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Volatile profiles of healthy and aflatoxin contaminated pistachios

M. Georgiadou [a], Chr. Gardeli [a], M. Komaitis [a], D.I. Tsitsigiannis [b], E.J. Paplomatas [b], K. Sotirakoglou [b], S. Yanniotis [a,*]

[a] Dept. of Food Science & Human Nutrition, Agricultural University of Athens, Iera Odos 75, Athens 118 55, Greece
[b] Dept. of Crop Science, Agricultural University of Athens, Iera Odos 75, Athens 118 55, Greece

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ABSTRACT

The volatile profile of four different groups of dried pistachios namely: H: healthy, NC: naturally contaminated with aflatoxin, AC: artificially contaminated with aflatoxigenic strains of the fungi *Aspergillus flavus* and ANT: artificially contaminated with non-toxigenic strains of the fungi *A. flavus*, was determined. The volatiles were isolated by the HS-SPME method and determined by GC–FID and GC–MS, whereas aflatoxin by HPLC. Seventy two volatile compounds were identified almost equally distributed among the above four studied groups. The predominant chemical compounds were monoterpenes, alcohols, ketones, aldehydes, esters and hydrocarbons. The monoterpenes, mainly determined as α-pinene and α-terpinolene were detected in all samples. Even though the general volatile profile was similar among groups, some differences were detected between healthy and contaminated groups of samples. When some key volatiles such as eight-carbon and seven-carbon alcohols and aldehydes were used along with the species-specific sesquiterpenes and the other terpenes detected, a correct classification was obtained in H, NC, AC and ANT groups, as was demonstrated by cluster and discriminant analyses. This evidence provides a potential tool for distinguishing contaminated samples on the basis of characteristic volatile patterns.

1. Introduction

The presence of aflatoxins represents a serious problem in cereals, nuts, dried fruits and other food products. Their elimination from food and feed is of great concern due to the fact that they are highly toxic, mutagenic, teratogenic and carcinogenic compounds which have been implicated as causative agents in human hepatic and extrahepatic carcinogenesis (Amaike & Keller, 2011). The causative pathogens are fungi of the genus *Aspergillus*, especially the species that most commonly belong to section Flavi such as *Aspergilllus flavus* and *Aspergillus parasiticus*. The toxigenic strains of these pathogens can produce aflatoxin as a secondary metabolite.

Pistachios are one of the major sources of aflatoxins (Annual Report 2011 European Rapid Alert System for Food & Feed — RASFF). In a previous work (Georgiadou, Dimou, & Yanniotis, 2012), conditions and handling practices in pistachios that affect aflatoxin contamination in every production or processing step have been studied. Results showed that pistachios could be contaminated with aflatoxin in every production stage, from maturity on the tree to storage, especially in heavily pest-damaged orchards or warehouses. Prevention strategies, as represented by Good Agricultural Practices, Good Manufacturing Practices and Good Storage Practices, are the primary means of defense. However, when preventive action cannot be achieved, a corrective action, such as sorting of contaminated or suspicious nuts, needs to be carried out to ensure that contaminated material does not enter the food chain (Yanniotis, Proshlyakov, Revithi, Georgiadou, & Blahovec, 2011).

Monitoring of the volatiles might be a good early indicator of quality deterioration and possible contamination, due to the fact that the volatile profile of a product is affected by spoilage. In the case of fungal spoilage of food items, fungi produce volatiles, as primary and secondary metabolites, some of which are common to many fungi and others seem to be unique for a species (Schnürer, Olsson, & Börjesson, 1999). Therefore monitoring of fungal volatile compounds could be used to detect fungal infection and identify specific fungi.

Kaminski, Libbey, Stawicki, and Wasowicz (1972) identified some odorous volatiles from steam distillates of *A. flavus* grown on moistened wheat meal, responsible for the characteristic mushroom odor, different from those produced by bacteria or the seeds themselves. The occurrence of these compounds in agricultural products could be an indication of contamination by molds in general, since the same compounds are produced by several fungi (Kaminski, Stawicki, & Wasowicz, 1974). Magan and Evans (2000) indicated that volatile profiles can be used rather than individual volatile compounds to classify fungi at a species level, as a combination of volatiles is often unique to each species. Other studies in the literature refer to the use of microbial volatile metabolites as a detection tool for the presence of fungal activity and mycotoxin production, mainly in cereals (Tuma, Sinha, Muir, & Abramson, 1989; Kaminski & Wasowicz, 1991; Larsen & Frisvad, 1995;

* Corresponding author. Tel.: +30 210 529 4703.
E-mail address: yanniotis@aua.gr (S. Yanniotis).

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Jelen & Wasowicz, 1998). Pasanen, Lappalainen, and Pasanen (1996) demonstrated some relationship between fungal volatiles and mycotoxins in grains. Further attempts to discriminate the mycotoxigenic and non-toxigenic fungal strains grown in liquid growth medium, cereals and peanut substrates by their volatile profile have been published by other researchers (Zeringue, Bhatnagar, & Cleveland, 1993; Keshri & Magan, 2000; Sahgal, Needham, Cabañes, & Magan, 2007; Jurjevic, Rains, Wilson, & Lewis, 2008). De Lucca, Boué, Carter-Wientjes, and Bhatnagar (2012) investigated the volatile profiles of toxigenic and non-toxigenic isolates of *A. flavus* grown on sterile and non-sterile cracked corn and identified volatiles unique to the aflatoxin producing isolates, which however were not uniformly produced in number and type by the tested *A. flavus* isolates.

Variation and disparity among results are noted by all authors. Apart from differences in analytical methods applied, a variation within strains of each fungal species has been also described, indicating metabolic differences and side-effects by the presence of other fungi (De Lucca et al., 2012) or other organisms, such as bacteria (Spraker et al., 2014). Moreover, the production of volatiles depends on the growth substrate (Sunesson et al., 1995) as it is affected by nutrient synthesis, water availability, temperature and culture age (Magan & Evans, 2000). The volatile profile of pistachios infected with *A. flavus* has not been studied. The aim of the present study was to investigate the volatile profile of healthy and aflatoxin contaminated pistachios and identify those volatiles that are characteristic to contaminated samples. The volatile compounds from four different groups of dried pistachios were identified namely: H: healthy, NC: naturally contaminated with aflatoxin, AC: artificially contaminated with aflatoxigenic strains of the fungi *A. flavus* and ANT: artificially contaminated with non-toxigenic strains of the fungi *A. flavus*.

2. Materials and methods

2.1. Pistachio samples and treatments

All samples used in this study were Greek dried pistachio nuts of the local variety "Aegina". The samples of the healthy (H) group were obtained from several cultivation areas of Greece during the 2010 harvesting period. The samples of the naturally aflatoxin contaminated group (NC) were obtained from several local processing units during handling or storage. The samples of artificially contaminated groups (AC and ANT) were obtained from the respective healthy samples after inoculation in the laboratory with *A. flavus* strains, as described in Section 2.2. Each sample weighing 0.5 kg was placed in a separate plastic bag, sealed and stored in a refrigerator until analyzed. More details for the samples are given in Table 1.

Table 1
Data for the dried pistachio samples.

Sample group	Sample name	Cultivation area	Harvesting time	Drying method
H: Healthy	H-1	Aegina	Normal	Sun
	H-2	Aegina	Normal	Hot air
	H-3	Kapandriti	Normal	Sun
	H-4	Makri	Normal	Sun
	H-5	Evia	Early	Sun
	H-6	Evia	Late	Sun
	H-7	Poros	Normal	Sun
	H-8	Attiki	Normal	Sun
NC: Naturally aflatoxin contaminated	NC-1	Aegina	Normal	Sun
	NC-2	Aegina	Normal	Sun
	NC-3	Markopoulo	Normal	Sun
	NC-4	Aegina	Normal	Sun
	NC-5	Aegina	Normal	Sun
	NC-6	Aegina	Normal	Sun
	NC-7	Aegina	Normal	Sun
	NC-8	Aegina	Normal	Sun
	NC-9	Aegina	Normal	Sun
	NC-10	Aegina	Normal	Sun
AC: Artificially contaminated with aflatoxigenic strains of *A. flavus*	AC-1	Aegina	Normal	Sun
	AC-2	Kapandriti	Normal	Sun
	AC-3	Makri	Normal	Sun
	AC-4	Evia	Early	Sun
	AC-5	Poros	Normal	Sun
ANT: Artificially contaminated with non-toxigenic strains of *A. flavus*	ANT-1	Makri	Normal	Sun
	ANT-2	Makri	Normal	Sun
	ANT-3	Aegina	Normal	Sun
	ANT-4	Evia	Normal	Sun
	ANT-5	Makri	Normal	Sun

2.2. Inoculation with A. flavus

The samples of AC and ANT group were artificially contaminated by inoculation with toxigenic and non-toxigenic strains of *A. flavus*, respectively. All strains (both toxigenic and non-toxigenic) were selected from the Greek *Aspergillus* collection held in the Laboratory of Plant Pathology of the Agricultural University of Athens and were pre-checked for their toxigenic ability using HPLC analysis (as described later). The non-toxigenic strains were further characterized genetically and molecularly with PCR (Georgiadou, Agoritsis, et al., 2012), while the toxigenic isolate was further checked by sub-culturing on 5/2 agar for five days in the dark at 31 °C to see if it is an L- or S-morphotype based on its sclerotia morphology (Cotty, 1989). The diameter of the sclerotia was measured using a compound microscope (N-400FL, Optika, Italy) equipped with ocular micrometer and found to have an average value of 235 ± 71 μm, characterizing this isolate as an S-morphotype (Cotty, 1989).

As shown in Table 1, five samples were artificially contaminated (AC) with an aflatoxigenic strain of *A. flavus* (referenced as D1-3 AF2) and five other samples (ANT) with non-aflatoxigenic strains of *A. flavus* (referenced as AF48-GR, AF54-GR, AF38-GR, AF27-GR and AF47-GR) selected from the Greek *Aspergillus* collection held in the Laboratory of Plant Pathology of the Agricultural University of Athens. Fungal cultures were grown at 28 °C on PDA (potato dextrose agar) and stored as glycerol stocks at −80 °C.

For the inoculation, the following procedure was used: for each treatment, 100 dried shelled pistachios from the respective healthy sample (H) were surface-sterilized by immersion in 10% sodium hypochlorite solution for 3 min, then momentarily dipped in distilled water, then in 70% ethanol, rinsed-off with distilled water and left to drain on a sterilized filtered paper. The pistachios were then inoculated by immersing them in a conidial suspension in H_2O treated with 0.01% Tween 80 with 10^6 spores/ml and shaking for 30 min at 80 rpm. The concentration of the fungal spores was calculated using a hemocytometer. Finally, the inoculated pistachios were placed in clear sterile plastic boxes on a wet sterilized filtered paper containing a water reservoir to ensure sufficient humidity and were incubated at 28 °C for nine days. This time period was considered sufficient for full growth of the *Aspergillus* strains that were used in this study, as it was determined from preliminary tests. After the incubation period the shells were removed and the kernels of each sample were kept in the freezer at −18 °C in plastic boxes. Before analysis, each sample was ground to powder.

In addition, two samples (H-3, H-4) of the healthy group (H) and one sample (NC-3) of the naturally contaminated group (NC) were also submitted to the abovementioned wetting and incubation treatment, in exactly the same way as the samples of AC and ANT groups, but without the fungal inoculum. This experiment aimed to assess any effect of the inoculation and incubation procedure on the volatile profile of the AC and ANT groups.

2.3. Aflatoxin analysis

The aflatoxin concentration of the contaminated samples of NC and AC groups was measured by HPLC. Eight grams of the finely ground pistachio kernels was removed from the total sample quantity of each sample for aflatoxin analysis. The method that was followed is