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MOLD EXTRACT COMPARABILITY, STABILITY AND COMPATIBILITY : COMPOSITIONAL AND IMMUNOCHEMICAL INVESTIGATIONS

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ABSTRACT

The diagnosis and treatment of mold allergies are complicated by the genetic diversity of individual fungal species and the influence of endogenous fungal proteases on extract compositions and potencies. Studies examining the biochemical comparability of mold extracts from different sources and their compatibility with other allergens in immunotherapy mixtures are essential to the clinical effectiveness of these products.

Compositional comparisons of extracts prepared from Alternaria, Aspergillus and Penicillium source materials by 7 U.S. allergen manufacturers revealed variable SDS-PAGE protein banding patterns and noticeable differences in total protein and carbohydrate concentrations. Alt a 1 levels in Alternaria extracts did not correlate closely with total protein levels or with IgE-binding potencies measured by ELISA inhibition. Immunoblot profiles of fungal extracts were more closely related to one another compared to SDS-PAGE patterns, suggesting that similar allergenic or antigenic epitopes may be retained in molecules of varying size. Parallel ELISA inhibition dose-response curves provided statistical evidence that a repertoire of IgE epitopes is conserved in many of these products. Variations in the potencies of fungal extracts from different manufacturers may result from differences in source materials and/or conditions of extraction and storage.

The stability and compatibility of allergen mixtures containing mold extracts were assessed after storage for up to 12 months at 2-8°C using specific immunoblot and ELISA procedures. Grass and mite allergens compromised by mixing with mold extracts were stabilized by glycerin. Alternaria extracts from different sources produced similar effects on grass allergens when added at comparable strengths. Degradative effects caused by Aspergillus or Penicillium products were more closely related to total fungal protein concentrations than to extraction ratios. Structural epitopes on cat, ragweed and fungal allergens were stable after mixing with protease-containing mold extracts, indicating the presence of natural protease inhibitors or allergenic protein sequences distinct from those recognized by these enzymes.

COMPLEXITY OF MOLD ALLERGIES

- **Multiple strains of individual fungal species with distinct morphologies, biochemical compositions and IgE-binding characteristics**
- **High rates of somatic mutation for many species may produce further variations in allergenic protein repertoires and/or prominent allergen concentrations**
- **Inconsistent or undetermined cross-reactivity patterns among common airborne fungi for many mold-sensitive patients**
- **Different types of allergic reactions or clinical conditions induced by the same organism may be linked to different fungal components/fractions**
- **Fungal extracts are not standardized to defined IgE-binding potencies nor major allergen concentrations**
- **Source material differences between allergen providers due to differences in starter cultures or strains, growth media, culture conditions (static/shaker), fractionation and processing steps**
- **Extract differences related to extraction methods, protease levels/activities and resulting stabilities**
- **Limited knowledge of prominent allergenic proteins implicated in allergic diseases for specific patient populations**
- **Limited availability of validated quantitative or qualitative assays for fungal extract components**

MOLD EXTRACT COMPARISONS

- **Aqueous and glycerinated concentrates from 7 U.S. manufacturers**

  Alternaria alternata (tenuis)
  Aspergillus fumigatus
  Penicillium notatum (chrysogenum)

  Protein and carbohydrate concentrations:
  Bradford and Phenol-sulfuric acid assays

  Protein compositional profiles: SDS-PAGE

  Protein reactivity patterns: Immunoblotting

  Allergenic potencies + epitope similarities:
  ELISA inhibition

  Alternaria Alt a 1 concentrations:
  MAb ELISA assay, INDOOR Biotechnologies kit

- **Extract strengths**

Manufacturer #	*Aqueous*	*Glycerinated*
1	1:10 w/v	1:20 w/v
2	1:10 w/v	1:10 w/v
3		1:20 w/v
4	1:10 w/v	1:10 w/v
5		1:10 w/v
6	1:5 w/v	1:10 w/v
7		1:10 w/v

ALTERNARIA EXTRACTS

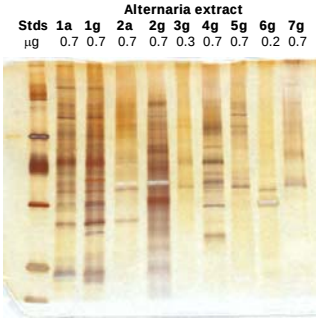
- **Compositions and potencies of aqueous (a) and glycerinated (g) Alternaria extracts sourced directly from allergen manufacturers 1-7 :**

Alternaria extract	*Protein mg/mL*	*Carbohydrate mg/mL*	*Carb-Protein ratio*	*Alt a 1 µg/mL*	*IgE-binding potency vs. 1g, 2g, 4g, 7g **	
					Mean	*Range*
1a	0.73	4.27	5.8	1.85	1.29	1.01-1.41
1g	0.68	3.54	5.2	0.95	1.28	1.00-1.54
2a	0.19	11.38	59.9	1.30	0.41	0.25-0.49
2g	0.26	13.69	52.7	2.75	0.55	0.26-1.00
3g	0.06	31.30	521.7	0.05	0.23	0.06-0.41
4a	0.03	17.14	571.3	ND	ND	ND
4g1	0.02	19.70	985.0	ND	ND	ND
4g2	0.18	3.27	18.2	0.04	0.67	0.28-1.00
5g	0.15	2.40	16.0	0.05	0.96	0.58-1.11
6a	0.08	33.79	422.4	ND	ND	ND
6g	0.03	16.65	555.0	2.04	0.03	0.01-0.04
7g	0.14	10.96	78.3	0.09	0.75	0.22-1.19
Range (X)	37	14	189	69	43	

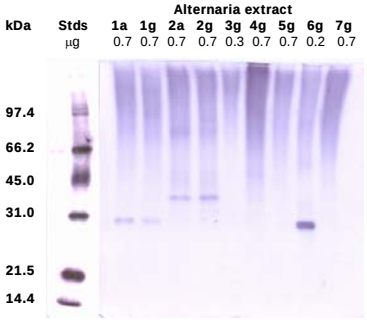
* Parallel dose-response curves (similar allergen compositions) observed for all analyses

- **SDS-PAGE gels visualized by silver staining at equivalent or maximum protein loads produced variable patterns for many Alternaria products. Blots probed with IgE or IgG antibodies showed that allergen epitopes may be present on molecules of similar or distinct size in these extracts.**

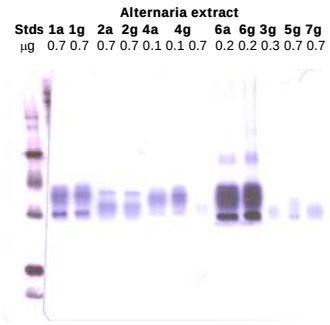
SDS-PAGE
Invitrogen silver stain



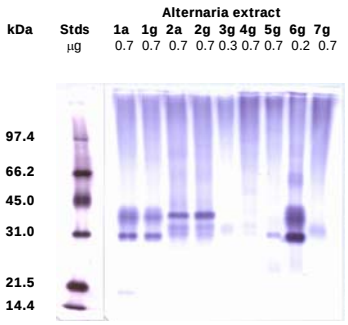
IgE immunoblot
Greer G003 human serum



Mouse Mc anti-Alt a 1 immunoblot
INDOOR Biotech Ab MA-121 lot 2427



Rabbit Pc anti-Alt a 1 immunoblot
Greer rabbit serum lot 060796



- **IgE-binding potencies do not correlate closely with Alt a 1 (major Ag) levels but are generally higher in extracts with elevated protein content. Multiple allergens are conserved across all extract pairs tested based on the parallelism (t test) of the ELISA inhibition dose-response curves.**

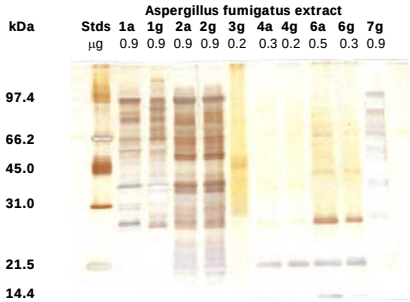
ASPERGILLUS EXTRACTS

- **Aspergillus fumigatus extracts possessed variable compositions and potencies, yet retained qualitatively-similar (parallel) allergenic protein constituents :**

Aspergillus extract	*Protein mg/mL*	*Carbohydrate mg/mL*	*Carb-Protein ratio*	*IgE-binding potency vs. 1g, 2g, 7g **	
				Mean	*Range*
1a	0.29	4.16	14.6	0.35	0.18-0.59
1g	0.18	1.64	9.1	0.47	0.19-1.00
2a	0.38	12.23	32.5	1.01	0.54-1.75
2g	0.56	11.77	20.9	2.17	1.00-3.97
3g	0.04	23.38	631.8	0.19	0.04-0.40
4a	0.05	10.94	218.8	0.06	0.02-0.14
4g	0.03	11.94	351.1	0.06	0.02-0.14
6a	0.09	28.78	323.4	0.13	0.05-0.22
6g	0.05	14.38	299.6	ND	ND
7g	0.17	9.97	58.0	1.27	0.28-2.53
Range (X)	19	18	69	36	

* Parallel dose-response curves (similar allergen compositions) observed for all analyses

- **Variable SDS-PAGE patterns were also observed for commercial Aspergillus fumigatus extracts**



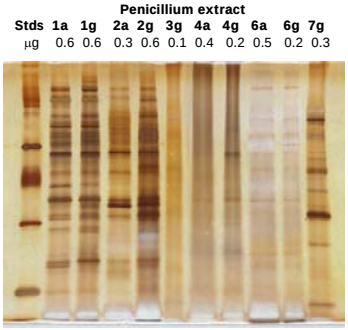
PENICILLIUM EXTRACTS

- **Penicillium notatum extracts also displayed variable compositions and IgE potencies. Several Penicillium products possessed significant differences in IgE epitope repertoires (extracts 1a/2a/2g vs. 7g) based on non-parallel ELISA inhibition potency curves :**

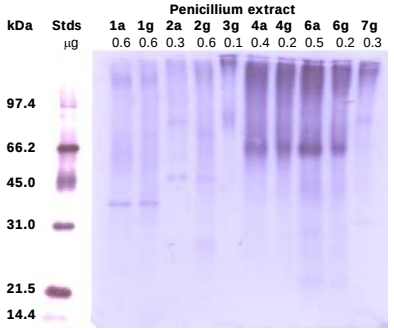
Penicillium extract	*Protein mg/mL*	*Carbohydrate mg/mL*	*Carb-Protein ratio*	*IgE-binding potency vs. 1g, 2g, 4g, 7g*	
				Mean	*Range*
1a	0.38	3.58	9.4	1.83	1.29-2.88
1g	0.24	1.82	7.6	0.76	0.48-1.03
2a	0.06	1.60	26.7	0.20	0.12-0.26
2g	0.13	2.36	18.2	0.65	0.41-1.00
3g	0.02	20.05	1002.5	0.33	0.18-0.46
4a	0.08	4.31	53.9	0.90	0.45-1.35
4g	0.04	3.86	96.5	0.95	0.52-1.58
6a	0.10	12.44	124.4	0.59	0.23-1.00
6g	0.05	4.82	96.4	ND	ND
7g	0.06	35.53	592.2	0.80	0.29-1.56
Range (X)	19	22	132	9	

- **Commercial Penicillium extracts produced distinct SDS-PAGE and immunoblot patterns**

SDS-PAGE
Invitrogen silver stain



IgE immunoblot
Greer ZM-P1 human serum



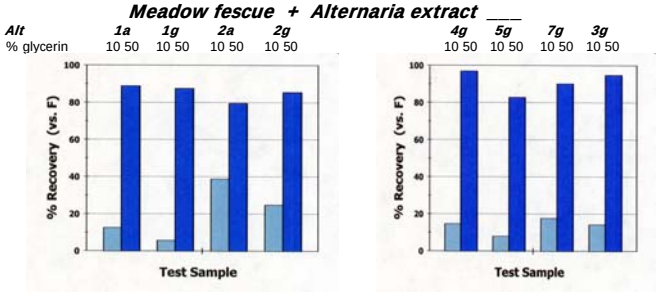
MOLD STABILITIES AND COMPATIBILITIES

- **Mix mold extracts with other allergens, with each product present at 1/10 of concentrate strengths**
  - ***Mold (Alt, Asp, Pen) + Grass (Meadow fescue)***
  - ***Mold (Alt, Asp, Pen) + Dust mite (D. farinae)***
  - ***Mold (Alt, Asp, Pen) + Cat***
  - ***Mold (Alt, Asp, Pen) + Short ragweed***
  - ***Mold (Alt, Asp, Pen) + Cockroach (Am/Ger)***
  - ***Mold (Alt, Asp, Pen) + other Molds***
- **Prepare identical extract mixtures with variable concentrations of glycerin (10-50%)**
- **Prepare individual extract dilutions at identical allergen and glycerin levels to serve as control samples**
- **Store mixtures and controls at 2-8°C for up to 1 year**
- **Analyze samples for specific allergenic activities**
  - ***Grass or Dust mite: Immunoblot, ELISA inhibition***
  - ***Cat or Short ragweed: Fel d 1 RID, Antigen E RID***
  - ***Mold or Cockroach: Immunoblot, specific Ag ELISA***
- **Stability/compatibility time points (storage periods) examined to date:**
  - ***Grass + Alt: 21, 118 d (Blots); 137 d (ELISA)***
  - ***Grass + Asp: 42, 119 d (Blots); 124, 368 d (ELISA)***
  - ***Grass + Pen: 42, 119 d (Blots); 124, 368 d (ELISA)***
  - ***D. far + Alt/Asp/Pen: 58 d (Blots); 69, 169 d (ELISA)***
  - ***Cat + Alt/Asp/Pen: 64, 143 d (RID)***
  - ***Ragweed + Alt/Asp/Pen: 65, 150 d (RID)***
  - ***Alt/Asp/Pen/Helminthosporium + other Molds (Alt, Asp, Pen, Helm, Hormodendrum, Epicoccum, Pullularia, Fusarium, Mucor): 60, 79, 275 d (Blots); 64, 343 d (ELISA)***
  - ***Alt/Asp/Pen + Cockroach: 10 d (Blots)***

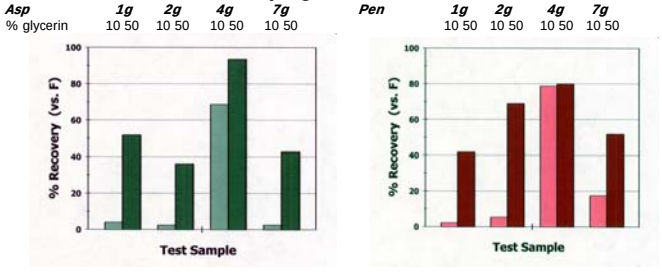
GRASS-MOLD MIXTURES

- **Grass allergen potencies are reduced significantly after mixing with mold extracts and storing for 4-5 months**

Meadow fescue + Alternaria extract ___

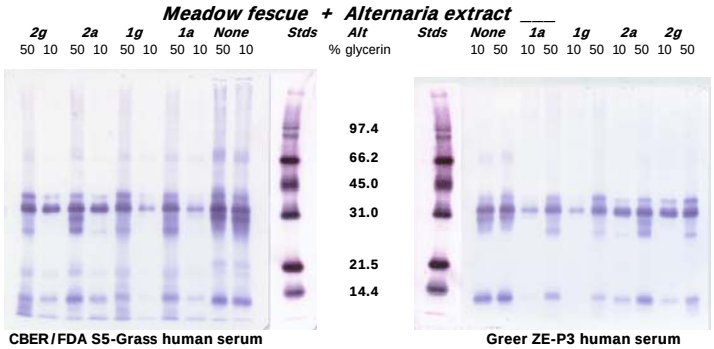


Meadow fescue + Aspergillus/Penicillium extract ___



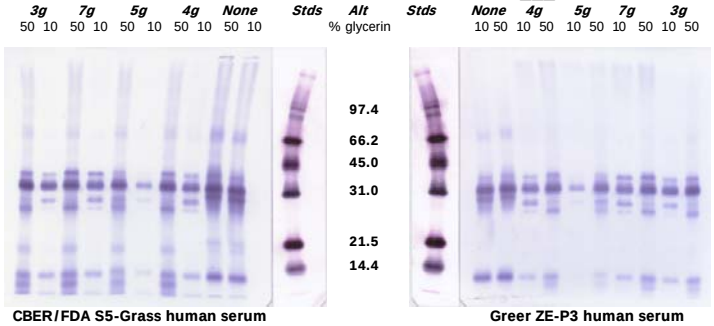
- **Grass-positive IgE immunoblots of grass-mold mixtures confirmed that reductions in meadow fescue allergen potencies after mixing with mold extracts involved numerous protein components, including group 1/5 allergens (30-35 kDa), group 4/13 allergens (50-60 kDa) and group 2/3/6/10/11 allergens (11-18 kDa)**

Meadow fescue + Alternaria extract ___



CBER/FDA S5-Grass human serum — Greer ZE-P3 human serum

Meadow fescue + Alternaria extract ___

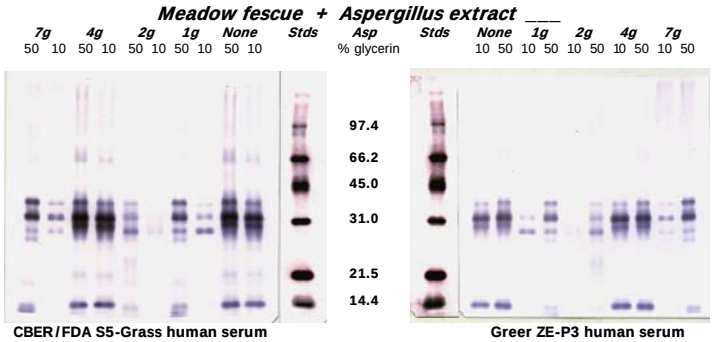


CBER/FDA S5-Grass human serum — Greer ZE-P3 human serum

Mixtures and controls stored for 118 days at 2-8°C

- **Alternaria extracts from different manufacturers at related w/v strengths produced similar levels of grass allergen instability based on human IgE ELISA inhibition and immunoblot analyses**

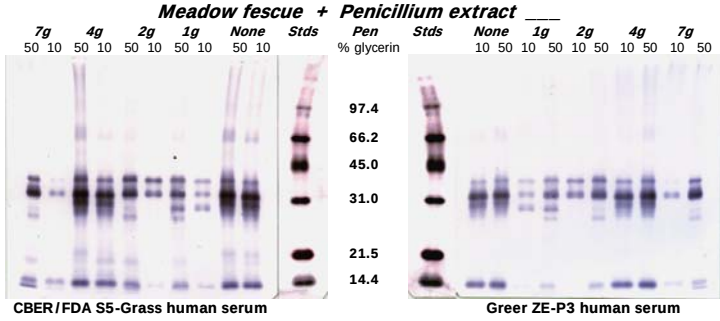
Meadow fescue + Aspergillus extract ___



CBER/FDA S5-Grass human serum — Greer ZE-P3 human serum

Mixtures and controls stored for 119 days at 2-8°C

Meadow fescue + Penicillium extract ___



CBER/FDA S5-Grass human serum — Greer ZE-P3 human serum

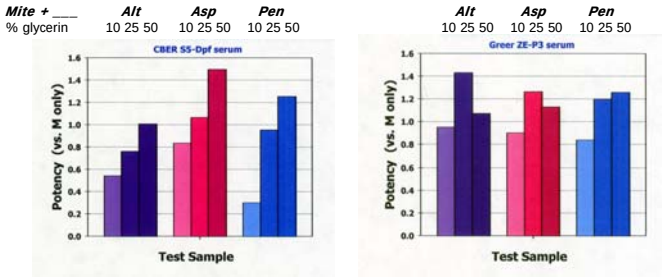
Mixtures and controls stored for 119 days at 2-8°C

- **Aspergillus extracts from different manufacturers destabilized grass allergens at levels related more to their total protein concentrations than to extraction conditions (w/v strengths)**

- **Penicillium extracts from different manufacturers also reduced grass allergen reactivities at levels corresponding to total protein content and not w/v strengths, similar to Aspergillus products**

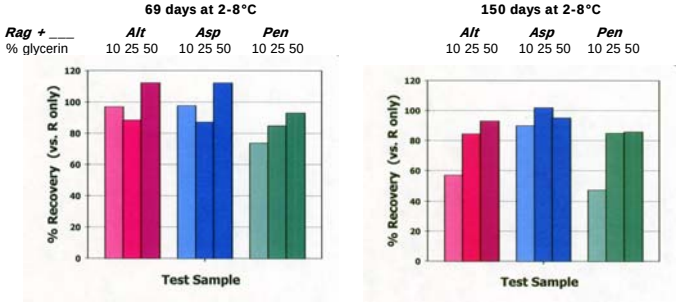
DUST MITE-MOLD MIXTURES

- **Mite allergen compatibilities with molds after storage for 69 days (2.3 months, S5-Spf serum) or 169 days (5.6 months, ZE-P3 serum) at 2-8°C vary considerably depending on the serum pool and fungal extract employed for these studies**



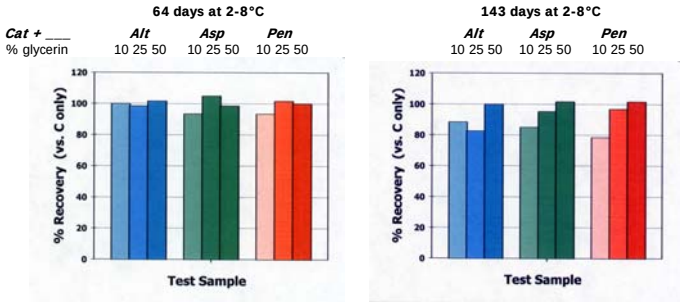
RAGWEED-MOLD MIXTURES

- **Short ragweed Antigen E stability was compromised by Penicillium and Alternaria but not Aspergillus at 10% glycerin levels after 150 days (5.0 months) at 2-8°C. Mixtures containing 25-50% glycerin retained high levels of Antigen E activity similar to mold-free controls.**



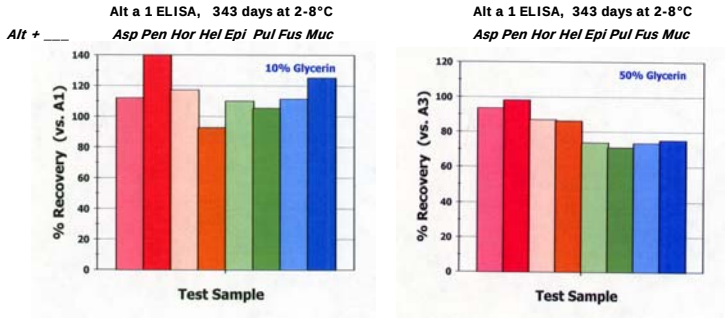
CAT-MOLD MIXTURES

- **Cat Fel d 1 activities were not compromised significantly after mixing with mold extracts and storing for up to 143 days (4.8 months) at 2-8°C**

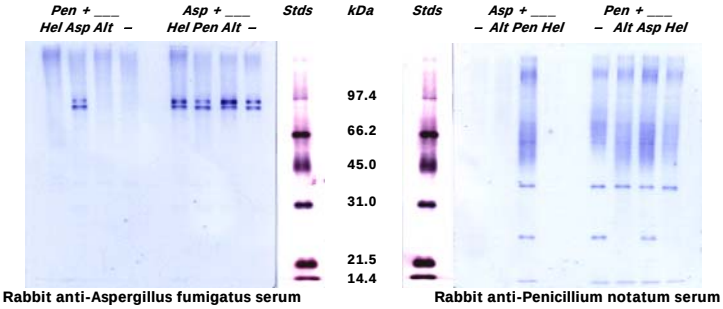


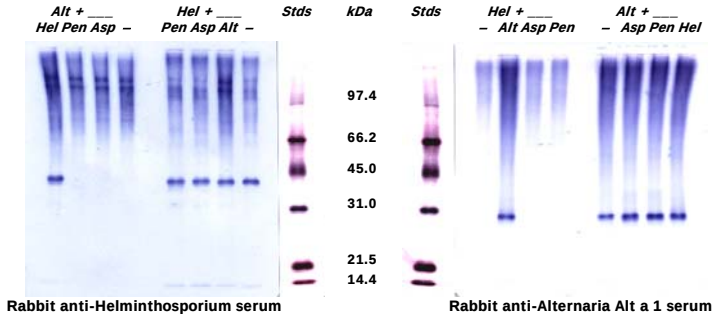
MOLD-MOLD MIXTURES

- **Fungal extracts appeared to be highly resistant to the proteases present in other mold extracts based on both ELISA (Alternaria Alt a 1) and immunoblot results (multiple fungi) after storage of mold-mold mixtures for up to 343 days (11.4 months) at 2-8°C**



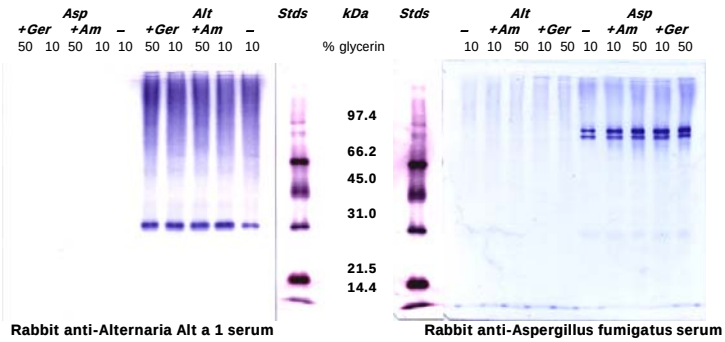
Mold-mold mixtures stored for 275 days at 2-8°C



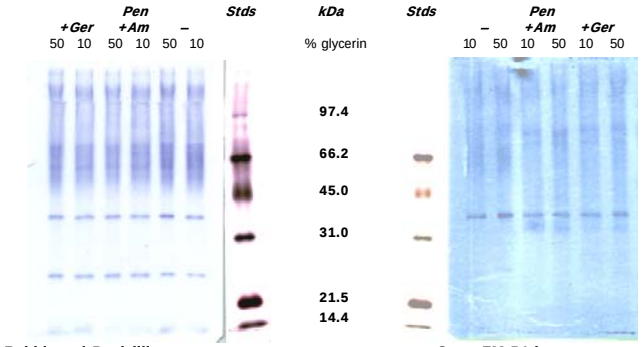


MOLD-COCKROACH MIXTURES

- **Short-term (10 day) exposures of mold antigens to cockroach extracts that contain highly-active proteases did not produce noticeable changes in protein structures and antibody-binding activities. Under these conditions, 80-90% of cockroach allergen potencies are destroyed by the actions of endogenous cockroach proteases.**



MOLD-COCKROACH MIXTURES



Rabbit anti-Penicillium notatum serum

Greer ZM-P1 human serum

CONCLUSIONS

- **Differences in allergen levels, stability (protein / carbohydrate content) and compatibility (protease activities) between fungal extracts from different sources contribute to the uncertainties and inconsistencies associated with the diagnosis and treatment of mold allergies**
- **The use of multiple extracts for a given organism / species may broaden the spectrum of allergenic epitopes presented to patients and improve the screening, identification and clinical management of fungal allergies**
- **Further studies on the IgE specificities of mold-allergic patients and cross-reactivity patterns relevant to their clinical sensitivities are in progress to support the validity of this approach**
- **Mold allergen compatibilities are influenced directly by glycerin in all cases examined in this study, and may be related to extract protein concentrations (Aspergillus), extraction ratios (Alternaria), protease activities (Penicillium), or other environmental / interactive factors**
- **Based on the data from this study and previous published reports, our current knowledge of extract compatibilities for immunotherapy can be represented as follows :**