Poster #944

Testing of Raw Materials Used for the Production of Allergenic Extracts

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Abstract

Background: Proposed thresholds for human exposure to pesticides, heavy metals, and mycotoxins are derived from animal models or formulas. While information on the concentration of environmental contaminants in pollen and mycotoxins in fungal raw materials is scarce, monitoring of these components is being requested by the European Medicines Agency to demonstrate that their levels are minimal. The purpose of this study was to determine types and levels of these products in pollen and fungal raw materials used for the manufacture of allergenic extracts.

Methods: 79 pollen lots were obtained from 18 collectors in the USA and Europe from 2003 to 2008. Thirty-three (41.8%) lots comprised 14 tree species, 16 (20.1%) included 7 weed species, and 30 (38.0%) were 8 grass species. These lots were tested for heavy metals (including As, Cd, Pb, and Hg) and 18 pesticides. Additionally, two lots each of known mycotoxin-producing fungal species grown in-house under controlled conditions were analyzed for mycotoxins. They were *Aspergillus flavus*, *A. fumigatus*, *A. niger*, *Penicillium chrysogenum*, and *Fusarium moniliforme*. Mycotoxins tested were aflatoxins, ochratoxin A, patulin, T2-toxin, and deoxynivalenol, as appropriate for each species.

Results: The levels of the heavy metals tested had skewed distributions, with most values below the assay detection levels. Two of the 79 (2.5%) pollen lots contained concentrations of Cd or Pb above the threshold for these components proposed by the European Phamacopoeia (7.5 mg/kg), at levels of 32.4 and 7.6 mg/kg, respectively. One of the 79 (1.3%) pollen lots contained a detectable concentration of one of the pesticides tested (dieldrin), at a level of 0.057 mg/kg, compared to a proposed threshold of 0.05 mg/kg of aldrin plus dieldrin. All of the mycotoxins tested were below the assay detection levels.

Conclusions: The levels of environmental contaminants in pollen and mycotoxins in fungal raw materials used at Greer Laboratories are negligible. Identification of trained pollen collectors and proper collection areas, combined with a testing sampling plan, should be the strategy to limit the concentrations of these components. Similarly, the selection of fungal strains and culture conditions, combined with a testing plan, should eliminate the risk of exposure to mycotoxins. Proposed thresholds of exposure to these components in humans should be based and supported by scientific evidence.

Objective

To determine concentrations of the following components in pollen and fungal raw materials used for the manufacture of allergenic extracts:

- Pollen: Pesticides and heavy metals
- Fungal materials: Mycotoxins

Introduction

Currently, FDA guidelines for pesticides and heavy metals in pollen have not been proposed. However, according to the European Medicines Agency (EMA) guidelines regarding pollen source materials, the content of relevant pesticides and heavy metals should be monitored in order to demonstrate that their levels are kept to a minimum in allergenic source materials (1).

The Greer Laboratories, Inc. (Greer) pollen collector network consists on a number of collectors, all of whom must become certified by showing proficiency prior to being allowed to provide Greer with pollen. Greer requests that pollen not to be collected near urbanized areas or major roads to minimize the contamination of pollen with pesticides and heavy metals.

However, Greer has been monitoring the types and levels pesticides and heavy metals in pollen since 2003, as per specific customers' requests. Pollen lots produced at Greer are tested in a commercial laboratory. Testing includes standard panels of common pesticides and heavy metals offered by several analytical laboratories.

A number of mycotoxins are produced by strains of different fungal species under particular growth conditions. Known mycotoxin-producing fungi are, but not limited to, *Aspergillus*, *Penicillium*, *Fusarium*, and *Alternaria*. While some of the mycotoxins produced by these fungi are well defined and can be tested in commercial laboratories, other mycotoxins are more obscure and commercial tests to measure them are not currently available. Important mycotoxins-producing fungi, mycotoxins produced, and tests available are listed on Table I

Table I: Most important mycotoxin-producing fungi, mycotoxin produced, and tests available

Fungus	Mycotoxins	Test Available
Fusarium	Fumonisines B1-B2	Yes
	DON	Yes
	T-2 toxin	Yes
	HT-2 toxin	No
	Zearalenone	Yes
Aspergillus	Ochratoxine A	Yes
Aspergillus flavus	Aflatoxins B1, B2, G1, and G2	Yes
Penicillium	Patuline	Yes

According to the EMA guidelines regarding fungal source materials (1), the strain/s of moulds used should be specified, the cultivation method should be described, and evidence should be provided that no detectable amounts of mycotoxins are produced by the moulds. The EMA also indicates that details on the composition of the cultivation medium should be provided, and that synthetic non-allergenic media free of animal-derived material should be preferably used. In addition, the EMA recommends that if strains of known mycotoxins-producing fungi are used, the amount of relevant mycotoxins should be quantified.

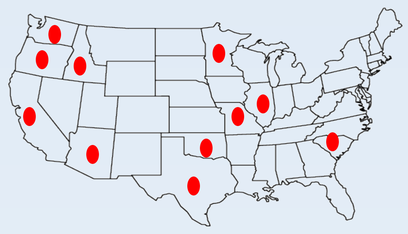
All of the 54 fungal species grown at Greer are strains obtained from accredited sources, including the American Type Culture Collection (ATCC) and Centraalbureau von Schimmelcultures (CBS) that do not produce mycotoxins under the specific growth conditions used. Greer's growth conditions for production of cellular and metabolic fungal materials include the use of non-allergenic media under shaking or static conditions, as appropriate.

Materials and Methods

Pollen Lots

- 79 pollen lots obtained by 18 certified collectors from 2003 to 2008 were tested.
- Thirty-tree samples (41.8%) were obtained from 14 tree species, 16 samples (20.1%) from 7 weed species, and 30 samples (38.0%) from 8 grass species.
- Pollen samples were obtained from different climatic areas in the USA and Europe (Figure 1).

Figure 1: Pollen Collection Areas in the USA



Fungal Cultures

- Two lots of each of the known mycotoxins-producing fungal species grown at Greer under controlled conditions were tested (Table II).

Table II: Lots tested for the presence of mycotoxins

Species	Strain	Grow Conditions	Mycotoxins tested
Aspergillus flavus	ATCC 11680	Shaking, Enriched Trypticase, 20-25°C	Aflatoxin B1, B2, G1, and G2
Penicillium chrysogenum (notatum)	ATCC 9178	Static, Enriched Trypticase 20-25°C	Patulin
Penicillium chrysogenum	ATCC 11709	Shaking, Enriched Trypticase 20-25°C	Patulin
Aspergillus fumigatus	SN-1 5167	Static, Enriched Trypticase 20-25°C	Ochratoxin A
Aspergillus niger	Lu1369	Static, synthetic 20-25°C	Ochratoxin A
Fusarium moniliforme	SN-1 6496	Static, Enriched Trypticase 20-25°C	T-2, Deoxynivalenol, 15 Acetyl-DON, 3 Acetyl-DON, Zearalenone, Fumonisins B1 and B2

Pesticide Testing of Pollen (Blue Ridge Laboratories)

- *Pesticides tested*: Dieldrin, alpha-BHC, alpha-chlordane, beta-BHC, delta-BHC, total DDT, dieldrin, endosulfan I, endosulfan II, endosulfan sulfate, endrin, endrin aldehyde, gamma-chlordane, heptachlor, heptachlor epoxide, lindane (gamma-BHC), methoxychlor, and tozaphene.
- *Sample preparation*: Pollen samples were extracted using a soxhlet apparatus and concentrated, according to laboratory standard procedures.
- *Spikes*: One matrix spike and one matrix spike duplicate per batch were used.
- *Analytical protocol*: EPA Method 8270C for semi-volatile analysis by gas chromatography with mass spectroscopy detection.
- *Detection limits*: 0.02 mg/kg, with the exception of alpha-chlordane and gamma-chlordane, which was 0.04 mg/kg and toxaphene, with a detection limit of 0.08 mg/kg.

Heavy Metals Testing of Pollen (Blue Ridge Laboratories)

- *Heavy metals tested*: As, Cd, Pb, and Hg
- *Sample preparation*: Pollen samples were solubilized according to laboratory standard procedures.
- *Spikes*: One matrix spike and one matrix spike duplicate were analyzed per batch.
- *Analytical protocols*:
  - As, Cd, and Pb: EPA Method 6010B for coupled plasma–atomic emission spectrometry.
  - Hg EPA Method 245.1 for cold vapor technique.
- *Assay detection limits*:
  - As, Cd, and Pb: 0.1 mg/kg
  - Hg: 0.005 mg/kg

Mycotoxin Testing (Romer Laboratories)

- *Sample preparation*: Ground samples were extracted 1:4 (w/v) in 84/16 acetonitrile/water for a minimum of one hour, and subsequently the supernatant was isolated by filtration.
- *Spikes*: Samples were spiked with known amounts of the mycotoxins tested.
- *Analytical protocols and detection levels*: Table III

Table III: Methods Used to Analyze Mycotoxins

Mycotoxin	Method	Detection Limit	Mobile Phase	Flow Rate (mL/min)
Aflatoxins B1, B2, g1, and G2	HPLC	0.005 mg/kg	5+1+1: water+acetonitrile+methanol	2.0
Patulin	HPLC	0.1 mg/kg	0.8% tetrahydrofuran in water	1.0
Ochratoxin	HPLC	10 µg/kg	54+45+1: water+acetonitrile+ acetic acid	1.0
Zearalenone	HPLC	0.5 mg/kg	52/47/1: water+acetonitrile+acetic acid	2.0
Fumonisin B1	HPLC	1.0 mg/kg	52/47/1: water+acetonitrile+acetic acid	2.0
Fumonisin B2	HPLC	1.0 mg/kg	52/47/1: water+acetonitrile+acetic acid	2.0
Mycotoxin	**Method**	**Detection Limit**	**Developer**	**Visualization**
T-2 toxin	TLC	0.5 mg/kg	25+15+1: methanol+water acetic acid	UV light
Deoxynivalenol	TLC	0.5 mg/kg	25+15+1: methanol + water + acetic acid	UV light
15-Acetyl-DON	TLC	0.5 mg/kg	25+15+1: methanol+water+acetic acid	UV light
3-Acetyl-DON	TLC	0.5 mg/kg	25+15+1: methanol+water+acetic acid	UV light

Statistical Analysis

- *Software*: Prism™ software (Prism Software Co., Irvine, CA).
- *Analysis performed*:
  - Descriptive statistics and normality tests were calculated.
  - Either continuous or discontinuous distributions were analyzed as appropriate, using parametric or non-parametric statistic tests.
  - Numerical results were also analyzed considering different variables, including the pollen collector and geographical collection area.

Results

Pesticides

- Only one of the 79 samples tested (1.3%) contained a detectable concentration of dieldrin, at a level of 0.057 mg/kg, just slightly above the proposed threshold concentration for this pesticide.
- This pollen sample was *Phleum pratense* (Timothy grass), and was obtained by collector #2 in Minnesota.

Heavy Metals

- The levels of all heavy metals analyzed had skewed distributions, with most values below the detection limits of the assays.
- Only two of the 79 pollen lots analyzed (2.5%) contained each concentrations of Cd or Pb above the proposed threshold levels for these components (7.5 mg/kg), at levels of 32.4 and 7.6 mg/kg, respectively.
- These pollen lots were obtained by collector #10 in Oklahoma and by collector #2 in Minnesota, respectively (Table IV).
- Another lot of the pollen species with levels of Cd above the proposed threshold was subsequently tested. The level of this component was below the assay detection limit.

Table IV: Heavy Metals below or above the Accepted Concentration Limits

Component	N	#(%) Positive	Minimum (mg/kg)	Maximum (mg/kg)	Threshold (mg/kg)	#(%) >Threshold	Collector # / Location	Species
Cd	79	9 (11.4)	0.0	32.4	<7.5	1 (1.3)	10/OK	*Cynodon dactilon*
Hg	43	2 (4.7)	0.0	0.03	<7.5	0 (0)	-	-
Pb	79	23 (29.1)	0.0	7.6	<7.5	1 (1.3)	2/MN	*Ambrosia artemisiifolia*

Mycotoxins

- All of the mycotoxins tested were below the assay detection limits.

Conclusions and Proposal

The levels of environmental contaminants in pollen and mycotoxins in fungal raw materials obtained at Greer are negligible. Based on these results, Greer does not consider that routine testing of all pollen lots for heavy metals and pesticides and fungal raw materials lots for mycotoxins is warranted. Greer proposes the implementation of the measures described below.

Pollen

Greer pollen collection policy will be reinforced for existing and new pollen collectors. While Greer doesn't consider that routine testing of all pollen lots is warranted, pollen lots will be monitored for the presence of commonly used pesticides and heavy metals to demonstrate that their level in pollen is kept at a minimum.

Fungal Raw Materials

Based on the facts that the fungal strains grown at Greer do not produce mycotoxins under the growth conditions used, and that the mycotoxins tested in two lots of known mycotoxin-producing fungal species were below the detection limits of the assays, each individual lot of fungal materials produced by the company should not be tested.

Greer will obtain mycotoxin testing results under the following circumstances:

- Once a year, one random lot of *Fusarium*, *Aspergillus*, *Aspergillus flavus*, and *Penicillium*
- If new strains of fungi are used at Greer
- The growth conditions are changed
- Additional testing becomes available, particularly to measure toxins produced by *Alternaria* spp.
- Any other circumstances that deem mycotoxin testing necessary

REFERENCE

1.Guideline on allergen products: Production and quality issues in evaluation of medicines for human use. European Medicines Agency, London November 20, 2008, EMEA/CHMP/BWP/304831/207.

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