and Payne (1975) reported that *Penicillium* spp. were also the most commonly isolated mycotoxigenic fungi. However, aside from *P. crustosum*, the profile of species identified by Wells and Payne (1975) from *C. mollissima* is different from what we observed on *C. sativa*.

In total, five mycotoxins associated with the more prolific mycotoxigenic fungi isolated in this survey, were identified from naturally colonized nut tissues. Chaetoglobosin A and to a lesser extent chaetoglobosin C, both produced by *P. discolor* (Frisvad et al., 1997), have been proven to be cytotoxic to human cell lines as well as having an acute toxicity when subcutaneously injected to mice (Umeda et al., 1975; Sekita et al., 1982). However, when administered orally, toxicity was found to be at least eight times lower than parenteral toxicity (Ohtsubo et al., 1978). Emodin is a commonly occurring genotoxic, diarrheagenic anthraquinone (Müller et al., 1996). Hasan (1998) demonstrated that emodin is produced in vitro in peanuts inoculated and incubated with an *Aspergillus wentii* strain. Moreover, Wells et al. (1975) found that emodin, present in culture extracts of an *A. wentii* strain isolated from weevil-damaged *C. mollissima*, induced a general debilitation and mortality when ingested by 1- and 12-day-old cockerels. Although emodin production has been identified as naturally occurring in numerous vegetable species (Müller et al., 1999) and fungal species isolated from food sources (Wells et al., 1975; Hasan, 1998), this is, to the best of our knowledge, the first report of emodin present in naturally contaminated food. Ochratoxin A is a regulated mycotoxin with nephrotoxic, immunosuppressive, teratogenic and carcinogenic properties (Smith and Moss, 1985; Frisvad, 1989a; Lea et al., 1989). Both emodin and ochratoxin A are produced by *A. ochraceus* (Frisvad, 1989b), which occurred at a high frequency in the sample from Nova Scotia (7%) but was detected only once in other samples. Penitrem A is a tremorgenic toxin known to be produced by *P. crustosum* (Richard and Arp, 1979; El-Banna and Leistner, 1988; Frisvad and Filtenborg, 1989). It is possible that other toxins produced by *P. crustosum*, such as roquefortine C, were also present but not detected using the extraction methods employed.

Because of the reported production of penitrem A within food commodities by *P. crustosum* (Richard and Arp, 1979; Richard et al., 1981), the high frequency with which this species was isolated from chestnut tissue (67%) is a cause for concern. Intracellular penitrem A production by *P. crustosum* is highest at 25 °C, but production does occur at refrigeration temperatures (El-Banna and Leistner, 1988). The mycotoxin penitrem A induces a neurological reaction defined by sustained tremors in chronic toxicosis and at acute doses, convulsions and death in a variety of animals (Richard and Arp, 1979; Richard et al., 1981; Peterson and Penny, 1982; Deschaux and Bizot, 1997; Breton et al., 1998). One case has been published involving penitrem A induced toxicosis following ingestion of nuts. A temporary, tremorgenic toxicosis (lasting 36 h) was reported in a dog following accidental consumption of mouldy walnuts (*Juglans* spp.) infected with *P. crustusom* (Richard et al., 1981).

Our results suggest that imported *C. sativa* nuts have high levels of mould colonization and may have significant contamination with mycotoxins. Because of the known toxicity of ochratoxin A and penitrem A, their presence in naturally colonized nuts might be an appropriate focus for future studies meant to evaluate exposure estimates under the normally seasonal consumption of this commodity. Since nuts are often cooked before they are shelled and eaten, mould colonies or tissue discoloration could easily be mistaken for overcooked or burned areas. We have

noticed that spoiled nuts often contain air pockets that cause them to float in water; indeed, this method is used in some nut orchards to remove spoiled nuts before shipping (Payne et al., 1972). As a precaution, consumers could be advised to purchase intact, heavy nuts and to remove contaminated nuts by floating them prior to roasting.

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