

## Current perspectives

# Manufacturing and standardizing fungal allergen products

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The importance of fungal allergen products in the practice of clinical allergy is frequently underestimated. A wide variety of fungal species have been demonstrated to elicit allergic symptoms and to sensitize patients. The quality of fungal allergen preparations might have a significant effect on the specificity and sensitivity of diagnostic tests. Varying degrees of cross-reactivity have been shown among fungal genera, and the clinical relevance of this cross-reactivity has been neither fully appreciated nor applied to clinical practice. In addition, an increasing number of potentially new fungal allergen sources for which commercial extracts are not available are being identified. Manufacturers of allergen products have the formidable task of providing quality fungal allergen extracts that are routinely used for specific allergy diagnosis and treatment in the clinic. Currently there are no standardized fungal allergen products available in the United States because of inherent difficulties with manufacturing and standardizing fungal extracts. Without intervention, progress will not be forthcoming. (*J Allergy Clin Immunol* 2004;113:210-5.)

**Key words:** Allergen standardization, fungal extracts

### MANUFACTURE OF FUNGAL EXTRACTS

Seven different US allergen product manufacturers offer a total of 236 unique fungal allergenic extract products representing 45 genera and 75 species of fungi (Table I). In addition, these manufacturers provide various mixtures of related and unrelated fungal species. The manufacturers' catalog listings are not exhaustive but do represent most of the fungal genera found in environmental surveys. The most influential studies that helped establish these offerings were made during the 1960s by a group known as the Association of Allergists for Mycological Investigations.<sup>6</sup> Fungal extracts representing the so-called "MMP molds" (the initials of 3 prominent members of the group) are still the most commonly used in clinical practice. The group also helped establish protocols for fungal allergen extraction, including the MMP extract, which was prepared from spent culture media that has been concentrated by means of evaporation or

#### Abbreviations used

ATCC: American Type Culture Collection

CBS: Centraalbureau voor Schimmelcultures

acetone precipitation, dialyzed, and dried by means of lyophilization.<sup>7</sup> Variations of this general procedure are still used by some manufacturers.

Source materials for fungal allergenic extracts are derived from stock cultures maintained by the manufacturer in a manner to ensure their purity and identity or are purchased from certified vendors of fungal cultures, such as the American Type Culture Collection (ATCC) or Centraalbureau voor Schimmelcultures (CBS). The criteria and specifications used to select the fungal strains vary depending on the manufacturer. Strains established for their inability to produce mycotoxins might be important criteria for species known to produce mycotoxins (ie, *Alternaria alternata*, *Aspergillus fumigatus*, *Aspergillus flavus*, *Fusarium verticillioides*, and *Fusarium culmorum*). Fungi rely on a high rate of somatic mutation and the production of heterokaryons by means of hyphal fusion to produce new gene combinations. Thus genetic stability of the stock cultures must be monitored and controlled by each manufacturer to ensure lot-to-lot consistency. Some strains are inherently less stable than others and not suitable for manufacturing use. A great diversity of strains are available from ATCC or CBS. For example, approximately 100 strains of *A alternata* and more than 200 strains of *A fumigatus* are available from ATCC or CBS. This diversity and variability of strains used by different manufacturers gives rise to the antigenic heterogeneity commonly observed with commercially available allergenic extracts.

A flowchart describing a typical production outline for fungal source materials is shown in Fig 1. Stock cultures are used to prepare seed cultures that in turn serve as inoculum for larger production-scale batch cultures. Some manufacturers use orbital shakers to produce submerged cultures that can increase growth rates and biomass production. Other manufacturers use static cultures and special growth media to maximize sporulation. The purity and identity of the cultures are confirmed at various production stages. The morphologic, biochemical, and antigenic characteristics of a given fungal strain are known to change with variations in the nutritional composition of the culture medium. Physical conditions, such as light and dark cycles, temperature, pH, and growth

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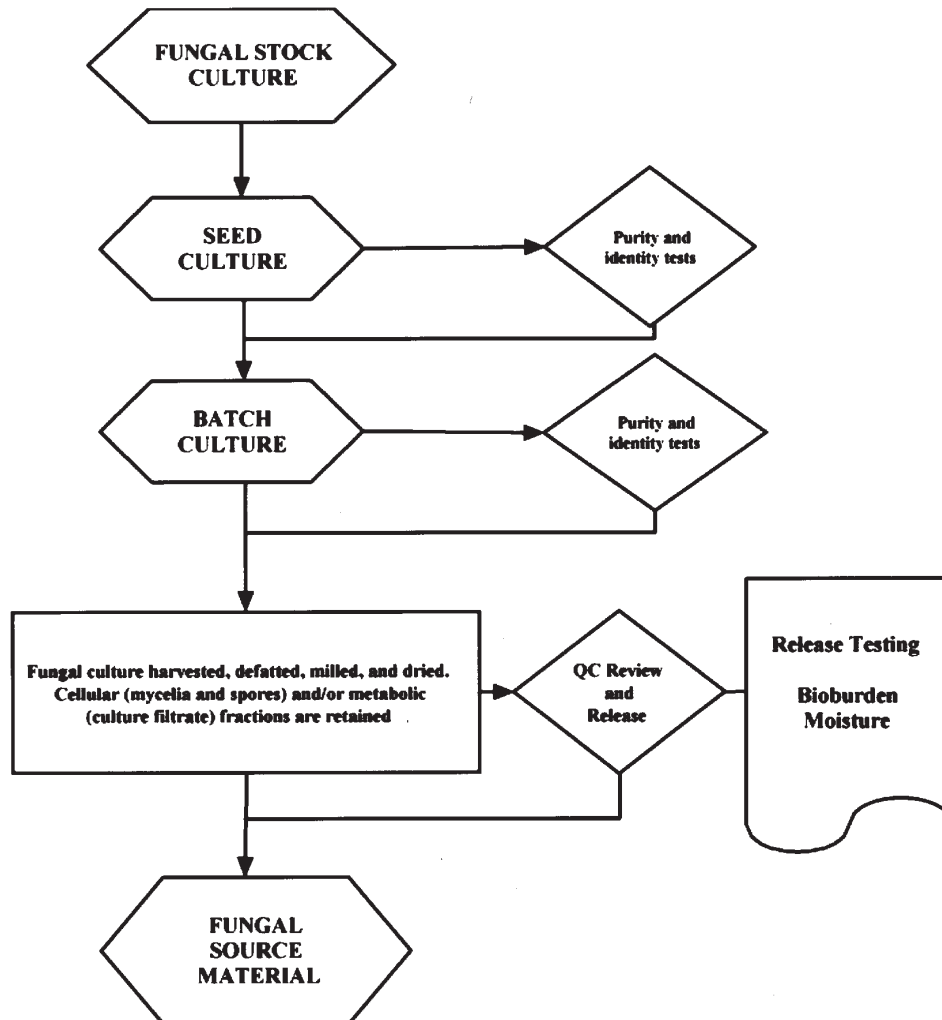


FIG 1. Production outline for fungal source materials. See text for details. QC, Quality control.

period, might be important for sporulation and might affect allergen production. Because no 2 manufacturers use the same culture medium or culture conditions, qualitative differences between products manufactured by different companies are expected, even when identical strains of fungal stock cultures are used.

Allergenic materials produced by fungi during cultivation are distributed throughout the culture in the form of spores, mycelia, and extracellular components secreted into the growth medium.<sup>8-11</sup> Manufacturers might retain the cellular (spores and mycelia), the extracellular (culture filtrate), or the whole culture during harvest. If culture filtrates are retained, nonantigenic culture media are used. If spores are retained, then they are rendered nonviable through chemical or physical means before transfer to the manufacturing (extraction) facilities. Various processing steps, including centrifugation, filtration, diafiltration, acetone precipitation, and lyophilization, are used to recover the allergenic materials from fungal cultures. Each manufacturer's process requirements and

specifications are unique and defined as part of their product and establishment licenses.

Fig 2 presents a typical production outline for fungal allergenic extracts. Fungal source materials are reconstituted from the lyophilized state or extracted in an appropriate buffer to recover the soluble allergenic materials. The allergenic potency of extracts can be influenced by a number of variables in the extraction procedure. The most important extraction parameters include the extraction buffer, pH, ratio, time, and temperature. The most common extraction buffer used by US manufacturers is Coca's extraction buffer, a slightly alkaline carbonate buffer developed to maximize protein recovery from allergen source materials. Manufacturers might add glycerin or phenol as preservatives. Extraction ratios are expressed as weight/volume or the weight in grams of the source material extracted to the volume in milliliters of extraction fluid. For example, a 1:10 wt/vol on a label designates an extract that was prepared with 1 g of source material for every 10 mL of extraction fluid. Some aller-

TABLE I. Commercially available fungal allergen extracts

Species	Synonym	Species	Synonym
<i>Acremonium strictum</i>	<i>Cephalosporium acremonium</i>	<i>Neurospora intermedia</i>	<i>Monilia sitophila</i>
<i>Alternaria alternata</i>	<i>Alternaria tenuis</i>	<i>Nigrospora oryzae</i>	
<i>Apiospora montagnei</i>	<i>Papularia</i>	<i>Nigrospora sphaerica</i>	
<i>Aspergillus fumigatus</i>		<i>Paecilomyces variotii</i>	
<i>Aspergillus niger</i>		<i>Paecilomyces clavisoris</i>	
<i>Aspergillus glaucus</i>		<i>Penicillium notatum</i>	
<i>Aspergillus terreus</i>		<i>Penicillium chrysogenum</i>	
<i>Aspergillus nidulans</i>		<i>Penicillium digitatum</i>	
<i>Aspergillus flavus</i>		<i>Phoma betae</i>	
<i>Aureobasidium pullulans</i>	<i>Pullularia pullulans</i>	<i>Phoma herbarum</i>	
<i>Botrytis cinerea</i>		<i>Phoma destructiva</i>	
<i>Candida albicans</i>		<i>Phycomyces blakesleenus</i>	
<i>Candida tropicalis</i>		<i>Pleospora herbarum</i>	<i>Stemphylium herbarum</i>
<i>Chaetomium globosum</i>		<i>Rhizopus oryzae</i>	<i>Rhizopus arrhizus</i>
<i>Cladosporium herbarum</i>		<i>Rhizopus stolonifer</i>	<i>Rhizopus nigricans</i>
<i>Cladosporium sphaerospermum</i>	<i>Hormodendrum hordei</i>	<i>Rhodotorula mucilagnosa</i>	<i>Rhodotorula rubra</i>
<i>Cladosporium cladosporioides</i>	<i>Hormodendrum cladosporioides</i>	<i>Saccharomyces cerevisiae</i>	
		<i>Scopulariopsis brevicaulis</i>	<i>Penicillium brevicaulis</i>
<i>Colletotrichum atramentarium</i>	<i>Spondylocladium atramentarium</i>	<i>Sporotrichum pruinosum</i>	<i>Chrysosporium pruinosum</i>
		<i>Stachybotrys chartarum</i>	
<i>Curvularia lunata</i>		<i>Stemphylium botryosum</i>	
<i>Drechslera specifera</i>	<i>Curvularia specifera</i>	<i>Stemphylium solani</i>	
<i>Epicoccum nigrum</i>	<i>Epicoccum purpurascens</i>	<i>Stemphylium sarcinaeforme</i>	
<i>Epidermophyton floccosum</i>		<i>Streptomyces griseus</i>	
<i>Fusarium solani</i>		<i>Syncephalastrum racemosum</i>	
<i>Fusarium moniliforme</i>		<i>Tetracoccosporium paxianum</i>	
<i>Fusarium vasinfectum</i>	<i>Fusarium oxysporum</i>	<i>Trichoderma harzianum</i>	<i>Trichoderma lignorum</i>
<i>Geotrichum candidum</i>		<i>Trichophyton mentagrophytes</i>	
<i>Gliocladium viride</i>	<i>Gliocladium deliquescens</i>	<i>Trichophyton rubrum</i>	
<i>Gliocladium fimbriatum</i>		<i>Trichophyton tonsurans</i>	
<i>Helminthosporium sativum</i>		<i>Trichothecium roseum</i>	<i>Cephalothecium roseum</i>
<i>Helminthosporium solani</i>	<i>Spondylocladium atrovirens</i>	<i>Verticillium albo-atrum</i>	
<i>Hypocrea rufa</i>		<i>Puccinia graminis tritici</i>	
<i>Microsporium canis</i>		<i>Ustilago maydis</i>	
<i>Mucor circinelloides</i>	<i>Mucor racemosus</i>	<i>Ustilago cynodontis</i>	
(formerly <i>lusitanicus</i> )		<i>Ustilago nuda</i>	
<i>Mucor circinelloides</i>	<i>Mucor mucedo</i>	<i>Ustilago avenae</i>	
(formerly <i>circinelloides</i> )		<i>Ustilago tritici</i>	
<i>Mucor plumbeus</i>		<i>Sphacelotheca cruenta</i>	<i>Sporisorium cruenta</i>
<i>Mycogone perniciosa</i>			

gens are rapidly eluted from fungal source materials, whereas others might require homogenization, shaking, or long extraction times for maximum recovery. There exists a tradeoff between maximum allergen recovery and the increased release of nonallergenic substances and proteases, which might degrade some allergens. Many different procedures have been devised to extract allergens from fungi, and no single process has been universally adopted by the industry.<sup>12</sup>

The extract is clarified by means of centrifugation, filtration, or both through a series of filters of decreasing porosity. The supernatant or filtrate might undergo further processing, such as dialysis to remove low-molecular-weight nonallergenic material and the addition of glycerin. The extract is rendered sterile by passing the extract through a sterilizing filter with a 0.2- $\mu$ m porosity. The clarifying and sterilizing filters used must be shown not to adsorb allergens onto their surface and not to contribute any residual material into the product. The sterile extract is quarantined until tested and released for additional aseptic processing. Quality control testing of

this sterile intermediate or bulk extract include tests for sterility, safety (toxicity in animals), pH, protein nitrogen units, and preservative (phenol or glycerin). The released bulk extract is aseptically diluted, mixed with other bulk products, or used without further processing to prepare stock concentrates, custom mixtures, or individual prescriptions for sale.

## COMPOSITION OF FUNGAL ALLERGENIC EXTRACTS

Fungal allergenic extracts are complex mixtures of proteins, glycoproteins, polysaccharides, and other substances. The mean protein and carbohydrate concentrations of selected fungal stock concentrates prepared by a single manufacturer at 1:10 wt/vol are presented in Table II. At equivalent weight/volume concentrations, the protein content varied approximately 7-fold. All extracts tested contained significantly higher concentrations of carbohydrate compared with protein, with the ratio ranging from about 5 to 25.

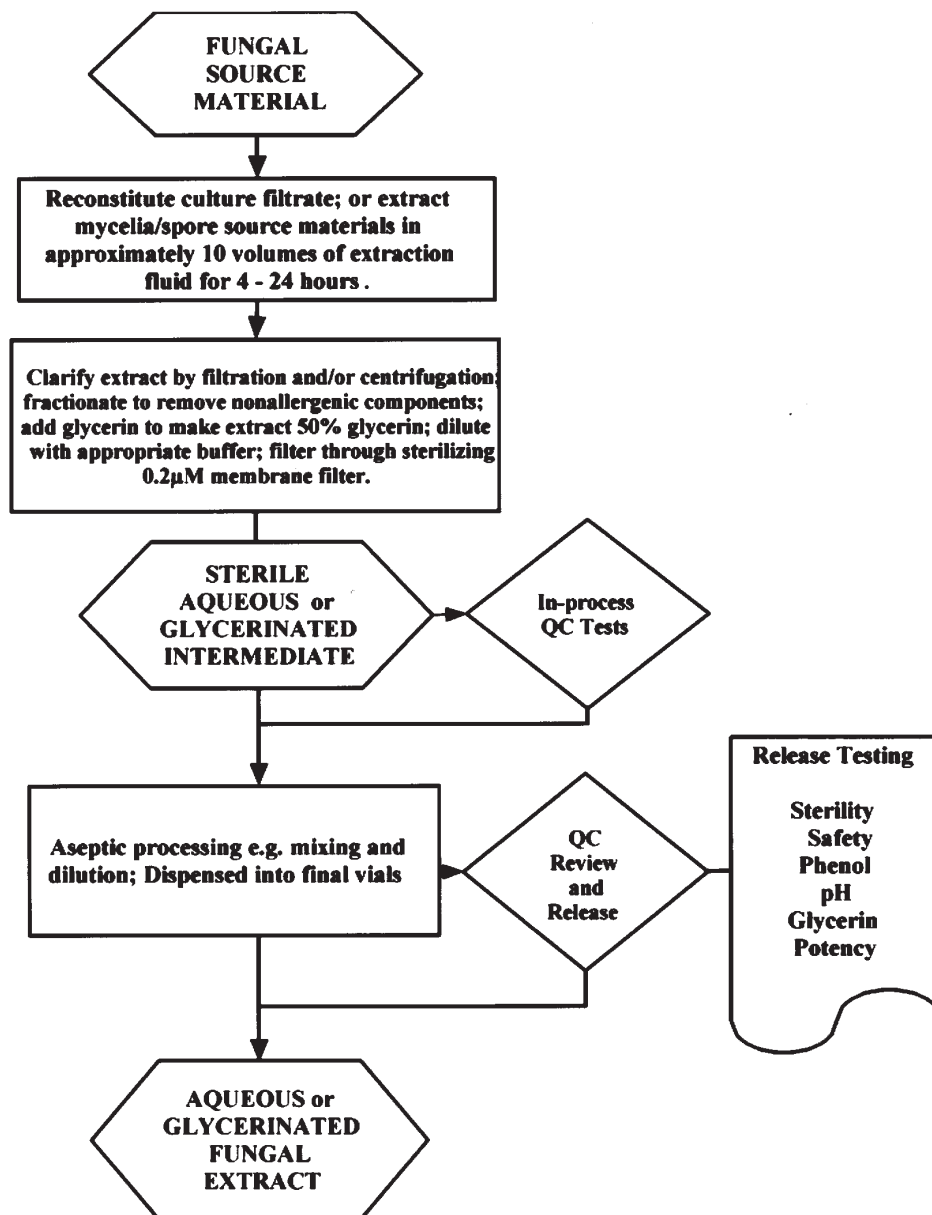


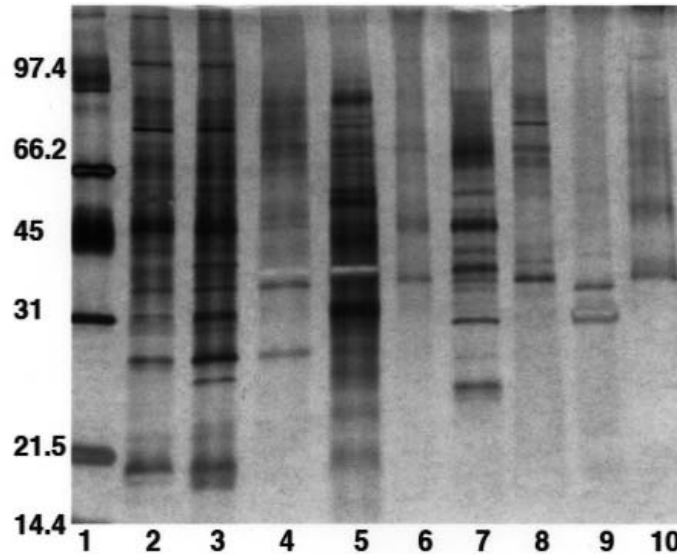
FIG 2. Production outline for fungal allergenic extracts. See text for details. QC, Quality control.

TABLE II. Protein and carbohydrate content of various fungal allergen products

Fungal species	Protein (mg/mL)	Carbohydrate (mg/mL)	Ratio
<i>Alternaria alternata</i>	0.73	4.27	6
<i>Helminthosporium sativum</i>	0.44	2.56	5
<i>Penicillium notatum</i>	0.38	3.58	9
<i>Aspergillus fumigatus</i>	0.28	4.16	15
<i>Cladosporium cladosporoides</i>	0.25	6.00	24
<i>Ustilago maydis</i>	0.10	2.38	24

The protein, carbohydrate, and Alt a 1 contents and the relative potencies of *A alternata* products manufactured by different companies are shown in Table III. When adjusted to equivalent weight/volume concentrations, the protein and carbohydrate contents varied by more than

100-fold. Similar variations in the protein and carbohydrate contents of commercially available fungal extracts derived from *Penicillium notatum*, *A fumigatus*, *Epicoccum nigrum*, *Helminthosporium sativum*, and *Aureobasium pullulans* were found (data not shown). The variability



**FIG 3.** SDS-PAGE profiles of commercial *A alternata* extracts: lane 1, molecular weight standards; lane 2, 1:10 wt/vol Greer aqueous; lane 3, 1:20 wt/vol Greer glycerinated; lane 4, 1:10 wt/vol Hollister-Stier aqueous; lane 5, 1:10 wt/vol Hollister-Stier glycerinated; lane 6, 1:20 wt/vol Allergy Labs glycerinated; lane 7, 1:10 wt/vol ALK glycerinated; lane 8, 1:10 wt/vol Allermed glycerinated; lane 9, 1:10 wt/vol Nelco glycerinated; lane 10, 1:10 wt/vol Antigen Labs glycerinated. Samples were loaded at 0.7  $\mu$ g of protein except for lanes 6 and 9, which were loaded at 0.3  $\mu$ g and 0.2  $\mu$ g, respectively, because of low protein content of the extracts. Components were visualized by means of silver staining.

**TABLE III.** Composition of *Alternaria alternata* products from different manufacturers

Manufacturer	Wt/vol	Protein (mg/mL)	Carbohydrate (mg/mL)	Alt a 1 ( $\mu$ g/mL)	Relative potency
Greer	1:10	0.73	4.27	1.85	1.334
Hollister-Stier	1:10	0.19	11.38	2.75	0.246
ALK-Abello	1:10	0.03	17.14	0.04	0.281
Allergy Labs	1:20	0.06	31.30	0.05	0.061
Allermed	1:10	0.15	2.40	0.05	0.581
Nelco Labs	1:5	0.08	33.79	2.04	0.012
Antigen Labs	1:10	0.15	10.96	0.09	0.219

ty in specific allergen content and ELISA inhibition potency of *A alternata* extracts from different manufacturers was consistent with that found in previous reports.<sup>13,14</sup> No significant correlation ( $r < .5$ ) was observed between the Alt 1 concentrations and the respective protein, carbohydrate, and relative potency values.

The compositional profiles evaluated by means of SDS-PAGE of various *A alternata* products manufactured by different companies are presented in Fig 3. No two company's products yielded comparable banding patterns. Similar profiles were evident between aqueous and glycerinated products manufactured by one company (lanes 2 and 3). In contrast, significant differences were evident between aqueous and glycerinated products manufactured by another company (lanes 4 and 5).

On the basis of compositional analyses, it becomes obvious that the different manufacturers' products are qualitatively different and that weight/volume designations do not reflect the relative antigen or allergenic activities of fungal allergen products. Such differences in the composition of fungal allergen products from the

same fungal species with identical weight/volume designations raise questions regarding their interchangeability.

Fungal allergen extracts are known to contain proteolytic activity that can reduce the allergenicity of other extracts when combined in therapeutic allergen vaccines.<sup>15-17</sup> Grass pollen extracts appear to be most susceptible to degradation, whereas others, such as short ragweed pollen extracts, are quite resistant. Increasing the glycerin concentration in the vaccines reduces the deleterious effect, but it has been recommended that fungal extracts be formulated in separate vials to maximize vaccine stability.<sup>18</sup>

### STANDARDIZATION OF FUNGAL ALLERGEN VACCINES

A goal of standardization is to ensure the lot-to-lot consistency of allergen products. Within the limitations imposed by each manufacturer's product licenses, this might be achievable for final products manufactured by a given company. Strict adherence to established manu-

facturing and quality controls for monitoring consistency can reduce product variability.<sup>19</sup> However, if standardization is to ensure consistency among products marketed by different manufacturers, then this goal does not appear possible for fungal allergen products. The marked differences in source materials and manufacturing procedures used, the lack of generally accepted potency assays, and the great variety of potentially allergenic fungal species have impeded any significant progress in fungal allergen standardization. Novel fungal standardization approaches have been suggested in the past, including the pooling of selected relevant strains to ensure an adequate complexity in composition.<sup>10,20</sup> This approach requires knowledge of most or all relevant allergens and the development of new quality control procedures. The establishment of a well-characterized reference preparation with a validated potency assay could facilitate allergen standardization efforts. In the 1980s, an international collaborative group developed a reference standard of *A alternata* under the auspices of the World Health Organization/International Union of Immunological Societies allergen standardization committee.<sup>21</sup> This initiative failed to gain wide acceptance by manufacturers, regulatory authorities, clinicians, and research laboratories. In 1997, the American Academy of Allergy, Asthma and Immunology Allergen Standardization Committee included *Alternaria*, *Cladosporium*, *Penicillium*, and *Aspergillus* species as priorities for allergen standardization efforts.<sup>22</sup> There have been no new initiatives after the publication of this position statement.

## CONCLUSIONS

Fungal allergen extracts produced by the different US manufacturers are highly variable in composition and should not be considered interchangeable. If one accepts this conclusion, then the total number of unique fungal allergen products marketed in the United States today exceeds 200. Standardization of fungal extracts or vaccines has been and will continue to be difficult because manufacturers use distinctly different strains, cultivation and source material processing methods, extraction processes, and quality control procedures. In contrast to other allergen sources, such as Hymenoptera venoms, pollens, dust mites, and cat dander, the limited knowledge of relevant allergens and the lack of generally accepted reference reagents and validated potency assays has impeded progress in developing a viable approach for standardizing fungal vaccines. Improvement in the safety, efficacy, and accuracy of fungal allergy diagnosis and immunotherapy will not be achieved without a coordinated effort by manufacturers, regulatory authorities, clinicians, and researchers.

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