FDA Briefing Document

190th Meeting of the Vaccines and Related Biological Products Advisory Committee

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Topic II: Advancing CBER's Allergen Extract Standardization Program

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GLOSSARY

CBER	Center for Biologics Evaluation and Research
cELISA	Competitive ELISA
CFR	Code of Federal Regulations
DNA	deoxyribonucleic acid
ELISA	enzyme-linked immunosorbent assay
FARE	Food Allergy Research and Education
FDA	U.S. Food and Drug Administration
HDM	house dust mites
ID50EAL	Intradermal Dilution for 50 mm sum of Erythema Intradermal Dilution determines the bioequivalent ALlergy Units
IgE	immunoglobulin E
LC/MS/MS	Liquid chromatography tandem mass spectrometry
mAb(s)	monoclonal antibody(ies)
MRM	Multiple Reaction Monitoring
PR10	pathogenesis-related protein family 10
PRM	Parallel Reaction Monitoring
RID	radial immunodiffusion
sELISA	sandwich ELISA
U.S.	United States

EXECUTIVE SUMMARY

The Center for Biologics Evaluation and Research (CBER) proposes a comprehensive modernization strategy for its allergen extract standardization program, which has remained fundamentally unchanged for over 25 years. Currently, only 19 allergen extracts are standardized in the United States (U.S.) (see Appendix 1), with the most recent standardizations completed in 1997-1998 for grass pollen extracts. This 25-year modernization gap has occurred despite remarkable advances in allergenic protein characterization, analytical technology development, and our understanding of allergen biology.

Pursuant to 21 CFR 680.3, CBER proposes to modernize its standardization approach through four key initiatives. First, we seek to replace outdated analytical methods with advanced immunoassay technologies, specifically transitioning from radial immunodiffusion (RID) assays to sandwich Enzyme-Linked Immunosorbent Assay (ELISA) for cat allergen standardization and implementing novel aptamer-based assays for ragweed pollen extracts. Second, we propose expanding the standardization program to encompass food allergens and additional environmental allergens using validated immunoassay templates. Third, we recommend implementing liquid chromatography tandem mass spectrometry (LC/MS/MS) for comprehensive characterization of complex allergen extracts that cannot be adequately represented by single-allergen measurements. Fourth, we advocate for optimization of house dust mite extract source materials to better represent clinically relevant allergen exposure.

The regulatory imperative for these changes has been underscored by recent critical safety failures. In 2022, four lots of peanut extract were recalled due to inadequate allergen content, resulting in false-negative diagnostic tests and subsequent anaphylactic reactions in peanut-allergic patients. Similarly, a 2023 recall of pecan extract revealed complete loss of immunoreactivity, leading to systematic false-negative diagnostic results. These incidents highlight the urgent need for comprehensive standardization to ensure patient safety and diagnostic reliability.

The proposed modernization strategy for allergen extract standardization is supported by extensive scientific validation, industry consensus, and demonstrated technical feasibility. All three U.S. allergen extract manufacturers have committed to continued product marketing following standardization implementation, despite the additional regulatory burden. The phased implementation approach, spanning five years, ensures minimal market disruption while maximizing public health benefits through enhanced product quality, clinical effectiveness, and patient safety.

REGULATORY FRAMEWORK AND CURRENT STATE

Allergen Extract Classification and Regulatory Authority

Allergen extracts are classified as biological products under FDA regulatory authority and serve dual clinical purposes in the diagnosis and treatment of IgE-mediated allergic diseases. For diagnostic applications, these extracts are administered via skin prick testing or intradermal injection to identify specific allergen sensitivities. Therapeutically, allergen extracts are used in subcutaneous immunotherapy to

desensitize patients to environmental allergens and reduce allergic symptoms. A critical safety restriction applies to food allergen extracts, which are approved exclusively for diagnostic skin prick testing due to the severe risk of anaphylaxis associated with intradermal or subcutaneous administration.

The regulatory framework governing allergen extracts establishes two distinct classification categories that reflect fundamentally different quality standards and clinical reliability profiles:

Non-standardized extracts, which constitute the majority of available products, operate without FDA-mandated quality standards for lot release. Their potency is expressed using protein nitrogen units per milliliter or extraction ratios, measurements that provide no assurance of protein integrity or immunological activity. This regulatory gap results in significant lot-to-lot variability and compromises clinical reliability.

Standardized extracts, currently limited to 19 products (see Appendix 1), operate under a comprehensive quality framework that includes common potency units referenced to FDA-designated standards, mandatory potency testing using FDA-provided reference reagents, and consistent quality standards across all manufacturers. This standardization ensures enhanced clinical reliability and safety through rigorous quality control measures that are absent from non-standardized products.

Historical Context, Standardization Gap, and Current State

The current standardization program reflects regulatory decisions made during the 1980s and 1990s when scientific understanding of allergen biology was limited. The last allergen extract standardizations occurred in 1997-1998 for grass pollen extracts, creating a 25-year modernization gap that has persisted despite extraordinary scientific advances. During this period, researchers have identified and characterized hundreds of allergenic proteins, determined three-dimensional protein structures, developed sophisticated analytical technologies, and established the availability of human monoclonal antibodies (mAb) from allergic donors.

The emergence of quantitative mass spectrometry platforms has revolutionized protein analysis capabilities, enabling comprehensive proteomic characterization that was previously impossible. These scientific developments create both an unprecedented opportunity and a regulatory imperative to modernize standardization approaches to improve public health outcomes. The current regulatory framework, while historically appropriate, no longer reflects the state of scientific knowledge or available analytical capabilities.

PROPOSED MODERNIZATION STRATEGY

Proposed Initiative 1: *Updating Major Allergen Potency Standardization*

Human Monoclonal Antibody-based Approach: Major Allergen of Cat Dander and Pelt Extracts

The current radial immunodiffusion (RID) assay for quantification of Fel d 1[1], a major allergen in cat dander and pelt allergenic extracts, presents multiple operational and technical challenges that compromise analytical efficiency and reliability. This labor-intensive methodology requires specialized expertise that is increasingly difficult to maintain, depends on equipment that is no longer manufactured, and provides limited precision and reproducibility compared to modern analytical standards. The extended analysis time required for RID also affects manufacturing efficiency and regulatory compliance timelines.

CBER proposes implementing sandwich ELISA technology using high-affinity human mAbs that offer significant advantages over current methods (see Appendix 2 for detailed review). These antibodies, derived from naturally allergic donors, ensure biological relevance through their origin in the human immune response to cat allergens. The high affinity characteristics result from in vivo affinity maturation processes, providing superior binding characteristics compared to traditional reagents. Additionally, these antibodies demonstrate consistent performance characteristics and offer unlimited supply potential through recombinant production technologies.

Comprehensive validation studies conducted by CBER have demonstrated strong correlation between sandwich ELISA and RID measurements, with correlation coefficients exceeding 0.95. The improved precision of the sandwich ELISA method shows coefficient of variation values below 10 percent, compared with greater than 20 percent for RID. Enhanced reproducibility across multiple operators and reduced analysis time from days to hours further support the technical superiority of this approach.

Implementation, pending committee endorsement and successful validation completion, represents a significant regulatory advancement—a transition from current bioequivalent allergy units per milliliter to mass concentration units expressed as micrograms per milliliter, using purified Fel d 1 as the reference standard. This change simplifies potency expression and improves international regulatory harmonization, e.g., harmonization with European Pharmacopoeia 10.4. Allergen Products, 01\2019:1063.

DNA Aptamer-based Approach: Major Allergen of Short Ragweed Pollen Extracts

The absence of available human mAbs specific for Amb a 1, a major allergen of short ragweed pollen, necessitates an innovative approach to ragweed pollen

[1] Allergen names are based on the scientific name of the plant or animal species; the first three letters are from the genus and the fourth letter is from the species. For example, the major allergen from the house cat, *Felis domesticus*, is Fel d 1. The number is chosen either in order of discovery of the protein unless the allergen is related (often cross-reactive) to another allergen from a different species, in which case it is often assigned the same number. For example, Der p 1 is a cysteine protease from the house dust mite *Dermatophagoides pteronyssinus*; cysteine proteases from other house dust mite species are assigned the number 1, and together they are referred to as Group 1 house dust mite allergens.

standardization. CBER has contracted for the development of DNA aptamer pairs that offer unique advantages over traditional antibody-based methods (see Appendix 2 for detailed review). These synthetic oligomers eliminate biological variability associated with antibody production, provide cost-effective manufacturing and quality control options, and achieve picomolar binding affinities comparable to high-quality antibodies without requiring animal or human-derived materials.

The aptamer selection process, in its final stages of completion, from ~10^{14} candidate sequences (1), represents a comprehensive screening approach that ensures optimal binding characteristics. The current development phase focuses on binding affinity optimization and identification of non-competitive aptamer pairs suitable for sandwich assay applications.

While aptamer technology is well-established in research applications, this represents the first regulatory implementation for allergen standardization, requiring comprehensive validation protocols to address this novel application. The risk assessment acknowledges this pioneering aspect while emphasizing the robust validation framework developed to ensure regulatory compliance and analytical reliability. Similar to cat allergen standardization, potency units will transition to mass concentration expressed as micrograms per milliliter using purified Amb a 1 as the reference standard.

Proposed Initiative 2: *Expanding Standardization Program to Include Food Allergens and Additional Environmental Allergens*

Food Allergens

Recent product failures have created an urgent clinical safety imperative for food allergen standardization. The 2022 peanut extract recalls involved four manufacturing lots containing less than 10 percent of expected allergen content, resulting in false-negative skin tests in peanut-allergic patients and subsequent anaphylactic reactions following peanut consumption[2]. The 2023 pecan extract recall demonstrated complete loss of immunoreactivity in distributed lots, causing systematic false-negative diagnostic results with delayed recognition due to the absence of standardization requirements[3].

The 2024 Food Allergy Research and Education (FARE) Clinical Development Day achieved unprecedented stakeholder consensus regarding the need for immediate food allergen standardization. Academic experts endorsed standardization as a critical safety measure, while all three U.S. manufacturers committed to continued product marketing post-standardization despite the additional regulatory burden. Patient advocacy groups prioritized safety considerations over potential cost implications, and CBER representatives confirmed technical feasibility for implementation.

[2] Voluntary Lot Withdrawals of Allergenic Extract – Peanut (Arachis hypogaea)- For Diagnostic Use Only, Manufactured by ALK-Abelló, Inc. for Increased Reports of False Negative Test Results | FDA

[3] Voluntary Lot Withdrawal of Allergenic Extract – Pecan nut (Carya illinoinensis) – For Diagnostic Use Only, Manufactured by ALK-Abelló, Inc. for Increased Reports of False Negative Test Results | FDA

Technical readiness for food allergen standardization is supported by the availability of human IgE mAbs for major food allergens (see Appendix 3 for detailed review). These include antibodies specific for peanut allergens Ara h 1, Ara h 2, Ara h 3, and Ara h 6, various tree nut species-specific allergens, milk allergens Bos d 4, Bos d 5, and Bos d 8, and egg allergens Gal d 1, Gal d 2, and Gal d 3. The implementation strategy employs a phased approach that prioritizes highest-risk allergens based on anaphylaxis frequency and documented extract quality issues.

Environmental Allergen Expansion

Structural biology advances have revealed significant homology among related environmental allergens, enabling efficient standardization approaches that leverage shared molecular characteristics (see Appendix 3 for detailed review). The pathogenesis-related protein family 10 serves as an exemplary case study, including major allergens from birch pollen, oak pollen, chestnut, European beech, hornbeam, and alder pollens. Sequence and structural analysis demonstrate greater than 80 percent amino acid sequence identity among family members, conserved three-dimensional protein folds, similar IgE-binding epitopes, and documented clinical cross-reactivity patterns.

We are evaluating two potential standardization strategies are under evaluation for the pathogenesis-related protein family 10 (PR10). The universal reagent approach would employ a single mAb pair for all PR10 allergens, while the hybrid approach would use a shared capture antibody with allergen-specific detection reagents. Clinical validation through cross-reactivity studies in birch and oak allergic patients supports the biological basis for either approach. The scientific opportunity presented by structural homology extends beyond the PR10 family to other allergen groups, potentially enabling efficient expansion of standardization to multiple related environmental allergens.

Proposed Initiative 3: *Complex Extract Characterization by Liquid Chromatography Tandem Mass Spectrometry (LC/MS/MS)*

Current Method Limitations

The existing standardization paradigm relies on measuring one or two major allergens, defined as those recognized by greater than 50 percent of sensitized patients, with the assumption that these measurements represent overall extract potency. This single-allergen approach has significant limitations when applied to complex extracts containing multiple major allergens of comparable clinical importance, significant inter-patient variability in allergen recognition, absence of clearly immunodominant proteins, and compositional differences not reflected in overall potency measurements.

Current approaches for complex extracts include Intradermal Dilution for 50 mm sum of Erythema determines the bioequivalent ALlergy units (ID50EAL) testing, which involves intradermal injection of serial dilutions in highly allergic subjects to determine the concentration producing 50-millimeter erythema response, with arbitrary assignment of 100,000 bioequivalent allergy units. The surrogate competitive ELISA (cELISA) uses pooled human sera from 10 to 15 allergic donors in a competitive

format between plate-bound reference extract and test extract, with inverse correlation between signal and extract concentration.

Critical limitations of these approaches include the ethical and practical infeasibility of ID50EAL testing for new standardizations, the inability of cELISA to detect compositional differences among extracts, human sera variability affecting long-term assay consistency, and the possibility that overall potency may not predict therapeutic efficacy for individual patients. These limitations compromise the ability to ensure consistent product quality and optimal therapeutic outcomes.

LC/MS/MS as a Transformative Analytical Platform

Liquid chromatography tandem mass spectrometry represents a transformative analytical platform that enables comprehensive proteomic characterization through systematic workflow processes (see Appendix 4 for detailed review). The technical approach involves proteolytic digestion of allergen extracts, liquid chromatography separation by hydrophobicity, tandem mass spectrometry for peptide identification, and database matching for protein identification and quantification.

Parallel reaction monitoring, an advanced quantification method using heavy isotope-labeled internal standards, provides absolute quantification of individual allergens with exceptional precision across six orders of magnitude. This approach enables reproducible measurements over extended time periods and comprehensive allergen profiling capabilities that far exceed current analytical methods. Optimization of house dust mite extract source materials (see Appendix 5 for detailed review) provides a proof-of-concept for implementing LC/MS/MS as an analytical platform.

The regulatory advantages of mass spectrometry implementation include detection of clinically relevant compositional differences, enhanced quality control and manufacturing oversight, objective instrument-based measurements, reduced dependence on biological reference materials, and international harmonization potential. Clinical benefits encompass personalized therapy guidance through component-resolved diagnostics, improved understanding of extract-to-extract variability, enhanced prediction of therapeutic efficacy, and better characterization of manufacturing consistency.

Proposed Initiative 4: *Optimization of House Dust Mite Extract Source Materials*

LC/MS/MS Proof-of-Concept Validation Studies

Comprehensive analysis of *Alternaria alternata* using liquid chromatography tandem mass spectrometry has revealed distinct proteomic profiles between spores and hyphae, with differential allergen distribution across developmental stages that creates optimization opportunities for allergen content enhancement (see Appendix 5 for detailed review). Commercial extract comparison among three U.S. manufacturer products demonstrated significant qualitative and quantitative variations, with the major allergen Alt a 1 primarily present in growth media rather than fungal biomass. Most clinically relevant allergens are concentrated in non-germinating spores, suggesting that current manufacturing approaches may not optimize allergen content for clinical effectiveness.

Optimization of House Dust Mite Extracts

House dust mite (HDM) source material analysis has revealed significant findings that challenge current manufacturing practices. Current production methods use mite bodies exclusively, excluding fecal pellets based on 1987 advisory committee recommendations that preceded current understanding of HDM allergen biology. Scientific rationale for reevaluation includes recognition that fecal pellets are the primary source of inhaled allergens in clinical exposure, contain concentrated digestive enzymes that are major allergens, and demonstrate size compatibility with lower airway deposition.

Comparative analysis using liquid chromatography tandem mass spectrometry revealed significantly higher concentrations of Der p 1 and Der p 23 in fecal pellets, distinct allergen profiles between body and fecal extracts, and evidence that current commercial extracts may not adequately represent clinical exposure patterns. Including fecal pellets in source material would better mimic natural allergen exposure and potentially improve both diagnostic accuracy and therapeutic efficacy.

VRBPAC VOTING QUESTIONS AND SUPPORTING EVIDENCE

Question 1: *Scientific Soundness of Mass Concentration Measurements*

The committee is asked: *Does measurement of mass concentrations by ELISA of their major allergens provide a scientifically sound approach for expressing and reporting potencies of cat hair and pelt allergen extracts, and of short ragweed pollen allergen extracts?*

Supporting evidence includes published validation data demonstrating strong correlation with correlation coefficients greater than 0.95 between sandwich ELISA and radial immunodiffusion methods, improved precision with coefficient of variation less than 10 percent versus greater than 20 percent for radial immunodiffusion, and international precedent through European Pharmacopoeia use of mass concentration units.

The simplified potency expression facilitates clinical interpretation while enhancing international harmonization potential. Mass concentration units represent standard practice for other biologic products including vaccines and therapeutic proteins, establishing regulatory precedent for this approach. Risk assessment indicates minimal transition risk given demonstrated method correlation and improved analytical performance compared to current methods.

Question 2: *Appropriateness of Revised Assays for CBER's Allergenic Standardization Program*

The committee is asked: Are the revised assays for cat hair/pelt and ragweed pollen allergen extracts scientifically appropriate templates for expanding CBER's allergenic standardization program to include major food allergens and environmental allergens?

Supporting evidence includes the availability of human IgE mAbs for major food allergens, structural homology among related environmental allergens supporting

shared reagent approaches, successful proof-of-concept studies demonstrating technical feasibility, industry consensus supporting expansion to food allergens, and clinical safety imperative based on recent product recalls.

Technical readiness is confirmed by reagent availability and analytical platform maturity, supporting immediate expansion initiation. Stakeholder support includes unanimous manufacturer commitment to continued product marketing post-standardization, demonstrating industry confidence in the proposed approach and willingness to invest in enhanced quality standards.

Question 3: *LC/MS/MS Analytics to Improve Product Quality*

The committee is asked: *Does LC/MS/MS technology, compared with the currently used analytic technology, provide sufficient fit-for-purpose analytical capability for better characterization of complex allergen extracts to improve product quality?*

Supporting evidence includes comprehensive proteomic characterization versus single-allergen measurements, detection of clinically relevant compositional differences among extracts, superior precision and reproducibility compared to biological assays, reduced dependence on variable human sera, and successful implementation in other FDA centers for protein characterization.

Regulatory precedent establishes liquid chromatography tandem mass spectrometry as the analytical standard for protein therapeutics in both CDER and CBER. Clinical relevance is demonstrated by compositional differences detected by mass spectrometry that correlate with variable patient responses to nominally equivalent extracts, supporting the clinical utility of enhanced characterization capabilities.

Question 4: *House Dust Mite (HDM) Source Material Optimization*

The committee is asked: *Do the available data support inclusion of both house dust mite (HDM) bodies and fecal pellets as source materials for HDM allergen extracts to more adequately mimic clinically relevant allergen exposure?*

Supporting evidence includes liquid chromatography tandem mass spectrometry analysis demonstrating 18-fold higher Der p 1 concentration in fecal pellets, recognition that fecal pellets are the primary source of inhaled allergens in natural exposure, size compatibility with lower airway deposition, enhanced representation of clinical allergen exposure, and potential improvement in diagnostic accuracy and therapeutic efficacy.

Exclusion of fecal pellets as HDM source material in 1987 predated current understanding of house dust mite allergen biology and clinical exposure patterns. Safety considerations indicate no additional safety risks, as fecal pellets are natural components of household dust allergen exposure that patients encounter in their daily environment.

CONCLUSION

CBER's proposed modernization of allergen extract standardization represents a comprehensive, science-based approach to addressing critical public health needs

that have been incompletely served by current regulatory norms. The integration of advanced analytical technologies, expanded standardization scope, and optimized source materials holds significant potential to enhance product quality, clinical effectiveness, and patient safety through evidence-based improvements to regulatory oversight.

The proposed initiatives are supported by strong scientific rationale based on 25 years of allergenic protein research that has transformed understanding of allergen biology and analytical capabilities. Demonstrated technical feasibility through proof-of-concept studies confirms the practical viability of proposed methods, while industry consensus and commitment to implementation ensures successful adoption across the manufacturing sector.

Regulatory precedent from other FDA centers and international authorities provides established frameworks for similar analytical improvements, while clear clinical imperative based on recent product safety failures demonstrates the urgent need for enhanced oversight. VRBPAC endorsement of these proposals would provide external scientific validity for CBER to modernize its allergen extract standardization program, ensuring continued public health protection while facilitating innovation in allergy diagnosis and treatment.

The transformation of allergen extract standardization from a static, largely outdated system to a dynamic, science-based regulatory framework represents a critical advancement in FDA's mission to protect and promote public health. Through these modernization efforts, CBER will significantly advance allergen extract regulation and more reliably ensure that patients receive the highest quality diagnostic and therapeutic products available, while enhancing public trust.

Appendix 1 – Current Standardized Allergen Extracts

Table 1. Standardized Allergen Extracts in the United States[a]

Allergen Extract	Potency tests[b]	Additional Lot Release Tests[c]	Labeled Unitage
Dust mite *(Dermatophagoides farinae)*	Competitive ELISA	Protein	AU/mL (equivalent to BAU/mL)
Dust mite *(Dermatophagoides pteronyssinus)*			
Cat pelt *(Felis domesticus)*	Fel d 1 (RID)	IEF Protein	BAU/mL 5-9.9 Fel d 1 U/mL = 5000 BAU/mL; 10-19.9 Fel d 1 U/mL = 10 000 BAU/mL
Cat hair *(Felis domesticus)*			
Bermuda grass pollen *(Cynodon dactylon)*	Competitive ELISA	IEF Protein	BAU/mL
Red top grass pollen *(Agrostis alba)*			
June (Kentucky blue) grass pollen *(Poa pratensis)*			
Perennial ryegrass pollen *(Lolium perenne)*			
Orchard grass pollen *(Dactylis glomerata)*			
Timothy grass pollen *(Phleum pratense)*			
Meadow fescue grass pollen *(Festuca elatior)*			
Sweet vernal grass pollen *(Anthoxanthum odoratum)*			
Short ragweed pollen *(Ambrosia artemisiifolia)*	Amb a 1 (RID)		Amb a 1 units
Yellow hornet (*Dolichovespula arenaria*)	Hyaluronidase and phospholipase activity		µg protein
Wasp (*Polistes* species)			
Honey bee *(Apis mellifera)*			
White faced hornet (*Dolichovespula maculate*)			
Yellow jacket (*Vespula* species)			
Mixed vespid (*Vespa* + *Vespula* species)			

[a]Adapted from Middleton's Allergy: Principles and Practice, Ninth Edition; 2020
[b]CBER supports these tests by distributing reference reagents to the manufacturers.
[c]Tests for informational purposes only.

Appendix 2 – Replacement of RID with ELISA

Replacement of radial immunodiffusion (RID) assays of currently standardized extracts with ELISA or aptamer-based enzymatic assays.

Some allergen extracts are standardized by levels of their dominant or "major allergen," which is traditionally defined as an allergen that is recognized by >50% of sensitized patients (2). The major allergens of cat dander and pelt and short ragweed pollen extracts are Fel d 1 and Amb a 1, respectively, which are currently measured with the RID assay, which is labor-intensive, cumbersome, and relies on equipment that is no longer manufactured. In 2011, the Allergenic Products Advisory Committee (APAC)[4] endorsed LIB's intent to transition to a sandwich ELISA (sELISA) in which the allergen is sandwiched between a plate-bound capture antibody and an enzyme-tagged revealing antibody. However, using the mAb available at the time, the assays were not suitably precise or reproducible, the change has not been implemented.

Recently, scientists from academia and industry have cloned allergen-specific IgE mAbs from highly allergic donors (3, 4). These mAb, whether as IgE or class-switched in vitro to IgG_4 (to use as a therapeutic agent) are inherently biologically relevant to human allergic disease and, as expected for antibodies that are the product of affinity maturation, are high affinity. Perceiving an opportunity for high quality reagents to advance CBER's standardization program, LIB tested and then licensed two of these high affinity human mAb to use in an sELISA for measurement of Fel d 1, and published data (Figures 1A-B) comparing measurements from the sELISA and RID assays (5). Since publishing those data, we have been improving the assay to meet the requirements of accuracy, precision, reproducibility, and robustness necessary to transfer the technology to the manufacturers and implement for lot release and labeling (Figure 1C). The process of validation, technology transfer to the manufacturers, and implementing the assay for lot releases will be presented to the committee.

For short ragweed pollen extract, human mAbs are not available. We therefore considered this an opportunity to generate a sandwich enzymatic assay in which, rather than mAb, the capture and revealing reagents are DNA aptamers. Aptamers, sometimes referred to as "chemical antibodies," are synthetically produced peptide or nucleic acid oligomers that bind to target molecules often with picomolar affinities. The process of identifying aptamer pairs involves rounds of selection from a starting pool of ~10^{14} randomly sequenced ssDNA oligomers (1). Advantages of aptamers over mAbs are that no animals or human blood are necessary, and synthesis of DNA aptamer pairs is cheap. In 2024, CBER contracted for identification of a pair of DNA aptamers that can function in an enzymatic assay. At time of submission of this document, candidates for aptamers have been identified. Pairs of aptamers for the assay will be selected based on affinity and after ensuring that they do not block each other.

Currently, the reference reagent to measure Fel d 1 and Amb a 1 is cat hair or short ragweed extract, respectively, purchased from one of the US allergenic extract

[4] APAC has since been disbanded, and its responsibilities have been incorporated into VRBPAC.

manufacturers. For the cat allergen sELISA and the ragweed pollen aptamer-based assay, the reference reagents will be purified Fel d 1 and Amb a 1 purchased from a qualified vendor (an extract will be included in the assay as a positive control). Consequently, rather than using the current unitage, BAU/mL and Fel d 1 units for cat extract and Amb a 1 units for ragweed, CBER proposes to simplify the potency units to the concentration (mcg/mL) of these allergen proteins.

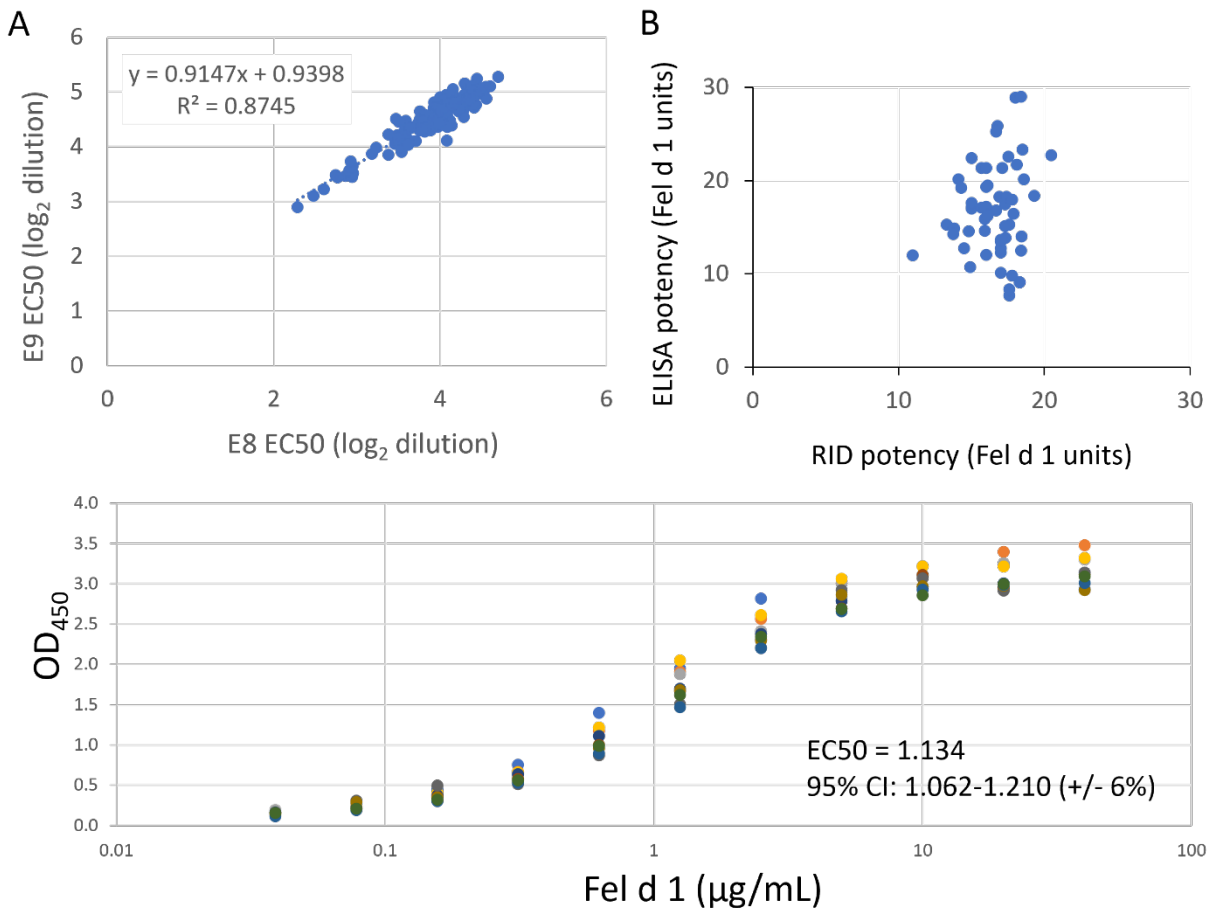


Figure 1. ELISA for cat allergen Fel d 1 using human IgE (class-switched to IgG4) mAbs. A: Ratio of potencies of two reference cat hair extracts is consistent over multiple replicates. B: Comparison of RID and ELISA values. C: Six replicates of naturally purified Fel d 1 showing increased precision after optimizing the sELISA (CBER Reference Reagent Laboratory, unpublished data). Figures A and B are from (5).

Appendix 3 – Food and Environment Allergen Extracts

Expanding CBER's standardization program to food allergen extracts and additional environmental allergen extracts.

Diagnosis and treatment of allergic disease with allergen extracts depends entirely on the quality of the extracts. Non-standardized extracts have no potency standards for lot release. Whether their unitage is mass/volume (protein nitrogen units/mL) or extraction ratio (w/v), there is no assurance that proteins are intact or immuno-active. Extracts with insufficient allergen are obviously ineffective but can also present a safety issue. In 2022, two lots of peanut extract had little allergen (Figure 2) (6); peanut-allergic patients were falsely informed that they were not allergic and experienced anaphylaxis after ingesting peanuts or food containing peanut protein[5]. In 2023, a manufactured lot of pecan extract was recalled because it similarly lacked immunoreactivity and was giving false-negative skin tests[6].

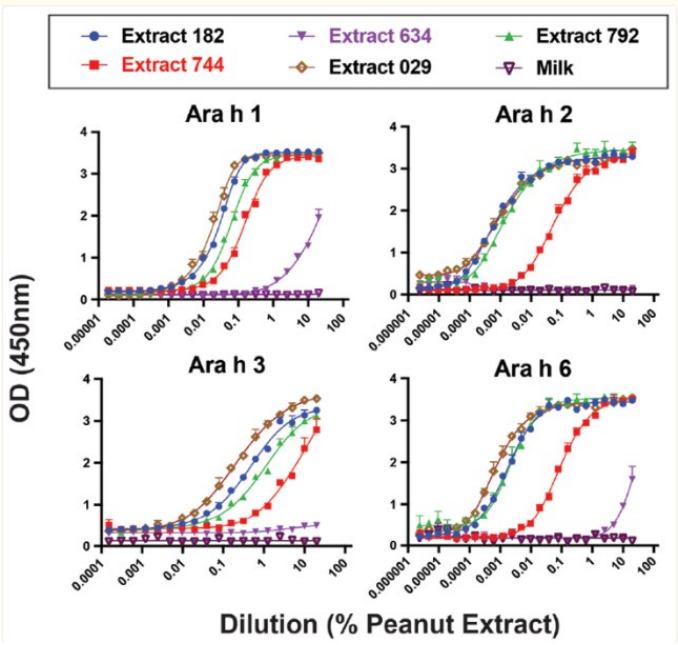


Figure 2. Peanut extracts with little or no immunoreactivity. ELISA using human IgE mAb binding to allergen proteins across two recalled lots of peanut allergen extract (lots 744 & 634) and three non-recalled lots of peanut allergen extract (lots 182, 029, & 792) for Ara h 1, Ara h 2, Ara h 3, Ara h 6. The dilution series for each extract begin with 1:5 diluted extract (20% dilution) followed by serial 1:2 dilutions. Note the variability of the three non-recalled lots in concentrations of Ara h 1 and Ara h 3. Copied from (6).

Following these events, Food Allergy Research and Education (FARE), a prominent advocacy non-profit, added a workshop focused on standardization of food allergen extracts to its 2024 "Clinical Development Day" meeting. Academic experts, two CBER representatives (RL Rabin and MB Strader) and each of the three US manufacturers attended the meeting. There was general agreement that standardization of food allergen extracts is necessary to ensure safe and effective diagnostic products, and that the necessary reagents—human IgE mAb—are

[5] Voluntary Lot Withdrawals of Allergenic Extract – Peanut (Arachis hypogaea)- For Diagnostic Use Only, Manufactured by ALK-Abelló, Inc. for Increased Reports of False Negative Test Results | FDA

[6] Voluntary Lot Withdrawal of Allergenic Extract – Pecan nut (Carya illinoinensis) – For Diagnostic Use Only, Manufactured by ALK-Abelló, Inc. for Increased Reports of False Negative Test Results | FDA

available and can serve that purpose. Most importantly, despite the additional burden for lot release, all three manufacturers welcomed standardization and committed to continue to market food allergen extracts after they are standardized.

In addition to food allergen extracts, we can also exploit current technologies and scientific knowledge to expand the number of standardized environmental allergens. For example, the major allergens of birch pollen (Bet v 1) and white oak pollen (Que a 1) have been cloned and their tertiary structure has been determined. These two allergens belong to the "Bet v 1 like," or PR10 family that is present in all oak pollens, chestnut, European beech, hornbeam and alder pollens (2). The sequence and structural similarities among these six PR10 family allergens are shown in Figures 3 and 4 respectively.

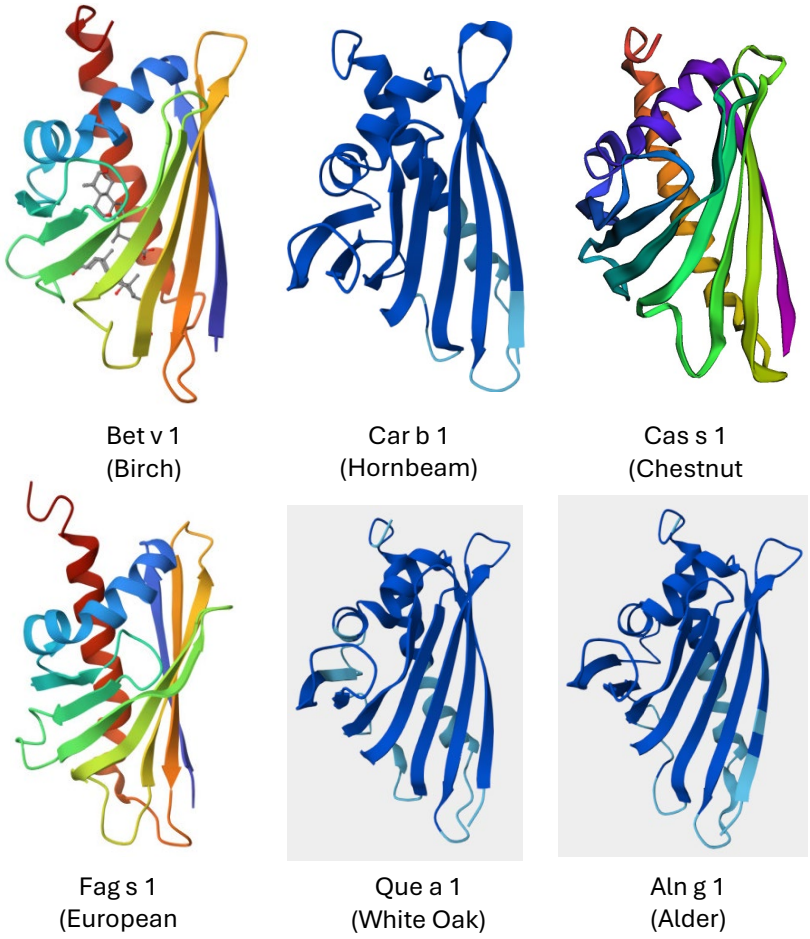


Figure 3. Structural similarity among six PR10 family allergens. (Car b 1 allergen structure determined with Alphafold; all others were determined experimentally.)

The implications of these high levels of sequence and structural similarity are that it may be possible that one pair of mAb or aptamers can be used to standardize multiple related extracts. Alternatively, one mAb may serve as a "capture" mAb, but we may require more than one "detection" mAb (or aptamer). The likelihood that this is a successful strategy is predicted by cross-sensitivities observed, for example, of patients who are allergic to both birch and oak pollens (7-9).

Figure 4. High sequence identity/similarity among six PR10 family allergens

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
Birch	Bet v 1	M	G	V	F	N	Y	E	T	E	T	T	S	V	I	P	A	A	R	L	F	K	A	F	I	L	D	G	D	N	L
Hornbeam	Car b 1	-	-	-	-	-	-	-	A	-	-	P	-	-	-	-	-	-	-	-	-	-	S	Y	V	-	-	-	-	K	-
Chestnut	Cas s 1	-	-	-	-	T	H	-	N	-	I	-	-	A	-	-	P	G	-	-	-	-	-	-	V	-	-	A	-	-	-
European Beech	Fag s 1	-	-	-	-	T	-	-	S	-	-	-	T	-	-	T	P	-	-	-	-	-	-	-	V	-	-	A	-	-	-
White Oak	Que a 1	-	-	-	-	T	-	-	S	-	D	A	-	-	-	-	P	-	-	-	-	-	-	-	V	-	-	S	-	-	-
Alder	Aln g 1	-	-	-	-	-	-	-	A	-	-	P	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	K	-

		31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60
Birch	Bet v 1	F	P	K	V	A	P	Q	A	I	S	S	V	E	N	I	E	G	N	G	G	P	G	T	I	K	K	I	S	F	P
Hornbeam	Car b 1	I	-	-	-	-	-	-	V	-	-	-	-	-	-	V	G	-	-	-	-	-	-	-	-	-	N	-	T	-	A
Chestnut	Cas s 1	I	-	-	L	-	-	H	-	-	K	-	A	-	I	-	-	-	-	-	-	-	-	-	-	-	-	-	T	-	G
European Beech	Fag s 1	I	-	-	-	-	-	-	-	-	K	-	S	-	I	-	-	-	S	-	-	-	-	-	-	-	-	-	T	-	G
White Oak	Que a 1	I	-	-	-	V	-	-	-	L	K	-	T	-	I	-	-	-	-	-	-	-	-	-	-	-	-	-	T	-	G
Alder	Aln g 1	L	-	-	-	-	-	E	-	V	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	T	-	-

		61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80	81	82	83	84	85	86	87	88	89	90
Birch	Bet v 1	E	G	F	P	F	K	Y	V	K	D	R	V	D	E	V	D	H	T	N	F	K	Y	N	Y	S	V	I	E	G	G
Hornbeam	Car b 1	-	-	I	-	-	-	F	-	-	E	-	-	-	-	-	-	N	A	-	-	-	-	-	-	T	-	-	-	-	D
Chestnut	Cas s 1	-	-	S	Q	-	-	-	-	-	H	-	I	-	-	I	-	Q	A	-	-	T	-	C	-	-	-	-	-	-	D
European Beech	Fag s 1	-	-	S	Q	-	N	-	M	-	H	-	I	-	-	I	-	N	A	-	-	T	-	A	-	T	L	-	-	-	D
White Oak	Que a 1	-	-	S	H	L	-	H	A	-	H	-	I	-	V	I	-	P	E	-	-	T	-	S	F	-	-	-	-	-	D
Alder	Aln g 1	-	-	S	-	-	-	-	-	-	E	-	-	-	-	-	-	R	V	-	-	-	-	S	F	-	-	-	-	-	-

		91	92	93	94	95	96	97	98	99	100	101	102	103	104	105	106	107	108	109	110	111	112	113	114	115	116	117	118	119	120
Birch	Bet v 1	P	I	G	D	T	L	E	K	I	S	N	E	I	K	I	V	A	T	P	D	G	G	S	I	L	K	I	S	N	K
Hornbeam	Car b 1	V	L	-	-	K	-	-	-	V	-	H	-	L	-	-	-	-	A	-	G	-	-	-	-	V	-	-	-	S	-
Chestnut	Cas s 1	V	V	N	E	L	-	-	-	-	-	Y	-	-	-	-	-	-	S	-	-	-	-	-	-	-	-	N	T	S	-
European Beech	Fag s 1	A	-	S	E	-	-	-	-	-	A	Y	-	-	-	L	-	-	S	-	-	-	-	-	-	-	-	S	T	S	-
White Oak	Que a 1	A	L	F	-	K	-	-	N	V	-	T	-	T	-	-	-	-	S	-	-	-	-	-	-	V	-	S	T	S	-
Alder	Aln g 1	A	V	-	-	A	-	-	-	V	C	-	-	-	-	-	-	-	A	-	-	-	-	-	-	-	-	-	-	-	-

		121	122	123	124	125	126	127	128	129	130	131	132	133	134	135	136	137	138	139	140	141	142	143	144	145	146	147	148	149	150
Birch	Bet v 1	Y	H	T	K	G	D	H	E	V	K	A	E	Q	V	K	A	S	K	E	M	G	E	T	L	L	R	A	V	E	S
Hornbeam	Car b 1	F	-	A	-	-	Y	-	-	-	N	-	-	K	M	-	G	A	-	-	-	A	-	K	-	-	-	-	-	-	-
Chestnut	Cas s 1	-	-	-	-	-	E	Q	-	I	-	E	-	K	-	M	-	G	-	-	K	A	A	G	-	F	K	-	-	-	A
European Beech	Fag s 1	-	-	-	-	-	-	-	-	I	-	E	D	-	I	-	-	G	-	-	E	A	S	G	I	F	K	-	-	-	A
White Oak	Que a 1	-	Q	-	-	-	-	F	Q	L	T	D	-	L	I	R	G	G	-	-	K	A	S	E	V	F	K	-	-	-	A
Alder	Aln g 1	F	-	-	-	-	-	-	-	I	N	-	-	-	I	-	I	E	-	-	K	A	V	G	-	-	K	-	-	-	-

		151	152	153	154	155	156	157	158	159	160
Birch	Bet v 1	Y	L	L	A	H	S	D	A	Y	N
Hornbeam	Car b 1	-	-	-	-	-	T	A	E	-	-
Chestnut	Cas s 1	-	-	-	-	-	-	-	-	-	-
European Beech	Fag s 1	-	-	-	-	N	P	A	-	-	H
White Oak	Que a 1	-	-	V	-	-	P	-	L	-	K
Alder	Aln g 1	-	-	-	-	-	-	-	-	-	-

Appendix 4 – LC/MS/MS Analytical Platform

Implementing tandem mass spectrometry to characterize complex allergen extracts.

In the U.S., 19 allergen extracts (see Appendix 1) are standardized for potency. Allergen extracts discussed in Proposed Initiative 1 are standardized by the quantity of one or two "major allergens," defined as an allergen to which >50% of patients are sensitive. For short ragweed pollen and cat hair, Amb a 1 and Fel d 1 are considered dominant such that their concentrations can represent extract potency. Similarly, hyaluronidase and phosphatase are major allergens that represent the potency of the six standardized Hymenoptera venoms. There are, however, allergen extracts that are not standardized by one or two allergen proteins, either because the allergens had not been identified or characterized at the time the extracts were standardized, because there are more than one or two major allergens that can serve as proxies for extract potency, or because variability among sensitive patients is such that there is no major allergen associated with that extract (Figure 5).

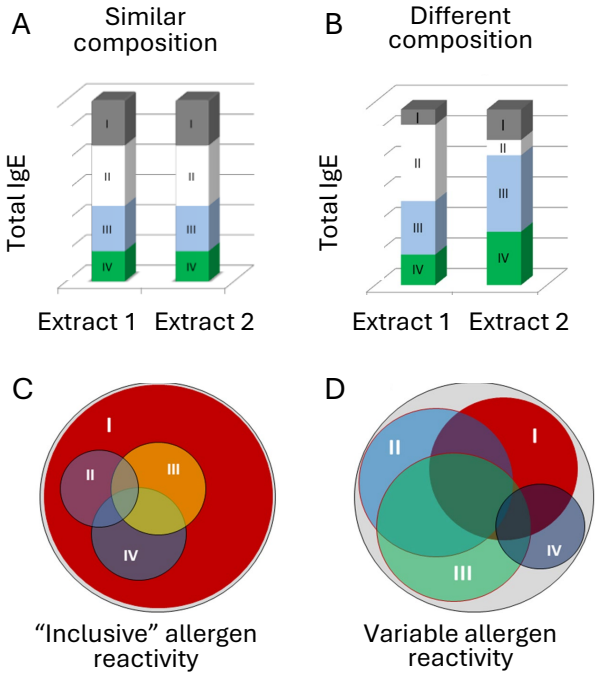


Fig. 5. Overall potency may not reflect compositional differences or correlate with efficacy of immunotherapy. (A and B) Hypothetical sets of extracts in which two extracts with identical overall potencies are compositionally identical (A) or different (B). (C and D) Profiles of allergen reactivity. (C) An 'inclusive profile', in which all patients are allergic to an immunodominant group 1 allergen, whose concentration may therefore represent the potency of the allergenic extract. Therapeutic potencies of cat hair and ragweed pollen extracts are represented by concentrations of Fel d 1 and Amb a 1, respectively. (D) A hypothetical profile of "variable reactivity," in which there is no immunodominant allergen. For these extracts, compositional differences among extracts shown in (B) may be therapeutically relevant (10).

Current state use of $ID_{50}EAL$ and cELISA.

Rather than measuring one or two allergen proteins, these complex allergen extracts were standardized in the 1980s and 1990s by Intradermal Dilution for 50 mm sum of Erythema determines the bioequivalent ALlergy units ($ID_{50}EAL$) testing, in which highly allergic individuals were injected with serial 3-fold dilutions of allergen extracts to determine the D_{50}—the dilution at which the sum of erythema was 50 mm (Figure 6). That concentration was arbitrarily assigned the value of 100,000 bioequivalent allergy units (BAU) (11). Eight grass pollen extracts are distributed in BAU potency units . Additionally, the two house dust mite extracts are distributed in allergy units (AU), which for practical purposes is equal to BAU.

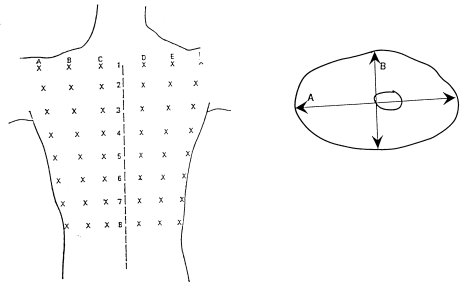


Figure 6. ID_{50}EAL testing to determine overall potency. Highly allergic subjects received injections of serial 3-fold dilutions of extract. The orthogonal diameters of the wheal and flare (inner and outer ovals, respectively) were measured. The concentration of allergen that elicited a 50 mm sum of diameters was arbitrarily assigned a value of 100,000 BAU.

Of course, ID_{50}EAL testing cannot be used for routine lot release. For this purpose, the surrogate assay is a competitive ELISA (cELISA), in which a plate bound reference allergen and the test extract (the lot of manufactured extract to be released) in solution compete for allergen-specific IgE. The source of the IgE is serum pooled from 10-15 allergic donors. In competitive ELISAs, the signal (IgE binding to the plate-bound reference extract) inversely correlates with the concentration of the extract in solution. In addition to reference extracts, LIB's Reference Reagent Lab purchases and distributes to the manufacturers three serum pools: one for Bermuda grass, one for the seven northern grass extracts, and one for the two house dust mite extracts.

In 2025, ID_{50}EAL testing to standardize currently non-standardized allergen extracts is impractical because it may be difficult to recruit investigators to conduct these studies, and because patients' sensitivities as well as extracts from different manufacturers may qualitatively vary such that no BAU value can be obtained. This was the case when CBER collaborated with the NIAID Inner City Asthma Consortium to standardize cockroach allergen extracts. As shown in Figure 7, the ID_{50}EAL tests varied between subjects, and for some subjects, responses varied among the extracts tested.

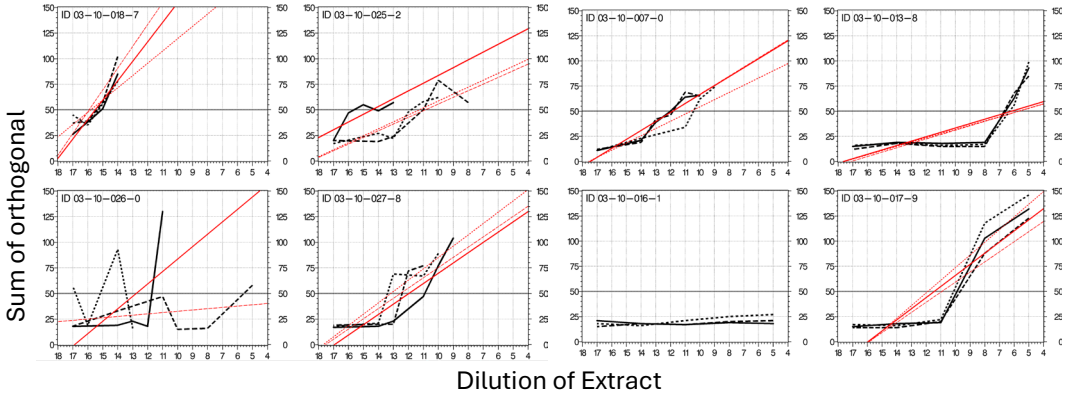


Figure 7. Failed attempt to standardize cockroach allergen extracts for overall potency. Each plot is an ID_{50}EAL test of a different cockroach-allergic subject. The lines represent extracts from each of three US cockroach extract manufacturers (Slater J with the NIAID Inner City Asthma Consortium, unpublished observations).

In addition, while ID_{50}EAL testing, and the surrogate competitive ELISA, provide a useful index of overall allergenic potency of complex allergen extracts, their value is limited because they do not detect compositional differences among extracts. As patients vary in their sensitivity (i.e., IgE reactivity) to different allergen proteins, compositional differences among the extracts may determine which extracts may be effective for particular subsets of allergic patients. Consequently, it is possible that despite identical overall potencies, different patients may respond differently to two nominally identically potent extracts (Figure 5). An additional limitation of cELISAs is that they may vary over time due to potential instability of human sera over time and variations in pooled sera that are collected to replace depleted or expired lots.

Rather than overall potency, quantification of all allergenic proteins and characterization of their variants may reveal important differences among the preparations that can inform patient care and benefit public health. Given the large number of allergenic proteins present in some complex allergen extracts, assessing their concentrations with multiple immunoassays that would require producing multiple mAb pairs is not feasible. Rather than immunoassays, CBER proposes that mass spectrometry (MS) to compositionally define complex allergen extracts will complement potency measurements with cELISA or measuring one or two representative allergens by sELISA.

Implementing tandem mass spectrometry to characterize complex allergen extracts.

Liquid chromatography tandem mass spectrometry (LC/MS/MS) is an analytical technique in which complex samples are first digested by proteolytic enzymes, which are then separated by hydrophobicity on a liquid chromatography column. The peptides are then directly fed into a mass spectrometer that measures the mass-to-charge ratio of ions which can then be translated into a precise mass of the peptide such that the amino acids that comprise the peptides can be identified. When matched to a proteome database, the peptide sequence can be identified and thus serve as a proxy for the presence of a given protein. When used to analyze allergen extracts, tandem mass spectrometry can characterize the proteome of the allergen extract and give values of relative quantity of the proteins in the extracts (Figure 8).

Mass spectrometry (MS) has been used to analyze allergen extracts before. Spiric et al used MS to show that isoforms of the major birch tree pollen allergen Bet v 1 were the reason that some extract preparations were not detected by mAbs that were being developed for standardization of that extract (12). Nolte et al reported variations in the ratio of two house dust mite allergens, Der p 1 and Der p 2 that are not quantified by the cELISA used to measure potency in the US (13).

As stated above, LC/MS/MS provides relative quantification data by identifying peptides that serve as proxies for the protein from which they are derived. When these peptides are unique to that protein, they may be used as surrogates for absolute measurement of allergen quantity in a method referred to as parallel reaction monitoring (PRM). For PRM, the surrogate peptide is synthesized with heavy lysine or arginine that include stable isotopes of nitrogen (N^{15}). The labelled surrogate peptide is mixed into the allergen extract before reduction/alkylation/ trypsinization. Since the heavy surrogate and natural peptides are chemically identical, they co-elute off the LC column. However,

because N^{15} increases the mass of the surrogate peptide, their mass/charge ratios differ, and the surrogate peptide has its own peak on the MS spectra. This peak provides a reference to calculate the precise concentration of the parent protein (Figure 9). PRM and its predecessor technology, multiple reaction monitoring (MRM) are incredibly precise and reproducible. Figure 10 is from five analyses over five days of a surrogate peptide for a protein in German cockroach allergen extract. Note the precision over a range of six orders of magnitude.

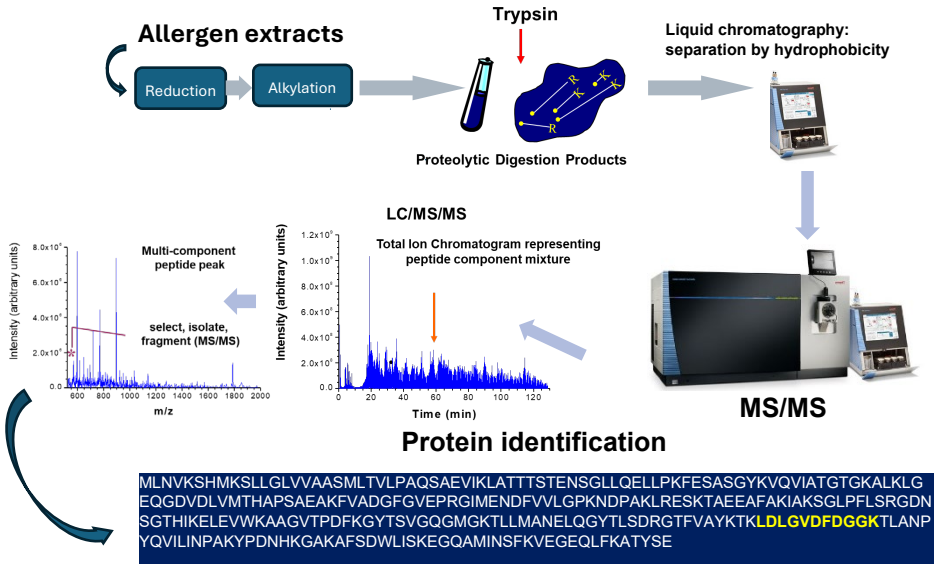


Figure 8. Workflow and output of LC/MS/MS. The allergen extract (or source material) is reduced, alkylated, and trypsinized. The peptides are run through a liquid chromatography column in which they are separated by hydrophobicity and fed into the mass spectrometer. The output is a total ion chromatogram and a combination of MS1 and MS2 spectra from which peptides are identified in context of their parent protein.

PRM is a recent development, but MRM was first used in 1991 to measure peptides from human pituitary extracts. In 2011, Seppala and colleagues used similar technology to both qualitatively and quantitatively characterize Phl p 1 and Phl p 5 in timothy grass pollen extracts—the first report of quantitative and qualitative information on complex allergen extracts, and the first use of stable isotope-labelled peptides as calibrants (14).

While compositionally defining complex allergenic extracts by LC/MS/MS and PRM requires substantial investment and is technically sophisticated, it may be ultimately cost effective compared to repetitive development of biological tests and continuous replacement of reference materials—pooled human sera in particular. In addition to ensuring product quality, PRM can inform practitioners of compositional differences among complex allergen extracts. Paired with novel serological testing platforms that quantifies IgE specific to a number of (for example) house dust mite allergens, PRM can facilitate a personalized approach towards allergen immunotherapy.

Our recent work on *Alternaria alternata* and *Dermatophagoides pteronyssinus* house dust mite extracts illustrate how LC/MS/MS with PRM may improve characterization of complex allergen extracts to improve their quality as diagnostic and therapeutic agents.

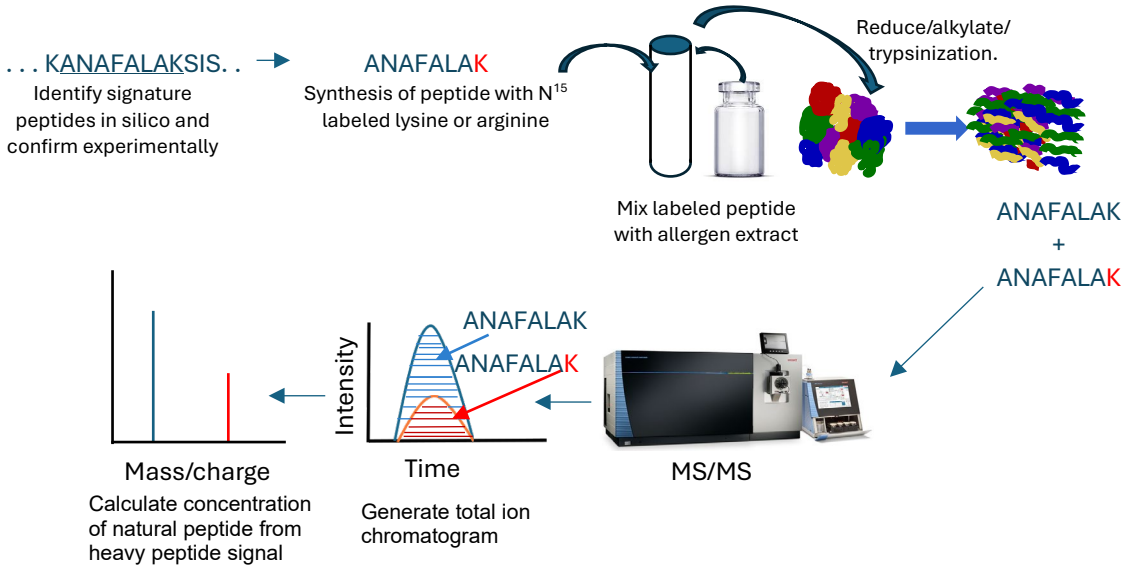


Figure 9. Parallel reaction monitoring allows for absolute quantification. A surrogate peptide (KANAFALAKSIS) is first identified in silico by simulating trypsin digestion, which cuts downstream of arginine or lysine. The surrogate is then verified experimentally with LC/MS/MS and synthesized with N^{15} labeled lysine or arginine. The labeled peptide is spiked at a known concentration into an aliquot of the allergen extract. The spiked extract then undergoes LC/MS/MS yielding thousands of peptides, including the natural peptide of interest from the parent protein and the labeled peptide that was spiked into the extract. The signal from labeled peptide is then used to calculate the absolute quantity of the natural peptide and thus the parent protein.

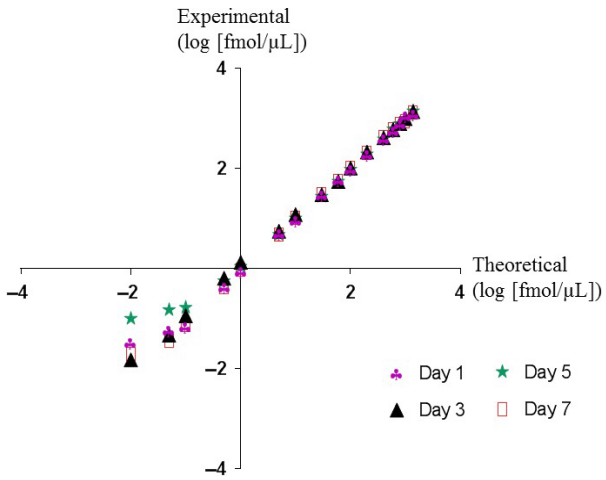


Figure 10. Precise quantification of a German cockroach (GCr) allergen in complex extracts using multiple reaction monitoring mass spectrometry (MRM MS) (15).

Characterization of Fungal Allergenic Extracts as Proof-of-Concept of LC/MS/MS Analytical Platform.

Alternaria alternata, a fungus found both indoors and outdoors (Figure 11), can elicit IgE-mediated respiratory diseases, including asthma and rhinoconjunctivitis. *Alternaria* allergy has been implicated in outbreaks of severe asthma attacks. The diagnosis of IgE-mediated allergy can be made by skin testing with a licensed allergen extract, or by measuring specific IgE in the serum. *A. alternata* allergen extracts are also used to treat *Alternaria* associated respiratory allergic diseases (16). Commercial *A. alternata* extracts in the United States are non-standardized and manufactured from varying combinations of hyphae, spore and spent media. Since the method of culturing *A. alternata* may vary among US manufacturers, their extracts may qualitatively differ.

Until our recent work, allergen profiles for *A. alternata* spore and hyphae were unknown. Since the mold life cycles are complex (Figure 11), we characterized allergen content variation between these cycles to potentially enhance manufacturing control over extract allergen content. Specifically, isolated spores and hyphae to investigate with LC/MS/MS differences between spore and hyphae proteomes and how allergens are distributed in *A. alternata*. We identified and quantitatively compared 4515 proteins to show that spores and hyphae express overlapping but distinct proteomes. We also compared the allergen profiles from the three commercial *A. alternata* extracts available in the U.S. (17).

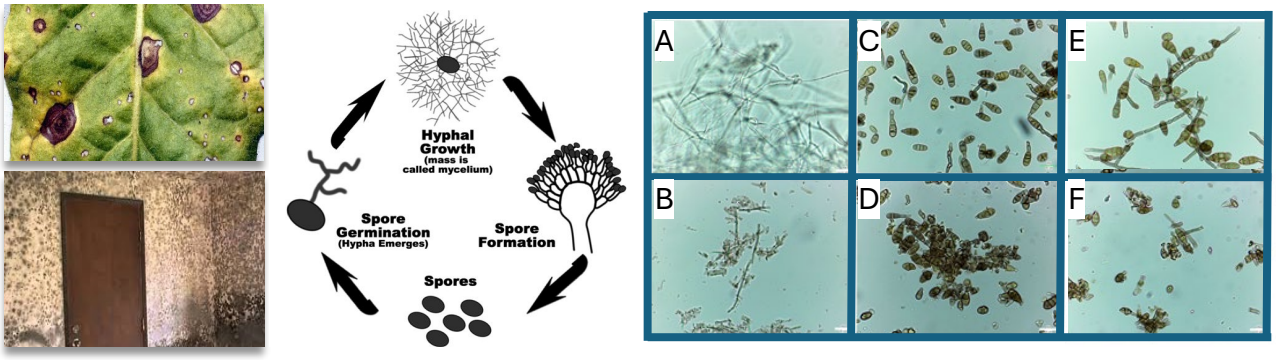


Figure 11. *Alternaria alternata* is a fungus found outdoors and indoors with a complex life cycle. Left: *A. alternata* on a leaf and in a poorly ventilated room. Center: Life cycle of mold. Right A and B: *A. alternata* hyphae and hyphal fragments, respectively. C and D: Non-germinating spores and fragmented non-germinating spores, respectively. E and F: Germinating spores and fragmented germinating spores, respectively.

Our key findings are (Figure 12 and Table 2):

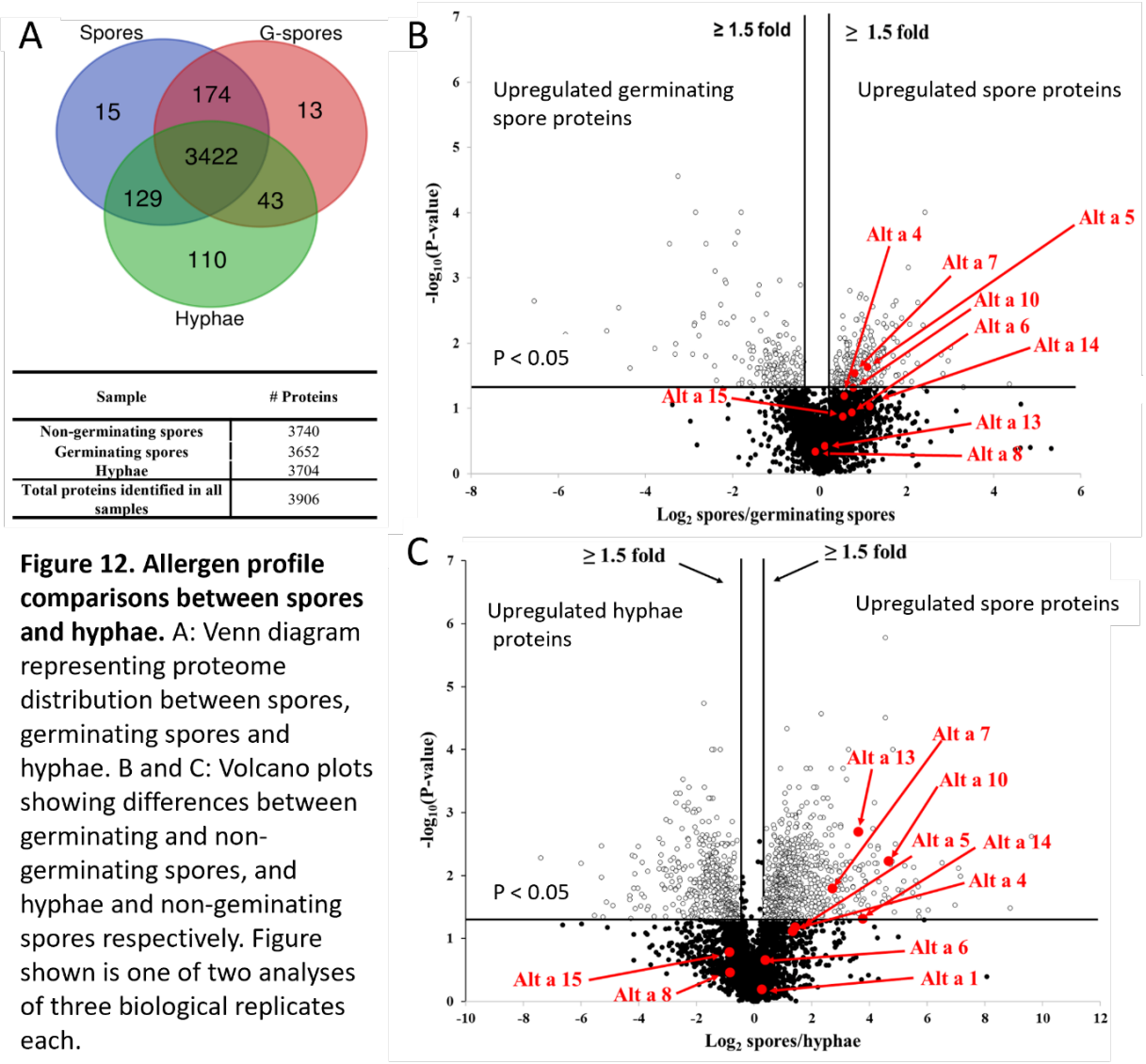
- In the non-germinating spore proteome, many upregulated proteins are functionally involved in cell wall synthesis, responding to cellular stress, and maintaining redox balance and homeostasis.

- The germinating spores contain high levels of proteases known to be virulence factors.
- The hyphal proteome includes cytosolic proteins mainly involved in pathways of cell metabolism.
- Most *A. alternata* allergens are more abundant in non-germinating spores, and many of these allergens serve known spore functions such as mitigating oxidative stress.
- The major allergen Alt a 1 is present at low levels in spores and hyphae and appears to be largely secreted into growth media.
- Allergen content quantitatively and qualitatively varies among the three US manufacturers.

Our study not only reveals quantitative information about allergen content but also provides an allergen content profile for each of the different life cycles of the mold. Since commercial allergen extracts are manufactured with varying combinations of hyphae, spore and spent media, this information will be useful for optimizing methods of allergen content enrichment to improve the quality of *A. alternata* allergen extracts.

Allergen	**ALK**	**GREER**	**JHS**
Alt a 1	2.6 ± 0.7	1.00 ± 0.40	2.0 ± 0.2
Alt a 3	1.0 ± 1.7	17.70 ± 17.10	2.0 ± 1.3
Alt a 6	-	6.84 ± 6.84	1.0 ± 1.0
Alt a 8	-	21.80 ± 19.50	-
Alt a 10	-	2.20 ± 1.10	-
Alt a 14	-	16.70 ± 8.30	-

Table 2. Relative abundance of known *A. alternata* allergens in U.S. commercial allergen extracts. Mean of normalized fold changes ± standard error of mean (SEM), of 9 lots, 3 lots each per manufacturer. Lots ranged from 130 to 796 days from date of manufacture on date of analysis. (-) not detected in those extracts; Allergens that are not listed were not detected in any of the lots tested.



Sample	# Proteins
Non-germinating spores	3740
Germinating spores	3652
Hyphae	3704
Total proteins identified in all samples	3906

Figure 12. Allergen profile comparisons between spores and hyphae. A: Venn diagram representing proteome distribution between spores, germinating spores and hyphae. B and C: Volcano plots showing differences between germinating and non-germinating spores, and hyphae and non-geminating spores respectively. Figure shown is one of two analyses of three biological replicates each.

Appendix 5 – House Dust Mite Extract Source Materials

Optimization of house dust mite extract source materials as proof-of-concept of LC/MS/MS analytical platform.

Dermatophagoides pteronyssinus and *Dermatophagoides farinae* are the predominant house dust mite (HDM) species in the northern hemisphere. Except for cold or dry regions, these HDM are ubiquitous indoor allergens. They are often the first respiratory allergens encountered by infants and are considered initiators of the "allergic march" from allergic rhinoconjunctivitis to allergic asthma. HDM eat epithelia shed by humans and their pets and live in upholstered furniture, carpets, and bedding[7]. HDM are coprophagic; their fecal pellets are structurally organized and contain digestive enzymes so that food within the ingested fecal pellets is absorbed for nutritional benefit. The fecal pellets are particularly important to allergic disease because these digestive enzymes are also allergens, and because unlike the HDM bodies, they are small enough to be inhaled into the lower airways (Figure 13) (18). As stated above, HDM allergen extracts are standardized for overall potency—which implies that they are qualitatively similar. However, the method of culturing HDM may differ among manufacturers, and thus their allergen extracts may qualitatively differ (19).

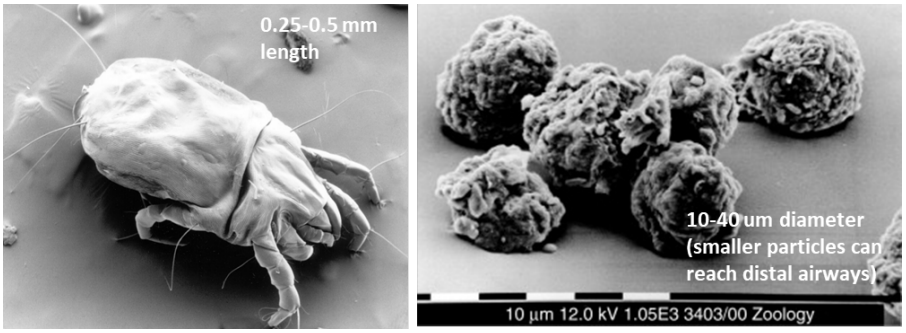


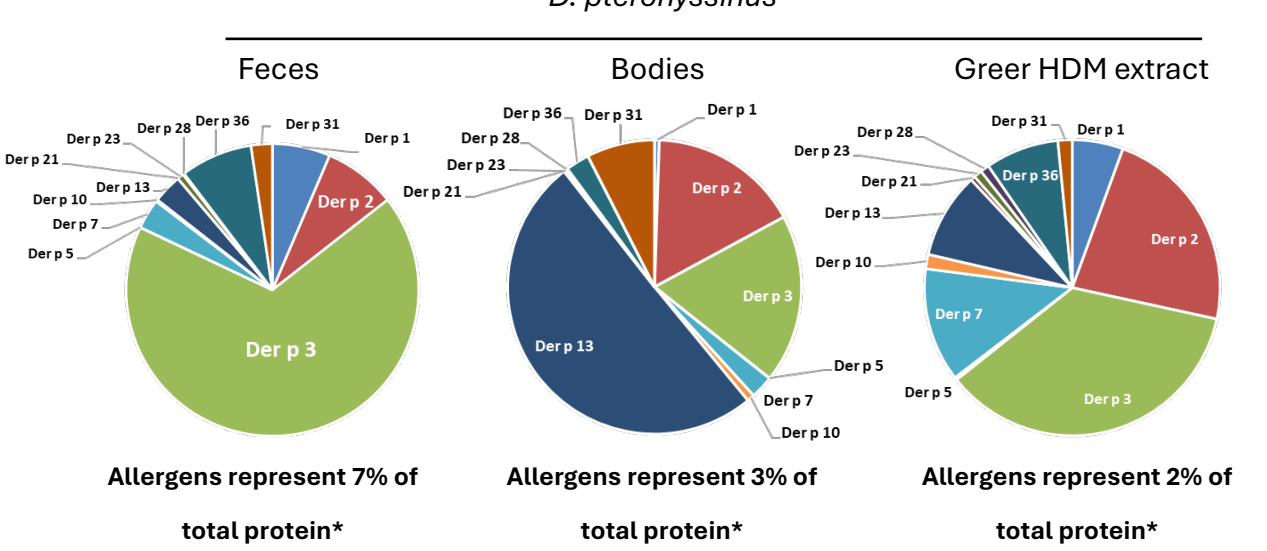
Figure 13. *Dermatophagoides pteronyssinus* body and fecal pellets (not to scale). Source: CSIRO ScienceImage 11085 and (20).

Another issue with HDM extracts is the source material. As a consequence of a decision made after a meeting of the Allergenics Products Advisory Committee in 1987, HDM extracts are derived from mite bodies, exclusive of the fecal pellets. However, in the intervening ~35 years, it has become evident that the fecal pellets are an important source of HDM allergens.

To investigate these issues, we performed LC/MS/MS on separate extracts from HDM bodies and fecal pellets. We also compared these two extracts to one lot of a manufactured *Dermatophagoides pteronyssinus* extract from Stallergenes-Greer (with the intention to analyze multiple lots of extracts from both species of HDM from all three US manufacturers). As expected, allergen content and distribution greatly differed between the fecal pellet and body extracts, and from either of those and the Greer HDM extract (Figure 14). Furthermore, the relative abundances of two of three dominant HDM allergens, Der p 1 and Der p 23, were higher in the fecal extracts (Table 2),

[7] It is estimated that ~10% weight of a 2-year-old pillow that hasn't been washed is mite bodies and feces.

demonstrating that including fecal pellets in HDM extracts will improve them as diagnostic and therapeutic agents.



* Based on summed allergen peptide ion current measurements

Figure 14. Distribution of HDM allergens in extracts from *Dermatophagoides pteronyssinus* feces and bodies, and in a manufactured *Dermatophagoides pteronyssinus* HDM extract. MB Strader, RL Rabin, and JE Slater; unpublished observations.

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