

Major Allergen Content In Allergen Immunotherapy Products: The Limited Value of Numbers

Running Title: The limits of major allergen numbers

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Abstract

The prevalence of allergic disorders drastically increased over the last 50 years that today they can be considered epidemic. At present, allergen-specific immunotherapy (AIT) is the only therapy targeting the underlying cause of allergic disorders, and its superior evidence is based on accumulated data from clinical trials and observational studies demonstrating efficacy and safety. However, several aspects remain unsolved, such as harmonization and standardization of manufacturing and quantification procedures across manufacturers, homogeneous reporting of strength, and also the establishment of international reference standards for many allergens. This article discusses the issues related to the measurement of major allergen content in AIT extracts, raising the question of whether comparison of products by different manufacturers are appropriate as basis to choose among the different AIT products. Allergen standardization in immunotherapy products is critical to ensure quality and thereby safety and efficacy. However, lack of harmonization in manufacturing process, allergen quantification (methodologies and references), national regulatory differences, clinical practice, and labeling shows that the comparison of AIT products solely based on major allergen amounts is not rationale and, in fact, impossible. Moreover, further inherent characteristics of products and their clinical use such as their state of extract modification, addition of adjuvant or adjuvant-system, route of administration (sublingual/subcutaneous) and cumulative dose as per posology (including the volume per administration) need to be taken into account, when rating the information given for a specific product. Finally, only convincing clinical data can serve as the product-specific evaluation, or the basis for cross-product comparability, for individual products.

Keywords (3-8): AIT. Major allergen content. Immunotherapy. Standardization methods. Quality. Adjuvants. Product comparability.

RESUMEN

La prevalencia de las enfermedades alérgicas se ha incrementado drásticamente en los últimos 50 años y hoy pueden considerarse una epidemia. Actualmente, la inmunoterapia específica con alérgenos (ITA) es el único tratamiento dirigido a la causa subyacente de las enfermedades alérgicas y su superioridad se basa en resultados de ensayos clínicos/estudios observacionales que demuestran su eficacia y seguridad. Pero quedan aspectos sin resolver, como la armonización y estandarización de los procesos de fabricación y cuantificación entre fabricantes, la declaración homogénea de la potencia y el establecimiento de estándares internacionales de referencia.

En este artículo se discuten aspectos relacionados con la medida del contenido de alérgenos mayores en los extractos de ITA, cuestionando si, como base para elegir entre productos, es apropiada la comparación entre diferentes fabricantes. La estandarización alérgica es crucial para asegurar la calidad y, por tanto, la seguridad y eficacia de la ITA. Sin embargo, la falta de armonización en los procesos de fabricación, la cuantificación alérgica, las diferencias regulatorias, la práctica clínica y el etiquetado, demuestran que comparar productos basándose únicamente en la cantidad de alérgeno mayor no está justificado y es imposible. Además, cuando se evalúa la información para un determinado producto, deben tenerse en cuenta las características propias de cada producto y su uso clínico, como el estado de la modificación del extracto, la adición de adyuvantes, la vía de administración y la dosis acumulada. Solo datos clínicos convincentes deben servir para la evaluación específica de cada producto o como base para la comparación entre productos.

Palabras clave: Inmunoterapia alérgica. Contenido de alérgenos mayores. Métodos de estandarización. Calidad. Adyuvantes. Comparación de productos.

1. INTRODUCTION

Allergy is the cause of an exaggerated response of the adaptive immune system to a usually harmless substance (allergen). Manifestations of allergy vary, and include medical conditions such as anaphylaxis, urticaria, allergic asthma, allergic rhinitis, eczema, contact eczema, serum sickness, and allergic vasculitis [1]. The prevalence of allergic disorders has increased significantly over the last 50 years that today they can be considered epidemic [2, 3]. Over decades, allergen immunotherapy (AIT) has been used for the treatment of allergic rhinitis and rhinoconjunctivitis, asthma, and venom allergy. At present, AIT is supported by accumulated data from large, double-blind, placebo-controlled clinical trials demonstrating efficacy and safety [4-11], as well as a high number of non-interventional studies, systematic reviews and meta analyses [12-14], and real-world evidence [15-19]. Among its benefits, it has been demonstrated that AIT may prevent the development of asthma in children with allergic rhinitis [8]. At present, this is the only therapy for allergies that target the underlying causes of the disease [7].

Currently, specialists can choose among several immunotherapy products available for the same allergen. The majority of allergen-based AIT products are derived from complex "Drug Substance" extracts; i.e. prepared from natural allergenic source materials such as pollen, house dust mites (HDM) or animal dander. These are complex due to the nature of allergen compositions within species with multiple allergen components that may vary over time due to environmental pressures. As such, they are categorized as inherently heterogeneous and variable in nature. Over recent decades, understanding the composition of allergen extracts and their component resolution has grown remarkably allowing manufacturers to extend the levels of allergen characterization and control, allowing homogeneity between batches that can be

manufactured consecutively and in line with the current state of the art guidance. While more products include quantification of specific allergens, product-specific standardization of “potency” (i.e., based on IgG from inoculated animals, again biased by the used allergen extract for immunizations, or IgE reactivity against human atopic sera pools) is routinely employed by manufacturers. These assays assess the accumulated capacity of all allergens within the mixture, as opposed to using monoclonal antibodies to measure the content of a single allergen. Hence these are expressed in “arbitrary” manufacturer-specific units relative to specific “in-house” allergen reference preparations [20]. As this is the sum of the binding affinity between multiple allergens and antibodies it cannot be measured in $\mu\text{g/mL}$. Therefore it is not possible to compare the “potency” of extracts between manufacturers.

The topic of product-specific standardization and cross-product comparability has been extensively discussed, harmonization of specifications used across all manufacturing processes among the various companies, and the establishment of standards have encountered difficulties, due to the variety of allergens, their sources and analytical methods. In this regard and in Europe, company-specific in-house validation methods shall ensure batch-to-batch reliability and comparability (product-specific standardization) [21-24]. However, content and composition of products from different manufacturers are not equal [25-27], and the measuring methods for concentration and activity differ substantially. Even though efforts to facilitate cross-product comparability are still ongoing [21, 22], many physicians – motivated by questionable marketing campaigns - tend to compare products from different manufacturers by exclusively relying on content of major allergens, which led to the belief “more is better”.

This article discusses the issues related to standardization, measurement of major allergen content and the shortcomings of using concentrations and biological activity for

comparing products, especially in view of personalized therapy and evidence-based medicine.

2. THE PITFALLS OF TRADITIONAL CONCEPTS – UNDERSTANDING PRODUCT STRENGTH

The key of AIT is administering *high doses* of an allergen to sensitized patients in order to increase their immunological tolerance to this allergen [28], the overall goal thus is disease-modification. However, it is not only relative high doses but also administration via a “non-natural” route: inhalant allergens primarily encounter mucosal surfaces of upper and lower airways whereas AIT is administered into subcutaneous tissue or under the tongue. In 1993, the European Academy of Allergy and Clinical Immunology (EAACI) defined AIT maintenance dose as “the highest tolerated dose by the patient without side-effects”, and this was based on the content of purified major allergens [29]. Thus, it was postulated for many years that a specific amount of a major allergen in each product corresponded with the optimal dose to reach a clinically relevant effect without causing unacceptable adverse events. This concept neglects the fact that the dose-response in drugs, but also in AIT, is not linear. Whatsoever, the concept of allergen concentration as a measure of suitability of an extract was rapidly taken up by the specialists but also marketing departments, not considering the aforementioned lack of homogeneous standardization of allergen extracts [21, 22]. It is also important to highlight that for many allergenic source materials (e.g. grass or HDM), a uniform consensus of what are considered immunodominant allergens are subject to different interpretations and may even depend on the population, its exposure to other allergens and the abundance. The erroneous use of this parameter to compare products was the consequence. Some SCIT (subcutaneous immunotherapy) and SLIT (sublingual immunotherapy) products with

variability in the content of the major allergen have proven efficacy in the context of clinical trials [30]; this indicates that allergen concentration is likely only one of several factors influencing efficacy of extracts. In fact, in the field of AIT, there is a limited number of products that have undergone well-designed dose-response-studies (phase II trials) [31]. In the present state of the art and regulatory context, allergen extracts should be evaluated individually by conducting rigorous clinical trials, to demonstrate efficacy [21, 32-34] and, in particular to establish the optimal dose defined by best balance between clinical efficacy and safety [31, 35].

It has to be emphasized that AIT is not comparable to traditional vaccination in immunologically naïve patients. In contrast, the pre-sensitized patients are treated with AIT formulations derived from materials they are already sensitized to, leading to the possibility of specific IgE mediated adverse events such as anaphylaxis. Therefore it could be argued that within future product design there is a moral imperative to deliver the lowest amount of allergen that would generate the optimal clinical effect as opposed to the highest concentration that is tolerated. This is particularly relevant for native AIT products where there is no attenuation of allergenicity.

3. KEY CONCEPTS REGARDING ALLERGEN CONTENT IN AIT PRODUCTS

Manufacturers have developed methodologies to assess complex allergen extract concentration, biological strength in terms of potency, as well as guarantee in-house batch-to-batch consistency and stability [36]. Allergen ordinance processes such as the TAO (Therapy Allergen Ordinance) [20, 37] initiated by the German Paul-Ehrlich-Institute (PEI) have been successful in increasing the requirements for product standardization and other countries are following. Initiatives such as the CMDh (Co-ordination group for Mutual recognition and Decentralised procedures – human) [38] provide legal framework

for regulators enforcing licensed therapies. Allergen extracts are medicinal products that should be regulated in accordance with applicable legislation in the US via the Food and Drug Administration (FDA) and the EU via the European Medicines Agency (EMA) (Directive 2001/83/EC) [24, 39].

3.1 Standardization of allergen extracts

In Europe [23], production of standardized extracts is mainly regulated by the *Guideline on Allergen Products: Production and Quality Issues* [22] and the *Monograph on Allergen Extracts* of the *European Pharmacopoeia* [40]. Allergen standardization ensures continued quality and reliability of each manufactured product. It concerns 1) the source, 2) the development of reference material and, 3) the measurement of quantity and activity (potency) of an allergen extract and the units (**Figure 1**). All these steps ensure the quality of each batch produced by a given manufacturer, but standardization is in-house, and variability in methodologies exists in all steps among manufacturers.

This process begins with the proper *identification* of the allergen, by means of controlled selection of the source material to be used in the preparation of allergen extracts (extraction process). Control of materials pertains to source and raw materials. The former refers to the natural moiety from which allergens are extracted such as biological samples or cell cultures for recombinant proteins. The latter includes solvents, media, chemicals used for extraction or cell culture reagents in the case of recombinant protein expression. Allergens derived from animal sources must comply with safety rules in order to exclude any source of infectious agents [41-43]. Monitoring raw materials should state references of material suppliers, a thorough material description, geographic location,

genus, species and type, cultivation and collection protocols, storage/shipping conditions and purification and manipulations must be registered [22].

Regarding the development of reference material, the US Food and Drug Administration develops and maintains US reference standards, and serum pools for manufacturers to perform lot testing [23, 44]. Whatsoever, this manuscript focusses on Europe: Harmonization and standardization across manufacturers have been considered in the EU, that supported the project CREATE (Certified References for Allergens and Test Evaluation) with the objective to develop international reference standards, from both purified natural and recombinant allergens, with verifiable allergen content [45, 46]. In this project, eight major allergens contained in the most frequent inhalant allergen sources were selected for such purpose: Bet v 1 (birch pollen), Ole e 1 (olive pollen), Phl p 1 and Phl p 5 (grass pollen), and Der p 1, Der p 2, Der f 1 and Der f 2 (HDM). The outcome of this project, after exhaustive characterization efforts and evaluation of ELISA (Enzyme-linked immunosorbent assay) data, Bet v 1 and Phl p 5 were identified as good candidates for further development in order to introduce an official biological standard(s). CREATE was followed by the Biological Standardization Programme (BSP090) of the European Directorate for Quality of Medicines and Healthcare. The somewhat sobering outcome of two decades of allergen standardization research were the establishment of two validated reference ELISAs - one for birch = Bet v 1.0101 [47] and one for grasses = Phl p 5.0109 [48]. Implementation of the Bet v 1 and Phl p 5a ELISA protocols as general chapters in the Ph. Eur. is currently in progress. Clearly, CREATE and BSP90 have unraveled the limitations of ELISA techniques [21], which are multifaceted including the problem of discrimination between isoforms as an example, and highlighting the complexities of achieving analytical standardization. Therefore, international standards are lacking for most allergens and manufacturers utilize their own in-house reference

preparations (IHRP) [49]. While this will enable cross-product comparability of birch pollen and timothy grass pollen allergen products based on major allergen content alone, it is important to re-emphasize the fact that single allergen concentration is likely only one among several factors influencing efficacy of extracts. Hence, the Paul Ehrlich Institute already back in 2016 stated [21]: “Despite the large progress in total allergenic activity determination and quantification of individual allergen molecules, the resulting values remain incomparable. Hence, today, allergists cannot decide for one or the other allergen product based on comparing contents of active ingredients or potency.”

3.2 Quantification of allergens

Quantification and verification of at least one major allergen during production is a quality requirement of regulatory entities [40, 50] and efforts are made to harmonize the quality of products [22]. However, considerable variability exists in measurement and reporting allergen extract strength.

3.2.1 Methods for quantification

Generally, the procedure involves *in vitro* testing. Once overall strength of the original extract is determined by using an *in vivo* method, this data is used to develop an IHRP to estimate the strength of extracts from other batches by an *in vitro* method. The IHRP can then be cross validated over time as the materials reach their expiry. ELISA is commonly applied as a measure of *in vitro* biological activity [51]. The measurement of micrograms of major allergen is only a standardized procedure for Bet v 1 and Phl p 5a (in development), and the results depend on the technique, the reference, and the antibodies used in case of e.g. immunoassays [52]. The results have proven to be variable from identical extracts. While ELISA is a universal, affordable, and sensitive

technique for detection of analytes such as proteins in allergen samples, its main limitation falls in the nature of the test itself. ELISA relies on the interaction between antibodies and specific epitopes on the surface of antigens present in allergens. In addition, monoclonal antibodies may not bind to all relevant iso-allergens and values obtained might differ from assays using polyclonal rabbit antibodies [53]. It has been shown that various assay variables may impact the result, and antibodies may vary across different laboratory protocols [52]. In addition, ELISA-based procedures cannot detect variations in allergen composition across different samples. This is important because patients may be tolerant to a specific subset of proteins but not to others in the same allergen extract (Figure 2). Finally, antibody-based approaches have also shown various shortcomings for quantification of allergoids, and adsorption of allergens to adjuvants may hinder the ability to create the required immunoconjugate by nature of the product conformation. Furthermore, two distinct allergoid studies [54, 55] demonstrated that the polymerization process disrupted conformational epitopes and led to the creation of new structures, harboring novel IgG epitopes. The formation of such may have the capacity to improve the immunological effects, such as blocking IgE epitopes or inducing novel IgE specificities potentially depending on the adjuvant used.

Mass spectrometry techniques are further developed to measure the strength of allergen extracts in a quantitative manner [23, 54, 56-59], but are currently too laborious and expensive for routine quantification and have lower throughput. The quality also depends on e.g. functional proteomic approaches to see which peptide chains are active, important, if proteolytic activity of pollen content is analyzed This allows identification of immunologically active pollen content and allergenicity beyond the known major and minor allergens.

This approach may also allow for “total” quantification of allergens, even from different species within a complex mix, due to its unrivalled specificity, which circumnavigates aforementioned issues associated with immunoassays. Moreover the way of quantification is variable with semi-quantitative techniques (ion-counting, spectral counting) or quantitative analysis by labeling samples with internal or external standards. Mass spectrometry will become an important technique in our field, but still needs to be further developed with regards to its interpretation, and harmonization across users, due to its limitations such as the large outcome datasets and their analysis, interpretation and graphical display [60].

3.2.2 Allergen concentration versus biological strength

Allergens are proteins made up of precisely folded polypeptides that trigger an immune response dictated by their surface-exposed epitopes, i.e. the specific region of an antigen targeted by an antibody. The structure of these epitopes, their level of exposure on the solvent accessible surface of the allergen, and the composition of the final product (e.g. adjuvants, modification) will strongly influence the biological strength (potency).

The labelled strength of allergen preparations in the EU are often expressed by proprietary units [26, 61-63]. Essentially, units are required to show that a product can be manufactured in a reproducible way. Biological units during development of specifications may be different from those used for labelling product strength, although there should be a consistent factor derived from an assay that is related to the final unitage of any particular product. Such final unitage must have a comprehensible scientific basis. For instance, correlation of *in vivo* biological standardization, by appropriate methods on the basis of skin reactivity test using methods such as those described by Turkeltaub [64] and Nordic Council of Medicines [65], and corresponding *in vitro* allergenic activity of the (first) IHRP should be described, with potency labelling

based upon *in vitro* testing. Each new IHRP is then prepared and compared against the previously assigned preparation, thus can provide an unbroken link back to original *in vivo* biological standardization studies. Manufacturers use several types of allergen units: histamine equivalent in prick testing (HEP), biologic or diagnostic unit (BU or DU), bioequivalent allergen unit (BAU), Therapeutic Unit (TU) or Standardized Unit (SU) [26, 62, 63]. However, regardless of the unitage employed, manufacturers may correlate *in vivo* standardization with *in vitro* testing using different factors, which results in arbitrary values assigned to the final product, and as such does not permit products to be compared between manufacturers for the purpose of comparing strength/content accurately [20]. A different approach reported was the computer-based comparison and correlation of SLIT solutions based on skin prick testing results with these AIT solutions [66].

Adjuvants or adjuvant systems (AS) are used in SCIT products to increase the clinical efficacy of treatment, and to reduce the number of doses needed to induce immune tolerance [67, 68]. The last decades have seen the development of two novel adjuvants used in AIT products as an AS: MATA (Modified Allergen L-Tyrosine Adsorbate)-MPL combines chemically cross-linked allergens (allergoids) adsorbed on the depot AS microcrystalline tyrosine (MCT) [69] in combination with Monophosphoryl Lipid A (MPL). Formulation and combinations of such additives matter [70], and characteristics such as the physico-chemical properties of depot-adjuvants such as MCT have an impact on immunological mechanisms [71]. In this regard, the variety of adjuvants in development, including immunostimulatory sequences and nanoparticles among others [68, 72] will further increase the differences in immune responses elicited by different products based on similar allergen extracts and content.

3.2.3 Allergen quantification and stage of manufacture

In addition to the different methodologies employed to quantify allergens, the manufacturing process step from which the obtained data is derived from, may vary. For instance, quantification in the final product presents problems: in native extracts, adjuvants or other additives interfere to some extent in several quantification tests. In the case of allergoids, this may not be possible due to the modification process. As such, allergen measurements may be derived from earlier stages of manufacture, or in essence, the last feasible time-point across the process. The methods used for polymerization (modification of the allergen extract to decrease IgE allergenicity but retain IgG immunogenicity) results in chemical cross-linking of allergens with glutaraldehyde, potassium cyanate or formaldehyde, resulting in antigen structure changes, e.g. disruption of conformational IgE epitopes [54, 73]. Noteworthy, the chemical modifications applied to these antigens have an impact on standardization protocols, as *in vitro* assays are disturbed by the reduced concentration of available epitopes for antibody binding and cross-linking. Therefore, tests such as the determination of total allergenic activity are usually performed during early targeting of drug substance strength in the manufacturing process, e.g. with the native allergen extract [44]. IgG potency methods for allergoids are “expected” albeit not essential part of the monograph/guidance.

Finally, conventional ELISA methods used for quantification have an additional limitation in case of mixed-species extracts, as an antibody approach may not discern between allergens from homologous species within the mix.

Interference of depot adjuvants added in the final product, such as aluminium compounds, should also be mentioned. The guideline states that testing should be at the latest and feasible stage of manufacture, but some content or potency assays may be

hindered once the product is adsorbed or absorbed to the adjuvant. Protocols including the information about timepoint and method for quantification are in-house and specifically designed for each extract or source material.

When formulating final products by diluting and combining active pharmaceutical ingredient (API) with depots, this in particular can cause issues for measurement of specific or total content due to the (desired) binding between protein and depot. Whilst this binding is desired pharmacologically to retard release of the API, it makes analysis difficult, variable and potentially impossible. When binding complex mixtures to aluminium hydroxide e.g., different binding efficiencies may be experienced, leading to potential variable results. For next generation depots such as MCT, methods have been developed to universally reverse binding (under non-pharmacological conditions) that allow consistent measurement of API improving the ability to characterize final Drug Products. Again, only allowing an in-house product-specific batch-to-batch comparison. If companies want to report major allergen content, they should therefore be obliged to clearly state at which manufacturing step these values were measured and which further manufacturing steps will follow after measurement.

4. WHAT IS THE REAL PURPOSE OF ALLERGEN QUANTIFICATION?

4.1 Verification of quality?

Product quality is the key element for any marketed drug. The *quality* of allergen extracts depends on source, extract purification, antigen composition, stability of product, and overall strength [62]. Every stage during manufacturing process until the final marketable product marketed in the EU must follow the guidelines set by the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH)

and the Committee for Medicinal Products for Human Use (CHMP) of the EMA [22, 23]. As said, quantification and verification of at least one major allergen is expected and the allergens relevant for the product have to be defined by the manufacturer [22, 40, 50] (Figure 2). In addition, clinical trials to evaluate the safety and efficacy of allergen extracts are done with a well-characterized and standardized product that needs to be reproducible batch by batch. This means validating an assay which is accurate and precise, including other important quality parameters outlined in ICH Q2R1, as such, provides a robust level of product-specific standardization. Logically, the manufacturers need to ensure that all subsequent batches comply with the specifications and the quality of the product tested, in which allergen quantification plays a role, otherwise the batch would not be released by authorities such as the PEI. But other aspects differing across products have an influence on the clinical outcome due to immunological mechanisms of action and are independent of quantity of major allergens. These characteristics include whether the allergen is native or an allergoid, the preparation is depot or non-depot, which adjuvant/AS was used, route of administration, posology (including dose volume and frequency) etc. [74], all of them carefully controlled and standardized regarding in-house procedures.

4.2 Check whether the product contains an optimal dose?

It is tempting to use the label information to check whether a product contains an “optimal” dose, especially if the clinician keeps the traditional definition of maintenance dose of EAACI as *“the highest tolerated by the patient without side-effects”* [29] in mind. As regulated, in vials of non-modified allergen preparations, the units must refer to total allergen-mediated activity (strength or potency) as, for example, measured via IgE or other competitive binding assays. As previously described, this introduces arbitrary factors associated with the unitage assigned. In addition, the individual amounts of each

allergen in the formulation will be stated in units of mass per volume. Micrograms of major allergen is the widely recognized method to express single allergen strength, and, in some cases, good correlation with biological strength has been reported [75-77]. However, as discussed, assay differences, procedures and extract variations influence the results of quantification [78-80] and seem to massively influence the major allergen concentrations reported by different companies [26, 52, 62]. Hence, using allergen content values to correlate “optimal strength” with units reported from another manufacturer is unwise and scientifically flawed [21].

Optimal dose for each product should be based on methodologically sound dose-finding phase 2 clinical trials that follow regulatory standards. In fact, phase 2 studies following EMA guidelines for new products and preparations to be licensed within the German Regulation for Therapy Allergens (TAO) are mandatory since 2008 [31, 37]. As a result, we have seen an increasing number of clinical trials in the field within the last years, most of them with shortcomings that have resulted in inability to define the optimal dose in a satisfactory manner [31]. However, some approaches were successful after careful trial design development, such as the definition of a statistically significant dose-response curve including a plateau to a birch allergoid [81], a grass allergoid [82] and a mite allergoid [83]. These trials showed the best suitable dose defined by the best possible reduction in conjunctival or nasal provocation test score, not limited by occurrence of adverse events. In fact, demonstrating a significant dose-response-relationship with an efficacy plateau means that efficacy **decreases** if allergen amounts are further **increased**! This aspect seen separately already shows the limitations of the “more is always better” approach.

Notably, the “optimal dosage” of a drug is not the most efficacious, it is the dosage with the optimal benefit-risk ratio. Therefore, dose-finding trials must not only be assessed on

the efficacy side. For the AIT field the optimal dosage of native allergen preparations was always defined by tolerability limitations. None of these products demonstrated an efficacy plateau. For allergoids this has been different: because of the modification process, the tolerability remains unchanged despite increasing the dose up to 7 times. Thus, the optimal dose in modern allergoids is defined by the efficacy plateau and not safety concerns [81, 82]. In any case, assessments are product specific, and the concept of *class effect* should be applied with caution to AIT products.

4.3 Cross product comparability?

It is of limited value to aim for cross-product comparability in the present state of the technique and regulations regarding production and testing. Finalized products are designed differently for various reasons and additives such as adjuvants, and the final preparation will result in a variety of products whose clinical characteristics cannot be attributed to major allergen content solely. Where harmonization is being progressed with respect to major allergen testing of Bet v 1 and Phl p 5a, such immunoassays are limited to one allergen at a time. Thus, allergen sources with several major allergens are difficult to standardize and better tools to do so are increasingly demanded. This single allergen approach for relevant allergen standardization again highlights the importance of considering these results in the context of the complete specification also including total allergenicity/antigenicity and allergen profile. One novel approach encompasses targeted proteomics (via mass spectrometry; MS) but additional research is needed to enable full implementation in allergen standardization [60] as further complex aspects need to be considered, such as the myriad MS technologies and methodologies, product matrices, validation and defining acceptance criteria. Currently no guidance endorses

any single approach nor provides any framework for data interpretation. Until these exist, the challenges related to cross-product comparability remain.

4.4 Thus, are major allergens not important at all?

It is intuitively right to state that an AIT product should contain the sensitizing allergens of an allergic individual. In fact, for some major allergens, such as Api m 10, the presence of such in final formulations was linked to treatment outcome in predominantly sensitized individuals [84]. Presence but also stability of such fragile major allergens might be critical in the final preparations [85, 86]. Therefore, the greatest value may not be the demonstration of a set quantity but the qualitative demonstration of presence over the applicable timeframe which is clinically important.

Despite the large progress in quantification of individual allergen molecules, resulting values remain incomparable between manufacturers. CREATE and BSP90 are a clear example, even after two decades of research and development, harmonizing the measurement of specific allergens by ELISA remains a challenge. Harmonization of total proteomic measurements is the new focus, but hurdles remain with this technology and the missing necessary guidance. Despite this, the myriad product-specific nuances with respect to product matrices and clinical use of AIT products does not change. Whether the amount of (major) allergen is sufficient in that preparation or not will be demonstrated in clinical trials and not in artificial units.

5. SUMMARY

The aim of this article was to review the issues related to the measurement of major allergen content in AIT extracts, and discuss whether comparison of products by different

manufacturers or calculating optimal doses based on concentration of single major allergens, could be the appropriate basis to choose among the different AIT products by clinicians.

Allergen quantification, and verification of at least one major allergen during production is a requirement of regulatory entities, including FDA and EMA [24, 39]. Those processes are both critical for the verification of quality of the AIT extract and the comparison between different batches in order to guarantee in-house batch-to-batch consistency. However, single allergen quantification alone cannot be used for cross product comparisons of different manufacturers due to the lack of international standards and their in-house preparation and quantification methodology (Table 1, Figure 3).

When deciding the optimal product for a particular patient, it is important to keep in mind that the biological strength or immune response elicited by the AIT product does not exclusively depend on a single allergen concentration. It is notably influenced by the epitope structure of the allergen and their level of exposure, also depending on potential chemical modification (allergoids) by natural matrix effects in the extract, and by the different adjuvants used in the AIT extracts to increase the clinical efficacy of treatment, and to reduce the number of doses needed to induce immune tolerance. Moreover, unitage reporting considerably varies among manufacturers, which does not allow to check if a product contains an optimal dose for a patient solely by evaluating allergen concentration, or by comparing different formulations (Table 1). As shown in Table 1, standardization of allergen is needed, but it is important to be aware of the limitations of the process.

6. CONCLUSION

Clinicians should be informed about the special circumstances regarding allergen quantification in AIT extracts. There needs to be a broader understanding about the pitfalls of comparing products based on allergen content, the use of this information as sole basis to estimate “optimal dose” or the suitability of an extract for a given patient. As suggested by Pfaar et al. in the German 2014 AIT guidelines [32], preparations with documented efficacy and safety in clinical trials meeting international regulatory standards or tradeable under regulatory ordinance should be used preferentially, and the specialists should bear in mind that, at present, any assessment of AIT preparation is source- and product-specific and similarities among products from different manufacturers in terms of the same content of major allergens cannot be anticipated. This review evaluates the special circumstances that clinicians face when comparing different AIT products, and describe why simply checking the content of single major allergens of a certain extract might not be a recommendable approach for achieving the best outcome for a patient (Figure 3).

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Table

Table 1. Checklist: When is allergen standardization and quantification of major allergen content the right method to use?	
✓ YES: Product-specific Standardization	✗ NOT: Cross-Product Comparability
<ul style="list-style-type: none"> ✓ Control of manufacture process parameters complying with EMA/FDA requirements. ✓ Control of product quality over shelf-life. ✓ To validate quality in-house and ensure reproducibility across batches. ✓ Ensuring consistent quality and, in turn, safety and efficacy. 	<ul style="list-style-type: none"> ✗ To compare contents of active ingredients or potency between products from different manufacturers in the absence of a harmonized procedure, and conclude whether the product contains an optimal dose. ✗ To compare arbitrary labelled potency units or allergen content between products. ✗ As a sole basis to select among the different AIT products available for a patient.

Figure Legends

Figure 1. A. Allergen extracts are manufactured following the guidelines set by the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) and the European Medicines Agency (EMA). Among several aspects, these regulate standardization of extracts with an official standard (if existing) or an in-house reference preparation (IHRP) validated by each manufacturer. **B.** Investigational products are manufactured according to the IHRP specifications; and clinical trials should be carried out for each individual product to test clinical efficacy, safety, dosing, and pharmacokinetic and -dynamic profile. **C.** The marketed product is produced in standardized reproducible batches. In the final product, allergen profile and quantification and biological potency are checked to match the specifications of the IHRP.

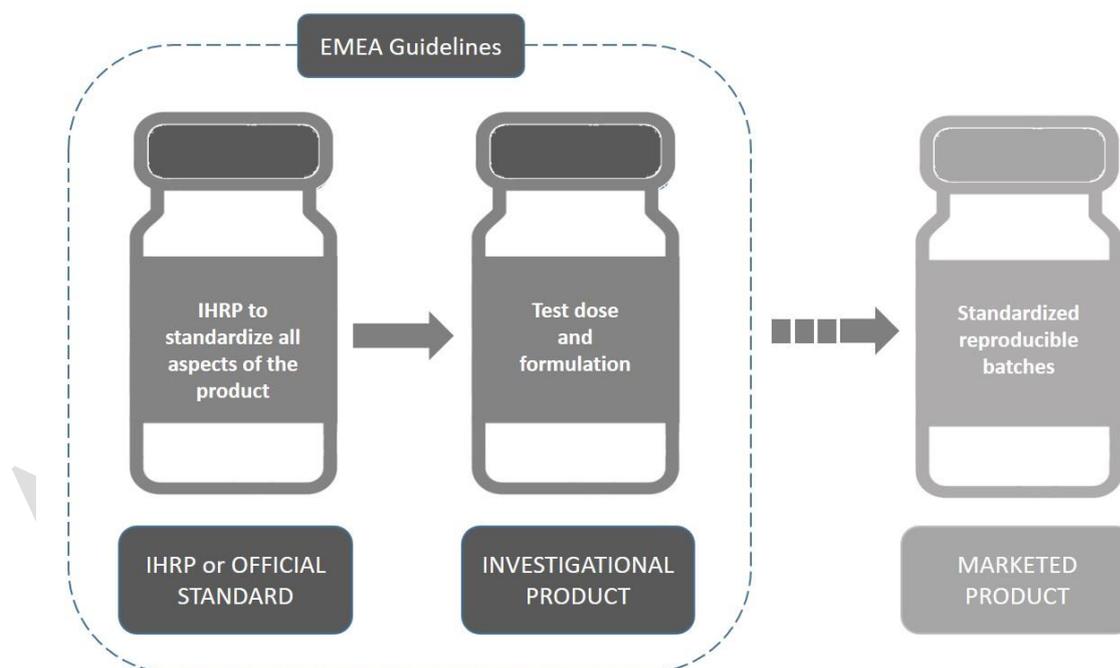


Figure 2. Theoretical example of challenges experienced when developing specifications to characterize and standardize AIT products. Note that this is a conceptual figure (All. x 4 = Allergen “x” 4, the theoretical major allergen). **A.** In general, each allergenic species contributes multiple allergens to the extract at ratios comparable to those that would be experienced by the patient under normal exposure. In some cases this exceeds 20 known allergens. **B.** A relevant/major allergen content assessment provides concise information about a specific allergen of interest that is shown to be relevant to 50% or more of sensitized patients but shows no additional data on other allergens. **C.** Patients who are allergic to the same source material may react both to different allergens in the mixture and, if allergic to the same allergen, with different intensities. Note that the major allergen content would be highly relevant for patient 2 but irrelevant to patient 3. **D.** A diversity of donors is useful in constructing an allergen specific donor pool that can be used for total allergenicity methods. These are critical for understanding allergy product relative strengths, they show however they provide information on how each individual allergen contributes.

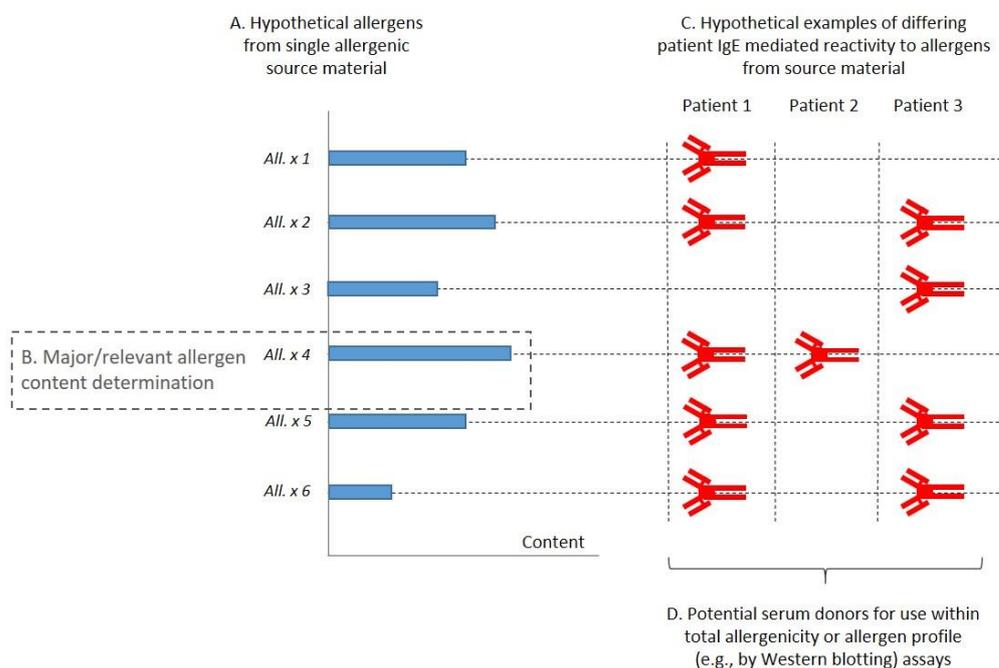


Figure 3. Representation of fundamental challenges regarding the models that summarize the characterization of AIT products. **A.** Indicates the imbalance of the complex reality of allergen characterization and translating this to a single number. **B.** Illustrates an appropriate balance of data required to make meaningful judgements regarding product characterization. (RoA = Route of Administration).

