

STABILITY, COMPATIBILITY AND
CROSS-REACTIVITY OF ALLERGENS :
IMMUNOCHEMICAL REACTIVITIES AND
PRACTICAL CONSIDERATIONS

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ABSTRACT

The effectiveness of testing and treatment procedures in the allergy clinic may be influenced significantly by the consistency and predictability of allergenic product compositions and potencies. To address this issue, the immunochemical characteristics of these products (thermal stabilities, compatibilities in treatment vaccines, cross-reactivities with related or unrelated allergens) have been examined using analytical methods specific for a diverse group of component proteins, including major allergens.

ELISA and immunoblot analyses of product stabilities in several diluents (HSA, 10% glycerin) at strengths as low as 1:10,000 w/v indicated that cockroach and short ragweed proteins were unstable to both high temperature (45°C, 113°F) and low temperature (-15°C, 5°F) incubations. *Alternaria*, ryegrass, fire ant and elm proteins exhibited low reactivities under some conditions. Dog epithelial proteins displayed no apparent changes in structure and potency. For most products, dilutions in 10% glycerin possessed similar heat stabilities and up to 100-fold increases in freeze-thaw stability compared to analogous dilutions in HSA.

Compatibility studies with vaccine formulations containing up to 3 of 6 common allergens (*Alternaria*, cat, cockroach, dog, dust mite and meadow fescue) confirmed the degradative effects of fungal and insect products on grass allergen reactivities but also demonstrated that many other combinations may coexist with no significant changes in component structures.

Allergenic cross-reactivities among grass pollens examined by competitive-binding immunoassays supported the close taxonomic relationships reported for these allergens. Within the *Pooideae* subfamily, grasses from different tribes (timothy/orchard, red top/sweet vernal) or from within the same tribe (timothy/red top, kentucky blue/meadow fescue) exhibited distinct IgE inhibitions with some sensitive subjects but not others.

The results of these studies may be useful to clinicians seeking to optimize the design, preparation and storage of their allergen dilutions and vaccines.

EXPERIMENTAL APPROACH

- Develop and validate sensitive immunoassays (ELISA, immunoblot) specific for a select group of extract protein/epitope structures
- Analyze immunochemical (IgE, IgG) integrity of extracts and major allergens during shipping, storage or intended use conditions
- Correlate direct and competition ELISA results (discontinuous epitopes, conformations) with corresponding immunoblot profiles (continuous epitopes, linear protein sequences)
- Determine sources of instability or incompatibility, and the predictability of cross-reactions based on taxonomic relationships
- Assess clinical utility of laboratory methods by comparisons with results from skin prick testing of sensitive subjects
- Identify materials and conditions producing optimal clinical and biochemical characteristics

MATERIALS AND METHODS

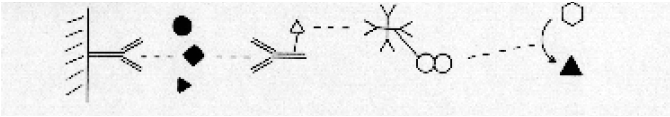
- Extract concentrates/diluents from Greer inventories Rabbit antisera (IgG), human allergic sera (IgE)
- Double-bind (sandwich) and inhibition ELISA analyses Direct/inhibition SDS-PAGE immunoblot procedures Skin prick/puncture tests using GreerPick device
- Individual protein (major allergen) analytes
Alternaria Alt a 1, Cat albumin, Dog albumin/Can f 1, Fire ant Sol i 3, Grass Group 1, Short ragweed AgE
- Multiple protein (extract) analytes
Alternaria, Cockroach, Dust mite D. farinae, Elm, Short ragweed, Temperate and subtropical grasses
- Skin prick/puncture testing
*Barbara Magera, M.D., Charleston, South Carolina
Bonita Wilson, M.D., Morganton, North Carolina*

STABILITY INVESTIGATIONS

- **Test extracts/analytes**
Alternaria, Alternaria Alt a 1, Cockroach, Dog albumin, Dog Can f 1, Elm, Fire ant Sol i 3, Grass Group 1 antigens, Meadow fescue, Short ragweed, Short ragweed Antigen E
- **Extract strengths**
1:10, 1:100, 1:1,000 and 1:10,000 w/v
- **Diluents**
HSA-Saline or 10% Glycerosaline
- **Methods of analysis**
Double-bind ELISA and SDS-PAGE immunoblotting
- **Incubation conditions**
Control temp. 3 days at 4°C (39°F)
High temp. 3 days at 23°C (70°F), 34°C (93°F) or 45°C (113°F)
Low temp. 1-2 Freeze-thaw cycles (-15°C/4°C)

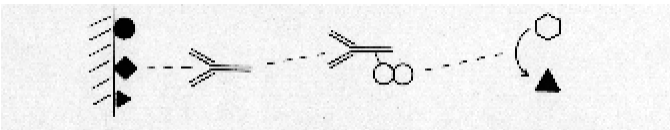
STABILITY COMPARISONS/CRITERIA

- **Double-bind ELISA**



Test extracts incubated under native, non-denaturing conditions
Sensitive primarily to conformational (discontinuous, 3D) epitopes
Confirmed using native vs. heat-denatured allergen samples
Stability defined as retention of $\geq 50\%$ of 4°C reactivities

- **SDS-PAGE Immunoblotting**



Test extracts denatured by heat and detergent treatments
Disrupts conformational binding sites, retains linear sequence epitopes
Stability defined as recovery of all major IgE+ or IgG+ bands (vs. 4°C)

- **Results**

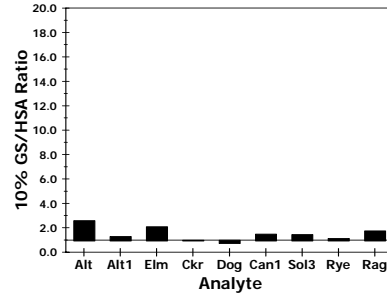
		Freeze/thaw stability	
		Stable	Unstable
Thermal stability	Stable	Alternaria Alt a 1 Dog albumin Dog Can f 1 Elm	Fire ant Sol i 3
	Unstable	Alternaria Italian ryegrass Grass Group 1 Ags Short ragweed	Cockroach Short ragweed AgE

- **Dilutions stabilized by HSA:**
Alternaria, Alternaria Alt a 1, Elm, Fire ant Sol i 3

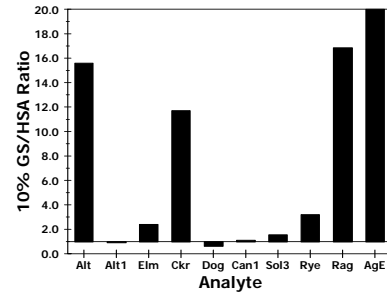
Dilutions stabilized by 10% Glycerin:
Alternaria, Alternaria Alt a 1, Elm, Fire ant Sol i 3, Cockroach, Italian rye, Grass Group 1 antigens, Short ragweed, Short ragweed Antigen E

10% Glycerin vs. HSA
Similar thermal stabilities, improved freeze-thaw stabilities

Thermal stability

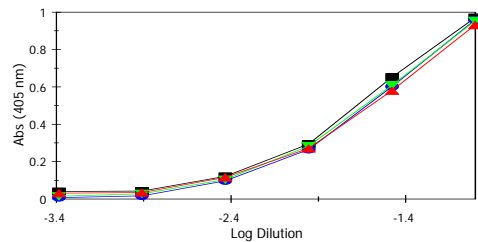


Freeze-thaw stability

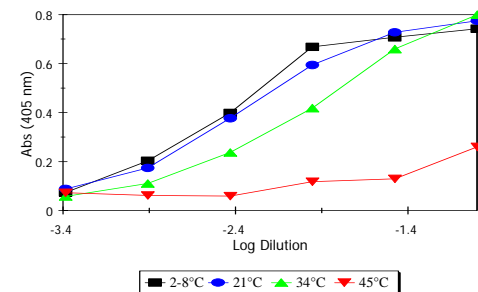


Thermal Stabilities of 1:1,000 w/v Extracts
ELISA Results

Alternaria, Alt a 1, Stable



Short ragweed, AgE, Unstable

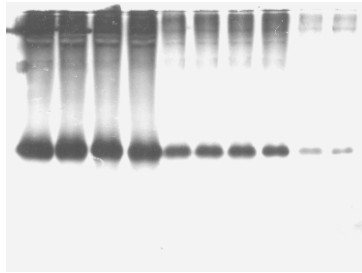


COMPATIBILITY SAMPLES/CRITERIA

Thermal Stabilities Immunoblot results

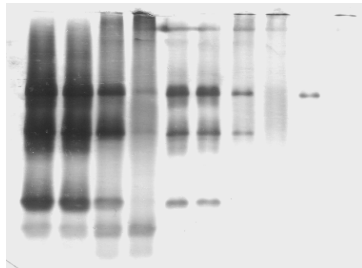
Alternaria, Alt a 1, Stable

w/v 1:10 1:100 1:1,000
°C 4 23 34 45 4 23 34 45 4 45



Short ragweed, AgE, Unstable

w/v 1:10 1:100 1:1,000
°C 4 23 34 45 4 23 34 45 4 45



COMPATIBILITY STUDIES

- **Test extracts/analytes**
Alternaria/Alt a 1 (A), Cat albumin (C), Cockroach (R), Dog albumin (D), Dust mite D. farinae (M), Meadow fescue (F)
- **Extract combinations and strengths**
*1-3 extracts per sample
10 mL volumes in 10-20 SEVs
Each component present at 1:10 dilution of glycerinated concentrate strength*
- **Diluent**
Normal saline
- **Methods of analysis**
*Double-bind ELISA and SDS-PAGE immunoblotting
Skin prick testing (Magera clinic)*
- **Storage and sampling conditions**
*Storage time/temperature 3 months at 4°C (39°F)
Test intervals 0, 1, 2 and 3 months*

- Test samples (mixtures) and controls (individual extracts (shadowed))

#	Extract	Alternaria (A)	Cat (C)	Dog (D)	M. fescue (F)	Mite (M)	Cockroach (R)
8	A	A	AC	AD	AF	AM	
4	C	AC	C			CM	
4	D	AD		D		DM	
6	F	AF			F	FM	RF
10	M	AM	CM	DM	FM	M	RM
4	R				RF	RM	R

#	Extract	C + M	D + M	F + M	F + M
8	A	ACM	ADM	AFM	
4	C	ACM			
4	D		ADM		
6	F			AFM	RFM
10	M	ACM	ADM	AFM	RFM
4	R				RFM

- **Extract compatibility criteria**
*ELISA: Retention of ≥ 50% of initial/control reactivity
SPT: Retention of ≥ 50% of control wheal diameters
Blots: Recovery of all major IgE/G+ bands (vs. control)*
- **Focus**
*Specific detection of individual extracts w/in mixtures
Instab due to endo- vs. exo-genous (mix) components
Influence of high [protease] of mold & insect extracts
Correlations between ELISA (3°), blot (1°), SPT results*

- ELISA/immunoblot results per test extract/analyte

High compatibility (mixtures) or stability (controls) samples

Alternaria (A)	Cat (C)	Dog (D)	M. fescue (F)	Mite (M)	Cockroach (R)
A ACM				M FM	
AD ADM				AM RM	RF
AF AFM	C	D	F FM	CM ACM	RM
AM				DM ADM	RFM

Samples displaying moderate compatibility/stability

Alternaria (A)	Cat (C)	Dog (D)	M. fescue (F)	Mite (M)	Cockroach (R)
AC	AC ACM	AD ADM		AFM	R
	CM	DM		RFM	

Mixtures exhibiting low (poor) compatibility

Alternaria (A)	Cat (C)	Dog (D)	M. fescue (F)	Mite (M)	Cockroach (R)
			AF AFM		
			RF RFM		

- ELISA/immunoblot correlations

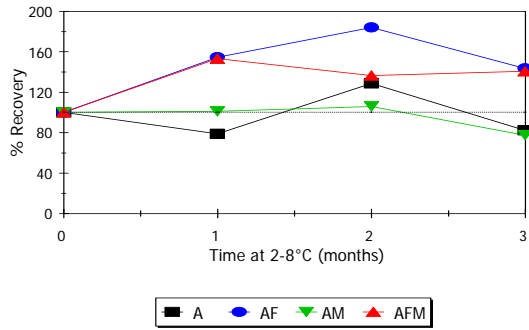
Analyte	Comparability of ELISA and blot results
Alternaria	Strong
Cat	Moderate
Dog	Strong
Meadow fescue	Strong
Dust mite D. farinae	Moderate
Cockroach	Strong

- Skin test results

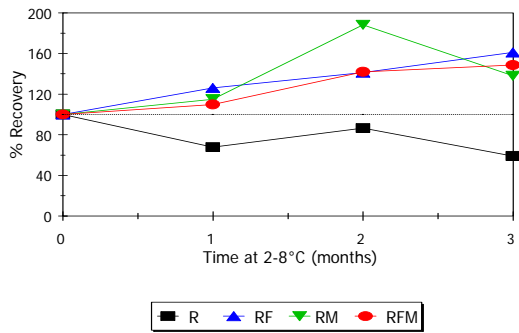
# of subjects tested with 19 test samples/controls	49
# of subjects with dominant skin reactions to one control allergen	13
# of subjects with dominant skin reactions to mite allergens	7
# of subjects with dominant skin reactions to fescue allergens	6
# of mite reactions reduced to < 50% of controls by cockroach	3/7
# of mite reactions reduced to < 50% of controls by Alternaria	1/7
# of fescue reactions reduced to < 50% of controls by cockroach	5/6
# of fescue reactions reduced to < 50% of controls by Alternaria	4/6

ELISA Compatibility Results

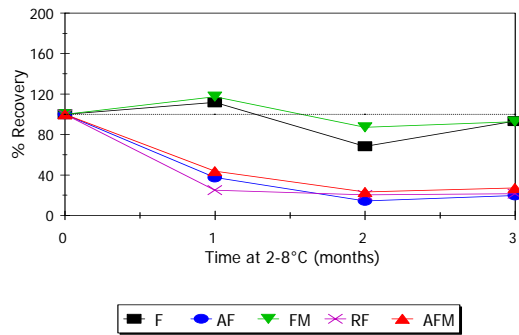
Alternaria



Cockroach



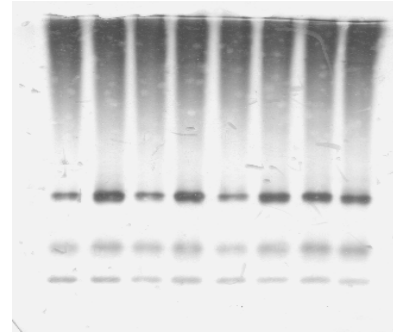
Meadow fescue



Immunoblot Compatibility Results

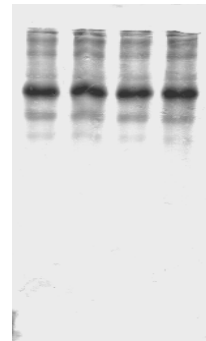
Alternaria, Rabbit anti-Alt a 1 serum

A AC AM AD AF ACM ADM AFM



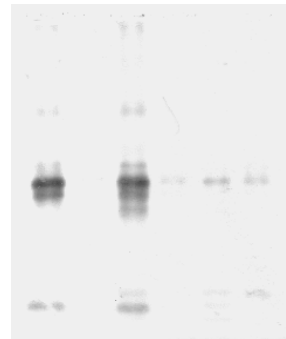
Cockroach, Rabbit anti-German cockroach serum

R RF RM RFM



Meadow fescue, Grass-positive human serum pool

F AF FM RF AFM RFM



CROSS-REACTIVITY RELATIONSHIPS

- Focus**
Clinical/biochemical cross-reactivities vs. predictions from taxonomy
Consistency of cross-reactions across subjects, extracts and analyses
Redundancies in common grasses
Optimization of Rx/Dx allergens

- Materials and methods**
Extracts Standardized and non-std grass pollens
Glyc. concentrates (1:20 w/v, 10-100K BAU/mL)
Subjects Grass-sensitive Greer employees
Analyses ELISA and SDS-PAGE immunoblotting
Skin prick testing (Wilson clinic)
Analysis modes Direct-bind
Reciprocal (cross-wise) inhibitions

- Grass species**
Temperate Kentucky blue (Kb), Meadow fescue (Mf), Orchard (or), Perennial rye (Pr), Red top (Rt), Sweet vernal (Sv), Timothy (Ti)
Subtropical Bermuda (Be), Bahia (Ba), Johnson* (Jo)*
** Non-standardized*

- Taxonomic relationships: Grasses in Gramineae family**

Subfamily	Tribe	Genus	Common name	Predicted similarity to Timothy
Pooideae	Agrostideae	Phleum Agrostis	Timothy Red top	High
	Poaceae	Poa Festuca Dactylus Lolium	Kentuckyblue Meadow fescue Orchard Perennial rye	Moderate
	Phlarideae	Anthoxanthum	Sweet vernal	
Chloridoideae	Chlorideae	Cynodon	Bermuda	Low
Panicoideae	Paniceae	Paspalum	Bahia	
	Andropogoneae	Sorghum	Johnson	

- Reactivity scales for ELISA, blot and skin test results**

Analysis	Unit of measure	Range of reactions			
		Grade 3	Grade 2	Grade 1	Grade 0
ELISA	Delta Abs 30'-5'	> 0.500	0.250-0.499	0.050-0.249	< 0.050
Blot	Band intensities	High	Moderate	Low	None
Skin test	Sum of erythema	> 100 mm	50-99 mm	1-49 mm	0 mm

- Subject reactivity profiles**

Category	Subject	Analysis	Reactivity scores with grass pollen extracts										
			Kb	Mf	Or	Pr	Rt	Sv	Ti	Be	Ba	Jo	
High +	G008	ELISA	3	3	3	3	3	3	3	3	3	3	3
		Blot	3	3	3	3	3	3	3	3	3	3	3
		SPT	3	2	2	3	3	3	3	3	2	3	2
Moderate +	G060	ELISA	3	3	3	3	3	3	3	3	2	2	
		Blot	3	3	3	3	3	3	3	3	3	3	
		SPT	2	3	2	3	3	3	3	3	3	3	
Moderate +	G020	ELISA	1	1	1	1	1	1	1	0	0	0	
		Blot	2	2	2	2	2	2	2	0	1	1	
		SPT	2	2	2	2	2	2	2	0	0	0	
Low +	G090	ELISA	1	1	1	1	1	1	0	0	0	0	
		Blot	2	2	2	2	2	2	2	1	1	1	
		SPT	1	1	2	2	2	2	2	0	1	2	
Low +	G033	ELISA	0	0	0	0	0	0	0	0	0	0	
		Blot	0	0	0	0	0	0	0	0	0	0	
		SPT	0	1	1	0	0	0	0	0	0	1	
Low +	G092	ELISA	0	0	0	0	0	0	0	0	0	0	
		Blot	0	0	0	0	0	0	0	0	0	1	
		SPT	0	0	0	1	0	0	0	0	0	1	

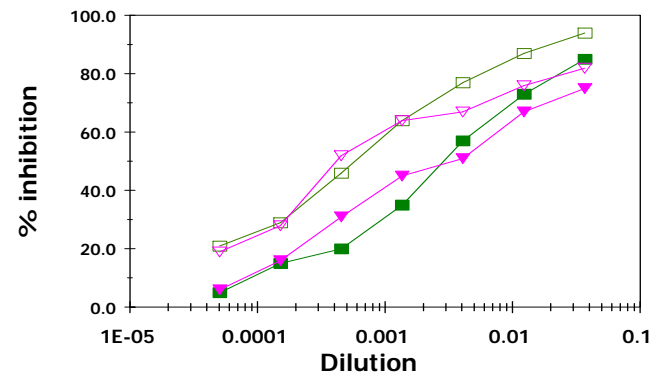
- Compositional similarities of IgE determinants in grass pollen extracts**

Similar: Parallel ELISA inhibition reactivities for inhibitors vs. solid phase allergens
Distinct: Non-parallel ELISA inhibition dose-response curves (paired t test)

Solid phase	Highest potency per mg protein (% of total)	# of subjects with cross-reactive vs. distinct grass reactions									
		Kb	Mf	Or	Pr	Rt	Sv	Ti	Be	Ba	Jo
Kb	29				1			1			
Mf	19				0			0			
Or	3	4	4		5	2	4	4			
Pr	16	3	3	2		2	1	2	1	1	0
Rt	3	4	4	5	6		4	7			
Sv	3	0	1			2		2			
Ti	26	1	1	0	1	2	2		0	0	1
Be	0	2	2	3	0	3	1		1	1	0
Ba	0										
Jo	0										

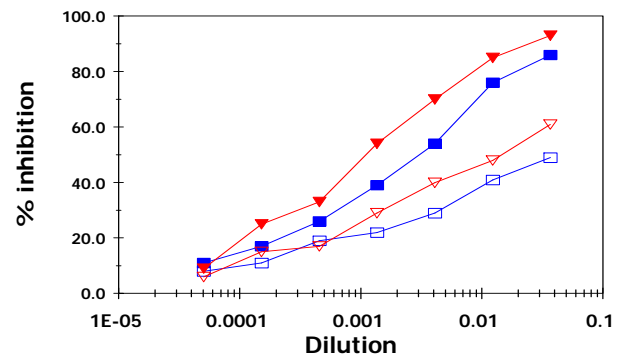
ELISA Inhibition Patterns Parallel vs. non-parallel responses

Timothy vs. Red top, Subjects G060, G067
Parallel



Legend:
■ Red top (reference, G060) ◻ Timothy (inhibitor, G060)
▲ Red top (reference, G067) ◻ Timothy (inhibitor, G067)

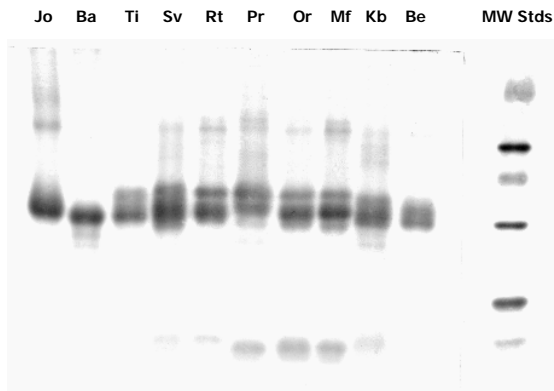
Red top vs. Timothy, Subjects G008, G060
Non-parallel



Legend:
■ Timothy (reference, G008) ◻ Red top (inhibitor, G008)
▲ Timothy (reference, G060) ◻ Red top (inhibitor, G060)

IgE Immunoblot Specificity Patterns

Serum G001 (Moderate +)

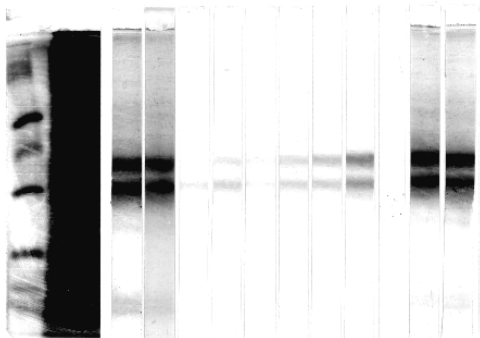


Immunoblot Inhibition Profiles

Inhibitors: Grass extracts at 100 µg/mL protein

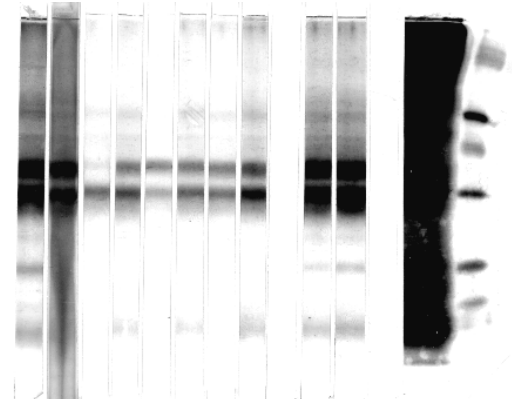
Serum G060, Timothy solid phase

Stds Au PC Be Kb Mf Or Pr Rt Sv Ti Ba Jo NC



Serum G008, Timothy solid phase

PC Be Kb Mf Or Pr Rt Sv Ti Ba Jo NC Au Stds



CONCLUSIONS

- Cockroach (high protease content) and short ragweed (low protease) extracts are unstable to low or high temperature exposures
 - Extracts diluted with HSA or 10% glycerin often possess equivalent or higher stabilities compared to 1:10 w/v concentrates
 - 10% glycerin dilutions provide similar thermal stabilities and improved freeze-thaw stabilities compared to analogous HSA dilutions
- Grass allergens are degraded after mixing with Alternaria or cockroach extracts (active proteases) but not with dust mite (inactive proteases)
 - Alternaria and cockroach antigens are protected by proteins from other extracts, consistent with the non-specific nature of mold/insect proteases
- Grass pollen sensitivities and allergenic cross-reactivities correlate closely between *in vivo* and *in vitro* test methods but are not always predicted by botanical or taxonomic relationships among grass species

PRACTICAL CONSIDERATIONS

- Reprepate unstable extract dilutions (1:100-1:10,000 w/v) every 2-3 months (buffered saline or normal saline diluent) or every 6 months (10% glycerin or HSA diluent)
 - Use glycerinated extracts whenever possible (glycerin protects unstable allergens from denaturation and inhibits protease activity)
- Keep molds and insects separate from pollens in Rx mixtures
 - OK to combine dust mites with pollens, cat/dog with molds
- Timothy allergens cover most (if not all) *Pooideae* grasses effectively and may also represent Bahia and Johnson allergens
 - Bermuda allergens are distinct from those of other grasses
 - IgE specificities (proteins) similar, epitopes may vary from patient-to-patient