

# Stability of Non-Standardized Tree Pollen, Weed Pollen and Dog Allergens After Mixing with Fungal or Insect Extracts

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## Abstract

**Introduction:** The biochemical compatibilities of allergens from diverse sources in extract mixtures are essential to formulation of stable and effective immunotherapy vaccines. Recent investigations from this laboratory on standardized allergen compatibilities with high-protease fungal or insect extracts provided new information incorporated in the 2007 immunotherapy practice parameter update. However, recommendations for extract mixtures containing non-standardized tree pollen, weed pollen and animal allergens remain conservative based on both insufficient data with these extracts and the reported instability of other (grass, ragweed) pollen allergens.

**Methods:** In this study, allergens from tree pollen (cottonwood, oak, maple), weed pollen (lamb's quarter, mugwort, english plantain) or dog (epithelia, hair/dander) extracts were analyzed after mixing with whole-body fungal (Alternaria, Aspergillus, Penicillium) or insect (American cockroach, German cockroach, fire ant) products and storing for up to 6 months at 2-8°C. The IgE-binding properties of these extracts were examined using both qualitative (immunoblot) and quantitative (ELISA inhibition) methods.

**Results:** Tree pollen and weed pollen allergen reactivities were compromised to a moderate or high degree by proteases present in most of the fungal or insect extracts included in this study. Dog allergens were degraded after mixing with most mold extracts but were more stable in general when combined with insect extracts. Allergen recoveries in most extract combinations improved noticeably with increasing glycerin concentrations, but allergen degradation was still evident even at 50% glycerin for several mixtures. Variable recoveries with human serum samples representing similar or different multi-allergen specificities confirmed the importance of examining extract compatibilities with several sera whenever possible.

**Conclusions:** Based on these results, it is clear that tree pollen and weed pollen allergens may be unstable if combined with fungal or insect extracts in immunotherapy vaccines. The current recommendation of separating pollen extracts from the high-protease fungal and insect products is thus supported. Dog allergens can also be degraded to some degree by mixing with certain mold or insect extracts at low (10%) glycerin concentrations.

## Materials and Methods

Allergen mixtures and control samples were prepared using the following glycerinated extract concentrates. All products were manufactured at Greer except AP Dog hair/dander (Hollister-Stier Labs, Spokane, WA):

Eastern cottonwood	<i>Populus deltoides</i>	1:20 w/v
Red maple	<i>Acer rubrum</i>	"
White oak	<i>Quercus alba</i>	"
Lamb's quarter	<i>Chenopodium album</i>	1:20 w/v
Common mugwort	<i>Artemisia vulgaris</i>	"
English plantain	<i>Plantago lanceolata</i>	"
Dog epithelia	<i>Canis familiaris</i>	1:20 w/v
AP Dog hair/dander	"	1:100 w/v
Alternaria (Alt)	<i>Alternaria alternata</i>	1:20 w/v
Aspergillus (Asp)	<i>Aspergillus fumigatus</i>	"
Penicillium (Pen)	<i>Penicillium notatum</i>	"
American cockroach	<i>Periplaneta americana</i>	1:20 w/v
German cockroach	<i>Blattella germanica</i>	"
Fire ant	<i>Solenopsis invicta</i>	"

Individual pollen or dog extracts were combined with individual fungal or insect products, with each extract component comprising 10% of the total volume (final concentrations = 1/10th of concentrate strengths).

All extract mixtures and individual extract controls were formulated to contain 10%, 25% or 50% glycerin final concentrations in 2.0 mL total volumes using specific combinations of Greer normal saline and 50% glycerol-normal saline diluents.

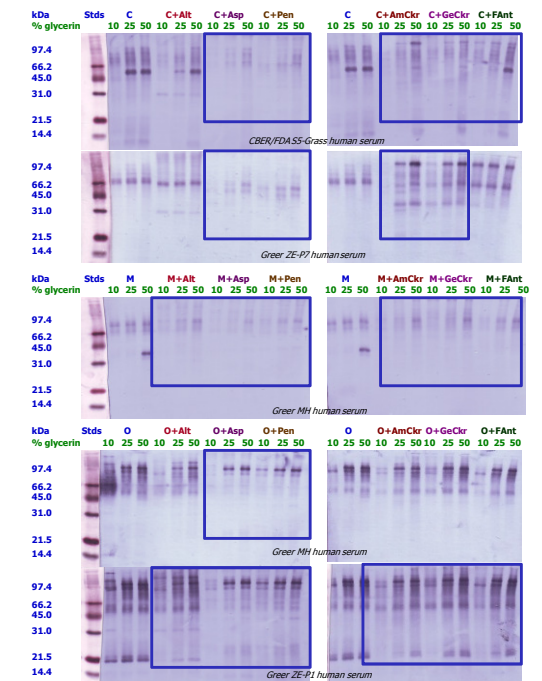
Immunoblot analyses typically included 2 different human allergic serum samples or pools. ELISA inhibition testing was performed on one of these sera for each allergen examined. Details of these procedures are described in Grier TJ et al, Ann Allergy Asthma Immunol 2007; 99 (2): 151-160. Allergen-specific ELISAs for dog Can f 1 were also performed using mouse or rabbit antibody reagents and allergen standards obtained from Indoor Biotechnologies (Charlottesville, VA) and Greer.

Mixtures and controls were stored at 2-8 degrees C for up to 6 months, and analyzed as follows:

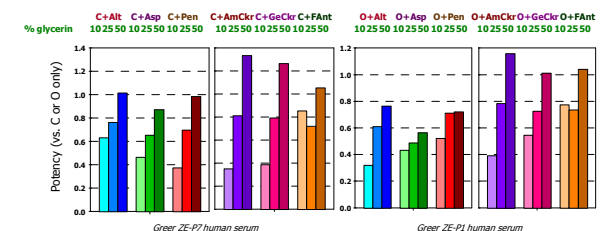
Allergen	Months stored at 2-8°C	
	Immunoblotting	ELISA
Eastern cottonwood	3.4	4.3
Red maple	3.4, 5.0	Not determined
White oak	3.6	4.7
Lamb's quarter	3.6	Not determined
Mugwort	4.3	Not determined
English plantain	4.3	Not determined
Dog epithelia	4.5	5.2, 5.5
Dog hair/dander	4.0	4.4, 5.4

## Tree Pollens ± Fungi or Insects

IgE immunoblot reactivities of Cottonwood (C), Maple (M) and Oak (O) allergens were compromised after mixing with most fungal or insect extracts at 10-50% glycerin. Aspergillus and Penicillium extracts produced the highest levels of degradation among the high-protease (fungal/insect) extracts used in this study.



IgE ELISA inhibition potencies of tree extracts in these mixtures were reduced to 30-80% of control samples (tree only) in 10% glycerin. Recoveries improved in 25% glycerin (50-80%) and 50% glycerin (60-130%).



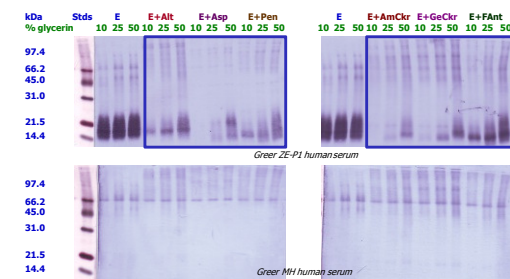
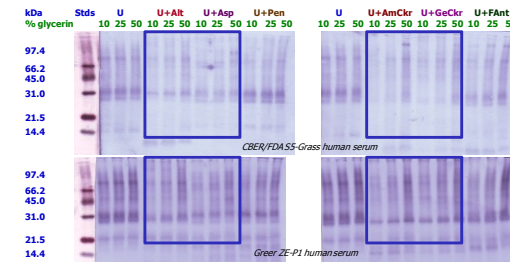
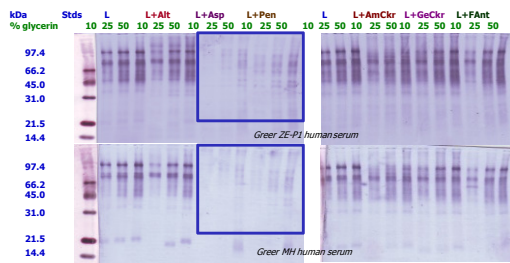
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## Weed Pollens ± Fungi or Insects

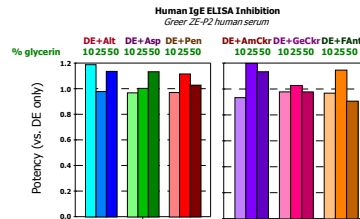
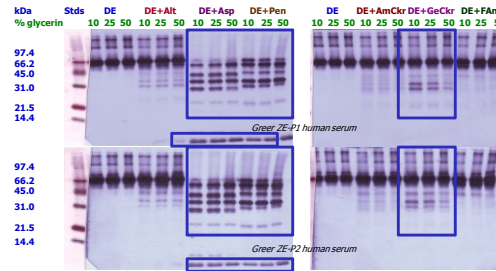
Lamb's quarter (L), Mugwort (U) and English plantain (E) allergens were altered to varying degrees after mixing with fungal or insect extracts. Results ranged from similar patterns with different sera (L, U) to very different patterns and recoveries with different sera (E). Considerable degradation was observed even at 50% glycerin for several extract combinations.



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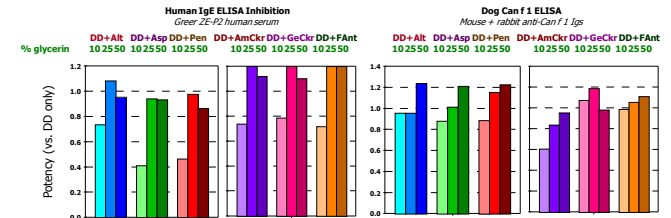
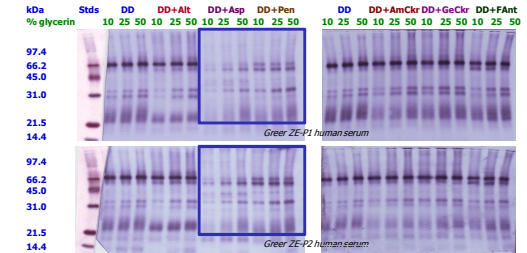
## Dog Epithelia ± Fungi or Insects

Dog epithelia (DE) allergens, primarily albumin (66 kDa), were hydrolyzed to discrete lower molecular weight fragments by several fungal and, to a lesser degree, insect extracts. Aspergillus and Penicillium extracts elicited the greatest degree of fragmentation on IgE immunoblots. DE fragments in all mixtures retained very high levels (> 95%) of quantitative IgE binding potencies based on ELISA testing.



## Dog Dander ± Fungi or Insects

Dog dander (DD) allergens, primarily Can f 1 (25 kDa) and albumin (66 kDa), were also sensitive to mixing with fungal or insect extracts. Aspergillus and Penicillium extracts altered both IgE immunoblot and IgE ELISA inhibition activities. Can f 1 ELISA activities were reduced by 40% after mixing with American cockroach extract but were not affected by the other high-protease extracts in this study.



## Conclusions

The observed instability of multiple tree and weed pollen allergens upon mixing with high-protease fungal or insect extracts supports the current immunotherapy practice parameter recommendation of maintaining these product groups in separate vaccine formulations. Dog allergens are also susceptible to protease degradation but retain high levels of allergen activities after storage for up to 6 months at 2-8 degrees C.

Differing recoveries with different test methods or sera illustrates the importance of a broad-based approach to compatibility or stability testing with allergens. Studies such as these contribute to a growing database of allergen extract interactions and support the development of optimal IT formulations and improved or standardized mixing practices.

## Compatibility Chart

Based on the results of these and previously published studies, the mixing compatibilities of allergen extracts are summarized below, with specific allergen combinations noted as compatible (green), risky (yellow) or not recommended (red).

Allergenic Extract	Protease-containing Extracts			
	Insects	Fungi	Mites	
Insects	⊗	⊕	⊕	<div style="display: flex; flex-direction: column; align-items: center;"> <div style="display: flex; align-items: center; margin-bottom: 5px;"> <span style="width: 15px; height: 15px; background-color: #90EE90; border: 1px solid black; margin-right: 5px;"></span> Compatible                 </div> <div style="display: flex; align-items: center; margin-bottom: 5px;"> <span style="width: 15px; height: 15px; background-color: #FFFF00; border: 1px solid black; margin-right: 5px;"></span> Risky                 </div> <div style="display: flex; align-items: center;"> <span style="width: 15px; height: 15px; background-color: #FF0000; border: 1px solid black; margin-right: 5px;"></span> Not recommended                 </div> </div>
Fungi	⊕	⊕	⊕	
Mites	⊗	⊗	⊕	
Pollens	⊗	⊗	⊕	
Cat hair/epithelia	⊕	⊕	⊕	
Dog hair/epithelia	⊕	⊗	⊕	