


ORIGINAL ARTICLE

Preparation of chemically stable allergen-specific sublingual immunotherapy from Egyptian allergens

Mohammed A. Abu El-Enin^{1,2} | Eman M. Rabie Shehab El-Din³ |
Heba Wagih Abdelwahab⁴  | Amina Abd El-Maksoud⁴ | Abeer M. Abd El-Aziz³ |
Mona I. Shaaban³ | Ahmed Nader Attia⁵ | Wafaa A. Aboukamar⁶ |
Sanaa Mohei-Aldin⁷ | Fathalla Belal⁸

¹Department of Medicinal Chemistry, Faculty of Pharmacy, Mansoura University, Mansoura, Egypt

²Department of Medicinal Chemistry, Faculty of Pharmacy, National University of Science and Technology, Nasiria, Iraq

³Microbiology & Immunology Department, Faculty of Pharmacy, Mansoura University, Mansoura, Egypt

⁴Chest Medicine Department, Mansoura University, Mansoura, Egypt

⁵Agronomy Department, Faculty of agriculture, Mansoura University, Mansoura, Egypt

⁶Department of medical parasitology, Faculty of medicine, Mansoura University, Mansoura, Egypt

⁷Microbiology & Immunology Department, Faculty of Medicine, Mansoura University, Mansoura, Egypt

⁸Pharmaceutical Analytical Chemistry, Faculty of Pharmacy, Mansoura University, Mansoura, Egypt

Correspondence

Heba Wagih Abdelwahab, Chest Medicine Department, Mansoura University, Mansoura, Egypt.
Email: Wagihheba84@gmail.com

Funding information

This study was conducted as a part of a research project financially supported by Mansoura University (code number: Mu-med-20-30).

Abstract

Background: The term “allergen extracts” refers to solutions of proteins or glycoproteins extracted from source raw materials.

Objectives: This study was planned to prepare chemically stable sublingual immunotherapy from different allergens in Egypt.

Methods: Allergen extraction from raw materials. The concentrated aqueous extract of each allergen was mixed with an equal volume of glycerol. The protein content of the preparations was determined using the modified Lowry assay method. The prepared allergens were stored for 9 months at 2–4°C. Samples were analyzed periodically (0, 3, 6, and 9 months of intervals) adopting the Lowry Assay method. Levels of specific IgE to *Chenopodium album* antigens were measured in patients' sera by ELISA.

Results: The concentration of all prepared allergens, as indicated by the concentration of the protein content, was found to decrease exponentially with time, implying first-order kinetics of degradation. From the values of the slopes of the log plot for each allergen, the half-life time ($t_{1/2}$) and ($t_{1/4}$) values were calculated. The expiration date was considered as the time after which the allergen loses 25% of its potency. The obtained values of $t_{1/4}$ vary according to the type of vaccine. The most stable one is that of *Chenopodium album* pollens (2.4 years) and the least stable is that of house dust Mites (9 months). The immunological characters of *Chenopodium album* extract were stable for at least 6 months.

Conclusion: Differences exist among allergen extracts made by multiple manufacturers. So, developments in studies on allergen preparation and characterization in a different locality are necessary.

KEYWORDS

allergen extract, chemical stability, Egyptian environment, Lowry assay, sublingual immunotherapy

1 | INTRODUCTION

Allergen immunotherapy (AIT) has been used to treat allergic diseases since the early 1900s.¹ The term “allergen extracts” refers to solutions of proteins or glycoproteins extracted from source raw materials.²

The allergenic content of the atmosphere varies according to climate, geography, and vegetation. The biological particles present in the atmosphere differ from place to place and today. They mainly depend on the season, vegetation growing in the surrounding locations, plant phenology, and meteorological conditions.³

The collection of raw materials for allergen preparation should be performed by qualified personnel, and appropriate measures should be taken to ensure correct characterization and quality check of source materials.²

The structure and properties of extract components may be influenced by many external factors, including allergen source (phylogenetic, geographic), pre-extraction procedures (milling, defatting), extraction conditions (time, temperature, pH, extraction solvent composition, degree of wetting, and mixing), post-extraction processing steps (filtration), and storage conditions.^{4,5}

Any protein in source material has the potential to elicit an IgE response; however, in practice, the IgE-binding capacity of an allergen extract is related to the content of one or a few significant allergens, i.e., allergens to which most patients react with IgE. It is, therefore, essential 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.⁶

Extracts prepared from a wide variety of allergenic raw materials are available at relatively low cost from numerous licensed manufacturers. However, differences may exist among products made by multiple manufacturers owing primarily to differences in raw material sources and extraction conditions.⁷ Studies addressing how antigens in extracts change under various storage conditions have provided general guidance for expiration dating, but the diversity of extracts and testing methods have been less than complete.⁸ Therefore, this study was planned to prepare chemically stable sublingual immunotherapy from different allergens in Egypt.

2 | METHODS

This study was conducted in the allergen immunotherapy unit, Chest Medicine Department, Pharmaceutical Analytical Chemistry department, Faculty of Pharmacy and parasitology department, Faculty of medicine, Mansoura University. The study was conducted within the Mansoura Institutional Research Board Ethics Committee (code number: PR.20.08.82).

2.1 | Collection of raw materials

Raw materials were collected from the rural area in Dakahlia Governorate, Egypt.

2.1.1 | Pollens

The pollens (*Cinnamomum camphora*, *Chenopodium album*, *Pluchea*, *Amaranthus caudatus*, *Arundo*, Cotton dust and hay dust) were collected from sources native to the Egyptian environment. The pollens were stored in sealed containers at 4°C or at -20°C. Pollen grains were harvested from common grasses, weeds, and trees. The maximum level of contamination with pollen from other species must be <1%. The pollens were free from flowers and plant debris of the same species (with a limit of 5% by weight in most cases).

The polliniferous material is collected during the pollen season. To procure pollen, dried flowers were crushed gently, and the pollen thus released was sieved through 100, 200, 300 mesh/cm² sieves. Pollen samples with a >95% purity were processed for allergen preparation.

Defatting

Pollens were defatted to remove the lipids and nonspecific irritants, using diethyl ether (3–4 times the volume of the material) by repeatedly changing the ether for a fresh lot till the ether became colorless. Defatted pollen was then dried in vacuum desiccators containing calcium chloride for 24–48 h, then stored in a dry airtight container at 4°C, till allergen extraction.

2.1.2 | Animal emanations

Source materials should be collected only from healthy animals at the time of collection. The animal is kept in a clean environment until source material is harvested by shaving hair and epidermis from different areas of the animal body. The source material must be free from visible traces of blood, serum, or other extraneous materials. Source materials used in allergenic preparations should consist of dander. Hair of bird feathers was used, and the feather's shaft was discarded.

2.1.3 | Mites

Mites were collected by vacuuming carpet or soft furnishings for 2–3 min with filter paper in a filter apparatus attached to the head of a vacuum cleaner pipe. The dust collected in the filter was placed in a mesh container secured at the top of a funnel; a 1.5-ml Eppendorf was placed at the base of the funnel and used to collect the mites after exposure of the dust to light and heat from a 60-W lamp for 30 min. Identification of mite species was performed using a stereomicroscope (MSC-ST40, Bioevopeak, China) using lactic acid for mite clearance.⁹ *Blomia tropicalis* was the predominance, and the remainder was a mixture of *Dermatophagoides pteronyssinus*, *Dermatophagoides farina*, and *Tyrophagus species*. The collected mites were cultured, dried, and stored at -4°C. The culture was maintained in flask plastic containers, with an opening at the top, covered by muslin to allow ventilation in an environmental incubator (GRW 500 CMP) at 25 ± 1°C, humidity 75 ± 5% r.h. and the darkness Source material was obtained from either whole mite cultures or mite bodies. The medium was made of a mixture of wheat germ

(Sigma, USA), baker's yeast (Biospringer, France), and human skin scales. The wheat germ was autoclaved at 121°C for 20 min, while the baker's yeast was heated at 122°C for 2–3 min then at 100°C for 15 min before using to avoid their allergic.¹⁰ Source material was obtained from either whole mite cultures or from mite bodies.

2.2 | Extraction of allergens

Allergenic proteins were extracted in phosphate-buffered saline (PBS, 0.1 M trisodium phosphate, 0.9 M NaCl, pH 7.2) 1:10 dilutions by continuous stirring for 18 h at 4°C. The extract was centrifuged at 13,751 g for 30 min at 4°C. Then, the supernatant was dialyzed in PBS and passed through 0.22- μ m Millipore filter (Seitz-filter Germany, or Millipore Corp.) with the aid of an electric compressor through 0.22/ μ m filters for sterilization and to remove contaminants. The sterility of extracts is checked for bacteria and fungi using suitable media as per the test requirements.

2.3 | Preparation of sublingual Immunotherapy

The concentrated aqueous extract of each allergen was mixed with an equal volume of glycerol, mixed well, and kept in closed containers protected from light.

2.4 | Determination of protein content

The protein content of the preparations was determined spectrophotometrically using the modified Lowry assay method.¹¹

Construction of Calibration graph:

Aliquots of bovine serum albumin (BSA), as standard protein, covering the working concentration range (9.0–70.0 μ g/ml) were transferred into a series of 5-ml volumetric flasks. Two milliliters of 0.2 M sodium hydroxide and 0.3 ml of reagent A (consisting of 6% sodium carbonate and 1.5% copper sulphate, using 3% sodium citrate as a solvent for both, in a ratio of 10:0.2) were added to each flask. After 10 min, 0.1 ml of reagent B "Folin-Ciocalteu's Phenol" reagent was added, and the solutions were mixed well. After 30 min, the volume was completed with distilled water, and the produced blue color was measured spectrophotometrically at 750 nm against an appropriate blank solution prepared simultaneously. The measured absorbance was plotted versus the final concentration in μ g/ml to get the calibration graph. Alternatively, the corresponding regression equation was derived.

In the same way, the samples were processed using aliquots covering the working concentration range. The nominal content of the allergen protein was determined either from the previously plotted calibration graph or using the corresponding regression equation. The protein nitrogen units (PNU) of the extracts were assessed after assessment of protein concentration (One PNU/ml is equivalent to 1×10^5 mg).¹²

2.5 | Chemical stability of the allergens and determination of their shelf life and storage conditions

The prepared allergens were stored for 9 months at 2–4°C. Samples were analyzed periodically (0, 3, 6, and 9 months of intervals) adopting the Lowry Assay method. The chemical stability of the preparation was assessed by applying the kinetics of the accelerated stability study.

Samples treated with 50% (v/v) glycerol were refrigerated at 4°C for the specified period, then left to acquire room temperature before analysis. The results obtained for each preparation were compared with freshly prepared ones (0 time).

2.6 | Immunological stability of *Chenopodium album* pollen

A skin prick test using the extract of *Chenopodium album* pollen grains (1:10 w/v) was performed on allergic patients. Skin reaction was graded compared to the negative control (glycerinated PBS) and positive control (histamine hydrochloride 1 mg/ml). Blood samples were collected from patients with positive skin tests sensitized to *Chenopodium album* extract. Levels of specific IgE to *Chenopodium album* antigens were measured in patients' sera by indirect Enzyme-linked immunosorbent assay (ELISA).¹³ Polystyrene microtiter plates (Nunc, Maxisorp, Denmark) were coated with 100 μ l of *Chenopodium album* pollen extracts (20 μ g/ml protein) in 100 mM carbonate buffer (15 mM Na₂CO₃ and 35 mM NaHCO₃, pH 9.6), and the plates were incubated overnight at 4°C. The plates were washed with TBS-T (Tris-buffered saline containing 0.05% Tween 20, pH 7.6) and blocked with 1% BSA for 2 h at room temperature. The coated wells were washed with TBS-T and incubated with the diluted patient's sera (1:10) (in duplicate) overnight at 4°C. Wells receiving buffer only were used as negative controls. After five washes with T-TBS, 100 μ l of diluted (1:1000) monoclonal antihuman IgE-alkaline phosphatase (Sigma-Aldrich, USA) was added to each well. The plates were further incubated for 2 h at room temperature. For color development, 100 μ l of 4-nitrophenol phosphate (1 mg/ml) (Sigma Aldrich, USA) was added to each well, and the plate was incubated at room temperature in the dark for 30 min. Optical density (OD_{405 nm}), two times greater than the median values of the negative controls, was assigned as a positive interaction. OD values of *Chenopodium album* extract stored for 6 months were evaluated and compared to freshly prepared extract.

2.7 | Dilution

Extracts that belong to a particular species and are obtained using sterile filtration are called pure extracts (used for research purposes). During diagnosis and treatment of allergies, dilution is necessary.

COCA's solution (1000 ml. contained 5-g NaCl, 2.75-g Na_2HCO_3 , and 4-g phenol in distilled water) is the diluent solution used for extracts dilution in our laboratory. Thereafter, it was mixed with an equal volume of glycerol to prepare sublingual drops.

3 | RESULTS

The absorbance of the colored products of all allergens was a linear function of their concentration over the range of 9.0–70.0 $\mu\text{g}/\text{ml}$. Standard calibration graphs were obtained upon plotting the absorbance versus the corresponding concentrations. Linear regression analysis of the data gave the following equations.

$$\text{Absorbance}(A) = 0.0575 + 0.00748X \quad R = 0.9989$$

Where X is the concentration of the allergen ($\mu\text{g}/\text{ml}$).

The analysis results of 12 different allergens adopting the modified Lowry assay method after 0, 3, 6 and 9 months of intervals were evaluated.

The concentrations of all prepared allergens (as indicated by the concentration of the protein content) were found to decrease exponentially with time, implying first-order kinetics of degradation (Figure 1).

The semi-logarithmic plots of the data according to the first-order kinetics gave straight lines for all allergens confirming that the degradation pathways obey first-order kinetics (Figure 2). From the values of the slopes for each allergen, the half-life time ($t_{1/2}$) values were calculated. The time after which the vaccine will be ineffective (expiration date) was considered as the time after which the allergen loses 25% of its potency ($t_{1/4}$). The results are also abridged in Table 1. The obtained values of $t_{1/4}$ vary according to the type of vaccine. The *Chenopodium album* (2.4 years) is the most stable one, while the least stable is that of H.D. Mites (9 months).

The results of immunological stability of *Chenopodium album* pollen extract (the most chemically stable allergen extract in this study) showed that the immunological characters of *Chenopodium album* extract were stable for at least 6 months compared to the fresh extract prepared under the same preparation and storage conditions (Table 2).

4 | DISCUSSION

Specific knowledge of the allergens in each environment is required to implement allergen-specific immunotherapy optimally. For example, the different distribution of house dust mites and their species in other geographical locations affect the allergens in the environment and their use.¹⁴

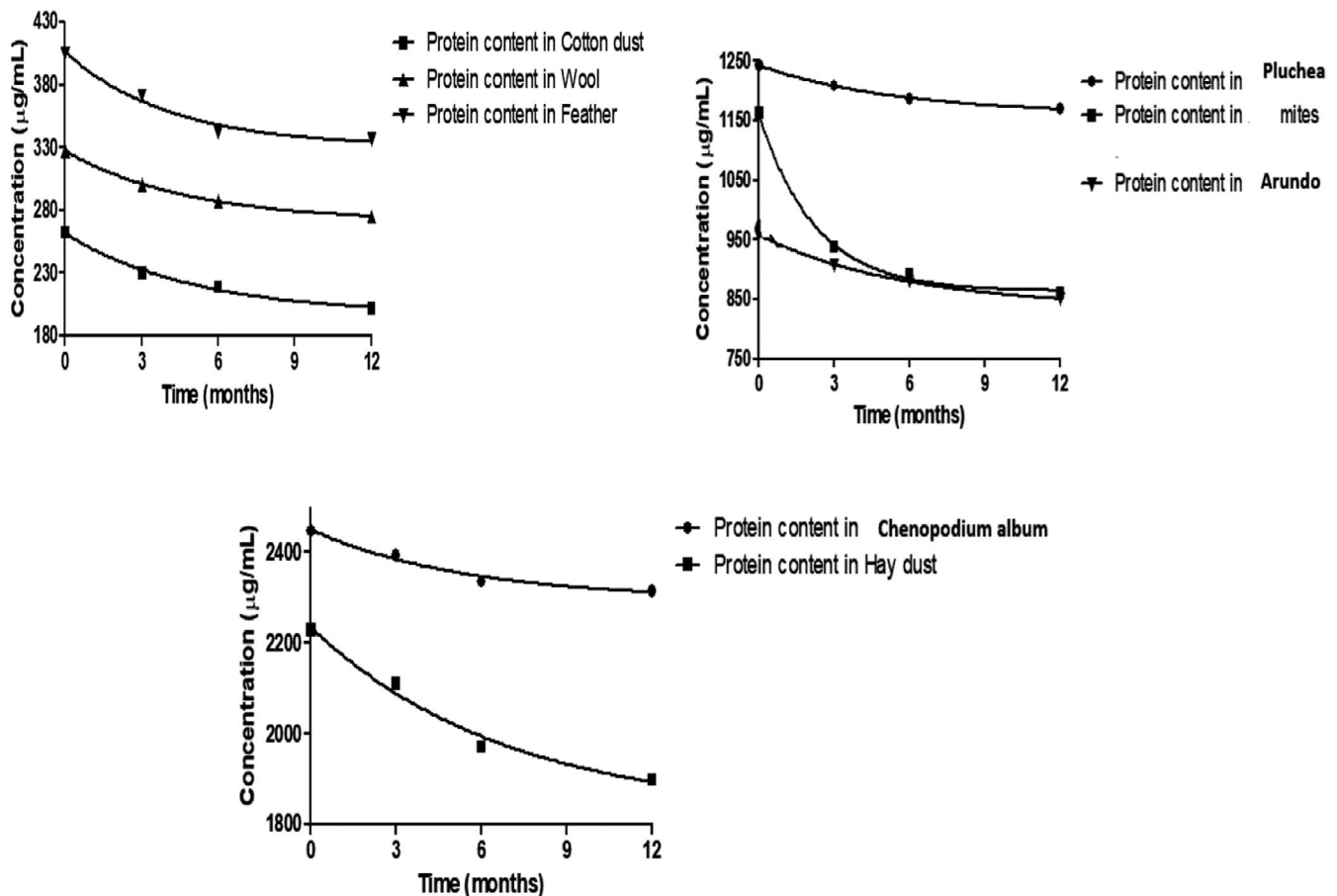


FIGURE 1 The allergen extract shows (follow) first-order kinetics where their concentrations decrease exponentially by time

FIGURE 2 The semi-logarithmic plots of first-order kinetics treatment of the data of some allergens. C, Initial concentration, Ct, Concentration after time t. (A) *Cinnamomum camphora*, (B) Pigeon, (C) *Chenopodium album*, (D) *Pluchea*

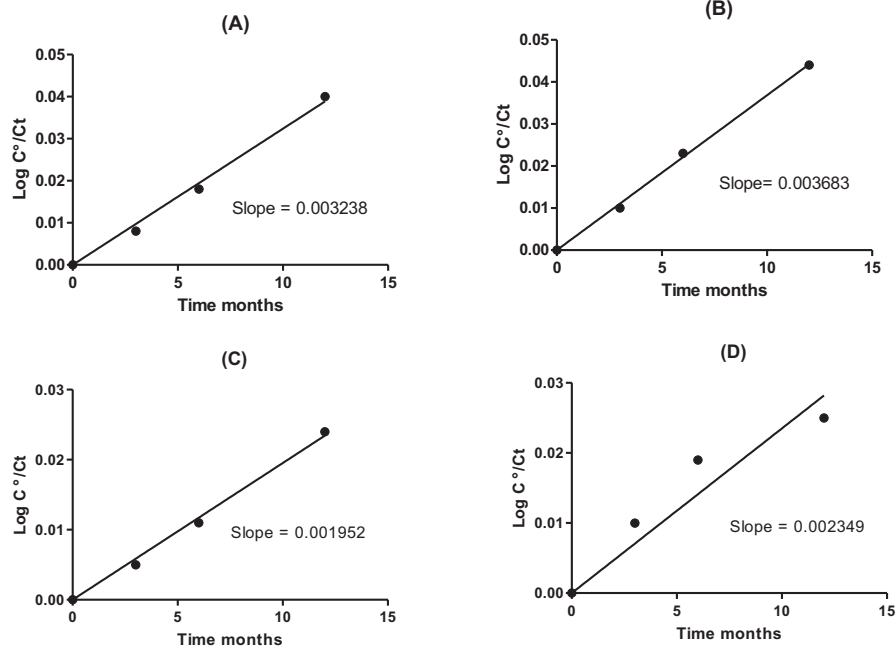


TABLE 1 Chemical stability of studied allergen extracts

Product of immunotherapy	K (month ⁻¹)	t _{1/2} (months)	t _{1/2} (years)	t _{25%} (months)	t _{25%} (years)
<i>Cinnamomum camphora</i>	0.0112	62	5.2	26	2.1
Pigeon	0.0131	53	4.4	22	1.8
<i>Chenopodium album</i>	0.0099	70	5.8	29	2.4
<i>Pluchea</i>	0.0105	66	5.5	27	2.3
House dust mites	0.0310	22	1.9	9	0.8
<i>Amaranthus caudatus</i>	0.0196	35	3.0	15	1.2
<i>Arundo</i>	0.0111	62	5.2	26	2.2
Hay dust	0.0148	47	3.9	19	1.6
Cotton dust	0.0243	29	2.4	12	1.0
Dog hair	0.0188	37	3.1	15	1.3
Wool	0.0167	42	3.5	17	1.4
Feather	0.0186	37	3.1	15	1.3

Note: t_{25%} and t_{1/2} are the time required for decomposition of 25% and 50% of the product, respectively.

TABLE 2 Immunological stability of *Chenopodium album* extract

Sample number of patient sera	OD 405 nm (mean ± SD)		
	Fresh samples	6 months	% Loss
1	0.068 ± 0.007	0.069 ± 0.02	0
2	0.036 ± 0.01	0.031 ± 0.01	11
3	0.035 ± 0.02	0.034 ± 0.01	1.9
4	0.111 ± 0.01	0.067 ± 0.01	39
5	0.376 ± 0.04	0.248 ± 0.08	34
Total loss			17.9

In Brazil, *Lolium multiflorum* is the leading cause of pollen allergy. However, other allergenic grass species grow haphazardly in city suburbs and on abandoned plots of land, such as *Anthoxanthum*

odoratum (sweet vernal grass) and *Cynodon dactylon* (Bermuda grass).^{15,16} So, studies on allergen sensitization and characterization have increased our understanding of the grass pollen sensitization process in insensitive individuals.¹⁶

In Egypt, for example, *Pluchea dioscoridis* (L.) DC. Occurs mainly in the Nile region. The pollen grains of *Pluchea dioscoridis* are monads, isopolar, radially symmetrical.¹⁷ Giant reed (*Arundo donax* L.) is recorded as a natural species along canals, roadsides, railways, and wastelands in the Nile region, oases of the Western Desert, Mediterranean coastal strip, Sinai, and all the desert of Egypt.¹⁸

So, in this study, preparation of chemically stable sublingual immunotherapy from common allergens in Egypt was conducted. The total protein content of the prepared extracts was quantified by the modified Lowry assay method using bovine serum albumin

(BSA) as a standard. The extracts in this study were stable for at least 9 months. The most stable one is the *Chenopodium album* (2.4 years), while the least stable is that of Mites (9 months).

Then, the immunological stability of the most chemically stable allergen in this study (*Chenopodium album*) was performed. Results of immunological stability showed that the immunological characters of *Chenopodium album* extract were stable for at least 6 months.

Although Lowry method uses BSA standards for calibration, which can be a source of error as the composition of the protein of interest may not necessarily match that of the protein standards, it is almost 100-fold more sensitive than measuring the absorbance of proteins in the UV region at 280 nm.¹⁹ Also, in this study, the volume was adjusted with distilled water, then the produced blue color was measured at 750 nm against an appropriate blank prepared simultaneously. The measured absorbance was plotted versus the final concentration in $\mu\text{g/ml}$ to get the calibration graph. These multiple sample dilutions covering the entire assay overcome the deviations from parallelism between extract samples and BSA standards and produce unbiased mean protein values.⁷

The Lowry method has been widely utilized for protein determination for several years due to its simplicity, sensitivity, and availability.²⁰ Proteins need to be extracted efficiently to ensure that an accurate representation of allergenic proteins from the source material is obtained. Usually, the main goal in optimizing protein extraction conditions is to get as much protein as possible.²¹

Wahl et al checked the reproducibility of the modified Lowry method with three independent measurements and concluded that protein measurements by the modified Lowry method of a six-grass pollen allergen extract in three different laboratories showed good reproducibility.²²

Allergen stability is the persistence of adequate quantities of relevant antigens in an allergen vaccine from the time of initial assay to the time of clinical use. Allergen extracts are prone to degradation by proteases when stored alone or mixed with other extracts and may contain unstable allergens. Various preservatives and stabilizers, such as ϵ -aminocaproic acid, sucrose, glycerol, human serum albumin, and phenol, have been reported either alone or in combination as maintaining the potency of allergen extracts.^{23,24}

The concentrated aqueous extracts in this study were mixed with an equal volume of glycerol. The protein content in prepared extracts was adequate from the initial assay to 6 and 9 months later. Also, according to Jeong et al.,²⁵ the addition of 50% glycerol and refrigerated storage temperature were essential factors for increasing the shelf life of protease-rich cockroach extract.

Glycerol is reported to interfere with the estimation of protein by the Lowry procedure. The color development is decreased by the presence of glycerol in samples. This problem may be overcome using appropriate blanks and calibration curves.²⁶

Several studies have studied the stability of allergens in distinct mixtures of immunotherapy vaccines. Those researches utilized commercial or noncommercial extracts.²⁷

The deterioration of allergen extracts with time has long been identified. Loss of potency is mainly due to adsorption of protein onto the surface of the vial or presence, in some allergen extracts, of proteases that can break down allergenic proteins.²⁸

However, standardization is not a precisely defined term, as different qualities of standardization are currently used by various laboratories and manufacturers of allergen extracts. It is therefore essential that even scientific laboratories describing new allergens, new aspects, or new processes establish internal standards that can be used and referenced in future work in a transparent manner. In Europe, each laboratory and manufacturer establish an in-house reference (IHR) preparation for each source material.⁶

Biological standardization, which primarily detects IgE-binding potency, is generally conducted to evaluate the quality of allergen extracts. For in vitro standardization, competitive IgE-binding inhibition test (e.g., ELISA) has proven reliable and is mostly applied to measure total allergenic activity. Numerous biochemical and immunological methods, such as SDS-PAGE, immunoelectrophoresis, immunoblotting, mass-spectrometric analysis, and proteomic approaches, could be made to verify and control the consistent composition and activity of allergen product. Nevertheless, IgE reactivity does not reflect the capability to cause allergic symptoms. The basophil activation test uses the similar mechanism as a skin test and is anticipated to consider the biological activity of allergens more directly, although it has not been commonly applied.²⁹

This study had some limitations such as immunological stability was conducted only on one allergen (*Chenopodium album*) and followed for 6 months only. Expansion of this study should include additional extracts.

5 | CONCLUSION

Differences exist among allergen extracts made by multiple manufacturers. So, developments in studies on allergen preparation and characterization are necessary.

Determination of the persistence of adequate quantities of proteins, by modified Lowry assay, in sublingual immunotherapy from the initial assay to about 9 months later provided general guidance for expiration dating.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

Derived data supporting the findings of this study are available from the corresponding author on request.

ORCID

Heba Wagih Abdelwahab  <https://orcid.org/0000-0001-8655-1820>

REFERENCES

1. Jutel M, Agache I, Bonini S, et al. international consensus on allergy immunotherapy. *J Allergy Clin Immunol*. 2015;136(3):556-568.
2. Cox L, Nelson H, Lockey R, et al. Allergen immunotherapy: a practice parameter third update. *J Allergy Clin Immunol*. 2011;127(1suppl):S1-55.
3. Galán C, Alcázar P, Oteros J, et al. Airborne pollen trends in the Iberian Peninsula. *Sci Total Environ*. 2016;15(550):53-59.
4. Van der Veen MJ, Mulder M, Witteman AM, et al. False-positive skin prick test responses to commercially available dog dander extracts caused by contamination with house dust mite (*Dermatophagoides pteronyssinus*) allergens. *J Allergy Clin Immunol*. 1996;98(6 Pt 1):1028-1034.
5. Wahl R, Oliver JD, Hauck PR, Winter HG, Maasch HJ. Comparison of *Alternaria tenuis* extracts prepared from different raw materials. *Ann Allergy*. 1989;63(6 Pt 1):527-531.
6. Larsen JN, Dreborg S. Standardization of allergen extracts. In: Jones MG, Lympny P, ed. *Allergy methods and protocols. Methods in molecular medicine*. Humana Press; 2008:133-145.
7. Grier TJ. Laboratory methods for allergen extract analysis and quality control. *Clin Rev Allergy Immunol*. 2001;21(2-3):111-140.
8. Plunkett G. Stability of allergen extracts used in skin testing and immunotherapy. *Curr Opin Otolaryngol Head Neck Surg*. 2008;16(3):285-291.
9. Soleimani-Ahmadi M, Zare M, Abtahi SM, Khazeni A. Species identification and prevalence of house dust mites as respiratory allergen in kindergartens of the Bandar Abbas City. *Iran J Allergy Asthma Immunol*. 2017;16(2):133-139.
10. Batard T, Hrabina A, Bi XZ, et al. Production and proteomic characterization of pharmaceutical-grade *Dermatophagoides pteronyssinus* and *Dermatophagoides farinae* extracts for allergy vaccines. *Int Arch Allergy Immunol*. 2006;140(4):295-305.
11. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. *J Biol Chem*. 1951;193(1):265-275.
12. May JC, Sih JT, Miller JR, Seligmann EB Jr. Optimization of parameters in protein nitrogen unit precipitation procedure for allergenic extracts. *J Allergy Clin Immunol*. 1979;63(2):87-97.
13. Sepulveda R, Longbottom JL, Pepys J. Enzyme linked immunosorbent assay (ELISA) for IgG and IgE antibodies to protein and polysaccharide antigens of *Aspergillus fumigatus*. *Clin Allergy*. 1979;9(4):359-371.
14. Thomas WR. Geography of house dust mite allergens. *Asian Pac J Allergy Immunol*. 2010;28(4):211-224.
15. Freidhoff LR, Ehrlich KE, Grant JH, Meyers DA, Marsh DGA. Study of the human immune response to *Lolium perenne* (rye) pollen and its components, Lol p I and Lol p II (rye I and rye II). I. Prevalence of reactivity to the allergens and correlations among skin test, IgE antibody, and IgG antibody data. *J Allergy Clin Immunol*. 1986;78(6):1190-1201.
16. Taketomi EA, Sopelete MC, de Sousa Moreira PF, de Assis Machado Vieira F. Pollen allergic disease: pollens and its major allergens. *Braz J Otorhinolaryngol*. 2006;72(4):562-567.
17. Shaltout K, Slima D. The biology of Egyptian woody perennials: 3. *Pluchea dioscoridis* (L.) DC. *Ass Univ Bull Environ Res*. 2007;10:85-103.
18. Eid E, Youssef M, Shaltout K. Population characteristics of giant reed (*Arundo donax* L.) in cultivated and naturalized habitats. *Aquat Bot*. 2016;129:1-8.
19. Shen CH. Quantification and analysis of proteins. In: Shen CH, ed. *Diagnostic molecular biolog*. Academic Press; 2019:187-214.
20. Everette JD, Bryant QM, Green AM, Abbey YA, Wangila GW, Walker RB. Thorough study of reactivity of various compound classes toward the Folin-Ciocalteu reagent. *J Agric Food Chem*. 2010;58:8139-8144.
21. Mihai DE, Pavaloiu RD, Sha't F, Pele M. effect of various extraction methods on peanut protein extraction efficiency. *Sci Bull*. 2020;xxiv:39-44.
22. Wahl R, Geissler W, Maasch HJ. Comparison of classical Lowry, modified Lowry, and a dye-binding assay for the estimation of protein in allergen extracts and influence of different parameters on the modified Lowry assay. *Biol Chem Hoppe Seyler*. 1985;366(10):979-984.
23. Van Hoeyveld EM, Stevens EA. Stabilizing effect of epsilon-aminocaproic acid on allergenic extracts. *J Allergy Clin Immunol*. 1985;76(4):543-550.
24. Saldotova LN, Paupore EJ, Burk SH, Pastor RW, Slater JE. The stability of house dust mite allergens in glycerinated extracts. *J Allergy Clin Immunol*. 2000;105:482-488.
25. Jeong KY, Lee J, Yuk JE, et al. Optimal conditions for the storage of German cockroach extract. *Mol Med Rep*. 2020;21(2):953-958.
26. Zishka MK, Nishimura JS. Effect of glycerol on Lowry and biuret methods of protein determination. *Anal Biochem*. 1970;34:291-297.
27. Grier TJ, LeFevre DM, Duncan EA, Esch RE. Stability of standardized grass, dust mite, cat, and short ragweed allergens after mixing with mold or cockroach extracts. *Ann Allergy Asthma Immunol*. 2007;99(2):151-160.
28. Nelson HS, Iklé D, Buchmeier A. Studies of allergen extract stability: the effects of dilution and mixing. *J Allergy Clin Immunol*. 1996;98(2):382-388.
29. Jeong KY, Hong CS, Lee JS, Park JW. Optimization of allergen standardization. *Yonsei Med J*. 2011;52(3):393-400.

How to cite this article: Abu El-Enin MA, Rabie Shehab El-Din EM, Abdelwahab HW, et al. Preparation of chemically stable allergen-specific sublingual immunotherapy from Egyptian allergens. *J Clin Lab Anal*. 2022;36:e24261. doi:[10.1002/jcla.24261](https://doi.org/10.1002/jcla.24261)