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Laboratory Methods for Allergen Extract Analysis and Quality Control

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Introduction

The development and implementation of reliable testing procedures are essential to the quality and consistency of many biotechnology products and processes. Complex and sensitive interactions often exist between a product or process step and the materials and methods used for their analysis. The optimization and validation of laboratory procedures whose results accurately reflect the biological activities of specific compounds or structures is a fundamental goal for virtually all areas of biomedical research and product development. In many of these areas, particularly pharmaceuticals, individual molecules serve as the active ingredients producing the desired physiological responses and clinical outcomes. The development of quantitative detection methods for these molecules follows established guidelines and acceptance criteria for analyte purity and stability, assay performance characteristics (accuracy, precision, reproducibility), and correlation with clinical results *(1–3).* The concentrations of other components present in these products, usually well-characterized, USP-grade inert materials (stabilizers, fillers), are controlled during the formulation process. Methods to measure the levels of these additives and their influence on the active ingredients are usually well-established and are relatively simple to perform. By comparison, allergen extracts are highly heterogeneous mixtures containing multiple active ingredients as well as nonallergenic components of unknown structure or concentration. The constituents responsible for allergic reactions represent a small percentage of the total extract composition, and their activities may vary considerably from patient to patient *(4,5).* Nonallergenic compounds may influence the structures and biological potencies of allergenic components in a productive manner through stabilizing interactions, in a destructive

manner by promoting degradative or conformational changes to critical allergenic determinants, or have no significant impact on extract properties. The complexities and uncertainties associated with allergen extracts present a considerable challenge to the development of meaningful analytical methods for these products.

In general, allergen extracts contain a wide variety of macromolecules (proteins, glycoproteins, polysaccharides, lipids, nucleic acids) along with low molecular weight metabolites, salts, and pigments. The structures and properties of extract components may be influenced by a large number of external factors, including allergen source (phylogenetic, geographic), pre-extraction procedures (milling, defatting), extraction conditions (time, temperature, pH, extraction fluid composition, degree of wetting, and mixing), post-extraction processing steps (filtration), and storage conditions *(6–14).* For cultured materials such as fungal organisms, dust mites, and selected insects, additional factors related to the inoculation, growth, harvesting, and maintenance of these cultures must be strictly controlled to produce extracts with consistent compositions and properties *(15,16).* Allergens from unrelated sources may possess similar biological functions, sequences, and structures consistent with their clinical sensitivities and cross-reactivities *(17).* To date, nearly all allergens have been identified as proteins or glycoproteins, and many possess distinct biological activities (hydrolytic enzymes) or physiological functions *(17,18).* Carbohydrate structures on glycoproteins or polysaccharides are capable of binding IgE but express low or insignificant cell-triggering activities compared to protein determinants *(19,20).* A wide variety of quantitative and qualitative methods are well-suited for physical and biochemical analyses of proteins and carbohydrates and are employed routinely to examine the composition, potency, and stability of allergen extracts.

Quantitative methods capable of measuring either multiple components (total extract potency, total protein concentration) or individual constituents (specific allergen or antigen concentration) are frequently employed as indicators of extract potency and stability. Potency tests rely on high-affinity binding interactions between allergens and antibodies derived from human allergic serums (IgE, polyclonal), animal serums (rabbit, goat, or sheep IgG, polyclonal), or hybridoma products (mouse or rat IgG, monoclonal). For a relatively small group of allergens, individual proteins have been shown to be important in a high percentage (> 75%) of human allergic reactions *(21,22).* The development and validation of quantitative immunodiffusion methods specific for two major allergens, cat hair/pelt allergen Fel d 1 and short ragweed pollen allergen Amb a 1 (Antigen E), have facilitated the standardization of cat and ragweed extracts in microgram per mL concentrations of these allergens. For most allergens, however, multiple components appear to

Table 1
Current Methods of Extract Standardization

Extract	Standardized units	Analyte(s)	Test method
Cat hair Cat pelt	BAU/mL (Fel d 1 U/mL)	Fel d 1	Radial immunodiffusion
Short ragweed	Antigen E U/mL (Amb a 1 μg/mL)	Antigen E (Amb a 1)	Radial immunodiffusion
Dust mites	AU/mL	Multiple allergens	ELISA inhibition
Grass pollens	BAU/mL	Multiple allergens	ELISA inhibition
Venoms	μg protein/mL	Multiple components	Ninhydrin total protein

play a prominent role in IgE-mediated reactions *(23,24).* In numerous studies reported to date, immunoblot analyses of extracts with human allergic serums reveal IgE-binding interactions with at least 5 distinct proteins for individual patients and 10–20 extract components across multiple patient samples. Standardization of dust mite and grass pollen extracts known to contain a diverse group of important allergens is based on a quantitative enzyme-linked immunosorbent assay (ELISA) inhibition method sensitive to structural changes in multiple extract components. Hymenoptera venoms also contain multiple allergens and are formulated based on total protein content. The laboratory methods used to assess the potencies of Food and Drug Administration (FDA)-approved standardized extracts are summarized in Table 1 *(25,26).*

Qualitative profiles or fingerprints of extract components, usually produced after electrophoretic separations, reveal the overall composition and concentration of proteins and glycoproteins in great detail, and serve as an effective complement to quantitative analyses. Components are separated in porous gels based on size, charge, or pH differences and produce distinct banding patterns for many allergen extracts. As a result, electrophoretic techniques such as isoelectric focusing (IEF), polyacrylamide gel electrophoresis (PAGE), and crossed immunoelectrophoresis (IE) are suitable methods for assessing the identity of an allergen extract. These procedures may also provide valuable insights into changes or differences in component structures or concentrations that cannot be deduced from other in vivo or in vitro analyses. IEF is currently used to confirm the presence of characteristic protein bands and patterns in standardized products.

The development and utilization of bioanalytical methods for multi-component analytes such as allergen extracts is a time-consuming and challenging process. The ability of a test method to detect only the target analytes among a highly heterogeneous mixture of components and produce unequivocal and reproducible results requires an intimate knowledge of the dynamic interactions between support materials, reagents, and test samples. Potential variations in each com-

ponent material, reagent, operator, and piece of equipment must be examined objectively and analyzed with respect to acceptance specifications, product requirements, and regulatory considerations. In addition, use of calibrated equipment and established data-reduction methods is essential during all phases of assay and product development. Once this knowledge is obtained, assay validation can be addressed in a systematic manner. The following discussion will focus on characteristics acknowledged as vital to effective analytical procedures.

Criteria for Effective Analytical Methods

The ability of laboratory procedures to produce meaningful results with complex test samples such as allergen extracts requires careful attention to, and validation of, a number of fundamental assay performance characteristics. These include, but are not limited to, the sensitivity, specificity, accuracy, precision, reproducibility, linearity, robustness, and instrument system suitability of a given procedure. Detailed discussions of validation characteristics can be obtained from published reports *(27).* Basic definitions and features of these parameters pertinent to allergen analyses are described in the following sections.

Characteristics

The essential properties of a validated test method can be divided into those pertaining to application, methodologic and performance characteristics *(28).* Application characteristics determine the suitability of a method for a particular laboratory environment, including sample size, batch size, turnaround time, equipment, and personnel needs. Methods-based features establish the optimal conditions for testing to satisfy specific requirements for analyte detection. Sensitivity is defined as the minimum quantity or concentration of detectable analyte. Detection limits are based on signal-to-noise ratios with cutoffs for most methods at values at least two to three times the background or baseline levels *(29,30).* Limits of quantitation for a given assay may be different than the detection limits based on the reliability and consistency of dose-response curves for standards or references at low analyte concentrations. Specificity is the ability of a method to measure the intended components and to discriminate between compounds of closely related structure that may be present in a test sample. Assay specificity may be controlled by reagent properties as well as assay format or configuration. Considerable knowledge and efforts are often required to establish specificities with low levels of false-positive (cross-reactions) and false-negative (interferences) reactions with minimal or

negligible effects on assay sensitivity. Linearity of responses is a critical characteristic that influences many performance-based parameters. Standard and sample measurements at multiple concentrations or dilutions are compared using graphs or spreadsheets, with linear character of their dose-response curves defined by line equation parameters (slope, y-intercept) and correlation coefficients derived from regression calculations. Many biochemical interactions produce sigmoidal or hyperbolic response curves indicative of an exponential dependence between signals and concentration. Transformations of data between linear and logarithmic values are performed to determine the dose-response combination (linear-linear, linear-log, log-linear, or log-log) providing the best fit. In general, at least four data points are required for construction of consistent standard curves, and sample analyses at multiple dilutions ensure that the slopes of standard and sample reactions are closely related.

Performance characteristics demonstrate the quality of the test methods and resulting data. Accuracy is the degree of comparability between samples and standards of known concentration or potency. The effects of impurities or endogenous sample components on the accuracy of an assay procedure must be established across the entire range of a standard curve, and are usually assessed by introduction of known quantities of these components into calibrated samples or controls. In general, assay accuracy is determined by independent testing of multiple sample concentrations in replicates of three or higher by one or more analysts. Precision is defined as the closeness of replicate test results performed by one or several technicians within an individual laboratory. Reproducibility is a measure of the agreement of sample values over an extended time period or between laboratories. Differences in parallelism or dilution recovery have a direct bearing on the consistency of intralaboratory and interlaboratory results.

Evaluations of assay precision or reproducibility also employ multiple dilutions and replicates of test samples, with at least six independent results required for a meaningful assessment of these characteristics. The robustness of a procedure is the ability to tolerate anticipated differences or changes in materials, conditions, equipment, or analysts. Comparisons of multiple lots of component materials are essential to item and vendor qualifications. This is particularly important for solid-phase materials or media employed to capture or adsorb target molecules and for biochemicals whose specific activity, purity, or degree of chemical modification play a fundamental role in subsequent binding interactions. Precise definition of optimal conditions and acceptability limits for all steps involving these components (concentration, composition, time, temperature, pH) is confirmed by a matrix of experiments capable of revealing both independent and interactive

characteristics. Stability considerations are also important to establish appropriate storage conditions and meaningful expiration dates for critical assay components.

Quality Management

Once a method is optimized and validated, standards of performance for personnel, methods, equipment, data analyses, and reports must be defined and implemented in order to maintain conformance to product requirements and specifications *(28).* Analytical criteria must be consistent with clinical performance characteristics, product claims, and federal regulations. Training and certification of laboratory technicians promotes high levels of intralaboratory accuracy and precision. Systematic surveillance and critical review of the performance of assay procedures ensures that the knowledge and skills obtained during training are maintained. Several examples include analysis of calibrated samples from internal or external sources, charting or tracking the reproducibility of test results over defined time intervals, and statistical derivations of acceptance ranges for normally distributed data based on action or alert limits (mean ± 2 standard deviations [SD]) and rejection or specification limits (mean ± 3 SD).

The limited group of assay methods approved by FDA for allergenic products (see Table 1) have been validated across multiple laboratories using common materials, references, and conditions. A wide variety of additional methods have been used for many years to isolate and characterize extract or raw material components, and provide valuable information and insights into the compositions and activities of these materials. Estimates of total extract potency may not correlate closely with specific component concentrations in the same extracts owing to the differences in proportion or requirements for productive binding to antibodies *(23,31).* Efforts to advance the characterization, consistency monitoring, and standardization of allergen extracts can benefit greatly by consideration, qualification, and validation of new procedures. Immunochemical methods are often developed in separate laboratories to measure related allergens, and it is now apparent that differences in the materials, reagents, or configurations used for these procedures can produce significant differences in test results by altering the structural requirements, affinity, or selectivity of antigen-antibody binding interactions. Development and comparison of multiple methods for individual extracts are desirable to identify in vitro methods that best correlate with in vivo (skin-test) reactivities of extraction products. In general, no single analytical method appears to provide optimal information or quality of results for all extracts and product groups. This observation is not unexpected considering the molecular complexity of extract preparations and differences in mechanism and

susceptibility observed for many of these methods. As a result, specific combinations of quantitative and qualitative methods measuring a broad spectrum of extract components provide an effective means of characterizing the biological activities of allergenic products *(5).*

Variables That Influence Allergen Analyses

As noted earlier, the true value and reliability of a laboratory test result is closely linked to the quality and consistency of the materials, reagents, analysts, and equipment employed to produce it. In many cases, assay variability may result from differences in any or all of these sources. Several studies investigating the origins of these differences across multiple test sites have shown that intralaboratory imprecision and material differences are responsible for a significant percentage of variable results *(28).* Many materials are designed and developed to accommodate a wide range of potential applications, and it is difficult to detect or predict inconsistencies critical to test methods with natural or synthetic materials from external sources. Communications and collaborations with manufacturers on technical issues promote the development of product and vendor qualifications consistent with end-user requirements and specifications. However, the dynamic relationships among many of the materials and reagents used in bioanalytical procedures require a more precise definition of the specific interactions that govern assay consistency and results. In this section, sources of variability pertinent to allergen extract analyses will be reviewed, including observations from our laboratory concerning the comparability of results for allergens analyzed by multiple methods or assay configurations.

Allergens and Antibodies

It is no surprise that the composition and consistency of allergen and antibody reagents contribute directly to the quality of analytical results. The presence of a select group of allergenic components in an extract is important to detect the diverse IgE specificities associated with human allergic reactions *(32).* The influence of nonallergenic constituents must also be addressed. Extracts prepared from a wide variety of allergenic raw materials are available at relatively low cost from numerous licensed manufacturers. Product lot consistency is usually very high for extracts prepared by a single manufacturer *(33).* However, differences may exist among products made by multiple manufacturers owing primarily to differences in raw material sources and extraction conditions *(34,35).* Both liquid-phase and freeze-dried extracts are popular sources of allergens for immunochemical applications. Although liquid allergens are viable for shorter time periods,

freeze-drying may induce irreversible structural changes to critical components or pH increases after reconstitution owing to removal of carbon dioxide. Use of buffered solutions and stabilizers such as glycerin are key to preserving the integrity of these products.

In contrast, antibodies possessing the specificity and reactivity desired for allergen analyses are much more difficult to obtain from commercial, clinical, or academic sources, and are frequently cost-prohibitive. Human plasmas containing high IgE levels to target allergens can be purchased from serological companies in large quantities. However, clinical histories and other vital information defining the allergic conditions of these donors are frequently incomplete or unknown. Construction of human serum pools whose IgE specificities reflect a broad spectrum of responses typical of an allergic patient population is a significant challenge. Qualifications of these pools are usually based on sensitivity and specificity criteria using both quantitative (ELISA) and qualitative (immunoblotting) analyses. Sourcing comparable serums to prepare replacement pools of equivalent immunochemical properties requires additional screening, pooling, and qualification testing. Serum references are often freeze-dried to promote long term stability; however, care must be taken to ensure that serum lipoproteins denatured during the freeze-drying process do not alter the solubility or structure of critical antibodies.

In addition to human serum-based reagents, rabbit polyclonal or mouse monoclonal IgG antibodies recognizing specific extract components or structural epitopes are sensitive probes of allergen structure and essential to immunodiffusion, immunoelectrophoresis, and ELISA analyses. However, animal antibodies often recognize distinct epitopes on allergenic proteins compared to human IgE, a situation that may produce different susceptibilities and test results for some extracts *(36).* Differences in the properties of these antibodies may also be related to immunogen purity, immunization conditions, antibody purity, or combinations of these factors *(37).* Some monoclonal antibodies (MAbs) thought to be mono-specific may display cross-reactions that compromise assay performance. Highly-specific MAbs are less sensitive in some assay formats owing to the limited availability of structural epitopes and are less durable than polyclonal antibodies (PAbs) in many cases owing to their purity *(31,38).* These limitations are often overcome by incorporation of two or more antibodies with different epitope specificities into an immunoassay *(39,40).*

Antibody reagents impart many of the critical performance characteristics to quantitative and qualitative assays, particularly avidity of allergen interactions and specificity of assays in various configurations. As a result, it is not surprising that rare and valuable antiserums developed in one laboratory are not readily available to other scientists inter-

ested in similar pursuits or goals. Although understandable in many respects, this situation is a major deterrent to the development of consensus methods of high quality and defined performance standards, and will be discussed in further detail later in this review.

Standards and References

The accuracy and precision of an assay result are highly dependent on the selection and qualification of the standard or reference materials that produce the desired dose-response characteristics *(41).* Selection criteria are usually based on both compositional requirements (purity, specific activity, presence or absence of defined components) and assay performance features such as analytical sensitivity, signal/background ratio, and consistency of responses at multiple concentrations spanning the dynamic range of the assay. The inclusion of positive and negative control samples is also important to provide independent measures of assay reliability.

Primary standards or references are the materials used to establish and validate a given procedure. The quantities of these materials available for routine use are often limited, if available at all, owing to the maturity of many methods and the use of these standards to certify analysts at multiple laboratories. To address this problem, secondary standards employed for daily use have been qualified by direct comparisons with primary standards or designated preparations *(14).* Assignment of concentration or activity values to new references must be performed consistently using defined guidelines and specifications *(42,43).* In spite of considerable efforts to demonstrate the identity of a new preparation with an existing one, small differences in their compositions or specific activities are virtually impossible to avoid. Qualifications of additional references based on comparisons with recently used preparations rather than a single designated lot may result in further changes in composition and reagent drift. This is particularly important for the extract and serum references used to standardize allergenic products. Current methods of extract standardization are based on both absolute (radial immunodiffusion) and relative (ELISA inhibition) assessments of allergen potency. If reference preparations experience noticeable drift, production lot extracts must drift in a similar manner to maintain assay results within the acceptance limits established for each product. It is ironic that efforts and practices intended to reduce lot-to-lot variability for standardized extracts may actually contribute variables that have a far greater impact on the long-term consistency of these products. For these reasons, it is important, whenever possible, to use the same lot of a standard or reference throughout the course of a prospective or time-related study such as product stability investigations. Consistent use of other material lots for immobilization and

detection steps in these methods may also be beneficial. Recombinant protein references are attractive alternatives often available in abundant supply but must possess structures and specific activities similar to those of native allergens and parallel dose responses with test extracts *(44–46)*.

Development of in-house references and test methods is useful to characterize and monitor the consistency of selected nonstandardized extracts, and may improve standardized extract properties by identifying differences in reference preparations that may not be apparent from functional tests. Owing to their complexity, allergen preparations exhibit distinct dose responses in some methods, particularly those in which the standard preparations are compositionally unrelated to the test extracts. In these cases, it is important to examine as many sample dilutions as possible spanning the dynamic range of the assay to eliminate potential differences and bias of results based on selection of single or fixed dilutions.

Homologous vs Heterologous Standards

In general, the compositions of standard or reference materials should closely resemble those of the samples to be analyzed. In a homologous test system, these materials would originate from the same source and possess similar concentrations or purities of active constituents. Most analytical methods produce precise and accurate results when homologous references are employed. By comparison, heterologous standard/sample combinations, derived from different sources or degrees of purity, are more likely to produce nonparallel dose-response curves and dilution recoveries. Rigorous validations are required to demonstrate the reliability of these tests.

A heterologous method currently used for FDA-approved standardized extracts is a gel diffusion enzyme activity assay for phospholipase, a major allergen involved in anaphylactic reactions to Hymenoptera venoms *(47)*. This assay employs honey bee venom standards to determine the phospholipase activities of honey bee and vespid (hornet, wasp, and yellow jacket) venom samples. Significant differences exist between the slopes of dose-response curves for honey bee venom standards or samples and those observed for vespid venoms (Fig. 1) *(48)*. Differences in vespid sample reactivities are much more difficult to detect, and interpolations from a honey bee standard curve yield inconsistent results for multiple dilutions of a given sample. As expected, phospholipase values produced by homologous combinations display improved parallelism and precision.

Quantitative immunoassays are particularly sensitive to differences related to allergen composition. Extracts derived from different fractions of allergenic source materials (animal hair/dander/epithelia)

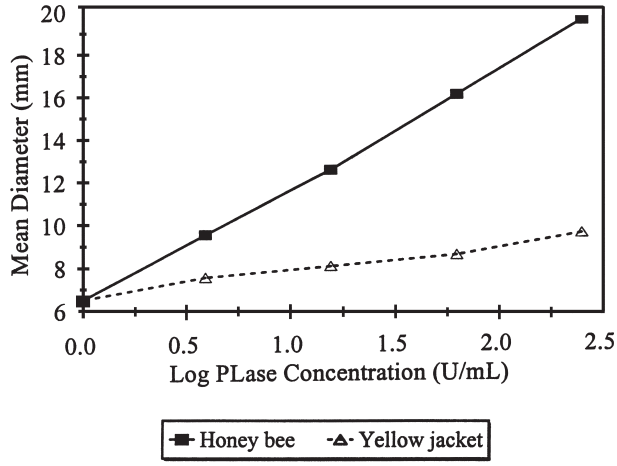

Fig. 1. Phospholipase dose-response curves for Hymenoptera venoms.

or laboratory cultures (fungal cells/culture filtrates, mite bodies/feces) may contain marked differences in the concentrations of distinct components, including major allergenic proteins *(49).* For example, dog hair/dander extracts contain moderate levels of Can f 1 but little, if any, albumin, whereas dog epithelial extracts possess high albumin concentrations but low levels of Can f 1. Consequently, analysis of one type of extract using a reference prepared from a different fraction or source of the same raw material can produce nonparallel dose-response curves and underestimated results. A similar situation exists with unstable allergens such as molds, insects, and grass pollens *(50,51).* Fungi and insects are rich sources of hydrolytic enzymes, which can impart major changes to the structure, properties, and stability of protein and glycoprotein components. Grass pollen allergens are also known to be heat-sensitive. Addition of glycerin to these extracts stabilizes allergenic constituents to both physical (adsorption to storage vessel surfaces) and biological (degradation by proteases or glycosidases) changes, and extraction of raw materials in glycerinated buffers is a logical approach to achieve desired product compositions and consistencies. However, for many raw materials, the solubilization of specific macromolecular components may be compromised by high glycerin concentrations, leading to compositional differences for these products based on the conditions of extraction, formulation, and storage. These changes have direct implications toward the qualification of reference extracts, the accuracy of analytical methods, and the clinical performance of these products. Complementarity of results from reciprocal or cross-wise analyses in which references and samples are substituted for each other in a single test format is a sensitive indicator of the overall compositions and specific activities of these materials.

Major allergen measurements frequently employ purified proteins as primary standards and crude extracts with assigned allergen values as secondary and daily-use standards. Standards calibrated by a separate laboratory or method must be used with caution owing to potential differences in materials and selectivities noted previously in this review. Comparisons of standard values and dose-response characteristics between multiple methods facilitates the correlation or normalization of test results.

Extract or Reagent Components

Numerous substances contained in allergen extracts and assay reagents play a major role in the development of well-defined, high-performance analytical methods. It is clear from the previous discussion that differences in macromolecular composition between test and reference allergen preparations may exert a strong influence on the parallelism, precision, and accuracy of extract quantitation. In addition, the presence of preservatives (phenol) or stabilizers (glycerin, human serum albumin [HSA], β-alanine) in allergy products may be incompatible with certain test methods. Phenol forms complexes or adducts with some allergens (e.g., latex) that may alter the structures required for IgE-mediated reactions. Glycerin is known to produce false-positive reactions in one protein assay method (ninhydrin) and false-negative or underestimated results in others (PNU) *(48).* Positive ninhydrin reactions are also produced by α-amino groups present in HSA and β-alanine.

The optimization of diluent components (buffers, salts, detergents, proteins, or serums) for allergen samples or antibody reagents is critical to development of test methods possessing appropriate specificity, analytical sensitivity, and reproducibility. In theory, the biological activity of a molecule is directly related to its availability for reaction. Proteins in nature are often complexed with other high or low molecular weight substances owing to their molecular size and ionic or hydrophobic surface properties. Shifts in the dynamic equilibrium between complexed and unbound proteins result in structures or conformations whose availability for reaction changes in response to the environment in which these components exist. Deviations from complete or optimal availability are attributed to environmental or matrix effects. Optimizing in vitro reactivities involves both the reduction or elimination of detrimental matrix effects and the promotion or augmentation of interactions that favor the expression of desired assay performance characteristics.

Matrix effects may also be important during raw material selection or assessment studies. Small-scale (less than 50 mL) extractions are frequently performed on raw material lots used to prepare standard-

Table 2
Influence of Coating Antibody Storage Buffer on Specificity of Egg White Double-Bind ELISA Analyses

Analyte	Extract	Storage buffer	Mean µg/mL	% of Egg white µg/mL
Egg white	Egg white	Borate, pH 8.4	3770	100
	Whole egg	Borate, pH 8.4	4585	122
	Egg yolk	Borate, pH 8.4	3229	86
Egg white	Egg white	PBS, pH 7.4	2966	100
	Whole egg	PBS, pH 7.4	1658	56
	Egg yolk	PBS, pH 7.4	615	21

ized products to determine extraction efficiencies and potencies of the component allergens. Differences in composition or specific activity between pilot-scale extracts and those prepared at full production scale (5–20 L) with the same raw materials may introduce matrix effects that influence the accuracy or predictability of these analyses. Linear scale-up of complex, multi-variable processes such as allergen extraction in which all material levels are increased proportionately does not always yield comparable compositions and properties. Understanding the critical factors controlling extraction efficiencies at multiple extraction scales and weight/volume ratios is essential to production of consistent extract compositions and effective assessment methods.

Several examples from our laboratory demonstrate that subtle differences in reagent lots, concentrations, or diluent components can produce significant changes in assay performance *(48).* One example demonstrates the importance of buffers and pH to the specificity of an antigen-antibody reaction. A double-bind (sandwich) ELISA procedure for egg white antigens measures the binding of egg extract components to rabbit anti-egg white antibodies immobilized onto polystyrene microtiter plates. A coating antibody preparation dialyzed into two different storage buffers (sodium borate at pH 8.4 and phosphate-buffered saline [PBS] at pH 7.4) and diluted with a common carbonate coating buffer (pH 9.6) was employed to assess the egg white protein concentrations of 1:20 (w/v) glycerinated egg white, whole egg, and egg yolk extracts using identical conditions and materials for all additional steps. The resulting data (*see* Table 2) shows that coating antibody stored in PBS produces the expected trend in egg white protein concentrations (egg white > whole egg > egg yolk), whereas borate-buffered coating antibody reveals only small differences in the egg white content of the three egg extracts. Differences in test results for individual extracts range from 27% for egg white to 526% for egg yolk.

Table 3
Influence of Coating Antibody Lot and Biotinylated Antibody Concentration on Reproducibility of Dog Albumin Double-Bind ELISA Analyses[a]

Analyte	Dog epithelia extract	Biotin Ab dilution	Mean µg/mL ± 1 SD Coat Ab lot A	Mean µg/mL ± 1 SD Coat Ab lot B	% of lot A
Dog albumin	Lot 1	1:5,000	601 ± 55	572 ± 82	95
	Lot 2	1:5,000	452 ± 193	439 ± 150	97
Dog albumin	Lot 1	1:1,000	883 ± 157	628 ± 89	71
	Lot 2	1:1,000	802 ± 157	573 ± 191	71

[a]Mean values derived from five independent assays and 15–20 sample dilutions performed by two technicians.

No significant differences in dose-response parallelism were observed between coating antibodies or egg extracts, suggesting that the observed differences in reactivity result from a matrix effect involving coating antibody storage buffers and microtiter plate surface.

Another example from our laboratory using a double-bind ELISA assay for dog albumin illustrates the potential for specific combinations of assay conditions to influence the consistency of test results *(48).* Qualifications of new reagent lots are usually performed in side-by-side comparisons with the lot currently in use or a designated reference preparation, keeping all other materials and conditions constant at their optimal levels. This approach is effective when the reagents being qualified interact exclusively with their target molecules. In some cases, however, interactions detected between materials and incubations from separate assay steps can be significant sources of assay variability and drift. These combinations do not always follow conventional wisdom or logic and are difficult to detect unless examined directly. Experimental design algorithms are useful tools for assessing interactive variables in a process or test method.

In the dog albumin double-bind ELISA study, a new lot of coating antibody was qualified against the current lot at two different concentrations of biotinylated anti-dog albumin antibody. Two 1:10 (w/v) aqueous dog epithelia extracts were employed as the allergen samples. The dog albumin levels of these extracts (*see* Table 3) are consistent at one concentration of biotinylated antibody (1:5,000 dilution), but noticeably different at a slightly higher concentration (1:1,000 dilution). Although the magnitude of these differences is relatively low, it is easy for interactive sources of reagent drift to go unnoticed and to accumulate over time into substantial variations in reagent performance or assay results. This is particularly important for assays measuring major dog allergens because of their potential use in the consistency monitor-

ing and standardization of dog extracts prepared from numerous raw material sources.

Assay Formats or Structural Requirements

Positive reactions in bioanalytical methods are produced by specific analyte structures or sequences recognized by the materials employed for a particular assay. Differences in the structural requirements of test methods measuring similar or identical substances can produce variable test results because of differences in the numbers of available reaction sites. These differences may result from matrix effects described above or from recognition of independent structures by distinct mechanisms *(31,36,38).* A classic example of mechanistic differences producing variable results can be found in quantitative methods for total protein concentration. The two protein methods used to analyze and release FDA-licensed allergen extracts are the ninhydrin and protein nitrogen unit (PNU) analyses. Ninhydrin (triketohydrindene hydrate) reacts specifically with molecules or protein hydrolysates possessing α-amino groups to produce a purple chromophore whose absorbance at 570 nanometers is directly proportional to the concentration of amino groups present in these samples *(52).* All amino acids except proline and hydroxyproline contain α-amino groups and exhibit similar degrees of reactivity with ninhydrin. As a result, proteins of varying composition possessing similar concentrations of total amino acids are expected to produce consistent ninhydrin results. By comparison, the PNU procedure involves precipitation of protein components with a strong acid (phosphotungstic acid) followed by quantitation of the nitrogen levels in these precipitates *(53).* One PNU corresponds to approx 10 ng of protein. The completeness and consistency of the acid-precipitation reactions are key to the effectiveness of PNU assays. The presence of glycerin (propanetriol) in allergen extracts interferes with PNU assays by inhibiting protein precipitation indirectly (altered structure and activity of surrounding water molecules) or directly (stabilization of protein surface structures via hydrogen bonding) *(54).* Other methods of protein quantitation established in many bioanalytical laboratories rely on visible dyes which bind to a limited group of amino acids (Bradford method) or oxidation-reduction reactions (Lowry and BCA methods) to produce colorimetric responses *(55–57).* It is not uncommon for these methods to produce protein values for a given sample that may differ from one another by 100% or more. Data from our laboratory (*see* Table 4) show that oxidation-reduction methods may overestimate extract protein concentrations, owing presumably to electron transfer reactions involving nonprotein components *(48).* These results are particularly compelling because of the relatively small

Table 4
Comparison of Ninhydrin, Bradford, and Modified Lowry Protein Results for Hymenoptera Venoms

Sample	Ninhydrin mg/mL	Bradford mg/mL	Modified Lowry mg/mL	Lowry/Bradford ratio
Yellow hornet	1.290	0.339	3.378	10.0
White-faced hornet	1.183	0.321	3.019	9.4
Honey bee	1.020	1.017	3.158	3.1
Yellow jacket	2.153	0.375	3.433	9.2
Paper wasp	0.813	0.383	3.310	8.6

number of components in Hymenoptera venom samples compared to a typical pollen or fungal extract. Deviations from parallelism between extract samples and pure bovine serum albumin (BSA) standards are also observed for many allergens, prompting the inclusion of multiple sample dilutions covering the entire assay dynamic range to produce unbiased mean protein values.

Whereas variations in protein assay results may be related to mechanistic differences, sources of immunoassay variability described in this section appear to be closely linked to the structural requirements for analyte recognition and detection in specific assay formats. Different configurations of materials and reagents can influence productive binding interactions by altering the number, exposure, availability, and specific activity of epitope structures. In nature, it is not unusual for the binding of an analyte at one site on a macromolecule to induce conformational changes that promote or restrict interactions at other sites *(52).* In the laboratory, antibodies have been shown to exert the same effects on multivalent or multideterminant antigens *(58).* Although it is likely that analysis of an individual sample by different methods may yield variable results, it is difficult in most cases to determine whether differences in materials, mechanism, format, or a combination of these factors represent the actual source of these variations.

Determining the origins of interassay variabilities is a fundamental goal for improving the reliability and clinical utility of in vitro tests for allergen extracts. An investigation conducted in our laboratory addressed this issue by focusing on comparisons of multiple assays for a single analyte protein (Alt a 1, a major Alternaria allergen) using the same allergen and antibody reagents in varying configurations *(59).* Development of consistent Alternaria extracts and assay results is particularly challenging owing to the heterogeneity of Alternaria strains and culture products from different sources *(60–62).* The antibodies employed for these studies were produced in rabbits to an 16–18 kilodalton hypoallergen fragment of Alt a 1 and employed for ELISA

Table 5
Alt a 1 Reactivities from Five Different Assays Using Identical Allergens and Antibodies

Analysis	Mean Relative Reactivities of Alternaria Extracts				
	M1	M1	M2	M3	M4
	Reference				
ELISA inhibition: Unmodified antibody	1.00	0.94	0.73	0.22	0.52
ELISA inhibition: Biotinylated antibody	1.00	3.10	2.93	0.78	3.54
Double-bind ELISA: Unmodified capture antibody, Biotinylated probe antibody	1.00	0.96	0.17	0.043	0.004
Radial immunodiffusion	1.00	1.00	0.26	0.00	1.60
SDS-PAGE immunoblot: 30–35 kd band intensity	++	++	+/-	+/-	++++

M1, Extracts prepared from raw materials derived from an in-house Alternaria strain.
M2, M3, M4, Extracts prepared from raw materials derived from external Alternaria strains with distinct physical and biochemical properties.

inhibition, double-bind ELISA, radial immunodiffusion, and sodium dodecyl sulfate (SDS)-PAGE immunoblot analyses of extracts from four distinct *Alternaria alternata* strains *(63,64).* Relative reactivities for each extract (designated M1-M4; *see* Table 5) were determined by parallel-line bioassay from multiple dose-response curves using an in-house, freeze-dried reference extract. Considerable differences in Alt a 1 reactivity were observed from one method to another, with only two assays (radial immunodiffusion and Western blot) displaying related patterns of reactivity for all five extracts. For assay systems employing a single antibody and allergen reagent, expressions of varying reactivities are likely to result from differences in adsorption or presentation of allergenic structures on solid phases, differences in the size, conformation, or determinant valency of target allergens, and changes in antibody specificity, avidity, or structural requirements for antigen binding in various assay formats. Ongoing studies comparing in vitro test results (*see* Table 5) with in vivo sensitivities of these extracts are critical to identifying the methods and extracts whose characteristics coincide closely with allergic patient reactions.

Basic Principles and Applications of Analytical Methods

The defining characteristics, advantages, and disadvantages of the quantitative and qualitative methods used routinely for protein and carbohydrate analyses are summarized below. Published reports rep-

resentative of the applications of these methods to analyses of allergen extracts and raw materials are also included. Further details on the materials, procedures, and equipment used for these assays are available from numerous reviews and books that address these subjects specifically *(65–70).*

Isoelectric Focusing

Electrophoretic separations of complex samples in polyacrylamide or agarose gels are perhaps the most widely used analytical procedures in protein biochemistry. The most common methods used to analyze or purify macromolecules are IEF and SDS-PAGE. IEF separations are based on the observation that most biological molecules carry a diverse group of ions in varying amounts within their native structures. At a given pH, proteins contain a variety of cationic and anionic charges attributed to their amino acid side chains. At a particular pH value or range, these positive and negative charges are balanced exactly, producing a net charge of zero and an inability to migrate in an electric field. In IEF, a pH gradient is established in a polyacrylamide or agarose medium by separation of carrier ampholytes, and molecules introduced into this medium will migrate according to their surface charges until they reach a pH at which no net charge remains. At this pH (isoelectric pH or pI), these components can no longer diffuse in either direction and become focused or condensed into a narrow zone. Differences in the types and numbers of charged groups on a molecule produce distinct pI values, and compositional differences result in unique IEF patterns or fingerprints for many samples after visualization of protein bands by Coomassie blue or silver staining. The pI values of sample components are estimated by linear regression analysis of their migration distances relative to standards proteins of established pI.

IEF procedures offer several advantages over SDS-PAGE or other methods. For both methods, gels can be prepared in the laboratory or purchased from commercial sources and can be adapted for immunoblotting procedures. IEF may be more effective at detecting small differences in protein structure or size related to electrostatic changes compared to SDS-PAGE, and is extremely useful for analysis of molecules present in numerous isomeric forms. The main drawback of IEF is a tendency to produce irregular, skewed, or wavy band patterns owing to sensitivities to reagents (age, oxidation) and samples (salts, precipitates). Precipitation is a significant issue because protein molecules at their isoelectric pH can not repel one another effectively and may aggregate to reduce exposure of hydrophobic regions to surrounding water molecules *(54).* IEF profiles of allergens have been reported for Alternaria *(61)*, short ragweed *(71)*, and timothy grass *(72)* samples.

SDS-PAGE

SDS-PAGE analyses provide an effective complement to data from IEF studies. In this method, molecules denatured by treatment with an ionic detergent (SDS) are separated in gels electrophoresed at a constant pH *(73).* Protein charge and size are directly proportional owing to the binding of consistent levels of SDS to protein sequences of a given length. The molecular weights of sample components detected after staining are calculated by regression analysis based on the migrations of molecular weight standards. SDS-PAGE patterns are less susceptible to buffer or sample interferences observed with IEF and routinely display near-perfect alignments of component bands. Chemical reduction of protein disulfide bonds with β-mercaptoethanol or dithiothreitol (DTT) facilitates analyses of oligomeric (multi-subunit) proteins, and reveals fine structural features such as varying levels of protein glycosylation in a predictable manner. Unlike IEF, components possessing similar molecular size but different charges are not likely to be resolved by SDS-PAGE. A combination of IEF and SDS-PAGE separations into a two-dimensional electrophoresis method is a popular and powerful alternative but remains prone to many of the same shortcomings as its component steps. One-dimensional SDS-PAGE has been effective for analysis of mold *(12,45,61)*, pollen *(71,74,75)*, dust mite *(76)*, insect *(77)*, and food (78) allergens. Two-dimensional IEF/SDS-PAGE profiles are reported for tree and grass pollens *(79,80)* and fire ants *(81).*

Immunoblotting

In an immunoblot procedure, sample components separated in IEF or SDS-PAGE gels are transferred by either electric or passive diffusion to nitrocellulose, polyvinylidene difluoride (PVDF), or nylon membranes and then examined for immunological reactivities with human or animal antibodies *(82).* The presence of bound antibodies is confirmed by incubations with a second antibody specific for the antibody source and subclass to be measured. Second antibodies are usually conjugated to enzymes such as alkaline phosphatase or peroxidase, which produce insoluble colored products with particular substrates. Washing of blot membranes with detergent solutions between incubation steps ensures that only high-affinity interactions are detected. In general, the analytical sensitivity of an immunoblot is directly related to antigen load, and although not quantitative in a formal sense, the intensities of individual bands on a blot are roughly proportional to antibody or antigen concentrations. Optimization of transfer media and conditions are often critical to consistent protein recoveries and overall assay sensitivity. The separation of a sample into multiple components prior to incubations with antibodies allows for a definitive identifica-

tion of reactive constituents. The specificity of a blot procedure may be compromised by a number of factors, including excessive antibody concentrations, nonspecific binding to unoccupied sites on the membranes, the presence of antibody complexes, or matrix effects involving probe reagents. SDS-PAGE immunoblotting has been performed extensively on a wide variety of allergen extracts and raw materials, including fungi *(12,45),* pollens *(72,83,84),* dust mites (72), epithelia *(49),* insects *(77),* and foods *(85).* IEF blots are much less common *(86).* Two-dimensional blots have been employed for grass pollen *(87)* and dust mite *(88)* investigations.

Rocket Immunoelectrophoresis

The semi-quantitative nature of immunoblots are shared by IE methods such as rocket immunoelectrophoresis, crossed immunoelectrophoresis (CIE), and crossed radioimmunoelectrophoresis (CRIE). In the rocket IE procedure, samples are applied to wells cut into horizontal agarose gels containing a specific antibody or serum and then electrophoresed to generate flame or rocket-shaped immunoprecipitates after protein staining *(89).* The area or height of the rockets are related to antigen concentration because antigen-antibody precipitates formed in the gel are redissolved by uncomplexed antigens or soluble antigen-antibody complexes until no soluble antigen remains. In most cases, polyclonal rabbit serums are preferred for IE assays compared to mouse or rat MAbs owing to their effectiveness in forming precipitates with antigens. Monospecific antibodies are desired but not required for rocket IE procedures as long as contaminating rocket patterns are minimal or or clearly distinguishable from those of the target allergens. The pH of the gel buffer must be maintained near the isoelectric point of the antibodies to immobilize the immunoglobulins during the electrophoresis step. Rocket IE assays may possess nanogram sensitivities for many antigens but may also be prone to irregular peak shapes and complex dose-response relationships. Reports of allergen analyses using this technique include studies with Alternaria *(61)* and house dust *(90)* extracts.

CIE and CRIE

CIE assays combine conventional agarose electrophoresis in one dimension with a perpendicular second-dimension rocket IE in antibody-containing gel to produce a detailed pattern of overlapping immunoprecipitates. As with rocket IE, peak areas in CIE are related to the concentrations of antigens recognized by the rabbit antibodies embedded in the second-dimension gel. The sensitivity and extraordinary resolving power of CIE provide considerable information concerning the compositions and immunoreactivities of antigen samples.

Precise identification and quantitation of antigenic components may be compromised by the complexity of peak pattern interpretations and the inability of some antibodies to recognize and precipitate all pertinent antigens to a similar degree *(5,66).* CRIE patterns reflect the IgE-binding activities of these precipitates and are produced by autoradiography of unstained CIE gels after incubations with allergic human serum and radiolabeled anti-human IgE *(91).* Additional complications to CRIE results include the potential masking of IgE binding sites by rabbit IgG or anti-immunoglobulins and nonspecific binding of the ^{125}I-labeled anti-IgE. Applications to allergen analyses are numerous and include studies with mold *(60),* mite *(92),* epithelia *(93)*, and grass-pollen *(94)* extracts.

Radial Immunodiffusion

Radial immunodiffusion (RID) analyses are widely used to quantitate both antibody or antigen concentrations *(66).* Similar to IE methods, immunoprecipitates formed in an antibody-containing agarose gel are proportional to the concentrations of target antigens in test samples. In RID, however, antigens migrate radially from wells cut into the gel based on passive diffusion rather than electrophoretic properties. As in IE, free antigen continues to redissolve precipitates and increase precipitin ring diameters until these reactions are complete. After removal of unwanted serum proteins from the gels, protein staining reveals the size and nature of the precipitation reactions. Standard curves relating ring diameters to the logarithm of antigen concentration produce consistent linear regression characteristics provided that diffusion takes place under fixed time and temperature conditions. Expensive or sophisticated equipment is not required for this assay, and very few steps or variables are involved. Unfortunately, this method is relatively insensitive compared to nearly all other procedures described in this review and requires considerable quantities of rabbit antiserum. The specificity of the serums must also directed against individual analytes in order to avoid misinterpretations caused by the presence of multiple antigen-antibody reactions and concentric rings in an RID gel. As noted earlier, RID is currently used to standardize cat and ragweed extracts based on Fel d 1 and Antigen E concentrations, respectively.

ELISA Inhibition

ELISAs are versatile methods used extensively to measure interactions between antigens and antibodies *(70).* The ability of microtitration plates prepared with polystyrene or related materials to adsorb a wide variety of polar and nonpolar molecules offers unparalleled flexibility for development of quantitative assay formats for both antigens and antibodies. Essential materials (plates, pipetters) and equipment (plate

washers, spectrophotometric readers) are common to all assays regardless of format.

The most common formats employed for antigen detection include direct-bind ELISA (antigen adsorption to solid phase probed with antibodies), ELISA inhibition (competition for antibody between soluble and immobilized antigens), and double-bind ELISA (capture of antigen by antibody bound to solid phase). Direct-bind ELISA is used frequently to titrate antigens or antibodies but may not produce accurate and consistent results unless all pertinent antigens are bound to the plates in an orientation conducive to antibody recognition. Quantitative values derived from inhibition and direct-bind ELISA are less reliant on the completeness of immobilization because their reactivities are defined by antigens in solution, but remain sensitive to compositional differences on the solid phase. As a result, these two methods are employed routinely for allergen analyses, with ELISA inhibition results serving as the basis for the standardization of dust mite and grass pollen extracts *(25,26).*

In an ELISA inhibition assay, a reference allergen is immobilized onto a plate and then incubated with a human or animal serum in the presence of variable concentrations of a test allergen. Competition between test and reference allergens for antibody-binding sites is dependent on the structural features and binding requirements of all three reactants. Test allergens of appropriate structure bind to antibodies in a dose-dependent manner and prevent them from binding to reference allergens on the solid phase. After a suitable incubation period, equilibration is established and antibodies not bound to the plate are removed by washing. Bound antibodies are then detected by incubations with probe reagents (e.g., anti-human IgE) labeled with an enzyme (alkaline phosphatase or horseradish peroxidase), which produces colored products with defined substrates. In ELISA inhibition, the absorbance values of test samples are inversely proportional to allergen concentration, and potency values are determined by comparison with the inhibitory properties of reference allergens subjected to the same conditions. Selection of optimal concentrations of test and reference extracts is essential to achieve inhibitions ranging from 10–90% of uninhibited controls. Dose-response curves for reference extracts are established by linear regression and relative potencies of test extracts and control samples are determined by parallel line bioassays. Statistical tests (paired t-test) are performed to determine if the degree of parallelism meets strict acceptance criteria. Multiple replicates of each test and reference extract are typically evaluated on a given plate to improve the accuracy and reliability of potency measurements.

ELISA inhibition assays are effective at assessing the properties of multicomponent analytes, and can produce consistent results over long

periods of time if material and reagent qualifications are performed to precise standards. The potential influence of solid phase material changes on test results must be monitored closely to avoid misinterpretations of relative potency or parallelism assessments. In addition, qualifications of new reference extracts are particularly important to this method because of their use as both solid-phase adsorbent and solution-phase control reagents. Published reports of ELISA inhibition results include studies with mold *(12,15)*, pollen *(95)*, ragweed *(71,95)*, and epithelia *(49)* allergens.

Double-Bind ELISA

Double-bind ELISA assays are based on the ability of multivalent antigens to interact with at least two antibody molecules simultaneously. In this format, antigen components are bound initially to antibodies (usually rabbit or mouse IgG) immobilized onto the microplate surface. The bound antigens are then detected by incubations with a second antibody of similar or distinct specificity which is labeled with a small molecule (biotin) or enzyme. The adsorption or coating of immunoglobulins onto microplate surfaces is based on noncovalent binding of antibody regions to surface structures possessing complementary electrostatic and hydrophobic properties. The number of attachment points between an antibody and surface is regulated by the structures and properties of both materials. The large size of IgG antibodies (150,000 daltons) facilitates multi-site binding but may result in complete, partial, or negligible antibody availabilities and activities (matrix effects) depending on the specific conditions employed.

In nearly all cases examined to date, double-bind ELISA assays provide extraordinary increases in analytical sensitivity (10–1,000 fold) compared to ELISA inhibition formats employing the same reagents. These increases may result from the preservation and orientation of antigen epitopes by antibody capture and from the amplification of assay signals owing to involvement of multiple recognition sites on antigen molecules. Assay specificity can be regulated by selection and order of antibody reagents, and sample interferences appear to be low relative to other assay formats. Use of epitope-specific mouse monoclonal capture antibodies with polyclonal rabbit probe antibodies is a convenient and cost-effective approach to achieving the desired balance of assay sensitivity and specificity. As with ELISA inhibition, reagent qualifications are critical to maintaining consistent assay performance, especially those involving coating antibody and microplate, as demonstrated previously (*see* Tables 2 and 3). Allergens analyzed by double-bind ELISA methods include Der 1 and Der 2 from dust mites *(96)*, Bet v 1 from birch pollen *(86)*, group 4 allergens from grass pollens *(84)*, and Fel d 1 from cat *(97)*.

Table 6
Comparison of Ninhydrin and Bradford Protein Results for Standardized Extracts[a]

Extract	Strength	Lots Tested	Ninhydrin mg/mL	Bradford mg/mL	Bradford/ Ninhydrin ratio
Bermuda	10,000 BAU/mL	2	3.31	1.25	0.38
Kentucky blue	100,000 BAU/mL	4	2.99	1.23	0.41
Meadow fescue	100,000 BAU/mL	4	1.05	0.45	0.43
Orchard	100,000 BAU/mL	4	3.02	1.71	0.57
Perennial rye	100,000 BAU/mL	3	3.75	1.30	0.35
Red top	100,000 BAU/mL	4	1.21	0.53	0.44
Sweet vernal	100,000 BAU/mL	3	2.31	0.99	0.43
Timothy	100,000 BAU/mL	4	4.12	2.45	0.59
D. farinae	10,000 AU/mL	3	1.89	0.71	0.38
D. pteronyssinus	10,000 AU/mL	3	2.62	0.60	0.23
Cat hair	10,000 BAU/mL	2	1.78	0.30	0.17

[a]Mean values derived from 2–8 independent measurements for each condition.

Protein and Carbohydrate Determinations

As noted earlier, protein analyses of allergen extracts may produce variable results owing to the presence of nonprotein reactants or interferences in extraction products and notable differences in mechanism between most procedures. However, it is possible in some cases for protein values from different tests to exhibit proportional reactivities. Extracts whose strengths are derived from direct protein measurements (PNU, ninhydrin) are not candidates for alternative methods owing to the obvious impact these changes would have on the concentrations and potencies of current products. For products whose protein concentrations are determined for monitoring or qualitative analyses, a more convenient protein method such as the 1 h Bradford dye binding assay may offer clear cost and time advantages over the 2-d, labor-intensive ninhydrin procedure as long as consistent reactivity ratios are observed. Table 6 summarizes the results of ninhydrin and Bradford protein determinations performed in our laboratory on recent lots of standardized products *(48).* Consistent reaction ratios are observed among the eight grasses, with cat and *Dermatophagoides pteronyssinus* extracts displaying the largest differences. Production of comparable electrophoretic or immunochemical test results for sample dilutions based on corresponding ninhydrin and Bradford protein values is necessary to confirm the suitability of this approach. Several references describe these assay differences in more detail *(98,99).*

Amino acid sequence determinations are essential to detailed structural comparisons of allergens from related sources as well as recombinant products. Examples of these analyses include investiga-

tions with tree pollen *(79)*, grass pollen *(74)*, insect *(100)*, venom *(101)*, and food *(78)* allergens. The homogeneity or purity of native and recombinant protein allergens can be assessed effectively by reversed-phase high performance liquid chromatographic (RP-HPLC) procedures *(71,74)*. Secondary structure analyses of these molecules can provide exquisite details of the α-helix, β-strand, β-turn, and random coil domains critical to the expression of allergenic activities *(102)*.

Carbohydrate analyses have been performed on glycoprotein allergens in order to elucidate the relationships between sugar structures and IgE binding. Compositional analyses are also conducted after hydrolysis to monosaccharides *(74)* or release of asparagine-linked sugars by enzymatic or chemical treatments *(61,83)*.

Conclusions and Future Directions

Determination of the compositions and properties of allergen extracts that coincide with their diagnostic and therapeutic efficacy remains a challenging endeavor. Over the past 10 years, contributions from academic, corporate, and clinical allergy professionals have enhanced our knowledge and understanding of allergen extract compositions and their relationships to clinical outcomes. Although considerable progress remains to be made, a core technology has emerged that should provide a solid foundation for meaningful improvements in allergen production, testing, and treatment.

In most instances, investigations into the physical and biochemical properties of allergens have focused on the protein and glycoprotein constituents rather than the methods used to analyze them. As a result, extract or allergen comparisons are prevalent in the allergy literature but method comparisons are relatively rare. Current quantitative (*see* Table 1) and qualitative (IEF) methods used for FDA-approved standardized extracts are validated across all allergen manufacturers. In general, qualitative analyses are reasonably consistent from laboratory to laboratory owing to the commercial availability of essential materials and equipment systems. By comparison, quantitative or semiquantitative procedures are much less consistent across laboratories and may employ different materials and assay configurations to measure the same analytes. Access to data from individual laboratories addressing the comparability of test results from multiple sites is also highly limited.

Although some analytical methods for allergen extracts are based on direct comparisons with a fixed reference preparation, others employ in-house materials calibrated against previous (and currently nonexistent) lots of reference allergens. Qualifications of new references against a secondary standard (current reference) creates the potential

for material differences and matrix effects to influence assay results. Differences in qualification or validation criteria may also contribute to incidental or unintentional interlaboratory variations. The development or adoption of universal primary allergen standards such as the reference extracts recognized by the World Health Organization (WHO) or other international bodies would be a positive step facilitating the comparisons and correlations of data from a large number of test sites or analysts *(103).* Recent developments in the production and characterization of recombinant proteins possessing primary sequences, three-dimensional conformations, and determinant structures comparable to those of corresponding native allergens support the use of these products as interlaboratory reference or quality control reagents *(4,104).*

It is clear that improving the definition, qualification, and availability of these references or other critical assay reagents can play a major role in minimizing variations in test methods or results related to material differences. Efforts focusing on interlaboratory standardization of qualification procedures and criteria for allergens, antibodies, probe reagents, and solid-phase materials are likely to provide even further benefits. Organized quality control programs governing the proficiency and management of test procedures, data reductions, statistical analyses, quality practices, reporting, and documentation are currently in place for clinical chemistry laboratories and may serve as a blueprint for similar objectives among allergy testing laboratories *(28,105,106).*

Owing to contributions from a wide range of allergy professionals, our collective knowledge of the biochemical and clinical properties of allergens continues to grow at a rapid pace. Development of effective analytical methods has resulted from the talents and efforts of a large number of individuals or research groups working independently in most cases. Collaborative investigations and objective interlaboratory comparisons possess great potential for establishing unified standards, criteria, and practices for the development and validation of test methods and allergy products whose essential properties are closely related to allergic patient sensitivities.

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