

Standardization of Allergen Extracts

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Abstract

Allergens are molecules with the capacity to elicit IgE responses in humans. When stimulated with allergens, most allergic patients respond with production of IgE specific for several proteins/allergens in the source material. The standardization of allergen extracts is essential in order to control variability and to achieve consistency and reproducibility in a clinical setting.

Because the IgE binding capacity of an allergen extract is related to the content of one or a few major allergens, it is important that the standardization procedure ensures consistency, not only in the overall IgE binding potency, but also in the content and ratio of individual major allergens. Owing to the complexity of allergen extracts, a key element in standardization of allergen extracts is the use of standards.

This chapter describes the principles for standardization of allergen extracts to be used by research laboratories. Other chapters in this volume describe methods in detail.

Key Words: Allergen; extracts; standardization; in vitro; in vivo.

1. Introduction

Allergen extracts are used for diagnosis and treatment of allergic diseases. In the manufacture of allergen extracts, it is essential to apply standardization in order to control the variability in naturally occurring source materials and achieve consistency and reproducibility for optimal safety and efficacy in clinical use. In the widest sense, standardization includes controlling the entire production chain of processes including qualifications of collectors of raw materials, establishment of robust and reproducible manufacturing procedures in compliance with 'good manufacturing practice' (GMP) regulations, and formulation of allergen extracts intended for clinical use in allergy diagnosis, in vivo as well as in vitro, and in allergen immunotherapy/vaccination. Standardization, however, is not a precisely defined term, as different qualities of

standardization are in current use by various laboratories and manufacturers of allergen extracts.

Allergens are molecules with the capacity to elicit IgE responses in humans. Inhalant allergens are proteins, readily soluble in water, and present in airborne particles. The particles carrying allergens are inhaled and deposited on the mucosal surfaces stimulating immunocompetent cells associated with the airway mucosa. When stimulated with low doses of allergens, most allergic patients respond with production of IgE specific for several proteins/allergens in the source material. A limited number of allergens, 'major' allergens (**1**), stimulate IgE production in a majority of patients; however, any antigen in a given source material has the potential to elicit an IgE response. It is therefore important to ensure that all protein antigens/allergens in the allergenic source material, to which humans are exposed, are contained in the raw material. Because the IgE binding capacity of an allergen extract is related to the content of one or a few major allergens, it is also important that the standardization procedure ensures consistency, not only in the overall IgE binding potency, but also in the content and ratio of individual major allergens.

Owing to the complexity of allergen extracts, a key element in standardization of allergen extracts is the use of standards (**2**). In Europe, each laboratory and manufacturer establishes In-House Reference (IHR) preparations for each source material. The IHR must be thoroughly characterized by in vitro methods as a basis for equilibration of subsequent batches, and the biological activity in humans should also be determined by in vivo methods. The IHR eliminates the need for in vivo methods in batch-to-batch standardization, which can be performed by comparing each and every new batch to the IHR using in vitro methods exclusively. Comparison of individual activities of different IHR preparations can be performed by use of International Standards (IS).

This chapter describes the principles for standardization of allergen extracts to be used by research laboratories. Other chapters in this volume describe methods in detail.

2. Materials

2.1. Selection of Source Materials

Inhalant allergens are present in airborne particles derived from natural allergen sources. These particles constitute the material to which humans are exposed, and the aim of raw material selection is to provide materials containing the same active allergens in a manageable form. In most cases, the optimal source material is rather obvious, but in some cases the allergen source is still debated (e.g., cat saliva/pelt/dander or mouse urine/dander).

The source materials should be selected with attention to the need for specificity and for inclusion of all relevant allergens in sufficient amounts (**2**).

The collection of the source materials should be performed by qualified persons, and reasonable measures must be employed to assure that collector qualifications and collection procedures are appropriate to verify the identity and quality of the source materials. This means that only specifically identified allergenic source materials that do not contain avoidable foreign substances should be used in the manufacture of allergenic extracts. Means of identification and limits of foreign materials should meet established acceptance criteria for each source material. Where identity and purity cannot be determined by direct examination of the source materials, other appropriate methods should be applied to trace the materials from their origin. This includes complete identity labeling and certification from competent collectors. The processing and storage of source materials should be performed to ensure that no unintended substances, including microbial organisms, are introduced into the materials. When possible, source materials should be fresh or stored in a manner that minimizes or prevents decomposition. Records should describe source materials in as much detail as possible, including the particulars of collection, pretreatment, and storage.

2.2. Specific Aspects of Source Materials

Specific aspects of the most important raw material categories are briefly discussed below.

2.2.1. Pollens

The natural sources of inhalant allergens from plants are the pollens. Pollen may be obtained either by collection in nature or from cultivated fields or greenhouses. The collection may be performed by several methods, such as vacuuming or drying flower heads followed by grinding. The pollen may be cleaned either by passing through sieves of different mesh sizes or by flotation. Finally, pollens are dried under controlled conditions and stored in sealed containers at -20°C . The maximum level of accepted contamination with pollen from other species is 1% by number. Pollen should be devoid of flower and plant debris, with a limit of 5% by weight. Pollens may show large variation in quantitative composition depending on season and location of growth, and in order to achieve a relatively constant composition, harvests from different years and sites of collection should, after thorough characterization *in vitro*, be pooled for the production of allergen extracts.

2.2.2. Acarids

House dust mites are grown in pure cultures. Source materials are either pure mite bodies (PMB) or whole mite cultures (WMC). Extracts based on WMC include material from mite bodies, eggs, larvae, and fecal particles as well as

mite decomposition material and contain all the material to which a mite-allergic patient is exposed under natural conditions. The culture medium should ideally be antigen free, or contaminants from the culture medium should be shown not to be allergenic. The PMB extract avoids extensive contamination with debris from the culture medium. Clinical trials comparing vaccines based on WMC and PMB extracts have shown similar clinical efficacy in specific allergy vaccination (3).

2.2.3. *Mammals*

Allergens of mammalian origin may be present in various sources (e.g., dander, serum, saliva, or urine). The allergens to which humans are exposed depend on the normal behavior of the animal and, therefore, cannot be generalized. In each case, the optimal source or mix of sources of allergens from mammals should be thoroughly investigated using a large panel of sera from allergic patients. Whether derived from dander or deposited from body fluids, most allergens are present in the pelt. Source materials should be collected only from animals that are declared healthy by a veterinarian at the time of collection. When animals that are killed are used, the conditions for storing should minimize postmortem decomposition until the source materials can be collected. The optimal source materials are often dander, because hair proteins are insoluble. Use of whole pelt would increase the proportion of serum proteins, which are generally of low allergenic activity.

Because of the quantitative differences in the yield of the various allergens from different dog breeds (4), a mixture of material from different breeds should be selected representing a balanced content of the major allergens (5).

2.2.4. *Insects*

The optimal source for insect allergens is dependent on the natural route of exposure (i.e., inhalation, bite, or sting). Where whole insects or insect debris are inhaled, the whole insect body is selected as allergen source. In the case of biting or stinging insects, saliva or venom, respectively, is the proper allergen source.

2.2.5. *Fungi*

Moulds should be grown under controlled conditions. The harvested raw materials should consist of mycelia and spores. Owing to difficulties in maintaining a constant composition of fungal cultures, it is recommended that extracts should be derived from several independent cultures of the same species. The primary inoculum should be obtained from established fungal culture banks, i.e., American Type Culture Collection (ATCC, <http://www.atcc.org/>) or Central Bureau Schimmelcultures (CBS, <http://www.cbs.knaw.nl/>), and the following batches should be derived from the same strain to secure a constant composition,

which may vary even under seemingly similar growth conditions (6,7). The cultivation medium should be synthetic or at least devoid of allergenic constituents (i.e., proteins). Culturing should be conducted under aseptic conditions to reduce the risk of contamination by micro-organisms or other fungi, and controls must include tests for suspected toxins.

2.2.6. Foods

Foods constitute a diversified area, and the supply of standardized allergen extracts is scarce. Foods are often derived from various subspecies, grown under a broad variety of conditions reflecting geographical variation. In addition, foods are often cooked prior to ingestion, and the cooking procedures may differ geographically. Consequently, the source of allergen exposure, qualitative as well as quantitative, is highly variable (8).

Ideally, source materials for food allergen extracts should reflect local subspecies, conditions, and habits for the cultivation, harvesting, storing, and cooking of the foods. However, ingested foods are increasingly derived from distant parts of the world. The best solution to these problems may be to combine materials from as many sources as possible, reflecting variation in as many parameters as possible.

The difficulties in producing consistent and reproducible food allergen extracts have resulted in many clinicians using untreated foods from retail trade for diagnosis by the prick-prick method (9). Examples are fresh fruit, cow's milk, and hen's egg. Undiluted cow's milk and hen's egg have the best documented diagnostic properties (10).

A further problem in food allergen extract production is the presence in many foods of natural or microbial toxins, pesticides, antibiotics, preservatives, and other additives that may be concentrated in the manufacturing process. The use of organic source material should therefore be preferred.

3. Methods

3.1. Preparation of Allergen Extracts

Allergen-IgE binding involves the interaction of molecular surfaces of allergens and IgE antibodies having contours exactly fitting each other (11). Because the structure of the molecular surface of the allergen and thus the IgE binding epitopes are vulnerable to protein denaturation, procedures used for the preparation and storage of allergen extracts/vaccines should avoid organic solvents, elevated temperatures, and extreme pH and ionic conditions. The extraction should be performed under conditions resembling the physiological conditions in the human airways (i.e., pH and ionic strength) and suppressing possible proteolytic degradation and microbial growth (12). The optimal extraction time is always a compromise between yield and degradation/denaturation of the allergens, and special attention should be drawn to the fact that different allergens

are released with different kinetics (**13**). In general, processing time should be minimized and extraction performed at low temperatures.

Low molecular weight, i.e., below 5000 Da, non-antigenic material should be removed from the extract by dialysis, ultra-filtration, or size exclusion chromatography. Any substance excluded from the final product should be shown to be nonallergenic. The production procedure should include procedures to measure and exclude below-defined thresholds for known toxins, viral particles, and free histamine or other physiologically active substances.

The final extract should be stored under conditions that impede deterioration of the allergenic activity either by lyophilizing the extract or by storage at low temperatures (i.e., -20°C to -80°C), possibly in the presence of stabilizing agents, such as 50% glycerol, non-allergenic proteins (e.g., certified human serum albumin), or other stabilizers.

3.2. IHR Preparations and Standards

3.2.1. IHR Preparations

A particularly important aspect of the standardization procedure is the IHR. In Europe, the IHR is prepared by the individual laboratory or manufacturer, whereas in the United States, the US Food and Drug Administration (FDA) authorizes general standards of some common allergens for the purpose. The IHR is used by manufacturers/laboratories for equilibration of the potency and composition of each batch of manufactured extract (**14,15**). By this procedure the batch-to-batch standardization can be performed by comparison to the IHR using in vitro techniques exclusively, and unethical use of in vivo methods can be avoided. The establishment of an IHR and subsequent production of new batches related to the IHR is illustrated in **Fig. 1**.

When a robust extraction procedure has been defined, three batches of the extract should be produced in order to verify consistency and reproducibility of the production processes. The three batches are compared, and if consistency is achieved one is selected to represent the new IHR, which is subsequently dispensed into freeze-dried aliquots of suitable size. The IHR should be carefully defined including assessment of dry weight, protein content and composition, major allergen content, and total allergenic activity by in vivo and in vitro methods (**Fig. 1**). The dispensing into aliquots can be based on dry weight, as other methods are less precise, e.g., protein by Lowry, or too expensive for routine use, e.g., automated amino acid analysis.

The determination of total allergenic activity by in vivo methods, most often by skin prick testing (SPT) (**16**), is not only laborious but also unethical to perform more than occasionally. Because the biological activity has been shown to correlate with the major allergen content (**17**), the use of major allergen determination with a validated assay is sufficient in combination with a specific

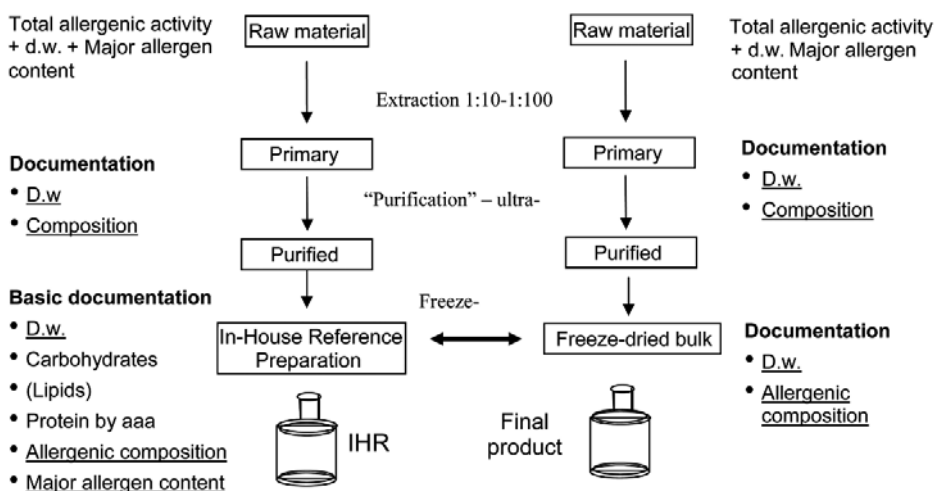


Fig. 1. Standardization of allergen extracts. The principle for establishment of an In House Reference (IHR) preparation to the left and standardization of subsequent batches, calibrated against the IHR. Different methods can be applied to determine the total allergenic activity in vitro and in vivo, major allergen content, and the allergenic composition.

IgE potency assay. If in vitro methods alone are used for the establishment of the potency of the IHR, comparison is best made with the IS.

3.2.2. International Standards

Allergen extract standardization ideally requires use of two types of standards for each source material, an IHR preparation as described above and an IS. IS of allergen extracts are obtainable from the National Institute of Biological Science and Control, NIBSC, London, United Kingdom, and were produced under the auspices of the WHO according to guidelines established by the Allergen Standardization Sub-Committee under the International Union of Immunological Societies (IUIS). IS enable comparison of specific activities of products from different manufacturers and can be used as calibrators by new producers and laboratories.

IS are available for the following allergen extracts: *Ambrosia artemisiifolia* (short ragweed) (18), *Phleum pratense* (timothy grass) (19), the house dust mite *Dermatophagoides pteronyssinus* (20), *Betula verrucosa* (birch) (21), and *Canis familiaris* (dog) (22). Additional standards were planned for the mould *Alternaria alternata* (23), for the grasses *Cynodon dactylon* (Bermuda grass) (24), and *Lolium perenne* (rye grass) (25), *Felis domesticus* (cat), and the house dust mite *Dermatophagoides farinae*, but unfortunately this initiative has stopped prematurely because of lack of general acceptance.

3.3. Units and Measure

The strength or potency of an allergen extract relies on the magnitude of the response it will elicit in human allergic subjects. Allergic patients respond individually to allergen extracts because they are sensitized to different allergens and the degree of sensitivity differs from patient to patient as well as for each allergen in the extract. Because allergen extracts from different producers differ in composition there is no straightforward relationship between potency and response when considering allergen extracts from different manufacturers. This paradox poses an inherent problem in the labeling of allergen extracts, as the potency of different extracts cannot be compared in a meaningful manner.

Direct skin testing of human allergic subjects is the predominant *in vivo* method for the assessment of allergen extract potency (26) and also constitutes the principle underlying the establishment of biological units of allergen extract potency. Patient selection criteria are obviously important, as all potency measures will be dependent on the patient panel. Several units are in use.

In Europe, SPT has been used for estimating the biological activity of allergen extracts. The aim is to equilibrate the potency between extracts of different inhalant allergen extracts (27). At least 20 consecutive patients attending a specialist clinic should be included. The concentration inducing a weal of the same size as that of histamine dihydrochloride, 10 mg/mL, is estimated by parallel line bioassay. The median concentration constitutes 10,000 biological units (BU) (28). This method was adopted by the Nordic Council on Medicines as the Nordic Biological Unit, HEP (29). With proper patient selection the unit has been shown to be reproducible between different regions of Europe (28). European manufacturers use their own company-specific units, most of which are based on the same method (30).

In the United States, the FDA uses a unit based on intradermal testing with the allergen extract and subsequent measurement of the flare rather than the weal size. The 'intradermal end point' is expressed as the number of threefold dilutions producing a summed erythema diameter of 50 mm. The mean value of 15 individuals defines the potency of the allergen extract, which is expressed in 'allergy units' (AU). More recently the CBER in the United States proposed the 'bioequivalent allergy unit' (BAU). The method for assigning BAU is named the ID50EAL method, i.e., 'intradermal dilution for 50 mm sum of erythema diameters determines bioequivalent allergy units' (31).

Labeling of the potency of allergen extracts based on the microgram amount or, more correctly, the millimolar concentration of major allergen has been proposed, as the major allergen content correlates with the biological activity (17). Using the same antibodies, IHR and methodology, and with similar extract composition, determination of major allergen content can replace other methods

for potency declaration. However, in most cases the composition of test materials and the specificity of anti-allergen antibodies vary between laboratories and the content of a single major allergen does not allow comparison of overall potency between marketed allergen extracts from different companies.

3.4. Stability Testing

Criteria, methods, and limits for stability should be established. In Europe, a total allergenic activity in the interval between 30 and 300% of the IHR is accepted, whereas the US authorities use limits of 50–200% of the labeled activity. Methods used to assess the potency should have a demonstrated higher precision, which is not always trivial. The reference should be the IHR stored at -70°C or lower. For stability assessment the extract should be stored at several different temperatures to assure safe storage at room temperature, in a refrigerator, and at -20°C . Accelerated degradation studies designed to measure the kinetics of breakdown of the allergen extract can be performed by incubation at multiple high temperatures.

3.5. Batch-to-Batch Control

Having established an IHR preparation, batch-to-batch standardization is performed by calibrating new freeze-dried batches in bulk with the IHR before dispensing into vials for distribution. Batch-to-batch standardization includes, apart from determination of dry weight, three steps:

1. Assessment of allergenic composition to ensure the presence in the final product of all allergens present in the source material. Techniques used include crossed (radio)-immunoelectrophoresis (CIE/CRIE)(32), sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE)(33), and immunoblotting (34), as well as isoelectric focusing (IEF)(35). CIE has the advantage of being a semiquantitative technique, and a reproducible precipitation pattern will ensure constant ratios between all major antigenic components.
2. Quantification of specific major allergens to ensure that essential allergens are present in constant ratios. Techniques used include quantitative immunoelectrophoresis (QIE)(32) and enzyme-linked immunosorbent assay (ELISA)(36).
3. Quantification of the total allergenic activity to ensure that the overall potency of the extract is constant. Techniques used include several variations of the radio-allergosorbent test (RAST)(37) and RAST inhibition assays.

In batch-to-batch standardization, *in vitro* laboratory techniques are used to compare individual batches to the IHR, which should be matched in every aspect, *see* **Fig. 2**.

3.6. Conclusion

Allergen extracts are complex mixtures derived from natural source materials and as such prone to natural variation. Standardization is necessary to control

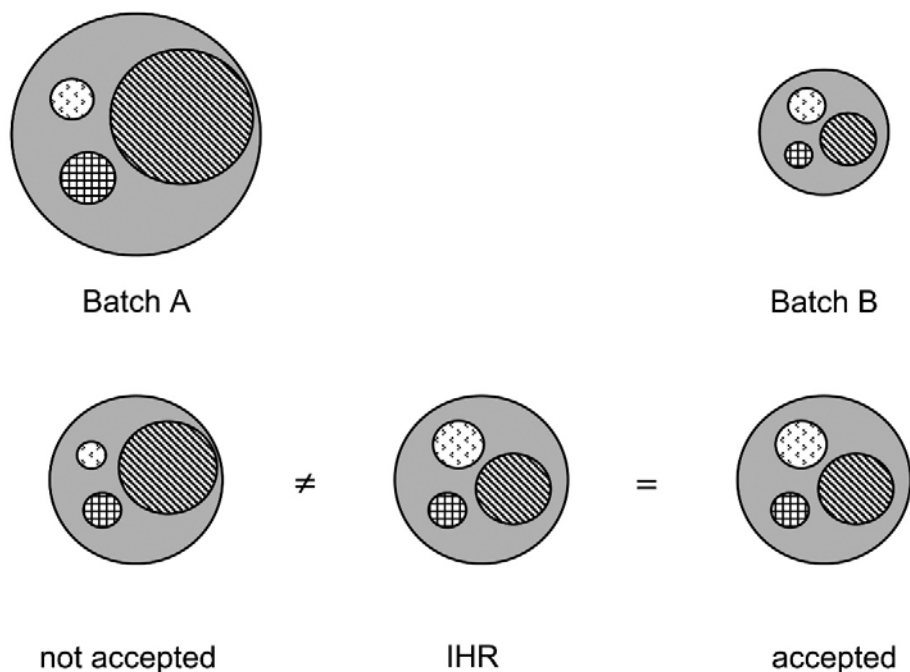


Fig. 2. Complexity of allergen extracts represented by a model with three major allergens. The area of shaded circles represents the relative potency of individual components. The area of outer circles represents the total allergenic potency of the extracts. The total allergenic potency of batches A and B may be adjusted by dilution or concentration, but still the composition of the extracts may vary stressing the importance of the measurement of individual components.

variation and ensure consistency and reproducibility for the safety and efficacy of specific allergy disease management. Batch-to-batch standardization is performed by comparison of new batches to established standards assessing complexity, major allergen content, and IgE binding.

3.7. Checklist for Standardization of Allergen Extracts

1. Establish criteria for raw material quality

Criteria should define standard procedures for collection and storage of raw materials and thresholds for contamination with bacteria, viruses, and suspected toxins.

2. Select supplier of raw materials

Suppliers of raw materials should document qualifications of experts, e.g., mycologists, acarocologists, and palynologists, and collection procedures to ensure optimal quality and absence of infectious particles.

3. Select relevant raw materials

Source materials from different suppliers and different origin should ideally be included in initial screenings.

4. Perform test extractions

Allergenic raw materials are complex. Empirical testing of the following parameters is essential: extraction buffer composition, time, and temperature.

5. Remove low molecular weight components by dialysis or filtration

Excluded material should be verified to be non-allergenic in IgE binding assays.

6. Establish criteria for batch-to-batch variability

Assess the robustness of the extraction procedure by performing three batches, and verify that the variability is below predefined limits.

7. Define the IHR

The IHR should be characterized with respect to dry weight, allergen complexity, major allergen content, and IgE binding potency. Biologic activity should ideally be determined by skin testing.

8. Assess stability of the IHR

Methods to be used and criteria for the precision of assays used for stability assessment should be established.

9. Batch control

Production batches should be produced according to the established production processes, and the freeze-dried product should be calibrated to the IHR assessing complexity, major allergen content, and IgE binding.

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