

Effect of dilution, temperature, and preservatives on the long-term stability of standardized inhalant allergen extracts

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Background: Although documented stability of allergens used for diagnosis is important, research in this area has been limited. Most studies on extract stability have been of limited duration and discrepancies have been reported between stability test results of in vivo and in vitro methods.

Objective: In this study we determined the stability of allergenic extracts, comparing the intracutaneous test and enzymallergosorbent test inhibition method and determining the effect of temperature, dilution, and preservatives.

Methods: Three formulations of timothy pollen, birch pollen, house dust mite (*D. pteronyssinus*) and cat dander extracts, as used for bronchoprovocation, skin prick testing and intracutaneous testing, were stored for 24 months at 6 °C. The influence of temperature on various formulations was determined using the enzymallergosorbent test inhibition technique during storage for up to 36 months.

Results: Most formulations were found to be stable for 24 (intracutaneous test) or 36 (enzymallergosorbent test inhibition) months at 6 °C. At 25 °C, most formulations showed a decrease in relative potency, which remained above the limit of 0.3 times the in-house-reference for the bronchoprovocation formulation of timothy pollen, birch pollen, and house dust mite and for the skin prick test formulation of cat dander.

Conclusions: Cat dander was remarkably stable at 6 and 25 °C in glycerine and birch pollen was very susceptible to phenol. This destructive effect of phenol could be prevented by adding human serum albumin. the discrepancy between in vivo and in vitro tests reported by others was confirmed for house dust mite and timothy pollen.

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INTRODUCTION

Skin tests are widely used for the detection of IgE-mediated allergy. It is important for scientific and routine diagnostic skin testing to use standardized allergen preparations, ie, with known composition, potency, and stability.¹ Research in this area, however, has been limited. Most studies of extract stability have been of limited duration.

The stability of allergen preparations can be estimated with different in vivo and in vitro methods. Two studies have compared the enzymallergosorbent test inhibition with skin test methods.^{2,3} Both authors found that the relative potency of pollen extracts was reduced in the enzymallergosorbent test inhibition method, while the skin test reactivity was unchanged. Several authors, however, have used enzymallergosorbent test inhibition for the evaluation of the stability of allergen extracts.

Pollen extracts have been reported to be stable for 3 to 15 months at 4 °C when dissolved in phosphate buffered saline (PBS) with 50% glycerol,^{4–7} whereas the aqueous form without glycerol was less stable.^{6,7}

For house dust mite extracts, conflicting results have been reported. Bousquet et al⁴ reported a stability of 1 month at 4 °C in 50% glycerol, whereas Naerdal et al⁸ reported a *D. farinae* extract to be stable for 8 months in saline containing 0.03% human serum albumin. No stability studies have been published concerning cat dander extracts.

In this study we compared the stability results of the intracutaneous test with the enzymallergosorbent test inhibition method using different concentrations of standardized timothy pollen, birch pollen, house dust mite, and cat dander extracts during 24 months of storage at 6 °C. The influence of temperature on various formulations of the mentioned extracts was determined using the enzymallergosorbent test inhibition technique during storage for a maximum of 36 months.

MATERIALS AND METHODS

Allergen Preparations

Two different concentrations of allergen extracts were prepared in PBS using different preservatives. The following formulations were tested: a high concentration containing 0.03% human serum albumin and 0.5% phenol, commonly used in bronchoprovocation (bronchoprovocation test formulation), a low concentration extract with the same preservatives routinely used in intracutaneous testing (intracutaneous test formulation), and a high concentration extract containing 50% glycerol and 0.5% phenol commonly used in skin prick testing (skin prick test formulation). Four extracts (ALK Benelux BV, Groningen, the Netherlands) were evaluated in this study: timothy pollen (*Phleum pratensis*) at

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10 and 1,000 BU/mL, birch pollen (*Betula verrucosa*) at 700 BU/mL or 70,000 BU/mL, house dust mite (*D. pteronyssinus*) (1,000 BU corresponds to 0.54 μ g *Derp*I) at 30 BU/mL or 10,000 BU/mL, and cat dander (1,000 BU corresponds to 0.31 μ g *Fel d* I) at 300 and 30,000 BU/mL. In a separate analysis of birch pollen skin prick test formulation, the effect of removal of phenol and addition or replacement by human serum albumin were also assessed.

Study Design

The stability of the bronchoprovocation test formulation and intracutaneous test formulation was determined with intracutaneous test and enzymallergosorbent test inhibition after storage for up to 24 (intracutaneous test) and 36 (enzymallergosorbent test inhibition) months at 6 °C and after up to 6 months (enzymallergosorbent test inhibition) at 25 °C. The stability of the intracutaneous test formulation of timothy pollen and house dust mite could not be determined using enzymallergosorbent test inhibition because the potency of these dilute extracts was below the detection limit of this method.

The potency (enzymallergosorbent test inhibition) of the skin prick test formulation was determined after storage for up to 36 months at 6 °C and after up to 6 months at 25 °C. In an additional study, the potency (enzymallergosorbent test inhibition) of the skin prick test formulation of birch pollen with or without phenol and/or human serum albumin was determined after storage for up to 3 months at 4 °C and at 25 °C.

Patients and Study Design for Intracutaneous Test

Three to five patients per allergen were recruited from the Allergy Outpatient Clinic (University Hospital of Groningen) with a RAST class of 3 or more and a proven sensitivity to heat labile allergens of timothy pollen, birch pollen, house dust mite, or cat dander. A quantitative intracutaneous test was used to determine the stability

of allergen extracts according to the procedure described by Turkeltaub et al⁹ All patients gave their informed consent to take part in this study.

Both concentrations of each allergen were tested after 0.5, 1, 2, 3, 6, 12, 18, and 24 months of storage. The results of the test solutions were compared with the results of corresponding freshly prepared solutions.

As described by Turkeltaub⁹ eight 3-fold dilutions of the low concentration (intracutaneous test formulation) were used for the quantitative intracutaneous test procedure. The high concentration (bronchoprovocation test formulation) was first diluted 100-fold. The highest concentration of the reference, giving an erythema on the arm between 60 to 80 mm, and the three following lower concentrations were skin tested in duplicate on the back. The lowest concentration of the reference extract giving an erythema of 45 to 95 mm was determined. The duplicate sample extracts were skin tested in the same concentration as the reference extract on the back. The relative potency was calculated by dividing the erythema size of the sample extract by the erythema size of the reference extract.

Intracutaneous Test

Intracutaneous testing was performed according to Dreborg's guidelines for skin testing.¹ Briefly, 0.03 mL allergen extract was injected into the skin of both forearms at volar sites (pretest) or into the skin of the back (stability test) at least 5 cm apart, using a 0.5 mL syringe (Monoject no. 8881-500501, Sherwood medical, St. Louis MO, USA). The results were recorded after 15 minutes by circumscribing the erythema in ink and transferring the resulting imprint to adhesive tape. The solutions were used at ambient temperature within seven days of reconstitution. Mean erythema sizes and their standard deviations were calculated using an SPSS/PC+ computer program.¹⁰

The positive control was histamine phosphate (base) 0.01 mg/mL (intracutaneous test). The diluent was used as a

negative control. Patients did not use antihistamines or oral corticosteroids during the 48 hours prior to skin testing, in accordance with the skin test standardization report.¹¹ No patient had used astemizole for at least 6 weeks.

The sum of the erythema diameters was calculated by adding the longest diameter to the orthogonal diameter measured at the midpoint of the longest diameter.

Enzymallergosorbent Test Inhibition

Enzymallergosorbent test inhibition was used for the measurement of the relative potency of the allergen preparations by comparison to an in-house reference, according to the procedure of Anderson and Baer.¹² The allergen preparations were measured directly without prior dilution. This method was validated for every allergen, performing four triplicate assays on two different days using duplicate dilution series of 0.5 mg/mL (= 33,320 BU/mL), 0.2 mg/mL (= 17,675 BU/mL), 1 mg/mL (= 114,689 BU/mL), and 1 mg/mL (= 4,206 BU/mL) of timothy pollen, birch pollen, house dust mite, and cat dander respectively.

The best-fit linear regression lines were calculated between 10% and 90% inhibition. Using Student's *t* test (*P* < .01) the regression lines of the reference and samples were checked for parallelism. The *C*₅₀ values (μ L inhibitor at 50% inhibition) were calculated using the equation of the regression line. This value was corrected for the concentration of the sample.

Finally, the mean relative potency and the coefficient of variation was calculated. The results were validated according to the guidelines described by Anderson and Baer.¹²

Analysis of allergen activity was also related to the note for guidance on allergen products (111/9271/90-EN) of the European Economic Community. The requirement for stability states that no less than 30% of the stated allergenic activity should be maintained at the end of shelf-life.

RESULTS

Enzymallergosorbent Test Inhibition

In Figure 1 the *in vitro* results for the various formulations stored at 6 or 25 °C are depicted for timothy pollen (1A), birch pollen (1B), house dust mite (1C), and cat dander (1D). All formulations, except for birch skin prick test formulation, maintained at least 30% of initial allergenic activity for 36 months at 6 °C. Birch skin prick test formulation was stable for

less than 6 months at 6 °C and less than 2 weeks at 25 °C. Another batch of birch skin prick test formulation was also studied using different combinations of preservatives (Table 1). A "conventional" skin prick test formulation containing 50% glycerol and 0.5% phenol was stable for less than 1 month at both temperatures. All other skin prick test formulation were stable for at least 3 months at 6 and 25 °C.

At 25 °C all formulations tested showed a decrease in relative potency, which stayed above the limit of 0.3 for the bronchoprovocation test formulation of timothy pollen, birch pollen, and house dust mite and for the skin prick test formulation of cat dander. The mean coefficient of variation (CV) of the enzymallergosorbent test inhibition method used was 11.6%, 14.1%, 8.8%, and 18.8% for house dust mite, timothy

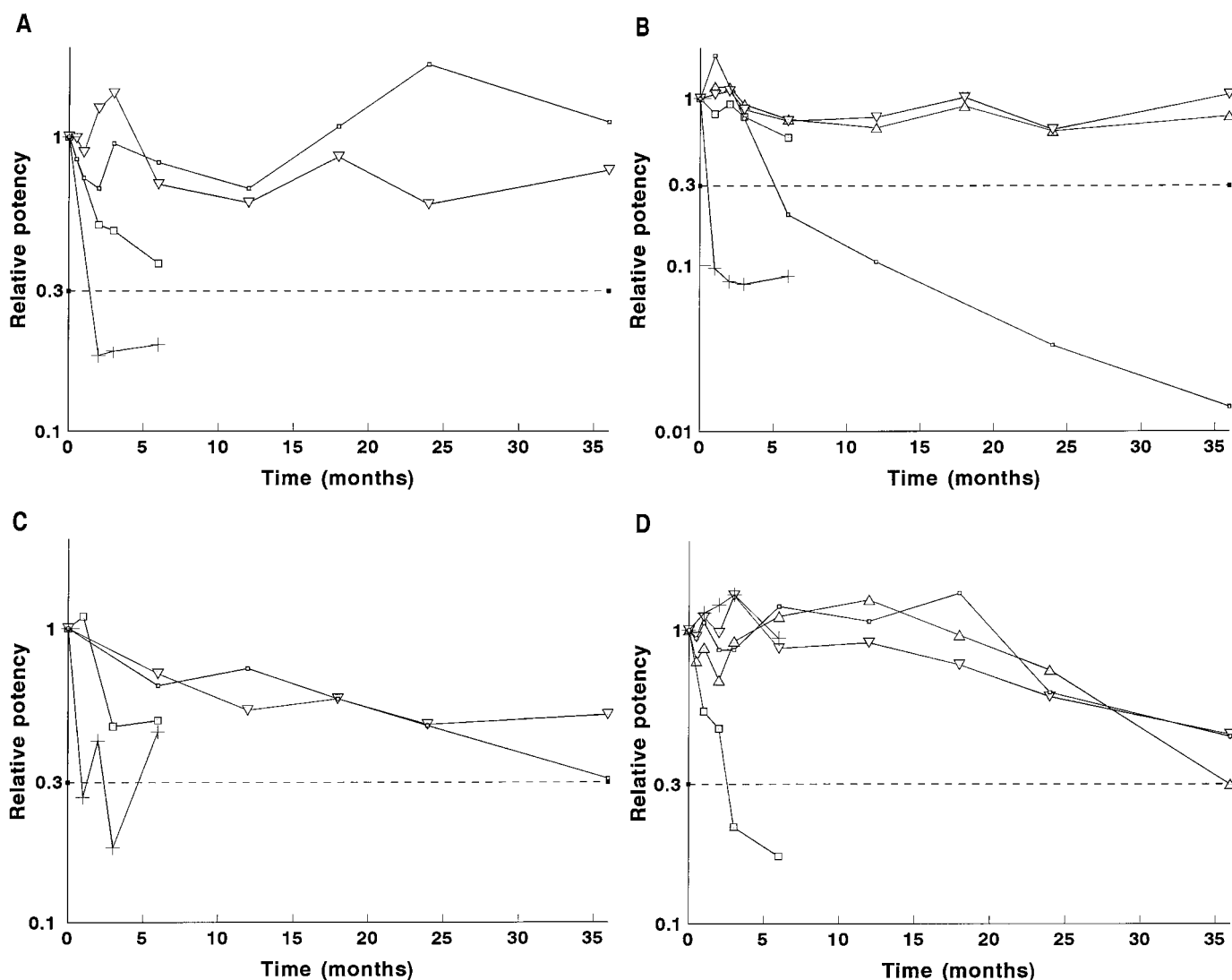


Figure 1. Results of the stability study for timothy pollen (A), birch pollen (B), house dust mite (C), and cat dander (D) as measured by enzymallergosorbent test inhibition for skin prick test formulation at 6 °C (□), skin prick test formulation at 25 °C (+), bronchoprovocation test formulation at 6 °C (▽), bronchoprovocation test formulation at 25 °C (□), and intracutaneous test formulation at 6 °C (△). The skin prick test and bronchoprovocation test formulation had a concentration of 1,000, 70,000, 10,000, and 30,000 BU/mL for timothy pollen, birch pollen, house dust mite, and cat dander, respectively, and the intracutaneous test formulation had a concentration of 700 and 300 BU/mL for birch pollen and cat dander, respectively.

pollen, birch pollen, and cat dander, respectively.

Intracutaneous Test

In Figure 2 the *in vivo* results show no decrease in relative potency during storage of 24 months at 6 °C for the bronchoprovocation test formulation and intracutaneous test formulation. The mean erythema for histamine was 28.5 mm (CV = 5.4% between patients) and the CV of the intracutaneous test method was below 12.0% for all allergens.

Table 1. Stability Results of 4 Different Birch Pollen Skin Prick Test Formulations

Formulation*	Relative Potency, 6 °C		Relative potency, 25 °C	
	1 month	3 months	1 month	3 months
1	0.0	—	0.0	—
2	1.11	1.23	0.93	1.23
3	1.48	1.74	1.49	1.31
4	1.63	1.35	1.77	1.37

* (1) Phosphate buffered saline (PBS) with 50% glycerol and 0.5% phenol, (2) PBS with 50% glycerol, (3) PBS with 50% glycerol and 0.03% human serum albumin, (4) PBS with 50% glycerol with 0.03% human serum albumin and 0.5% phenol.

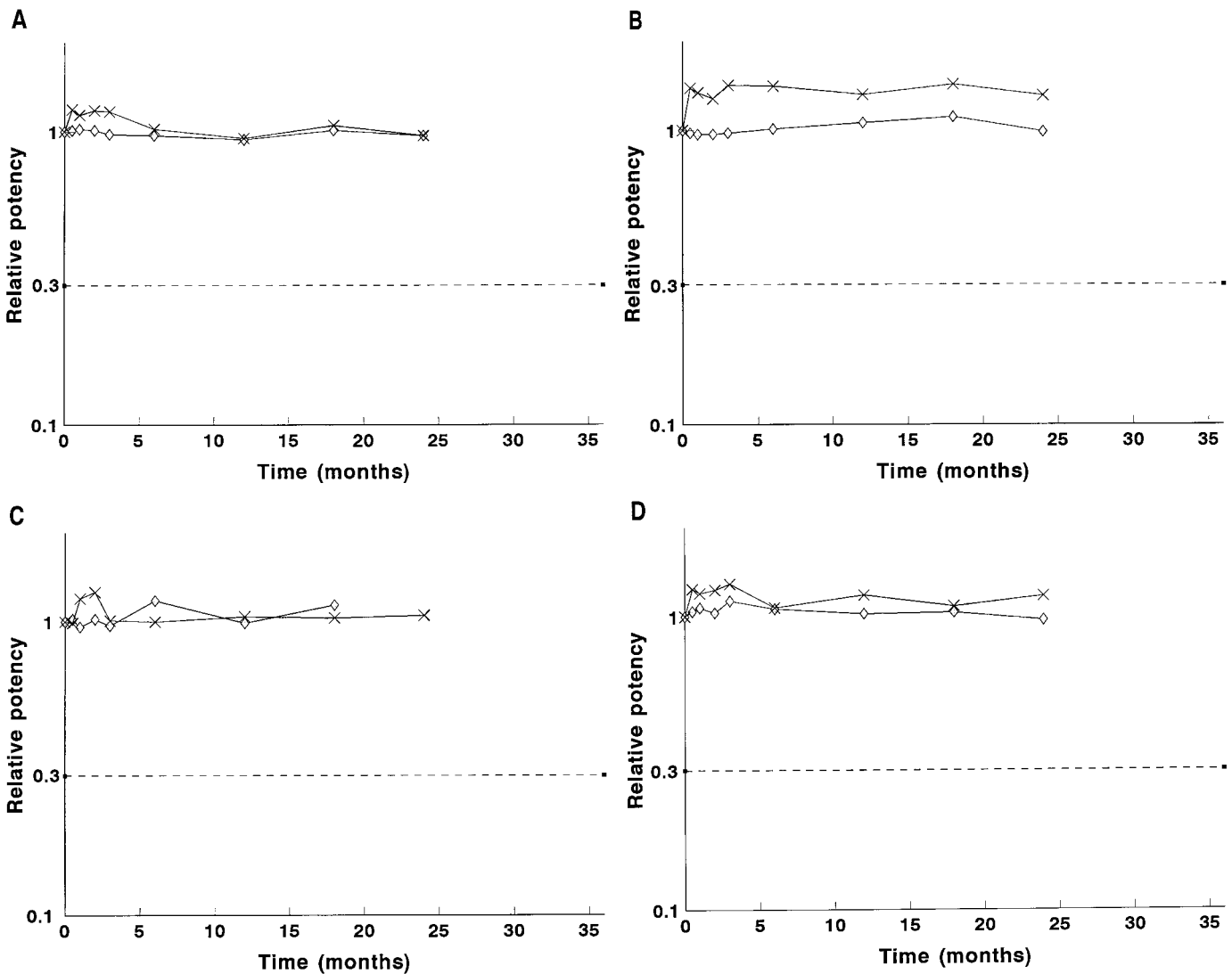


Figure 2. Results of the stability study for timothy pollen (A), birch pollen (B), house dust mite (C), and cat dander (D) as measured by the intracutaneous test method for bronchoprovocation test formulation at 6 °C (x), and intracutaneous test formulation at 6 °C (◊). The bronchoprovocation test formulation had a concentration of 1,000, 70,000, 10,000, and 30,000 BU/mL and the intracutaneous test formulation had a concentration of 10, 700, 30, and 300 BU/mL for timothy pollen, birch pollen, house dust mite, and cat dander, respectively.

DISCUSSION

All preparations tested, except for birch pollen skin prick test formulation, were stable for 36 months at 6 °C, which is much longer than reported by others.⁴⁻⁸ As there was no difference in stability between the bronchoprovocation test formulation and intracutaneous test formulation, the 100-fold dilution of these preparations did not seem to influence stability.

Birch pollen was found to be stable for only 5 months in the skin prick test formulation, whereas the aqueous formulations (bronchoprovocation test formulation and intracutaneous test formulation) were stable for 36 months. The reason for this poor stability is not known, but seems to be related to the 0.5% phenol in combination with 50% glycerol. For this reason we also dissolved the freeze dried material of birch pollen in PBS with various combinations of preservatives. The results indicated that all other skin prick test formulation were stable for at least 3 months. Removing the phenol resulted in increased stability. When human serum albumin was added to the skin prick test formulation, the destructive effect of phenol was diminished. Human serum albumin thus seems to have a protective effect in this preparation. Others have reported pollen preparations to be stable for 6 to 18 months in 50% glycerol^{4,6,7,13,14} but the destructive effect of phenol on birch pollen has not been reported previously.

At 25 °C, decline of the relative potency in the skin prick test formulation preparation is seen not only for the birch preparation but also for the timothy and house dust mite preparations. The activity of the skin prick test formulation of timothy and house dust mite preparations was completely lost within 1 to 2 months, whereas the aqueous formulation (bronchoprovocation test formulation) was more stable at 25 °C. The skin prick test formulation of cat dander was very stable even at 25 °C, when compared with the other allergens, whereas the bronchoprovocation test formulation of cat

dander lost activity after for 2 to 3 months at 25 °C. The bronchoprovocation test formulation of cat dander, however, was stable for 36 months at 6 °C. Possibly, the cat dander preparation is sensitive to phenol at elevated temperatures, an effect which is not seen in combination with glycerol.

All bronchoprovocation test formulation preparations had a relative potency higher than $0.5 \times$ standard after 36 months. This seems adequate for routine use but for research purposes, where allergen quantities must be as uniform as possible, use of a lyophilized extract to be reconstituted on the day of challenge will generally be preferable.

The bronchoprovocation test formulation and intracutaneous test formulation were more stable than reported by others. Center et al⁶ reported that the aqueous form of antigen E (major allergen of ragweed pollen) lost 50% of the initial activity after 5 months' storage at 4 °C and that phenol in aqueous extracts is destructive to antigen E.

Nelson et al⁷ concluded that 50% glycerin was superior to all other additives in maintaining stability of pollen extracts after storage of 12 months at 4 or 17 °C. Human serum albumin increased the stability of allergen extracts. The deleterious effect of phenol was minimal at high concentrations of allergens, but more marked in diluted solutions.

The results of the cat dander preparations could not be compared with the results of others, since no stability data of cat dander extracts have been reported.

The discrepancy between the intracutaneous test and enzymallergosorbent test inhibition results reported by others^{2,3} was partly confirmed in our study. Franklin et al² reported that the skin reactivity to a ragweed extract was unchanged after storage during 18 months at 4 °C, whereas the antigen E content decreased to 40% of the initial value and the RAST inhibitory capacity decreased minimally. Hoek³ reported that a *Lolium perenne* extract showed no change in the skin test reactivity after 197 days of storage at 4

°C, whereas the relative potency in the RAST inhibition was reduced to 0.1 after 90 days. Our results show that in both tests birch pollen and cat dander bronchoprovocation test formulation and intracutaneous test formulation were stable for 24 months. The bronchoprovocation test formulation of house dust mite and timothy pollen was also stable for 24 months in the skin test method, whereas the relative potency decreased to about 0.5 in the enzymallergosorbent test inhibition method. The stability of the intracutaneous test formulation of house dust mite and timothy pollen was only determined with the intracutaneous test method, because the potency of these extracts was below the detection limit of the enzymallergosorbent test inhibition method.

The reason for the discrepancy between these results could be that the enzymallergosorbent test inhibition procedure is more sensitive in detecting a decrease in potency of heat labile allergens, which does not correspond with a decrease of the biologic potency. The patients used for the intracutaneous test method, however, were selected on the basis of sensitization to heat labile allergens. Another reason could be that the serum pool used for the enzymallergosorbent test inhibition was obtained from other patients than used for the intracutaneous test. It is possible that the serum pool used in the enzymallergosorbent test inhibition method contained IgE antibodies to allergens for which the patients used for the intracutaneous test method were not sensitive.

The reproducibility of the enzymallergosorbent test inhibition assay was good and comparable to or better than the results of others.¹⁵⁻¹⁷

We conclude that the enzymallergosorbent test inhibition method does not overestimate the biologic activity of the tested allergen extracts and therefore this method probably can be used for stability studies of allergen extracts.

In summary, this study adds the following information to this area of research. Most formulations were found

to be stable for 36 months at 6 °C. At 25 °C most formulations showed a significant decrease in relative potency. Cat dander was remarkably stable in glycerine. Birch pollen was very susceptible to phenol. This destructive effect of phenol could be prevented by adding human serum albumin. The discrepancies between in vivo and in vitro tests reported by others was confirmed for house dust mite and timothy.

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