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Control Process for Manufacturing and Standardization of Allergenic Molecules

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Abstract It is widely accepted that the success of the allergen immunotherapy (AIT), beyond clinical parameters such as dose, dosage regimen, or compliance, depends on the quality and composition of the final products used in the vaccines. Allergenic vaccines are pharmaceutical preparations derived from the natural sources which contain the allergenic components responsible for allergic sensitization. The selection of the appropriate allergenic sources must be a requirement. They suffer a dramatic transformation during the manufacturing process which renders a biologically standardized final product. The inclusion of the appropriate control analyses in the manufacturing process has demonstrated to be an efficient method to guarantee the quality and homogeneity of the final product as well as being a very useful tool for saving time and money. In this context, in the last years, the Regulatory Agencies have released specific guidelines to guarantee the manufacturing of the most appropriate products for the treatment of patients.

Keywords Allergy vaccines · Standardization · Process control · Raw material · Allergenic sources · Allergens · Allergen extracts · Final product

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Introduction

The European Pharmacopoeia (EP) [1••] defines the allergenic extracts or allergenic vaccines as pharmaceutical preparations derived from naturally occurring source materials which contain the allergenic molecules responsible for allergic sensitization. In general terms, allergens can be defined as proteins or glycoproteins with a molecular weight range from 6–8 kDa to 100 kDa. However, during the last years, it has been demonstrated that not only proteins but also other substances such as polysaccharides, peptides, or even chemical compounds are responsible to induce allergic reactions when they are combined with the appropriate carriers.

The allergenic extracts constitute the core for the diagnosis of allergic sensitization and allergen immunotherapy (AIT). AIT is an immunomodulatory treatment recognized as the best option for the treatment of allergic diseases and the only method to prevent the onset of asthma in patients suffering from allergic rhinoconjunctivitis. It is recognized worldwide that the success of the AIT depends on the quality of the composition and the dose of the allergenic vaccine beside other clinical factors such as compliance or the appropriate selection of the treatment. Many different in vitro and in vivo experiments have been designed and carried out in cell cultures, animal models, or patients (clinical trials) with the objective to identify and clarify the mechanism of action of AIT and to investigate the relevance of biological units and the amount of major and minor allergens needed.

For over a hundred of years, allergenic vaccines have been manufactured from the extraction of natural allergenic sources such as pollen grains and mite cultures. The aim of this manufacturing process was to produce enriched protein solutions containing a high proportion of the water-soluble allergens present in the raw material. This concept remains similar since 1911 when the first allergenic vaccine, an aqueous

solution of grass pollen, was used for the treatment of allergic patients [2, 3]. However, the methodology for the extraction, purification, or preparation of allergenic vaccines has been sophisticated and industrialized through the years. Moreover, with the release and coming into force of specific guidelines for the allergen manufacturing process and the new regulatory requirements of international agencies published in the last 5–6 years, the concept of natural and allergenic vaccines is being modified and adapted to the modern pharmaceutical products standards. Additionally, new products are being developed under the umbrella of immunological-based concepts such as peptide-based vaccines, molecules based on biotechnological models such as recombinant allergens or DNA vaccines or considering the pathophysiological mechanism of the disease such as biologics or monoclonal antibodies which are currently under preclinical and/or clinical development [4, 5].

Finally, the concept of standardization is one of the current cornerstones of AIT and allergenic vaccines. This concept became a reality during the 1970s when the FDA released the criteria of potency and allergen composition with the objective to establish a method for the estimation of the clinical relevance of allergenic vaccines and to guarantee the safety and efficacy of the product.

The European Guideline in Allergen Extracts

From 2007, the European Medicines Agency (EMA) was working in a new guideline for allergen extracts which was published and came into force in November 2009 [6••], replacing the previous guideline released in 1996. The objective of this new version was to provide the principles and guidance for manufacturing and quality control of allergen products of biological origin, including allergen extracts from natural source materials, which are the current base for the manufacturing of AIT. Moreover, the document also contained the recommendations for the production of allergenic extracts based on recombinant proteins, although these products are not a commercial reality yet. Although the document constitutes only a recommendation for the manufacturing of allergen extracts, many points contained within it have been transposed to the EP and it has become a reference document for Regulatory Agencies of different countries in Europe and the model for the industry.

The guideline established some concepts such as the homologous groups for the classification of different allergen sources [7•], the mixtures of allergens, and the comparability between the different steps of the manufacturing processes. Additionally, the document is focused on the description, characterization, and control of the active substances, intermediate products, and finished products which constitute the cornerstone of the allergen extracts.

Process Control Tools in Allergenic Vaccines

In the case of allergenic vaccines, it is not possible to determine beforehand the final product consistency, homogeneity, or the clinical efficacy and safety when a raw material is selected for the manufacturing process. For this reason, the control and optimization of analyses carried out during the process usually provide an advantage, since data are collected directly during the process, increasing process quality, confirming the homogeneity of different intermediate products and reducing the risk of rejection of final products due to nonconformities, and reducing, therefore, the process time and costs.

In 2004, the FDA released a guideline for the industry describing the regulatory framework for the development and implementation of innovative pharmaceutical developments, manufacturing, and quality assurance. This guideline was in line with the concept of the definition of a link between the design of new products and processes and an effective control of all critical quality specifications [8]. In that sense, allergenic products are not an exception. Allergenic vaccines are obtained after different critical steps and modifications of the raw material, often influenced by the intrinsic variability of the characteristics of these products. For that reason, process monitoring and control strategies are essential for the achievement and maintenance of the desired consistency and homogeneity of the final products.

According to the recommendations of the abovementioned guidelines, a process control must include:

- The identification and quantification of key/critical parameters of the raw material as a critical start point for the production of a high-quality product.
- The design of a process control system that allows real time or near real time monitoring of all critical attributes
- The design of a process control system that allows adjustments to ensure control of all critical attributes.

The Manufacturing Process of Allergenic Vaccines

From nature to patients or, in other words, from raw material to AIT, allergenic sources suffer an important transformation consisting mainly in the extraction of the allergenic relevant molecules (usually mixed with other molecules), adjustment of the concentration based on allergen standardization, and manufacturing of clinically efficacious and safe final products. This process is the result of different steps, including different "in process" control analyses which will guarantee a consistent and high-quality product with the appropriate dose for the treatment of patients without undesirable systemic reactions, including potentially life-threatening anaphylaxis.

Allergenic Source Material

The source material constitutes the starting point of the process for the production of allergenic vaccines [9•]. Its quality and characteristics have a critical importance. Allergenic sources are defined by their origin, nature, method of collection, method of production, and pretreatment. Control methods of the source material and acceptance criteria are major issues, and the investment of efforts and controls at this stage is key to guarantee the quality of the final product and is defined in the EP. The acceptance criteria defined for the use of raw material must include parameters that ensure the consistency of the source material from a qualitative and quantitative point of view, must guarantee the identity and purity of the raw material, and must include limits for microbial and/or foreign particle contamination.

Another important issue for raw material is the stability and the storage conditions. Most raw materials, and specially pollens, are seasonally collected and may be used several months or even years after the collection period (Table 1). The definition of appropriate and controlled storage conditions must be clearly established.

The most consumed allergenic sources for the production of allergenic vaccines include (Table 1):

- Pollens. They are obtained from natural or cultivated plants and can be collected by vacuum or water setting. The most critical issues are the correct identification of the species and the contamination with foreign pollens or mould spores (<1 %). Both parameters are crucial for the quality of the raw material and must be determined and accepted prior to the inclusion of the raw material in a manufacturing process. Another important parameter is the variability of the pollen depending on climatic conditions. Temperature, humidity, position of the flowers in plants, orientation, environmental contamination, diseases, or age of the plants may also influence the allergen composition and concentration [10, 11]. Keeping in mind the above detailed potential high variability, it is accepted that homogeneous lots may be generated through the mixing of lots from different years or locations [12]. Finally, the content of pesticides, solvents, or heavy metals must also be monitored and minimized.
- Mites. The origin of mites for the production of allergen extracts are mite cultures. They are produced in large quantities under optimal conditions of temperature and humidity, using specific culture media with the less possible allergenic constituents. The absence of contamination with other mite species is a key point that must be always confirmed before the selection of the raw material. Although mites are produced under controlled conditions, the variability of the raw material arises from the composition of the culture. Culture medium and conditions may influence the growth and development of mites and the final composition which includes eggs, fecal particles, adults, or immature individuals that may modify the allergen content [13]. The homogeneity of the raw material, established by the composition, must be controlled.
- Moulds. They are typically cultured from well-defined strains and in standardized conditions to guarantee the

Table 1 Description of the different types of raw materials, parameters, and variable which must be considered before the approval and process in control parameters to be analyzed

Source material	Causes of variability	Parameters to consider	"In process" control
Pollens	• Climatic conditions • Areas of collection • Varieties • Contamination by foreign contaminants • Contamination by other parts of the plants	• Variability of the potency and protein content	• Levels of pesticides, solvents, and heavy metals • Identity • Purity • Foreign contaminants
Mites	• Growth conditions • Presence of different components and biological stages • Culture medium • Contamination by other mite species	• Allergenicity of the medium	• Identity • Homogeneity • Purity
Moulds	• Strain • Composition of metabolic or somatic components • Culture medium • Contamination by foreign strains	• Allergenicity of the culture medium	• Identity • Absence of mycotoxins • Homogeneity and protein content • Purity
Epithelia	• Breed or variety • Type of product, origin, and collection method • Sexual conditions	• Health conditions	• Viral contamination • Homogeneity • Purity
Foods	• Breed or variety • Ripening degree • Origin • Processing stage	• Adequate for human consumption	• Homogeneity • Purity

uniformity across different lots [14]. The selection of somatic or metabolic parts of the moulds may influence the final composition. It is, therefore, critical to establish the percentage of each part to guarantee the "lot to lot" uniformity. Culture conditions must also be controlled, since they may influence the allergen content [15] or biological activity of the raw material and may modify or induce the production of mycotoxins which must be minimized.

- Epithelia. Raw materials from mammals or birds are directly obtained from dander, hair, or feathers, although skin-derived allergen sources are known by a wide variety of terms including epithelium(a) [16]. These structures contain different relevant allergens form epithelia, saliva, liquids secreted by sebaceous glands, or even urine. One of the main issues for this kind of raw material is the lack of uniformity. It has been demonstrated, for example in dogs, that there is an important variability when hair or epithelia is collected from different breeds [17]. In cats, it has been demonstrated that the release of allergens (e.g., Fel d 1) varies between males and females [18]. The shaving process for the collection of hair may also modify the homogeneity. A close shaving collects more proteins related to albumin while a superficial shaving collects more lipocalins. In conclusion, the homogeneity of the epithelial raw material must be deeply analyzed prior to the production of allergen extracts. The avoidance or inactivation of any transmission of viral diseases such as bovine spongiform encephalopathies must be considered. Source animals must be in perfect health conditions as certified by qualified veterinarians.
- Foods. Although AIT for foods is not commercially available yet, with the exception of SLIT for peach allergy in Spain [19], the need to develop homogeneous and high-quality food allergenic extracts is unquestionable not only for diagnosis but also for further development of standardized products for the induction of oral tolerance or for further development of AIT. The homogeneity of this raw material constitutes a real challenge [20]. Origin, species, varieties, storage conditions, ripening degree, or cooking stage among others are some of the factors responsible for a great variability of the raw material which must be addressed [21, 22]. All these parameters must be considered and the protein profile and content determined. In all cases, the products must be adequate for human consumption.
- Venoms. The most common insect venoms belong to Hymenoptera (Families Apidae and Vespidae). The venoms contain a mixture of proteins responsible for the allergenic response, and the raw material consists of the unprocessed venom obtained from the insects by different methods [23]. For the manufacturing of allergenic vaccines, the origin of the source material must be clearly identified. The conservation, storage, contaminants, and protein composition must be specified. Currently in the USA, venoms are standardized according to the hyaluronidase activity using a FDA standard which is used as positive control.

From Raw Material to Active Substance

Extraction Process

In general terms, the manufacturing process to obtain protein-enriched allergenic extracts consists on the homogenization and dissolution of the raw material in the appropriate buffer and the later collection of proteins/allergens in aqueous solution (Table 2). This process is usually carried out by shaking or smooth magnetic stirring of the solution. Along the process, the most appropriate methods must be used for the collection and preservation of the allergenic material. Optimal conditions on parameters such as the composition of the buffer, the ratio solid raw material/volume of solvent, temperature, or time must be maintained [24, 25]. The exhaustive compliance with these requirements will determine the success of the extraction process and, in consequence, the quality and homogeneity of the final protein solution.

For years, allergen extractions have been performed without considering the specific particularities of each allergen source. However, during the last decade, many changes have been implemented in the allergen extraction process with the objective to assure the highest quality of the solution and the most efficacious systems of extraction. Different studies have shown that the extraction from the same raw material subjected to a preliminary pretreatment with specific solvents or adapted methods render different protein and allergen content, allergen composition, and significant differences in the final potency of the extracts (Fig. 1) [26].

From Enriched Protein/Allergen Extract to the Active Substance

The step from the selection of the raw material to the collection of an enriched protein solution, as described above, is usually similar between different manufacturers, with specific strategies of extraction. At this point, extracts are usually filtered or centrifuged to eliminate the solid material of the allergenic source and dialyzed in order to remove low molecular weight components or salts added with the buffer solution. Although this is a fairly simple step, the conditions and materials used can determine the final composition of the product. The conditions of centrifugation, such as temperature or speed, may reduce the proportion of proteins. More than 10 years ago, when small proteins such as LTPs or polcalcins had not been associated to allergic sensitizations, they were

Table 2 Description of the process steps and process control parameters to take into account in the manufacturing process of allergenic vaccines

Process step	In process control	Techniques
From raw material to enriched protein solution	• Quality of the raw material (variability, stability, storage conditions) • Preparation of the raw material (Defat, chemical treatment, physical treatment) • Buffer solution physical parameters (temperature, pH, time, shaking conditions)	• Karl Fischer • Microscopy
Native extract (active substance)	• Water content • Protein and allergenic profile • Allergen identification • Major allergen content • Biological potency • Protein content • Physicochemical parameters	• Karl Fischer • 1-D and 2D SDS-PAGE • IEF • Western blot • CIE/CRIE • Mass spectrometry • ELISA sandwich • ELISA inhibition tests
Polymerized extract (active substance)	• Water content • Protein and allergenic profile • Molecular distribution • Allergen identification • Major allergen content • Biological potency • Protein content • Physicochemical parameters	• Karl Fischer • 1-D and 2D SDS-PAGE • IEF • Western blot • CIE/CRIE • HPLC • Mass spectrometry • ELISA sandwich • ELISA inhibition tests • Bradford/Lowry/BCA colorimetric test • Kjeldahl
Final product (from native active substance)	• Biological potency • Stability • Immunogenicity • Protein content • Alum content • Physicochemical parameters	• ELISA inhibition • Bradford/Lowry/BCA colorimetric test • Kjeldahl
Final product (from polymerized active substance)	• Biological potency • Stability • Immunogenicity • Protein content • Alum content • Physicochemical parameters	• ELISA inhibition • Bradford/Lowry/BCA colorimetric test • Kjeldahl

Karl Fischer technique is used for the determination of water content

IEF isoelectric focusing *CIE*/*CRIE* crossed immunoelectrophoresis/crossed radioimmunoelectrophoresis, *Kjeldahl* quantitative determination of nitrogen in chemical substances, *BCA* bicinchoninic acid assay

removed from the extract due to the cutoff size of dialysis membranes. In other occasions, the nature and composition of filters may interfere in the filtration process retaining some specific proteins and, as a consequence, increasing the variability of the products and reducing the proportion of proteins. But, in general, specific controls and analysis are similar. However, from this point until the production of the active substance, there are a wide variety of processes which will depend on the type of active substance that manufacturers need to obtain.

In some cases, the active substance is the native extract which contains the allergens. The concentration of native extracts is adjusted according to their biological potency (the standardization will be discussed later in detail) in the appropriate solution (aqueous or glycerinated extracts) in biological units if they are biologically standardized or according to the protein concentration (mg/ml or Protein Nitrogen Unit (PNU)) if they are not biologically standardized. As we will describe later, a new method has been proposed in the last years trying to include the major allergen concentration as a new method for allergen standardization. In other cases, the native extract is freeze-dried and stored in vacuum conditions for the time determined in the stability studies until it is dissolved in the appropriate solution and when concentration is required; or it is prepared in a solid presentation after the addition of the corresponding excipients. On the other hand, in many occasions, especially in Europe, native extracts are considered as an intermediate substance. In some cases, the native extract will be subjected to an additional process of purification and concentration prior to an ulterior modification, such us

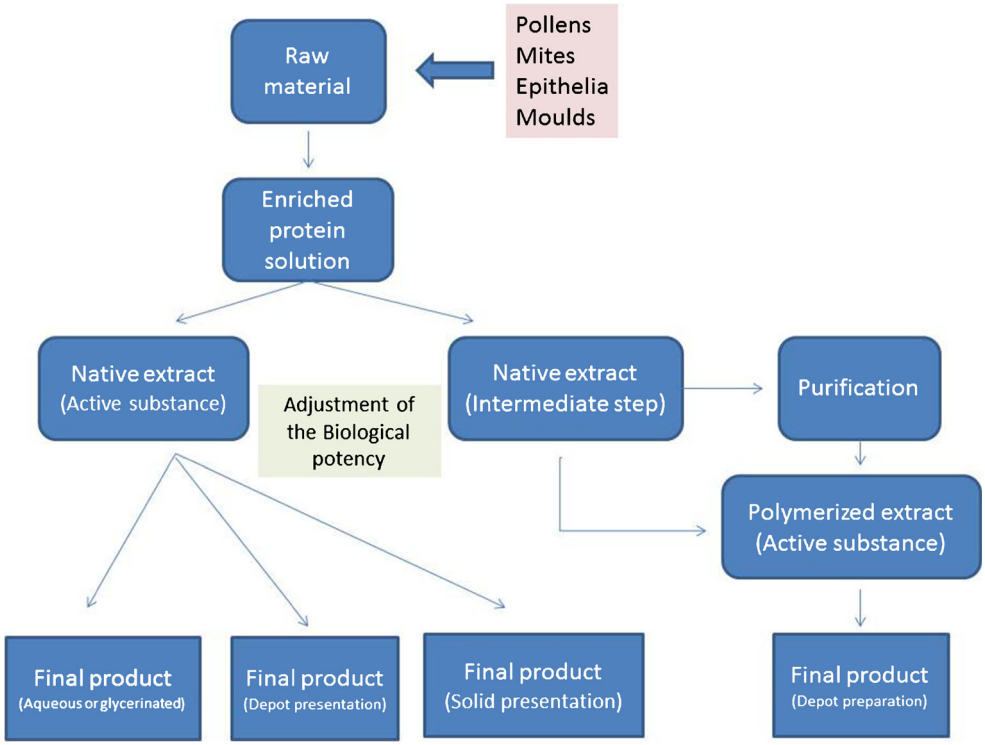

Fig. 1 Manufacturing process of allergenic vaccines. The scheme explains the different steps from raw material until the final products ready to be used in patients. Depot preparations are physically modified products which contain salts (Alum hydroxide is the most frequently used). The objectives of the modification are to increase the immunogenicity of the proteins, enhance the stability of the product, and reduce the induction of adverse reactions

polymerization. The most known process is termed depigmentation, whereby irrelevant substances with low molecular weight are removed and the protein/allergen concentration increased [27]. In other cases, native extracts will be polymerized directly in the presence of formaldehyde or glutaraldehyde [28].

The objective of the polymerization is to generate high molecular weight chains of allergens with a reduced IgE binding capacity when compared to their corresponding native extracts [29•, 30]. Depigmented or not, these polymerized products are the active substance which can be maintained in solution or in freeze-dried conditions. However, polymerized extracts require additional steps and process control which are crucial for the consistency and homogeneity of the resulting product. The concentration/volume of formaldehyde or glutaraldehyde added to the allergenic solution, pH of the solution, rate of incorporation, temperature, or duration process may influence the final quality and consistency of the active substance (in that case, polymerized allergen extract).

In Process Control and Allergen Extract Characterization

Allergenic sources suffer a significant transformation during the manufacturing process. Many parameters may influence the active substance final quality and batch-to-batch consistency. As a result, different acceptance criteria must be fulfilled on the different steps on the process from raw material to allergen extracts (native or polymerized, depending on each manufacturer). With this objective, the European Directorate for the Quality of Medicines (EDQM) and the EMA released new editions which came into force in 2010 and 2009, respectively, with the objective to guarantee an accurate characterization of the active substance prior to the production of the final product.

The acceptance criteria must include, as a minimum, the following parameters: appearance and description, identity, purity and impurities, total allergenic activity, and major allergen quantification. The final objective of the determination of these parameters is to demonstrate the consistency of the process and that the manufacturing process does not alter the presence of allergens which constitute the immunological unit of efficacy. The presence of allergens and its demonstration and confirmation by analytical studies has become a key issue. This is probably the reason why the presence and concentration of individual allergens has become critical as well as the protein profile of the extract or the biological potency, either on polymerized or non-polymerized extracts [31•]. Clinically translated, currently, it can be considered that biological potency is related to the safety of the product, and the protein and allergen content and profile are correlated to the efficacy of the product.

In consequence, it is clear that all the abovementioned parameters and analyses should preferably be measured and identified in native allergen extracts. However, it is known that some of these parameters are difficult or even impossible

to characterize in polymerized products. For this reason, the EP incorporated a premise specifying that if a specific control test cannot be applied to the active substance (specifically in polymerized extracts), it should be analyzed at intermediate manufacturing stages and at the latest stage of the manufacturing process (Table 2).

Standardization of Allergenic Extracts

Allergen standardization is a major issue in the development and use of allergenic vaccines. As a result of the high variability of the raw material and the differences in IgE binding capacity of different lots, the standardization of allergenic extracts based on the capacity of the extracts to bind IgE is crucial for the consistency of the product and in consequence for the efficacy and safety of the product. Undoubtedly, the most critical parameter in allergen standardization is the selection of the product of reference, also known "In House Reference Preparation" (IHRP). This product will be used as the reference for the successive batches and will guaranty their homogeneity. The IHRP must be a common extract, accurately characterized and compliant with all the specifications established for the active substance. Evidently, there must be a specific IHRP for each allergen extract.

The EP and the guideline of allergens require that the IHRP must be characterized by the protein content and protein profile. Allergenic components must also be detected, and the characterization of the allergenic components may include identification of relevant allergens. Determination of the content of relevant allergens must be also performed. Additionally, the biological potency of the first IHRP must be determined by in vivo and in vitro techniques [32•]. It is also recognized the necessity to renew periodically the IHRP, and for that reason, the biological activity of future IHRPs must be adapted and corrected compared to the initial IHRP.

In Europe, there is no homogeneity in the selection of the IHRP, and each manufacturer has developed its own IHRPs, units, and characteristics. However, in the USA, the Food and Drug Administration (FDA) provides all the information needed for allergen standardization and the references. In an effort to unify the standardization of allergenic extracts in Europe, the project CREATE [33] tried to modify the European system of standardization from the measurement of total allergenic activity to the measurement of major allergen content [34]. The results have had only limited success until now because allergen extract continues to be characterized by biological potency, and the manufacturers continue using their own units. However, the EDQM developed two validated assays during the BSP090 [35•] study for the quantification of the major allergens Bet v 1 and Phl p 5a using as standards of the assays, the recombinant forms of both allergens and produced under GMP conditions. Both reference standards are routinely used for the measurement of these two major allergens. The four major allergens of mites (Der p 1, Der p 2, Der f 1, and Der f 2) are currently listed as possible candidates.

The allergen standardization in the USA regulates the units in which allergenic extracts are expressed [36•]. All the products are released and labeled in common bioequivalent allergy units (BAUs), based on intradermal testing [37], due to the existence of a national potency reference preparation. On the contrary, in Europe, there is not a common unit, and each manufacturer has their own reference preparations and products are labeled with different units, making the comparability and interchangeability of products [38] difficult.

The Final Product

The characteristics of the final product in allergenic vaccines may vary significantly depending on the type of AIT or the clinical practice in different countries [39]. In Europe, final products are usually individual vials which contain the final composition (one allergen extract or mixtures) prepared by manufacturers specifically for one patient. In American countries, this concept is significantly different because final products are prepared as bulks, and practitioners may prepare the appropriate prescription for patients containing an allergen extract or a mixture of different allergen extracts [40]. Additionally, in Europe, subcutaneous AIT usually consists on a final product, native or polymerized extract, adsorbed onto alum hydroxide, while sublingual AIT consist on glycerinated solutions which contain the active substance or, in less proportion, solid tablets available, for the moment, exclusively for grass allergy.

With these differences, control of the finished product and appropriate specification must be adapted to the characteristics of each country. However, the premise established for the active substance remains valid, and when it is not possible to analyze the final product, the specifications must be defined at the latest stage prior to the modification step.

In case of alum adsorbed products, the efficacy and stability of the adsorption must be determined and the alum or adjuvant salt measured. This is a critical step because not only the concentration but also the physicochemical properties of the alum, the conditions for adsorption, or the method must be controlled. For the analyses of the adsorbed product, the total soluble protein and the final potency in the supernatant must be estimated. For non-modified preparations such as glycerinated products for sublingual AIT biological potency, protein content and major allergen concentration must be determined. For allergen mixtures, potency must be performed for each individual allergen active substance in the mixture. Finally, sterility must be guaranteed in all parenteral preparations [41].

Validated Assays

A process is generally considered well understood when all critical sources of variability are identified and explained, when the variability is controlled by the process, and when the quality of a product can be accurately and reliably predicted. These three premises are usually controlled with validated systems.

On the other hand, the validation of analytical methods following international guidelines [42] demonstrates the reliability of a specific method for the determination of a parameter. The main characteristics of a method to ensure the acceptability of the performance and the reliability of analytical results includes selectivity, lower limit of quantification, the response function and calibration range (calibration curve performance), accuracy, precision, matrix effects, and stability of the analyte(s).

Conclusions

Vaccines constitute a key point in AIT and are evident requirement from any regulatory agency. Allergy vaccines are manufactured from natural allergenic sources which have an intrinsic variability in their protein composition. For that reason, the establishment of specifications and a deep characterization of the raw material will increase the homogeneity of the final products. Additionally, the long manufacturing processes, with different steps and times, do not favor the reduction of the variability but increase the probability to modify the protein and allergenic composition. Exhaustive control-in-process from the early stages and a deep standardization of the manufacturing methods and reagents contribute to the consistency of the final product, to the reduction of variability and may alert from any deviation, saving time and money, and reducing the number of rejections or no conformities. Finally, it is widely recognized that allergen standardization is the cornerstone of the allergy vaccines. Although different efforts have been made to unify the system and units of standardization, nowadays, important differences exist between American and European methods. These differences are even bigger among different European manufacturers which make the comparability of products difficult. Undoubtedly, the priority is focused in the prestige of the AIT and in the benefit for allergic patients.

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Compliance with Ethics Guidelines

Conflict of Interest Jerónimo Carnés, Victor Iraola, Mayte Gallego, and José Ramón Leonor are employees of Laboratorios LETI S.L.U.

Human and Animal Rights and Informed Consent This article does not contain any studies with human or animal subjects performed by any of the authors.

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