

Allergen Compatibilities in Extract Mixtures

Robert E. Esch, PhD^{*}, Thomas J. Grier, PhD

KEYWORDS

• Allergenic extracts • Stability • Compatibility

INTRODUCTION

A working knowledge of allergen extract stabilities and compatibilities can influence clinical decisions related to developing specific allergen panels for skin testing and optimizing named-patient immunotherapy formulations.^{1,2} Inclusion of stock or customized extract mixtures is not uncommon in United States allergy clinics. In most cases, these mixtures consist of extracts from phylogenetically related species that exhibit near-complete allergenic cross-reactivity because of the presence of homologous allergens. The most common examples are the house dust mites mixture containing equal parts *Dermatophagoides farinae* and *D pteronyssinus*; and the ragweed mixture containing short ragweed (*Ambrosia artemisiifolia*) and giant ragweed (*A trifida*). These and other mixtures of allergenic extract are often used for skin testing and treatment in place of extracts derived from a single species. Broader mixtures covering multiple non-cross-reactive allergens have also been incorporated into treatment regimens, but their prudent use is essential to improving the diversity and effectiveness of allergen-specific immunotherapy.

Allergenic extracts lose their allergenic activity during storage and handling, sometimes in complex ways, making it difficult to identify all of the mechanisms involved in a particular situation. Early studies on allergen stability involved thermal stability, dilution, and adsorption to glass surfaces.^{3–5} Because allergic patients are not sensitized to the same allergens, an extract may appear to retain its potency when evaluated in patients who are reactive to heat-stable allergens, whereas loss of potency may be evident in patients who are reactive to heat-labile allergens.⁶ This differential heat sensitivity of different allergens can be extended to other physical conditions that are known to affect allergen structure and function. Thus, studies have been conducted that evaluated the effect of pH or diluent used to prepare extracts, use of

The authors are employees of Greer Laboratories, Inc, a manufacturer and supplier of allergen products.

Research and Development, Greer Laboratories, Inc, 639 Nuway Circle, Lenoir, NC 28645, USA

* Corresponding author.

E-mail address: esch@greerlabs.com

Immunol Allergy Clin N Am 31 (2011) 227–239

doi:[10.1016/j.iac.2011.02.009](https://doi.org/10.1016/j.iac.2011.02.009)

immunology.theclinics.com

0889-8561/11/\$ – see front matter © 2011 Elsevier Inc. All rights reserved.

preservatives, and dilution, and all have been shown to have an important role in extract stability.⁷⁻¹¹

The kinetics of denaturation of an allergen in purified form is different when compared with the same allergen present in an allergen extract. The thermal decay of purified Amb a 1 occurred as a first order reaction, whereas the same allergen as a component of an extract decayed through a more complex reaction.¹² Many enzymes, especially proteases, were known to be present in allergenic extracts,^{13,14} but their role in extract stability became apparent only after extracts with high protease content were mixed with extracts with allergens that were susceptible to proteolytic degradation.¹⁵

Stability is an essential requirement for diagnostic and therapeutic allergen preparations that are routinely diluted, used in multiple-dose vials, and stored for extended periods. Manufacturers' concentrates should be stable at refrigeration temperatures for perhaps several years, and should be stable for periods at higher temperatures that may be encountered during shipment and routine use. Expiration dating based on potency tests is a requirement for standardized extracts licensed in the United States. The dating periods verify that the labeled potency accurately reflects the allergenic activity of the vial contents under specified conditions. Unfortunately, validated potency tests are available only for standardized extracts, and for most commercial allergen products, which are not standardized, expiration dating is based on the rule that gives 36 months for 50% glycerinated extracts and 18 months for extracts containing less than 50% glycerin.

Once the products leave the manufacturer and enter the clinic, the products are frequently diluted or combined with other extracts, sometimes from another manufacturer. The stability of these mixtures is extremely difficult to predict because the combinations vary widely, the diluent used may vary from clinic to clinic, and storage conditions can also vary. Thus, the compositional heterogeneity of allergen extracts and the added complexity of multiextract mixtures present a formidable challenge to optimization and validation of stability-indicating *in vivo* or *in vitro* assays displaying appropriate sensitivity and specificity levels. To date, owing primarily to the small number of published studies on extract stability and compatibilities, no consensus exists on the best available experimental approaches, methods, and criteria for studies of this type.

POTENCY TESTS FOR ALLERGEN PRODUCTS

Various methods have been proposed for the potency testing of diagnostic and therapeutic allergen preparations. They range from the measurement of overall allergenicity using quantitative skin testing or competition immunoglobulin E (IgE) enzyme-linked immunosorbent assays (ELISAs) to immunoassays for specific allergen measurements.¹⁶⁻¹⁹ Each of these methods has advantages and disadvantages. Measurements of overall allergenicity are important for labeling purposes but may lack the sensitivity to detect the degradation of selected allergens in an extract or mixture of extracts. Skin testing, when conducted intradermally, can have the sensitivity needed to assess the potency of highly diluted extracts but must be performed in several patients to account for their differences in sensitivities to different allergenic components. In multicenter studies, skin testing at each clinic can yield interesting but conflicting data based on these differences in subject reactivities or allergen exposures.

In the United States, standardization of commercial allergen extract potencies through skin testing involves only the initial (primary) reference extract preparation.

Subsequent batches of reference antigen and all production lots from licensed manufacturers undergo *in vitro* potency testing, with labeled values based on immunochemical equivalence to the current reference extract. Selection of appropriate and consistent allergic serum pools for competition IgE ELISAs becomes crucial to the accuracy of both lot-release and stability testing of products analyzed in this manner. Failure to maintain well-characterized reference reagents or a drift in their performance characteristics can have a significant impact on the product's clinical efficacy or safety.

Methods for measuring overall allergen activity have limitations in evaluating more complex, multiextract mixtures. Physical interactions between extract constituents in mixtures can lead to unpredictable or inconsistent interpretation of results. For this reason, immunochemical assays offering exquisite allergenic specificity and moderate to high analytical sensitivity have emerged as the preferred methods for most extract compatibility investigations. Allergen-specific monoclonal and polyclonal antibodies can be produced in large quantities, and various immunoassay formats can be configured without the need for a sophisticated laboratory. The analytical performance of these assays can vary depending on the selection and qualification criteria for component reagents, incubation conditions, and data output and reduction procedures.

Small differences among procedures used in different laboratories can result in significant variability in assay results, particularly for sensitive methods requiring relatively high (>1000-fold) sample dilutions to produce parallel dilution-response curves within the desired dynamic range. Monoclonal antibody-based immunoassays can be highly sensitive and reproducible but, paradoxically, can also be too specific to yield clinically relevant information.

Therefore, care must be taken when selecting the suitable method for stability testing, and more than one method is almost always required to generate useful information. Regardless of the methods selected, they must be validated for their intended use and the test results must be carefully interpreted before applying them in clinical settings.

ALLERGEN EXTRACT COMPATIBILITY STUDIES

Since 1990, data and methodologies from eight studies^{15,20–26} on allergen extract compatibility have been reported. The specific extract combinations and analytical methods examined in these studies are described in chronologic order in **Tables 1** and **2**, respectively. Unpublished results summarized in meeting abstracts or comments in publications displaying no experimental details and data were excluded from this discussion.

Seven of the eight published studies^{15,20,21,23–26} on allergen compatibility were conducted in the United States using source materials, extract concentrates, and diluents produced by multiple manufacturers licensed by the US Food and Drug Administration (FDA). Initial investigations were focused on the IgE-binding potencies of perennial ryegrass extracts based on observations that grass pollen allergens were susceptible to potency reductions during exposures to elevated temperature or after mixing with certain fungal extracts.

Other grass extracts (timothy, meadow fescue) known to be highly cross-reactive with ryegrass were included in subsequent studies^{21,24,25} to confirm allergen susceptibilities within a model homologous group for specific grass products used in many allergy clinics across the United States. The wide range of other extracts evaluated for compatibility in the United States studies also reflects the broad spectrum of

Table 1 Extract combinations included in eight published studies on extract compatibility			
References	Year	Target Extract	Companion Extracts
Esch ¹⁵	1991	Perennial ryegrass	Cocklebur, sagebrush, marsh elder, ragweed, oak, lamb's quarter, elm, or pigweed Cat epithelia, dog epithelia or goat epithelia Moth, caddisfly, dust mite <i>Dermatophagoides farinae</i> , dust mite <i>D pteronyssinus</i> , or American cockroach <i>Alternaria alternata</i> , <i>Aspergillus fumigatus</i> , <i>Penicillium notatum</i> , <i>Fusarium moniliforme</i> , <i>Mucor racemosus</i> , <i>Aureobasidium pullulans</i> , <i>Paecilomyces varioti</i> , or <i>Microsporium canis</i>
Kordash et al ²⁰	1993	Perennial ryegrass	Hickory, <i>D farinae</i> , cockroach mix or <i>Helminthosporium interseminatum</i>
Nelson et al ²¹	1996	Timothy grass	<i>Alternaria alternata</i> , <i>Cladosporium herbarum</i> , <i>Penicillium notatum</i> , American cockroach, dust mite mix, or mold mix
		Bermuda grass	<i>Alternaria alternata</i> , <i>C herbarum</i> , <i>Penicillium notatum</i> , American cockroach, dust mite mix, or mold mix
		Short ragweed	<i>Alternaria alternata</i> , <i>C herbarum</i> , <i>Penicillium notatum</i> , American cockroach, dust mite mix, or mold mix
		White oak	<i>Alternaria alternata</i> , <i>C herbarum</i> , <i>Penicillium notatum</i> , American cockroach, dust mite mix, or mold mix
		Box elder	<i>Alternaria alternata</i> , <i>C herbarum</i> , <i>Penicillium notatum</i> , American cockroach, dust mite mix, or mold mix
		Russian thistle	<i>Alternaria alternata</i> , <i>C herbarum</i> , <i>Penicillium notatum</i> , American cockroach, dust mite mix, or mold mix
		Cat	<i>Alternaria alternata</i> , <i>C herbarum</i> , <i>Penicillium notatum</i> , American cockroach, dust mite mix, or mold mix
		<i>D farinae</i>	<i>Alternaria alternata</i> , <i>C herbarum</i> , <i>Penicillium notatum</i> , American cockroach, dust mite mix, or mold mix
Hoff et al ²²	2002	Timothy grass Birch	<i>Fusarium culmorum</i> <i>F culmorum</i>
Meier et al ²³	2006	Mountain cedar	Fire ant
Grier et al ²⁴	2007	Meadow fescue grass	Dust mite <i>D farinae</i> or dust mite <i>D pteronyssinus</i> <i>Alternaria alternata</i> , <i>Aspergillus fumigatus</i> , <i>Aspergillus niger</i> , <i>Penicillium chrysogenum</i> , <i>Penicillium notatum</i> , <i>Bipolaris sorokiniana</i> , <i>C herbarum</i> , <i>C sphaerospermum</i> , <i>Epicoccum nigrum</i> , <i>F moniliforme</i> , <i>F solani</i> , <i>Mucor plumbeus</i> , or <i>Aureobasidium pullulans</i>

(continued on next page)

Table 1
(continued)

References	Year	Target Extract	Companion Extracts
		Short ragweed	<i>Alternaria alternata</i> , <i>Aspergillus fumigatus</i> , <i>Penicillium notatum</i> , American cockroach, German cockroach
		Cat	<i>Alternaria alternata</i> , <i>Aspergillus fumigatus</i> , <i>Penicillium notatum</i> , American cockroach, German cockroach
		<i>D farinae</i>	<i>Alternaria alternata</i> , <i>Aspergillus fumigatus</i> , <i>Penicillium notatum</i> , American cockroach, German cockroach
Rans et al ²⁵	2009	Timothy grass	Fire ant
		Short ragweed	Fire ant
		Cat	Fire ant
		<i>D pteronyssinus</i>	Fire ant
Grier et al ²⁶	2009	Dog epithelia	<i>Alternaria alternata</i> , <i>Aspergillus fumigatus</i> , <i>Penicillium notatum</i> , American cockroach, German cockroach, fire ant
		Dog hair/dander	<i>Alternaria alternata</i> , <i>Aspergillus fumigatus</i> , <i>Penicillium notatum</i> , American cockroach, German cockroach, fire ant

allergens recognized by many atopic patients and the resulting use of diverse multiextract mixtures for immunotherapy. The single European study²² used freeze-dried target pollen extracts (timothy grass, birch) mixed with a protease-rich *Fusarium* extract prepared by the investigators from commercial source materials cultured from a defined strain.

Of the seven United States reports,^{15,20,21,23–26} four were authored or coauthored by scientists from one extract manufacturer (Greer, including the authors of this article),^{15,24–26} and one study was conducted by investigators from a different extract supplier (Hollister-Stier, formerly Miles Allergy Products).²⁰ Familiarity and years of experience with the in vitro potency tests listed in **Table 2**, combined with access to or synthesis of specialized allergen and antibody reagents used for these methods, have facilitated the execution of extract compatibility studies in these laboratories. Recent investigations have included mixtures prepared with extracts from multiple United States manufacturers at variable glycerin and human serum albumin (HSA) concentrations. Incorporation of systematic sample designs and diverse test methodologies will continue to drive progress toward improved analytical and clinical assessments of more complex extract mixtures.

The pioneering studies of Esch¹⁵ and Kordash²⁰ examined the properties of perennial ryegrass extracts mixed with a wide variety of allergenic products known or suspected to possess hydrolytic enzymes capable of altering protein structures and associated allergen activities. Both studies reported proteolytic enzyme activities of the companion extracts using chromogenic substrate (Azocoll) or casein gel digestion methods. Esch compared extracts prepared with multiple pollen, epithelia, insect, and fungal source materials and found protease concentrations ranging from less than 5 µg trypsin equivalent units per milliliter (eight pollens, three epithelia, one insect, three fungi) to more than 4000 units per milliliter (the fungus *Microsporium canis*). Extracts from one insect (American cockroach) and three other fungi (*Fusarium moniliforme*, *Aspergillus fumigatus*, and *Penicillium notatum*) possessed moderate protease levels between 150 and 250 trypsin equivalent units per milliliter.

Table 2

In vivo and in vitro analytical methods included in eight published studies on extract compatibility

References	Year	Target Extract	Analysis Method
Esch ¹⁵	1991	Perennial ryegrass	IgE ELISA inhibition IgE SDS-PAGE immunoblotting Protease assays
		Companion extracts	
Kordash et al ²⁰	1993	Perennial ryegrass	ID ₅₀ EAL quantitative intradermal skin testing IgE RAST inhibition SDS-PAGE HPLC
		Companion extracts	Protease casein gel clearing assays
Nelson et al ²¹	1996	Timothy grass Bermuda grass Short ragweed White oak Box elder Russian thistle Cat <i>D farinae</i>	IgE ELISA inhibition IgE ELISA inhibition IgE ELISA inhibition IgE ELISA inhibition IgE ELISA inhibition IgE ELISA inhibition IgE ELISA inhibition IgE ELISA inhibition
Hoff et al ²²	2002	Timothy grass Birch <i>Fusarium culmorum</i>	IgE, Phl p 1 mIgG, and Phl p 5 mIgG SDS-PAGE immunoblotting Rat basophil leukemia cell-mediator release assay SDS-PAGE IgE, Bet v 1 mIgG, and Bet v 2 mIgG SDS-PAGE immunoblotting Rat basophil leukemia cell-mediator release assay SDS-PAGE Gelatinase zymography and PepTag protease assay
Meier et al ²³	2006	Mountain cedar	SDS-PAGE
Grier et al ²⁴	2007	Meadow fescue grass Short ragweed Cat <i>D farinae</i>	IgE ELISA inhibition IgE SDS-PAGE immunoblotting Amb a 1 radial immunodiffusion Fel d 1 radial immunodiffusion IgE ELISA inhibition IgE SDS-PAGE immunoblotting
Rans et al ²⁵	2009	Timothy grass Short ragweed Cat <i>D pteronyssinus</i>	IgE ELISA inhibition IgE SDS-PAGE immunoblotting SDS-PAGE Amb a 1 radial immunodiffusion SDS-PAGE Fel d 1 radial immunodiffusion SDS-PAGE IgE ELISA inhibition SDS-PAGE
Grier et al ²⁶	2009	Dog epithelia Dog hair/dander	IgE ELISA inhibition IgE SDS-PAGE immunoblotting Dog albumin rIgG ELISA IgE ELISA inhibition IgE SDS-PAGE immunoblotting Can f 1 mIgG/rIgG ELISA

Abbreviations: HPLC, high-performance liquid chromatography; ID₅₀EAL, Intradermal Dilution for 50 mm sum of Erythema diameters determines bioequivalent ALLergy units; mIgG, mouse immunoglobulin G; RAST, radioallergosorbent test; rIgG, rabbit immunoglobulin G; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

A direct correlation was observed between total protease levels and the reductions of IgE-binding potency of perennial rye extracts in mixtures with these fungal extracts after storage for 1 month at refrigeration temperatures (4°C).¹⁵ Recoveries below 50% of ryegrass control (unmixed) were found in all mixtures containing fungal extracts with protease levels at or greater than 20 units per milliliter. Glycerin stabilized grass allergens mixed with fungal extracts at moderate protease levels but did not produce a substantial improvement in ryegrass potency after mixing with the protease-rich *M canis* extract. Changes in IgE binding to ryegrass allergens that had been mixed with several fungal extracts were also observed on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) immunoblots (Western blots) at levels consistent with the protease activities of the fungal products.

Kordash and colleagues²⁰ studied the effects of fungal (*Helminthosporium interseminatum*), insect (cockroach mix), and dust mite (*D farinae*) extracts on perennial ryegrass allergens using a combination of in vitro and in vivo analyses. Consistent with the results of the Esch study,¹⁵ companion extracts possessing relatively high proteolytic enzyme activities (fungal, insect) produced significant (10- to 40-fold) decreases in ryegrass extract potency determined using IgE radioallergosorbent test (RAST) inhibition assays and intradermal skin test titrations. Degradation of grass allergens with *Helminthosporium* extract was more pronounced at all time points tested with both methods compared with cockroach extract. High levels of glycerin (50% volume to volume [v:v]) improved ryegrass allergen recoveries in these mixtures relative to comparable formulations in HSA-saline diluent. Mixing with dust mite extract or a hickory extract control had no apparent effect on ryegrass extract potencies after storage for up to 90 days at 2°C to 4°C.

Inclusion of skin testing in extract compatibility studies is an attractive approach but presents numerous challenges. In the study by Kordash and colleagues,²⁰ subjects selected for testing with specific ryegrass extract mixtures were required to exhibit negative skin puncture tests for the various companion extracts to isolate reactions to grass allergens only. In relatively simple mixtures, enrolling subjects that meet these criteria is feasible, but in mixtures of increasing complexity or compositions with airborne allergens prevalent in many geographic regions, subjects with discrete sensitivities to only target extracts become more difficult to qualify. Differences in IgE specificities to allergens or distinct epitope regions recognized by these subjects could also impact the magnitude, consistency, or significance of test results. Collaborative investigations among scientists and clinicians proficient at prick and intradermal skin test procedures are needed to incorporate these important in vivo evaluations into future studies of extract stability and compatibility.

In 1996, Nelson and colleagues²¹ examined the IgE-binding reactivities of various low-protease extracts (grass pollen, weed pollen, tree pollen, dust mite, or cat) after mixing with certain high-protease products (American cockroach, *Alternaria alternata*, *Cladosporium herbarum*, or *P notatum*). Two-component extract mixtures and target extract controls were prepared through adding 5 mL of glycerinated dust mite *D farinae* extract or 1 mL of aqueous (or lyophilized, reconstituted) low-protease extract concentrates to 1 mL of high-protease extracts, followed by dilution to a total volume of 10 mL with the same aqueous diluent (HSA-saline) used for reconstitution steps. The resulting solutions contained either 25% glycerin final concentrations (*D farinae*) or no glycerin.

After storage for 3 months at 4°C, samples were analyzed with ELISA inhibition using serum pools prepared from patients exhibiting strong positive skin test reactions to the target allergens. Significant levels of allergen degradation were observed in at least one mixture for all target allergens except *D farinae* and ragweed. *Alternaria* and American

cockroach affected the highest number of allergens, whereas *Penicillium* and dust mite mix (*D. farinae*/*D. pteronyssinus*) had no significant impact on any allergens tested. Timothy grass and box elder allergens were compromised by individual fungal and cockroach extracts, with others degraded by fungi only (Bermuda grass, white oak, cat) or cockroach only (Russian thistle).

Allergic reactions to birch pollen or timothy grass pollen are prevalent in northern and central Europe, and are frequently included in immunotherapy vaccines for sensitive patients residing in these regions. Hoff and coworkers²² at the Paul Ehrlich Institute in Germany assessed the compatibilities of low-protease, aqueous birch and timothy grass allergens with a protease-rich aqueous *F. culmorum* extract after storage of product mixtures and controls for up to 60 days at 6°C. The protease content of the three extracts was confirmed with zymography (gelatinase activity) and chromogenic peptide hydrolysis (tryptic, chymotryptic and pepsin-like protease or peptidase activities). Rapid and complete degradation of birch or timothy extract reactivities by *Fusarium* extract components was shown using SDS-PAGE immunoblotting with target extract-specific IgE-positive human serum pools or monoclonal IgG antibodies recognizing prominent individual birch allergens (Bet v 1, Bet v 6) or timothy allergens (Phl p 1, Phl p 5). Mediator release assays using rat basophil leukemia cells also revealed time-dependent reductions of birch or timothy allergen activities after mixing with the *Fusarium* extract, with recoveries of approximately 25% and 4% of initial or control values, respectively, after 60 days at 6°C.

In certain areas of the United States, it is not unusual for unique combinations of allergens to comprise the dominant airborne sensitivities and desired extract mix formulations for immunotherapy. Imported fire ant stings are a major cause of allergic reactions in the southern United States, and mountain cedar pollinosis is also prevalent in several of these states, including Texas. Meier and colleagues²³ investigated the allergenic compatibility of extracts from these two sources using gel electrophoresis (SDS-PAGE) methods, with visualization of component protein bands by Coomassie blue dye staining. Aqueous extract mixtures stored for up to 180 days at 4°C displayed no apparent changes in band patterns or staining intensities during this period relative to analogous aqueous control extract samples. Although one mountain cedar and three fire ant bands were detected in these samples, the IgE-binding characteristics of these bands were not determined. The lack of apparent degradation of mountain cedar proteins by a whole-body fire ant extract supports combination of these products into a single immunotherapy vaccine for appropriate patients.

The high- and low-protease extracts included in the five studies summarized earlier^{15,20-23} were typically obtained from a single licensed or non-licensed commercial source per study, which varied between investigators. In these studies, the stabilizing effects of glycerin were assessed at only one concentration (25% or 50%) above those usually found in most patient mixtures. In several investigations, the laboratory methods performed were different from those recognized and regulated by FDA for lot release, stability testing, and potency labeling of United States standardized extracts, with limited or no information available on the quantitative and qualitative relationships between differing analyses of similar or identical target extracts and mixtures.

In 2007, Grier and colleagues²⁴ incorporated licensed product lots from multiple United States manufacturers, variable glycerin concentrations for mixtures with single-extract controls at equivalent glycerin content, and quantitative compendial FDA potency methods in combination with qualitative allergen profiles to examine the compatibilities of low-protease standardized extracts (meadow fescue grass;

dust mite *D farinae* and *D pteronyssinus*; cat; short ragweed) with protease-rich non-standardized fungal and cockroach products. The IgE-binding potencies and banding patterns of meadow fescue grass allergens were not altered significantly in mixtures with dust mite products from five different commercial sources at two concentrations (1000 and 3000 AU/mL) within practice parameter guidelines for maintenance immunotherapy after storage for up to 12 months at 2°C to 8°C. When present in similar mixtures with fungal or cockroach extracts, grass allergens were highly susceptible to protease-related degradation under most conditions examined.

Among the 13 fungal extracts (24 product lots) combined with meadow fescue extract at 10% glycerin final concentrations, more than half of these products (and 75% of fungal product lots) produced rapid (1 month) and significant levels of grass allergen degradation (>50% reduction of control extract potency) at 2°C to 8°C. The lowest recoveries were associated with products derived from three of the most prevalent, allergenic, and clinically important mold species (*Penicillium*, *Aspergillus*, *Alternaria*). Comparable mixtures and controls at 10% and 50% glycerin levels prepared with these fungal extracts from three manufacturers possessed improved grass allergen compatibilities at 50% glycerin, with recoveries closely related to the compositions and protein concentrations of the fungal products.

The compatibilities of dust mite, cat, and short ragweed allergens mixed with *Alternaria*, *Aspergillus*, *Penicillium*, American cockroach, or German cockroach extracts were also examined. Dust mite *D farinae* allergen recoveries in most of these mixtures were relatively high when measured with one human serum pool but significantly lower when tested with a second pool. Differences in the IgE specificities of the two serum pools on immunoblots were consistent with the ELISA inhibition potency reductions observed using the second pool. If these analyses had been conducted with only one of the two serum pools, a different interpretation of the experimental results would likely have been reported. The importance of defining the essential properties of key assay reagents and understanding the consequences of variations in these characteristics on study outcomes cannot be understated. *Alternaria* and *Penicillium* extracts degraded short ragweed allergen Amb a 1 in 10% glycerin after 5 months at 2°C to 8°C, but higher glycerin levels (25%, 50%) afforded near-complete protection. Short ragweed allergen was stable after mixing with *Aspergillus*, American cockroach, or German cockroach extracts and stored at 2°C to 8°C. Cat allergen Fel d 1 was compatible with all companion fungal and insect extracts at 2°C to 8°C, even at minimal (10%) glycerin concentrations.

A follow-up to the Meier study reported by Rans²⁵ examined the compatibilities of fire ant extracts with standardized cat, ragweed, dust mite *D pteronyssinus*, and timothy grass products. In addition to SDS-PAGE, methods of analysis included the identical cat, ragweed, dust mite, and grass allergen potency tests described in the Grier study.²⁴ Timothy grass allergens were degraded rapidly (within 1 month) at 4°C by fire ant extract. Cat (Fel d 1), short ragweed (Amb a 1), and multiple *D pteronyssinus* allergens remained stable when mixed with fire ant extracts and stored for up to 6 months at 4°C.

Dog allergen stabilities in extract mixtures were assessed for the first time in 2009 by Grier and coworkers.²⁶ Clinically important and compositionally distinct dog epithelia and dog hair/dander extracts from different commercial sources were included in this investigation to reflect current use of both product types for allergen immunotherapy. Dog extracts were combined with high-protease fungal extracts (*Alternaria*, *Aspergillus*, or *Penicillium*) or insect products (American cockroach, German cockroach, or fire ant) and evaluated after storage for up to 12 to 15 months at 2°C to 8°C. Dog allergen recoveries in these mixtures were determined with IgE ELISA inhibition and

immunoblotting using test samples and assay procedures analogous to those described in previous studies from this laboratory. Dog epithelia extracts retained moderate to high levels of IgE-binding potency (67%–113% of controls) in all mixtures at 10%, 25%, or 50% glycerin concentrations after storage for up to 13 months at 2°C to 8°C, comparable to recoveries observed for cat extracts under comparable mixing and incubation conditions.

Although partial degradation of intact (66 kDa) dog albumin structures was detected on immunoblots, productive IgE and anti-albumin IgG binding interactions were retained to some degree with these fragments. Dog hair/dander extracts exhibited lower recoveries of IgE ELISA inhibition activity in mixtures with *Aspergillus*, *Penicillium*, or *Alternaria* extracts at 10% or 25% glycerin (28%–61% of controls) after up to 15 months at 2°C to 8°C. Fungal extracts at 50% glycerin and all three insect extracts at 10%, 25%, or 50% glycerin had no appreciable impact on dog hair/dander allergens. IgE immunoblot profiles were consistent in most cases with ELISA inhibition potencies. Can f 1 ELISA reactivities of several dog hair/dander extract mixtures varied noticeably from those determined with ELISA inhibition. The largest discrepancies were observed for mixtures containing *Aspergillus* or *Penicillium* (2.5-fold higher Can f 1 recoveries) and American cockroach (2.3-fold higher ELISA inhibition recoveries). The nature of these differences remains to be determined but is believed to be related to variations in antibody specificity, allergen presentation, or both, in the antibody-detection (ELISA inhibition) and antibody-capture (Can f 1 ELISA) immunoassay formats. These data show the potential bias or preference that methods of this type can exhibit in analyses of heterogeneous extract mixtures, and shows the importance of evaluating multiple, diverse test methodologies whenever possible to provide critical and objective assessments of target allergen activities that can be compared and correlated with clinical outcomes.

In the eight compatibility investigations summarized,^{15,20–26} extracts were combined to produce final product concentrations similar to those recommended in subcutaneous immunotherapy practice parameters for maintenance vial formulations (1:10 v:v dilutions of extract concentrates in most studies). These allergen concentrations also approximate the maximum tolerated dose levels for 0.5-mL injections in sensitive patients at allergy clinics in many regions of the United States.

Antibody-capture immunoassays such as ELISA inhibition are capable of quantifying allergen activities in mixtures diluted 10- to 100-fold higher than those reported in these studies, and are under investigation to document extract compatibilities at vial strengths used during the buildup or dose escalation phase of immunotherapy. Antibody-capture immunoassays exhibit a wide range of analytical sensitivities. Radial immunodiffusion assays offer very limited application to testing of extract mixtures diluted beyond the 10-fold dilutions of concentrates in the published studies. Two-site allergen-specific ELISA methods often exhibit dose-response characteristics suitable for detecting microgram levels of allergens at 1000-fold and higher dilutions of extract concentrates.

These methods and reagents were developed primarily for semiquantitative measurements of environmental allergens, and although their usefulness for extract compatibility studies seem significant, verification of the binding site specificities of these tests in relation to IgE-specific assays of the same allergens and their ability to detect comparable modified or denatured allergen structures is essential for them to be recognized and adopted as reliable indicators of extract stability. Improved availability of rare reagents and calibrated allergen standards currently used exclusively by selected laboratories is required to facilitate these critical evaluations and method comparisons. The long-term stability of these materials under appropriate

storage conditions also must be evaluated, with precise limits established for validity and reproducibility.

Based on the results from the eight published studies, and extrapolations of these data to extract combinations from the same or closely related phylogenetic sources, a chart summarizing the mixing compatibilities of allergens from the major extract categories (excluding foods) was created (**Fig. 1**). Product combinations characterized as stable (green shading) have been shown to retain near-complete levels of target allergen activities ($\geq 70\%$ recoveries compared with control samples) under refrigerated storage conditions (2°C – 8°C , 36°F – 46°F) at low (10%) glycerin concentrations. Mixtures represented as risky combinations (highlighted in yellow) were those exhibiting noticeable degradation of target allergens (50%–70% recoveries vs controls) during storage at 2°C to 8°C , which was prevented or improved substantially at higher (25%) glycerin levels. In unstable allergen combinations (shaded in red), target allergen potencies were not protected or stabilized under most conditions, and were often reduced significantly ($<50\%$ recoveries relative to controls) within 3 months when stored continuously at 2°C to 8°C . Clearly, the enzymatic activities that impact allergen stability and compatibility in the most significant manner are the endogenous proteases in fungal and insect extracts.

Among commercial extracts manufactured and licensed in the United States, all other product groups seem to contain much lower levels of active proteolytic enzymes. Based on the elevated levels of proteolytic enzymes in many fungal and insect extracts, physical separation of pollen, dust mite, and animal allergens from these protease-rich products in treatment mixtures has been recommended in previous and current immunotherapy practice parameter guidelines.^{27,28} Some exceptions to this designation have been observed (meadow fescue grass mixed with several fungi, mountain cedar mixed with fire ant). Mountain cedar pollen proteins seem to lack the specific amino acid sequences required for recognition and catalysis by fire ant proteases. Reduced enzyme levels or activities in certain fungal products are likely responsible for the favorable grass allergen recoveries in these mixtures.

Proteins in many fungal extracts seem to be resistant to the action of endogenous proteases in these products. By comparison, cockroach extract proteases are capable of degrading prominent IgE-binding cockroach proteins in product solutions

Target extracts ↓	Stability after mixing and storage with companion extracts ...								
	Fungi	Insects	Dust Mites	Ragweeds	Grasses	Trees/ Weeds	Dog	Cat	Venoms
Fungi	Green	Green	Green	Green	Green	Green	Green	Green	Red
Insects	Green	Yellow	Green	Green	Green	Green	Green	Green	Red
Dust Mites	Yellow	Yellow	Green	Green	Green	Green	Green	Green	Red
Grasses	Red	Red	Green	Green	Green	Green	Green	Green	Red
Trees/Weeds	Red	Red	Green	Green	Green	Green	Green	Green	Red
Ragweeds	Yellow	Green	Green	Green	Green	Green	Green	Green	Red
Dog	Yellow	Yellow	Green	Green	Green	Green	Green	Green	Red
Cat	Green	Green	Green	Green	Green	Green	Green	Green	Red
Venoms	Red	Red	Red	Red	Red	Red	Red	Red	Green

Fig. 1. Compatibility chart for subcutaneous allergen extract immunotherapy.

at reduced (10%–25%) glycerin levels stored at 2°C to 8°C. The compatibilities of high-protease extract mixtures (fungi with fungi, fungi with insects, insects with insects) are believed to be favorable but require further study to determine the actual recoveries of important allergens in these products when combined in specific mixtures at defined glycerin and HSA concentrations.

Regular, intermittent, or inadvertent exposures to ambient temperatures (20°C–25°C, 68°F–77°F) promote protease-catalyzed degradation events in extract mixtures, and can further reduce allergen recoveries in risky combinations to those categorized as unstable. Because glycerin typically provides concentration-dependent and time-dependent improvements in allergen stability for many risky or unstable mixtures, inclusion of higher glycerin levels or storage for shorter periods at 2°C to 8°C are options that some allergists have adopted to minimize the numbers of formulations and injections administered to certain patients. Hymenoptera venoms contain active hydrolytic enzymes, including proteases, but are usually formulated and injected separately from airborne or environmental allergens and are designated in red (not recommended) on [Fig. 1](#) for this reason.

SUMMARY

Considerable progress has been made over the past 20 years on the stability and mixing compatibility of allergenic extracts. Analysis of various allergens and mixtures has generated consistent results for similar or related products and established a solid foundation for future studies with new or more complex allergen combinations. Continued attention and efforts focused on improving the selectivity and robustness of immunochemical reagents and the characterization of calibrated universal standards will support validation of high-performance analytical methods. Collaborative investigations among clinicians, scientists, and extract manufacturers will ensure that appropriate extracts and methods are examined, that valid results are obtained and analyzed, and that correlations to clinical outcomes are reported. Incorporation of systematic approaches using matrix-based and higher-level (factorial) study designs will promote adoption of consistent acceptance criteria for stable, risky, and unstable allergen extract combinations in immunotherapy mixtures. Technological advances will continue to fuel development of robust immunochemical testing platforms and methodologies displaying subtle but meaningful differences in allergen-binding requirements.

REFERENCES

1. Esch RE. Allergen immunotherapy: what can and cannot be mixed? *J Allergy Clin Immunol* 2008;122:659–60.
2. Nelson HS. Specific immunotherapy with allergen mixes: what is the evidence? *Curr Opin Allergy Clin Immunol* 2009;9:549–53.
3. Arbesman CE, Eagle H. The thermolability of ragweed pollen extract and its corresponding regain. *J Allergy* 1939;11:18–27.
4. Hjorth N. Instability of grass pollen extracts. *Acta Allergol* 1958;12:316–35.
5. Center JG, Shuller N, Zelenick LD. Stability of antigen E in commercially prepared ragweed pollen extracts. *J Allergy Clin Immunol* 1974;54:305–10.
6. Baer H, Anderson MC, Hale R, et al. The heat stability of short ragweed pollen extract and the importance of individual allergens in skin reactivity. *J Allergy Clin Immunol* 1980;66:281–5.
7. Anderson MC, Baer H. Antigenic and allergenic changes during storage of a pollen extract. *J Allergy Clin Immunol* 1982;69:3–10.

8. Franklin RM, Baer H, Hooton ML, et al. The stability of short ragweed pollen extract as measured by skin test and antigen E. *J Allergy Clin Immunol* 1976;58:51–9.
9. Norman PS, Marsh DG. Human serum albumin and Tween 80 as stabilizers of allergen solutions. *J Allergy Clin Immunol* 1978;62:314–9.
10. Nelson HS. The effect of preservatives and dilution on the deterioration of Russian thistle (*Salsola pestifer*), a pollen extract. *J Allergy Clin Immunol* 1979;63:417–25.
11. Nelson HS. Effect of preservatives and conditions of storage on the potency of allergy extracts. *J Allergy Clin Immunol* 1981;67:64–9.
12. Hiatt CW, Baer H, Hooton ML. Kinetics of thermal decay of antigen E from short ragweed pollen. *J Biol Stand* 1977;5:39–44.
13. Baranuik JN, Esch RE, Buckley CE. Pollen grain chromatography: quantitation and biochemical analysis of ragweed pollen solutes. *J Allergy Clin Immunol* 1988;81:1126–34.
14. Bousquet J, Guerin B, Hewitt B, et al. Analysis of commercial pollen extracts by enzyme determination I. comparison of RAST-inhibition assay and enzyme titration for orchard grass, rye grass and short ragweed pollen extracts. *Ann Allergy* 1980;45:310–5.
15. Esch RE. Role of proteases on the stability of allergenic extracts. *Arb Paul Ehrlich Inst Bundesamt Sera Impfstoffe Frankf A M* 1992;85:171–9.
16. Esch RE. Evaluation of allergen vaccine potency. *Curr Allergy Asthma Rep* 2006;6:402–6.
17. Grier TJ. Laboratory methods for allergen extract analysis and quality control. *Clin Rev Allergy Immunol* 2001;21:111–40.
18. Grier TJ, Hazelhurst DM, Duncan EA, et al. Major allergen measurements: sources of variability, validation, quality assurance, and utility for laboratories, manufacturers, and clinics. *Allergy Asthma Proc* 2002;23:125–31.
19. Morrow KS, Slater JE. Regulatory aspects of allergen vaccines in the US. *Clin Rev Allergy Immunol* 2001;21:141–52.
20. Kordash TR, Amend MJ, Williamson SL, et al. Effect of mixing allergenic extracts containing *Helminthosporium*, *D. farinae*, and cockroach with perennial ryegrass. *Ann Allergy* 1993;71:240–6.
21. Nelson HS, Ikle D, Buchmeier A. Studies on allergen extract stability: the effects of dilution and mixing. *J Allergy Clin Immunol* 1996;98:382–8.
22. Hoff M, Krail M, Kastner M, et al. *Fusarium culmorum* causes strong degradation of pollen allergens in extract mixtures. *J Allergy Clin Immunol* 2002;109:96–101.
23. Meier EA, Whisman BA, Rathkopf MM. Effect of imported fire ant extract on the degradation of mountain cedar pollen allergen. *Ann Allergy Asthma Immunol* 2006;96:30–2.
24. Grier TJ, LeFevre DM, Duncan EA, et al. Stability of standardized grass, dust mite, cat, and short ragweed allergens after mixing with mold or cockroach extracts. *Ann Allergy Asthma Immunol* 2007;99:151–60.
25. Rans TS, Hrabak TM, Whisman BA, et al. Compatibility of imported fire ant whole body extract with cat, ragweed, *Dermatophagoides pteronyssinus*, and timothy grass allergens. *Ann Allergy Asthma Immunol* 2009;102:57–61.
26. Grier TJ, LeFevre DM, Duncan EA, et al. Stability and mixing compatibility of dog epithelia and dog dander allergens. *Ann Allergy Asthma Immunol* 2009;103:411–7.
27. Li JT, Lockey RF, Bernstein IL, et al. Allergen immunotherapy: a practice parameter. *Ann Allergy Asthma Immunol* 2003;90:1–40.
28. Cox LS, Li JT, Nelson HS, et al. Allergen immunotherapy: a practice parameter second update. *J Allergy Clin Immunol* 2007;120:S25–83.