Studies of allergen extract stability: The effects of dilution and mixing

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Background: *However potent the allergy extracts provided by manufacturers, they are subject to deterioration with storage, especially after dilution or mixture with other extracts.*
Objective: *This study was performed to assess separately the deterioration during storage in allergen extract potency caused by dilution or by mixture with allergen extracts that have been reported to contain proteases.*
Methods: *To assess the effect of dilution, three serial 10-fold dilutions of cat, short ragweed, Bermuda grass, and* Dermatophagoides farinae *extracts were prepared alone or combined with other extracts. They were stored at 4° C for 3 and 12 months. To assess the effect of mixing with other extracts that have been reported to contain proteases, extracts of timothy grass, Bermuda grass, short ragweed, Russian thistle, white oak, box elder,* D. farinae, *and cat were stored alone or combined with one or more extracts of American cockroach,* Alternaria *spp.,* Cladosporium *spp.,* Penicillium *spp., and a house dust mite mix for 3 months at 4° C.*
Results: *Bermuda grass, cat, and house dust mite extracts incurred significant loss of potency at all dilutions with storage. Short ragweed was stable at all dilutions. Potency of extracts of timothy grass, Bermuda grass, Russian thistle, white oak, box elder, and cat were all reduced by combination with one or more extracts potentially containing proteases. Only short ragweed and* D. farinae, *which was in a final concentration of 25% glycerin, were resistant.*
Alternaria *extract was most frequently responsible for loss of potency, followed by cockroach and* Cladosporium *extracts. Combination with extracts of* Penicillium *and a house dust mite mix did not reduce the potency of any extract.*
Conclusions: *Both dilution alone and mixture with extracts reported to contain proteases caused loss of potency of most extracts tested. Ragweed was uniquely resistant under both conditions of storage. (J Allergy Clin Immunol 1996;98:382-8.)*

Recently, attention has been focused on the quality of allergen extracts used for skin testing and allergen immunotherapy. Advances have included the establishment of international and national standards and increasing availability of extracts standardized for potency and batch-to-batch reproducibility.[1-3] However potent and reliable the extract provided by the manufacturer, it is still subject to loss of potency after purchase, particularly because of factors such as dilution, storage at improper temperatures, or mixture with other allergen extracts.[4-6] It is the purpose of this article to examine the effect on extract potency of two of these variables of extract storage: dilution and mixture with other allergen extracts that have been reported to contain proteases.[7-11]

Abbreviations used
AU: Allergen unit
BAU: Bioequivalent allergen unit
HSA: Human serum albumin

METHODS

Extracts

The extracts that were tested for preservation of potency under varying conditions and their stock potency were: white oak, box elder, Bermuda grass, short ragweed, and timothy grass—all 100,000 allergen units (AU)/ml (ALK, Milford, Conn.); Russian thistle, 100,000 AU/ml (Miles Inc. Allergy Products, Spokane, Wash.), and 1:10 wt/vol (Berkeley Biologicals, Berkeley, Calif.); cat hair and pelt, 10,000 BAU/ml, and *D. farinae,* 10,000 AU/ml (Miles Inc. Allergy Products). Except for

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Received for publication Mar. 14, 1995; revised Nov. 14, 1995; accepted for publication Nov. 17, 1995.
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0091-6749/96 $5.00 + 0 **1/1/70869**

two lots of Russian thistle, a single lot of each of these allergen extracts was used throughout the study.

The extracts that were used as potential sources of proteases and their stock concentrations were American cockroach, 1:10 wt/vol (Berkeley Biologicals); *Alternaria tenuis,* 1:10 wt/vol; *Penicillium notatum,* 1:10 wt/vol; *Cladosporium herbarum,* 1:10 wt/vol (Greer Laboratories, Lenoir, N.C.); and house dust mite mix (*D. farinae,* 5000 AU/ml, and *D. pteronyssinus,* 5000 AU/ml; Miles Inc. Allergy Products). For the first three of these allergen extracts, three different lots from a single commercial source were used at different times, although for the house dust mite mix, two different lots were used.

All extracts were either purchased in a lyophilized state and reconstituted in phenol-saline solution containing 0.03% human serum albumin (HSA) or were purchased in phenol-saline solution, with the exception of *D. farinae* and house dust mite mix, which were provided in 50% glycerin.

All dilutions were prepared with normal saline solution containing 0.03% HSA and 0.4% phenol (phenol-saline-HSA).

Extract mixes

Two different allergen extract mixes were used in the studies.

Allergen extract mixture 1. The first mixture was used in the studies on the effect of dilution on extract stability. This mixture consisted of a total volume of 10 ml containing 1 ml of the stock concentration of the allergen extract under study, plus 1 ml each of the stock concentration of *D. farinae/D. pteronyssinus* mix, cat, Bermuda grass, white oak, orchard grass, timothy grass, short ragweed, box elder, and Russian thistle extracts.

Allergen extract mixture 2. The second mixture was used in the mixing studies. This consisted of 1 ml each of the stock concentrations of *Penicillium, Cladosporium, Alternaria,* and American cockroach extracts plus 5 ml of phenol-saline solution, which was then added to 1 ml of the stock concentration of the allergen extract being studied.

Study design: Dilutions

Cat, short ragweed, Bermuda grass, and *D. farinae* extracts were tested in this study. Each extract was set up at a 1:10 dilution of the stock concentration diluted in phenol-saline solution with 0.03% HSA; this was designated *single.* Additionally, 1 ml of the stock concentration of the allergen extract was combined with 9 ml of allergen extract mixture 1 listed above; this was designated *mixture.* Two further 10-fold dilutions of both the single and the mixture were prepared with phenol-saline-HSA. These were designated *1:100* and *1:1000,* respectively.

The dilutions of the extracts were prepared 12 and 3 months before analysis for residual potency. They were stored at 4° C from the time of preparation until they were assayed. At the time of analysis, fresh extract dilutions of both the single allergen extract and the allergen extract mixture were prepared for comparison of potency.

Study design: mixing

To study the effect of mixing with extracts reported to contain proteases, the stock concentration of 1 ml of the allergen extract of interest (target allergen extract) was diluted in 9 ml of phenol-saline-HSA as a control. For comparison, 1 ml of the stock concentration of allergen extract being tested was combined with 1 ml each of *Penicillium, Cladosporium, Alternaria,* or American cockroach extract; or it was combined with 1 ml each of all four extracts (allergen extract mix 2). Because of the results with *D. farinae* in the dilution study, for testing with house dust mite mix or with *D. farinae* 5 ml rather than 1 ml of the stock concentration was used in the mixing studies. In each preparation phenol-saline-HSA was added in the amount required to yield a final volume of 10 ml.

The vials were stored for 3 months at 4° C. They were then analyzed by comparing the allergen diluted with phenol-saline-HSA with that mixed with the extracts potentially containing proteases.

Tests of extract potency

Extract potency was tested by inhibition ELISA with materials purchased from Sanofi Diagnostics Pasteur, Inc. (Chaska, Minn.). A pool of serum containing specific IgE was prepared for each allergen extract by combining 8 to 12 serum specimens from individuals with a strongly positive prick skin test response to that allergen extract. Controls for each allergen extract included the commercial allergen disk to which saline solution, but no specific IgE-containing serum, was added as the negative control and the commercial allergen disk to which specific IgE-containing serum and saline solution, but no allergen extract, were added as the positive control.

The ELISA was performed as follows. (1) Commercial allergen disks, 25 μl of allergen extract or saline solution and 25 μl of the pooled serum containing specific IgE antibodies to that allergen, were placed in the wells of microtiter plates, which were incubated at 37° C for 90 minutes. (2) Three milliliters of saline solution was added to each well, and after 5 minutes, the liquid was aspirated (this was repeated three times). Commercial enzyme conjugate (3.5 μl) was added to each well. (4) The plates were stored overnight at 4° C. (5) Disks were then washed as in step 2 with saline-Tween. (6) One hundred microliters of para-nitrophenylphosphate substrate was added, and the plates were incubated at 37° C for 1 to 2 hours. (7) The plates were read at an optical density of 405 nm.

Each assay was run in five separately prepared replicates.

TABLE I. Effect of dilution and storage on extract potency

Allergen extract	Dilution of allergen extract 1:10	1:100	1:1000
Bermuda	10,000 AU/ml	1000 AU/ml	100 AU/ml
Overall	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$
	Single fresh = mix fresh	Same as 1:10	Same as 1:10
	Both fresh > mix 3 mo & 12 mo	Same as 1:10	Same as 1:10
	Both fresh > single 12 mo	Same as 1:10	Same as 1:10
Stored extract	$p = 0.0004$	$p = 0.0127$	$p = 0.0045$
	Mix 3 mo = mix 12 mo	Same as 1:10	Same as 1:10
	Both mix > single 12 mo	Same as 1:10	Same as 1:10
Cat	1000 BAU/ml	100 BAU/ml	10 BAU/ml
Overall	$p = 0.0039$	$p < 0.0001$	$p < 0.0001$
	Single fresh > mix fresh, and mix 3 mo & 12 mo, and single 12 mo	Single fresh = mix fresh	Same as 100 BAU/ml
		Both fresh > mix 3 mo, mix 12 mo, & single 12 mo	Same as 100 BAU/ml
Stored extracts	*$p = 0.2147$*	$p = 0.0007$	$p = 0.2394$
	No difference	Single 12 mo > mix 3 & 12 mo	No difference
Short ragweed	10,000 AU/ml	1000 AU/ml	100 AU/ml
Overall	$p = 0.0897$	$p = 0.0527$	$p = 0.2625$
Stored extracts	$p = 0.1503$	$p = 0.6065$	$p = 0.5099$
D. farinae	1000 AU/ml	100 AU/ml	10 AU/ml
Overall	$p = 0.0022$	$p = 0.6009$	$p = 0.7930$
	Single fresh = mix fresh & 3 mo	No detectable activity in fresh preparations	No detectable activity in fresh preparations
	Single fresh > single 12 mo & mix 12 mo		
Stored extracts	$p = 0.0046$	No detectable activity in fresh preparations	No detectable activity in fresh preparations
	Mix 3 mo > mix 12 mo & single 12 mo		

Stock extracts were diluted 1:10, 1:100, and 1:1000 in phenol-saline-HSA (single) or a mixture of other extracts (mixture). Extract potency was compared with fresh dilutions by inhibition ELISA for single extracts after 12 months and extracts diluted in mixtures after 3 and 12 months. Overall is the statistical significance of differences among all five (two fresh, one 3 months, and two 12 months). Stored compares only single at 12 months and the extract diluted in the mixture after 3 and 12 months.
Mix, Mixture.

Statistical analysis

The overall measure of significance for each allergen extract at each dilution was determined by a one-way analysis of variance. The differences among means were then compared by using Tukey-Kramer HSD (honestly significant difference) with an alpha of 0.05. This multiple comparison procedure was used to provide suitable protection against inflation of the experimentwise error rate caused by the pairwise comparisons of means.

RESULTS

Effects of dilution

Four extracts were studied for the effect of storage at dilutions of 1:10, 1:100, and 1:1000 of the stock concentration provided by the allergen extract company. The four extracts (Bermuda grass, cat, *D. farinae,* and short ragweed) differed in the degree to which their potency was preserved under these circumstances (Table I).

There was a very significant loss of potency in all three dilutions of Bermuda grass extract ($p < 0.0001$ for each). For each dilution (10,000 AU/ml, 1000 AU/ml, and 100 AU/ml), the freshly prepared single and mixed allergen extracts were significantly more potent than were the mixes after 3 months and both the single and mixes after 12 months. Also, for all three dilutions, the single allergen extract after 12 months was significantly

less potent than the mixtures of allergen extracts after 3 months and 12 months (Fig. 1).

The results with the cat extract were similar to those with Bermuda grass. The overall loss of potency at the 1000 BAU/ml dilution was $p = 0.0039$, although for the 100 BAU/ml and 10 BAU/ml dilutions, the overall level of significance was $p < 0.0001$. Again, the pattern was fairly consistent with freshly prepared single cat extract at 1000 BAU/ml and both single cat extract and cat extract in the allergen mixture at 100 BAU/ml and 10 BAU/ml, showing significantly more potency than any extracts that had been stored at 4° C for either 3 or 12 months. For cat extract, there was not a consistent difference between the single extract and the allergen extract mixture in regard to residual potency after 3 and 12 months.

Ragweed did not significantly lose potency at any dilution during storage. When *D. farinae* extract (10,000 AU/ml) was tested at the same dilutions as the other three allergen extracts, there was so little allergenic activity present that a significant decline could only be demonstrated for the most concentrated specimen (1000 AU/ml). For this concentration, there was evidence of a significant loss of potency in the 12-month specimens compared with both the fresh and the 3-month preparations.

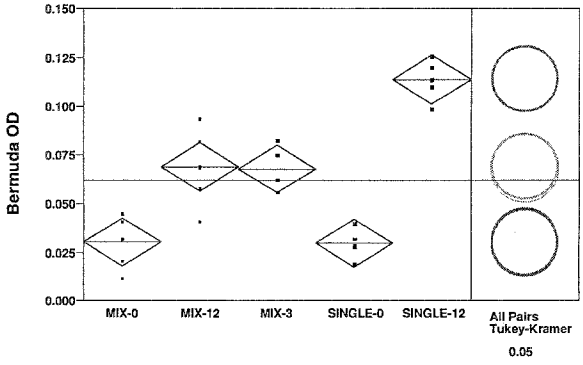

FIG. 1. Effect of conditions of storage on the residual potency of Bermuda grass, 10,000 AU/ml. Bermuda grass was diluted in phenol-saline-HSA *(Single)* or in a mixture of other allergen extracts *(Mix)* and stored at 4° C for 3 and 12 months. Stored and fresh dilutions were compared by inhibition ELISA. Indicated on the left are the means and pooled standard deviations for each condition of storage. On the right are the results of the Tukey-Kramer analysis. Pairs of means are not significantly different at the 0.05 level, if the corresponding comparison circles are nested or if they overlap with an outside angle of greater than 90 degrees. *OD,* Optical density.

Effect of mixing

The results of combining allergen extracts with other extracts that have been reported to contain proteases are summarized in Table II. In these studies the comparison is between the potency of allergen extracts that have been stored alone at a 1:10 dilution of the stock concentration and the same concentration of the allergen extract stored in combination with one or more extracts typically containing proteases.

The deleterious effects of mixing with *Alternaria* and cockroach extracts on timothy grass extract (Fig. 2) and of mixing with *Alternaria* extract on Bermuda grass extract and the lack of effect of any of the extracts on the potency of short ragweed extract (Fig. 3) appeared statistically quite convincing and were not repeated.

The effect of the extracts potentially containing proteases on the other allergen extracts was less clear-cut, and hence these studies were repeated but, because of lack of availability, not necessarily with the same lot of fungal or insect extract. Although there was some variability in the replicate studies, perhaps because of lot-to-lot variation in protease content of these extracts, the overall patterns appeared similar (see Table II). Most extracts tested were susceptible to deterioration from the addition of one or more extracts. Only short ragweed appeared to be convincingly resistant. *D. farinae* extract appeared to be nearly as resistant; however, because this extract was only available in 50% glycerin and because it was tested at only a 1:2 dilution of the stock, compared with the 1:10 dilution for the remaining extracts, it may be that the resulting 25% glycerin concentration protected the mite allergen extract from the effects of the other extracts.

It is evident from Table II that the allergenic proteins of different allergen extracts differ in their susceptibility to mixing with different fungal and insect extracts. *Alternaria* extract had the broadest activity, reducing the potency of five of the target allergen extracts; cockroach extract affected three allergen extracts, and *Cladosporium* extract affected one. *Penicillium* and *D. farinae/D. pteronyssinus* extracts did not affect the potency of any of the target extracts. Again, the failure to observe an effect from the house dust mite mix may have been related to the final concentration of 25% glycerin.

The effect of fungal and insect extracts on the

TABLE II. Effects of mixing on allergen extracts

Target extract	Alone	Combined with						
		ALT	CLADO	PCN	CR	Mix	Mite mix	Overall *p* value
Timothy grass	0.19	0.65	0.21	0.23	0.68	0.54	0.08	<0.0001
	(0.01)	(0.04)	(0.02)	(0.02)	(0.08)	(0.11)	(0.02)	
p vs alone	—	<0.05	NS	NS	<0.05	<0.05	NS	
Bermuda	0.09	0.57	0.19	0.12	0.15	0.45	0.09	<0.0001
	(0.00)	(0.04)	(0.05)	(0.01)	(0.02)	(0.03)	(0.01)	
p vs alone	—	<0.05	NS	NS	NS	NS	NS	
Ragweed	0.20	0.23	0.18	0.18	0.17	0.13	0.25	=0.6371
	(0.06)	(0.06)	(0.05)	(0.03)	(0.02)	(0.02)	(0.06)	
p vs alone	—	NS	NS	NS	NS	NS	NS	
Russian thistle								
First run	0.36	0.46	0.34	0.35	0.54	0.50	0.34	<0.0001
	(0.02)	(0.02)	(0.01)	(0.02)	(0.05)	(0.03)	(0.03)	
p vs alone	—	NS	NS	NS	<0.05	<0.05	NS	
Second run	0.07	0.08	0.07	0.07	0.07	0.08	0.07	=0.0065
	(0.00)	(0.00)	(0.00)	(0.00)	(0.00)	(0.00)	(0.00)	
p vs alone	—	NS	NS	NS	NS	<0.05	NS	
White oak								
First run	0.31	0.36	0.30	0.31	0.33	0.33	0.32	=0.0046
	(0.01)	(0.01)	(0.01)	(0.01)	(0.00)	(0.01)	(0.01)	
p vs alone	—	<0.05	NS	NS	NS	NS	NS	
Second run	0.12	0.14	0.13	0.15	0.15	0.20	0.13	
	(0.01)	(0.00)	(0.00)	(0.01)	(0.00)	(0.04)	(0.00)	
p vs alone	—	NS	NS	NS	NS	<0.05	NS	
Box elder								
First run	0.12	0.17	ND	ND	0.17	0.17	0.11	<0.0001
	(0.00)	(0.01)	—	—	(0.01)	(0.01	(0.00)	
p vs alone	—	<0.05	—	—	<0.05	<0.05	NS	
Second run	0.012	0.13	0.11	0.13	0.15	0.12	0.10	=0.0200
	(0.00)	(0.01)	(0.01)	(0.01)	(0.02)	(0.00)	(0.00)	
p vs alone	—	NS	NS	NS	NS	NS	NS	
D. farinae								
First run	0.11	0.12	0.05	0.23	0.13	0.07	ND	=0.0053
	(0.03)	(0.04)	(0.02)	(0.05)	(0.01)	(0.01)	—	
p vs alone	—	NS	NS	NS	NS	NS	—	
Second run	0.14	0.15	ND	0.17	ND	ND	ND	=0.4853
	(0.01)	(0.02)	—	(0.01)	—	—	—	
p vs alone	—	NS	—	NS	—	—	—	
Cat								
First run	−0.01	−0.01	0.05	−0.01	−0.01	−0.01	−0.01	=0.0018
	(0.00)	(0.01)	(0.02)	(0.00)	(0.00)	(0.00)	(0.00)	
p vs alone	—	NS	<0.05	NS	NS	NS	NS	
Second run	−0.04	0.00	−0.12	ND	ND	ND	ND	=0.0004
	(0.00)	(0.04)	(0.02)	—	—	—	—	
p vs alone	—	<0.05	NS	—	—	—	—	

Values are expressed as means ± SEM (optical density after 3 months of storage at 4° C).

Alt, Alternaria; Clado, Cladosporium; PCN, Penicillium; CR, cockroach; *Mix,* mixture of *Alternaria, Cladosporium, Penicillium,* and cockroach; *Mite Mix, D. pteronyssinus/D. farinae; NS,* not significant; *ND,* not done.

potency of each other was also examined. There was no evidence of reduction in potency of cockroach, *Alternaria, Cladosporium,* or *Penicillium* extracts as a result of admixture with the remaining extracts (results not shown). However, the cockroach extract did show a significant loss of potency at 3 months, compared with freshly diluted extract ($p < 0.0001$), whether stored alone or in mixture (results not shown).

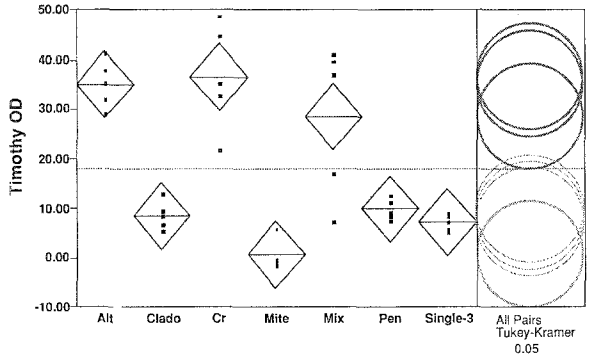

FIG. 2. Effect of protease-containing extracts on timothy grass, 10,000 AU/ml. Indicated on the left are the means and pooled standard deviation for each condition of storage. Indicated on the right are the results of the Tukey-Kramer analysis. Pairs of means are not significantly different at the 0.05 level, if the corresponding comparison circles are nested or if they overlap with an outside angle of greater than 90 degrees. *OD,* Optical density; *Alt, Alternaria tenuis; Clado, Cladosporium herbarum; CR,* American cockroach; *Mite, D. farinae; Pen, Penicillium notatum* (all at 1:10 dilution of stock except *D. farinae,* which was 1:2 dilution); *Single,* dilution in phenol-saline-HSA alone; *Mix,* mixture of other allergen extracts.

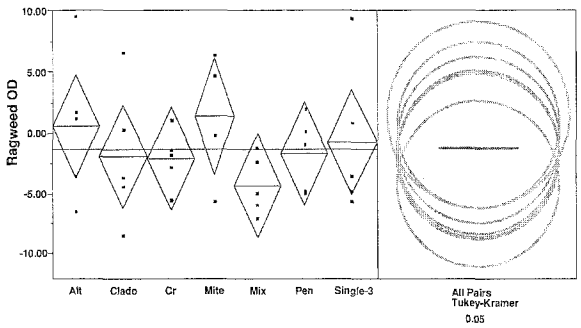

FIG. 3. Effect of protease-containing extracts on short ragweed 10,000 AU/ml. Indicated on the left are the means and pooled standard deviation for each condition of storage. Indicated on the right are the results of the Tukey-Kramer analysis. Pairs of means are not significantly different at the 0.05 level, if the corresponding comparison circles are nested or if they overlap with an outside angle of greater than 90 degrees. *OD,* Optical density; *Alt, Alternaria tenuis; Clado, Cladosporium herbarum; CR,* American cockroach; *Mite, D. farinae; Pen, Penicillium notatum* (all at 1:10 dilution of stock except *D. farinae,* which was 1:2 dilution); *Single,* dilution in phenol-saline-HSA alone; *Mix,* mixture of other allergen extracts.

DISCUSSION

Although it is important to be able to obtain allergen extracts of high and consistent potency, it is also important to maintain their potency after they are diluted for intradermal skin testing or in an allergen immunotherapy extract. In the formulation of allergen treatment sets for immunotherapy, it is common to combine several different extracts.

The deterioration of allergen extracts with time has long been recognized. Loss of potency is greater in dilute extracts and with storage at higher temperatures.[4] Loss of potency in dilute extracts is largely due to adsorption of protein onto the surface of the vial. This loss can be decreased by the addition of extra protein to the extract, either by the inclusion of other allergen extracts or by the addition of HSA.[4, 5]

Recently, it has been recognized that another cause for loss of potency is the presence in some allergen extracts of proteases that can break down allergenic proteins. Proteases are present in extracts of fungi[10] and also extracts of cockroaches and house dust mites.[9-11] Proteases are conspicuously absent from pollen and dander extracts.[10] Grass extracts appear to be particularly susceptible to the deleterious effect of proteases.[7-10]

Loss of potency with storage of extracts at unrefrigerated temperatures may occur through activity of proteolytic enzymes. In this situation addition of 50% glycerin, a recognized enzyme inhibitor,[10] has been found to be particularly effective.[4, 5]

To assess the effect of dilution, we first examined the loss of extract potency of Bermuda grass, cat, *D. farinae,* and short ragweed at three 10-fold dilutions of the stock extract. We found the loss of potency under these conditions was greater for grass and cat extracts than for ragweed extract. We also confirmed that the loss was less when the allergen extract was combined with other extracts rather than when diluted in phenol-saline-HSA alone.[4] This effect of other extracts presumably reflected the protective effect of the additional protein contained in the added extracts. Conspicuous was the lack of potency of the *D. farinae* extract. Only the 1000 AU/ml fresh preparation had detectable allergenic activity in the ELISA-inhibition system. This indicates that *D. farinae* is biologically less potent than its current AU designation would suggest.

For our studies of the affect of mixing allergen extracts, we did not directly measure protease content. However, we used extracts of fungi and insects, which have been demonstrated by others to contain proteases.[9-11] These studies of mixing allergen extracts with potential protease-containing

extracts revealed a broad and varying pattern of susceptibility to loss of potency. Extracts of *Alternaria tenuis* most frequently had an effect, followed by American cockroach and *Cladosporium herbarum* extracts. Only short ragweed extract appeared to be completely resistant to the effects of mixing with this group of fungal and insect extracts. *D. farinae* extract demonstrated inconsistent susceptibility; however, *D. farinae* was in solution in 25% glycerin, which may have resulted in an inhibitory effect on protease activity.[4, 5, 10]

Repeated assays over time necessitated use of more than one lot of some of the fungal extracts. These different lots yielded somewhat different results. This is consistent with reports of lot-to-lot variability in protease activity of the same extract.[9] This study used only the inhibition ELISA to determine residual extract potency. Other studies, however, have used a similar in vitro method and showed that the results correlated quite well with skin test and histamine release assays.[9, 12]

In conclusion, this study confirmed that both dilution and mixture with other allergen extracts presumed to contain proteases can lead to a significant loss in allergen extract potency. It has demonstrated that different allergen extracts vary in the degree to which they are adversely affected by these conditions of storage. It would appear prudent, on the basis of these data to: (1) reconstitute dilutions of stock extracts used for intradermal testing and dilutions of maintenance allergen treatment sets at intervals of at least 3 months and (2) avoid mixing *Alternaria, Cladosporium,* cockroach, and perhaps other fungal extracts with pollen extracts, unless the extract contains glycerin. The concentration of glycerin required has not been examined in this study; however, the data suggest that 25% glycerin may have had some protective effect.

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