Allergologia et immunopathologia
Sociedad Española de Inmunología Clínica, Alergología y Asma Pediátrica
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ORIGINAL ARTICLE

OPEN ACCESS

Comparative analysis of seven standardised commercially available grass extracts for sublingual immunotherapy in liquid formulation

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Received 31 October 2025; Accepted 5 January 2026
Available online 1 July 2026

KEYWORDS
allergen;
sublingual immunotherapy;
grass pollen;
allergenic extracts;
clinical efficacy

Abstract
Sublingual immunotherapy with allergens requires high allergen doses to achieve the desired clinical efficacy. Given the current differences among products on the market, this study aims to compare seven therapeutic sublingual grass pollen extracts to quantify the Group 5 allergen concentrations, assess their biological activity and characterise allergenic profiles. The extracts were analysed by protein quantification (Bradford method), characterisation of the protein profile (SDS-PAGE) and determination of biological activity (ELISA inhibition and immunoblotting). Major allergens (Group 5) were also quantified using a sandwich ELISA. Differences were observed between the various allergenic extracts. Protein content ranged from 27 to 231 µg/mL, with Apioral Forte, SlitOne Ultra and Sublivac showing similar protein profiles. These extracts also displayed the highest biological activity, while the quantification of major Group 5 allergens was greatest in Apioral Forte, Sublivac and Tol Forte. The differences observed between extracts may have a relevant clinical impact. It is essential to have standardised, comparative analytical methods that allow specialists to select the most appropriate treatment.

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https://doi.org/10.15586/aei.v54i4.1612

Introduction

IgE-mediated respiratory diseases are highly prevalent, affecting over 150 million people in the European Union. It is estimated that by 2025, more than 100 million people in Europe will be affected by bronchial asthma.[1] Although treatment of these diseases is based on three key pillars (preventive environmental control measures, symptomatic treatment and specific allergen immunotherapy), allergen immunotherapy (AIT) is the only aetiological treatment capable of modifying the course of IgE-mediated allergic disease and preventing the onset of bronchial asthma in patients with allergic rhinitis.[2]

Currently, the main routes of administration for AIT are subcutaneous (SCIT) and sublingual (SLIT). While the mechanism of action of AIT is well established, SLIT requires the administration of a higher quantity of allergen to achieve the desired efficacy.[3]

Allergenic extracts are complex mixtures of proteins, glycoproteins, polysaccharides and low molecular weight components.[4,5] Scientific literature has revealed significant differences between manufacturers (recommended daily dose and frequency, quantification of major allergens, biological standardisation units, etc.),[6,7] which presents a serious challenge for clinicians seeking to identify the most suitable product based on the profile and needs of each patient.

Grass pollen is one of the main aetiological agents of IgE-mediated respiratory diseases,[8] making it vital for clinicians to understand the characteristics of the available products to provide the most appropriate treatment for their patients through sublingual immunotherapy (SLIT).

One of the main challenges with published data is that values obtained using different methods and/or analytical standards are not always comparable. Therefore, in this study, we selected seven SLIT grass pollen products marketed in Spain and analysed their allergenic profile, Group 5 major allergen content and allergenic activity using the same methods and analytical standards for each of the objectives described.

Materials and Methods

The products compared are listed in Table 1, along with the main characteristics of the extracts as published by the various manufacturers in the SEAIC immunotherapy pharmacotherapeutic guide.[9]

Pharmacotherapy Guidelines of Immunotherapy. Available in: https://www.seaic.org/inicio/guia-farmacoterapeutica-de-inmunoterapia

All products were obtained from a community pharmacy. To avoid bias in the analysis, the products were coded from 1 to 7 and presented to the researchers in a blinded format, preventing identification of each product during the analytical process. All samples were stored in the same location under controlled conditions with temperature monitoring, thereby ensuring cold chain maintenance. Unblinding of the samples was carried out once the analysis had been completed, to allow transparent presentation of results without compromising the objectivity of the study.

Table 1 Products analysed in the study: Major allergen concentration and biological activity.

Product	[MA] µg/mL	Biological activity
Apioral Forte	Group 5: 43.4	6000 TBU/mL
SlitOne Ultra	Group 5: 34	300 SRU/dosis
Sublivac	Group 5: 17.5	10,000 AU/mL
Oraltek	Undetermined	30,000 UT/mL
Beltaoral Pro	Undetermined	2 RC/mL
Tol Forte	Undetermined	300 HEP/mL
Staloral 300	Group 5: 10.5	300 IR/mL

The analytical methods used were validated in accordance with ICH Q2 guidelines.[10] As part of this validation, the precision and accuracy of the standards were verified in the presence of the matrix or excipients, with all products analysed showing equivalent composition at this level. The methods were deemed valid for analysis, and analytical controls were implemented in parallel as criteria for validating the assays.

Quantification of total proteins

Total protein content was determined using the Bradford method with Coomassie Brilliant Blue G-250. BSA (ThermoScientific Albumin Standard) was used as the analytical standard. A calibration curve with seven serial dilutions of the standard, prepared in duplicate, was included, ranging from 200 to 5 µg/mL. Samples were analysed at a ½ dilution in PBS, in duplicate. Readings were taken at 595 nm, and results were calculated using SoftMaxPro GXP 5.4.2 software.

SDS-PAGE – protein profile

Protein separation was performed using SDS-PAGE in denaturing conditions. An 8–16% MiniProtein TGX gradient gel from Bio-Rad was used along with the Precision Plus Protein Standards molecular weight pattern, from 10 to 250 kDa, from Bio-Rad. Electrophoresis was performed with 20 µL/well for each sample before centrifugation at 10,000 rpm/10 min, and the samples were diluted to 2:3 with loading buffer. The electrophoresis was run in the Tetra System by Bio-Rad, at 200 V for approximately 25 min. The gel was stained using Coomassie Blue, Bio-Rad QC Colloidal Coomassie. The results were analysed using the TotalLab Quant computer system, version 13.2.

Biological activity

Biological activity was determined using an ELISA inhibition assay, taking the WHO international standard for *Phleum pratense* (85/520, NIBSC) as reference.

Polystyrene Maxisorp plates (Nunc) were coated with 500 IU/well of the WHO *P. pratense* reference standard. A pool of sera from allergic patients was inhibited using the standard

and the seven samples (centrifuged at 10,000 rpm for 10 min), with 11 serial 1:3 dilutions in 1% BSA/PBS-Tween, in duplicate. The bound IgE was detected via a secondary antibody, rabbit antihuman IgE, Dako A0094, labelled with Biotin (1/1000 v/v in 1% BSA/PBS-Tween), followed by streptavidin-HRP, Sigma S5512 (1/1000 v/v in 1% BSA/PBS-Tween). Detection was performed with OPD (Sigma P6412), and readings were taken at 492 nm with a reference filter at 595 nm.

Results were calculated using semilogarithmic linear regression of the dilution curves against optical density, with parallel line analysis. A relative slope between 0.7 and 1.3, between the reference and each sample, was used as the acceptance criterion.

Immunoblot – Allergenic profile

Protein separation by SDS-PAGE was carried out under denaturing conditions using an 8–16% Mini-PROTEAN® TGX gradient gel from Bio-Rad. The Precision Plus Protein Standards marker (10–250 kDa, Bio-Rad) was used, and electrophoresis was performed with 20 µL/well of each sample diluted 2:3 with loading buffer. The proteins were transferred via Bio-Rad Trans-Blot® Turbo™ Transfer System 25 V/10 min, to PVDF membrane, Bio-Rad Trans-Blot® Turbo™ Mini PVDF Transfer Pack.

For immunoblotting, the membrane was incubated with a pool of sera from grass pollen-allergic patients (1:10 v/v in 1% milk/TBS-Tween), followed by a biotin-labeled anti-IgE secondary antibody (Dako A0094; 1:5000 v/v in 1% milk/TBS-Tween), and then streptavidin-HRP (Sigma S5512; 1:20000 v/v in 0.3% gelatin/TBS-Tween). Detection was performed by chemiluminescence (Bio-Rad Clarity™ Western ECL Substrate). The results were analysed using the TotalLab Quant computer system, version 13.2.

Quantification of major allergens (Group 5)

The seven products were analysed after centrifugation at 10,000 rpm for 10 minutes, using eight serial 1:2 dilutions in 1% BSA/PBS-Tween, in duplicate. The allergenic reference extract IHR was included as a secondary standard (quantified against the primary standard ST PP5 from Indoor Biotechnologies, traceable to the Ph. Eur. CRS standard Y0001566).

Quantification was performed using a sandwich ELISA (Indoor Biotechnologies) on polystyrene Maxisorp plates (Nunc), with the capture antibody mAb 1D11, anti-Phl p 5 (1:1000 v/v in 1% BSA/PBS-Tween), and the biotinylated antibody Biotin Bo1 anti-Phl p 5 (1:1000 v/v in 1% BSA/PBS-Tween), followed by streptavidin-HRP, Sigma S5512 (1:1000 v/v in 1% BSA/PBS-Tween). Detection was carried out using OPD, Sigma P6412, and readings were taken at 492 nm with a reference filter at 595 nm.

Results were calculated using semilogarithmic linear regression of dilutions against optical density and parallel line analysis, with a relative slope between 0.8 and 1.2, between the reference line and each sample used as the acceptance criterion.

Results

Total proteins/Protein profile (SDS-PAGE)

The total protein results are shown, where differences between the various products can be observed, with total protein concentrations ranging from 27 µg/mL (Oraltek) to 231 µg/mL (Apioral Forte) (Figure 1). These findings are confirmed by the densitometric analysis (Figure S1), which shows that the Apioral Forte, SlitOne Ultra and Sublivac extracts displayed similar profiles, with greater band intensity around the molecular weights (MW) of approximately 34 and 12 kDa. By contrast, Tol Forte and Staloral 300 exhibited intermediate profiles, while Oraltek and Beltaoral Pro showed the weakest signal peaks.

Biological activity

The Sublivac extract stands out with the highest biological activity (31,686 IU/mL), also displaying the highest

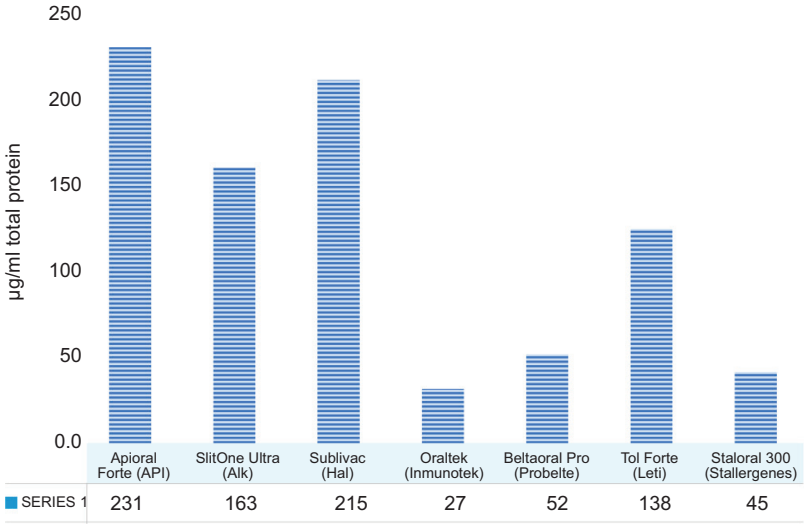

Figure 1 Total proteins for each product.

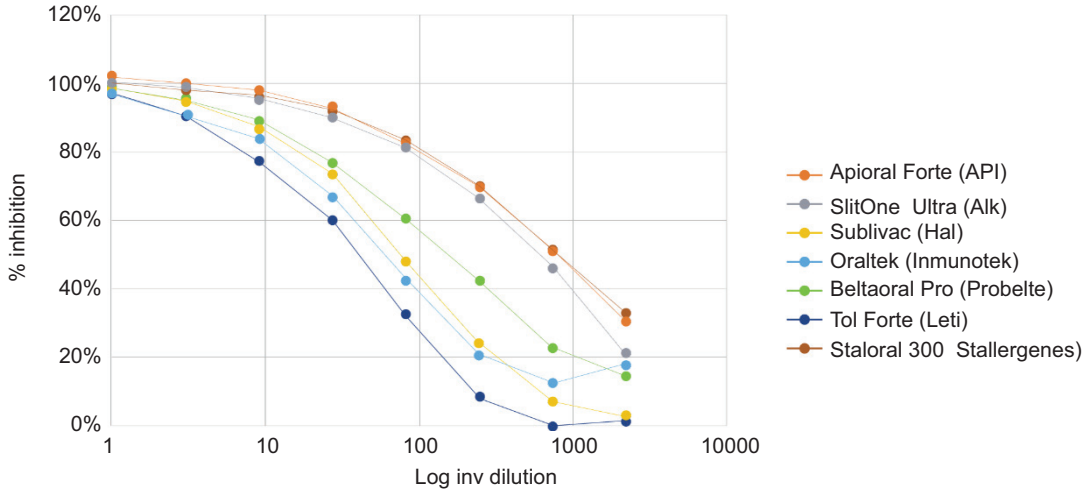

Figure 2 Biological activity of each product.

quantification of Group 5 allergens (Figure 2). These results correlate directly with the total biological activity (Figure S2) for each of the products tested, allowing for comparison using the same unit (IU) against the WHO standard. Apioral Forte, SlitOne Ultra and Sublivac showed the highest biological activity, while Oraltek and Beltaoral Pro presented the lowest. Tol Forte and Staloral 300 displayed intermediate to high biological activity, with a lower percentage of inhibition.

Allergenic profile

Clear differences were observed in the band definition of various products (Figure 3). These differences were also evident in the band density within the 32–38 kDa range, corresponding to the MW of the major Group 5 grass allergens (Figure 4). The products showing the strongest signals were once again Apioral Forte, SlitOne Ultra and Sublivac, with the most intense bands.

Quantification of major allergens (Group 5)

The highest Group 5 allergen concentration was found in the Sublivac extract (72.8 µg/mL), followed by Apioral Forte (43.3 µg/mL) and Tol Forte (32.7 µg/mL) (Figure 5). This is confirmed by the greater band density in the regions corresponding to an MW between 32 and 38 kDa observed in the immunoblot.

Discussion

Spain's geographical characteristics mean that large areas of the country have a continental climate, where pollen is the most prevalent aetiological agent in IgE-mediated allergic respiratory diseases. Among these, wild grasses are the most relevant allergens.[11] For this reason, our study selected seven SLIT allergenic extracts consisting of mixed grasses, marketed in Spain by different pharmaceutical companies specialised in the production of pharmaceutical products for both the diagnosis and treatment of such diseases. All of these are native extracts without physical or chemical modifications that could affect their immunogenicity and/or allergenicity. As finished pharmaceutical products, they can be subjected to in vitro analytical testing with full reliability, and the results obtained are reproducible and comparable.

Our study reveals significant differences between the various products analysed. For example, in terms of group 5 major allergen quantification, Sublivac had the highest level, nine times greater than Oraltek, which had the lowest. This was followed by Apioral Forte and Tol Forte. There were also marked differences in biological activity, with Apioral Forte, SlitOne Ultra and Sublivac showing the highest activity. This finding indicates that biological activity is also influenced by the presence and representation of both major and minor allergens.

Our results are in line with those of other researchers. Focke et al.,[12] in their comparative analysis of four commercial *Phleum pratense* extracts, they identified significant differences among them, both qualitative (detection of different major and minor *Phleum* allergens) and quantitative (concentration of major allergens Phl p 1, Phl p 2 and Phl p 5, and their biological activity). Similarly, Sander et al.[13] observed significant differences in protein concentration and allergenic profile in SLIT extracts, which directly affected the allergen dose administered. When Mösges and Cols[14] compared the biological activity of several commercial SLIT allergenic extracts, they also observed significant differences in biological activity and were able to generate individual regression curves to enable adequate comparison of the extracts used. On the other hand, Schmidt et al.[15] published a comprehensive proteomic analysis of three *P. pratense* extracts and observed differences in the protein composition and isoforms of major allergens, concluding that these differences could significantly impact their therapeutic effectiveness.

A limitation of some published studies may be the use of a single analytical technique, which provides only partial information. Moreno et al.,[16] in their comparative analysis of

several therapeutic extracts containing *Dermatophagoides* mixtures marketed for SLIT, strengthened their conclusions by using two techniques, thereby validating their results. In our study, a similar strategy was followed, incorporating multiple validated analytical techniques. Suitability controls were also included to ensure traceability and robustness of the data obtained. Taken together, our methodological approach minimised potential analytical bias, providing a solid basis for the comparative interpretation of the results.

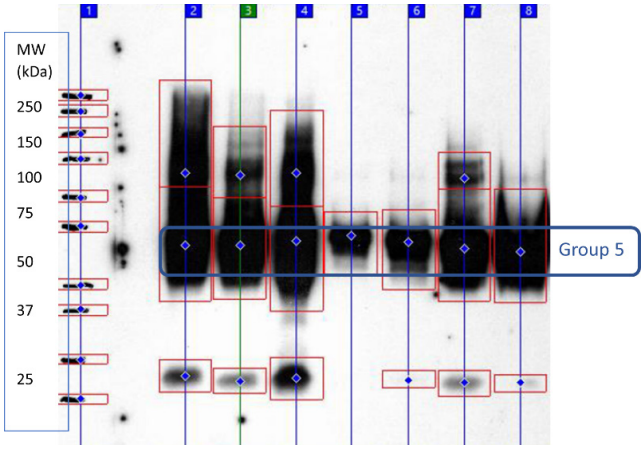

Figure 3 Allergenic profile by immunoblot of the products analysed. 1. Molecular weight standard. 2. Apioral Forte; 3. SlitOne Ultra; 4. Sublivac; 5. Oraltek; 6. Beltaoral Pro; 7. Tol Forte; 8. Staloral 300.

The European Medicines Agency (EMA)[17] has published guidelines regulating how allergenic extracts must be manufactured and standardised for diagnostic and therapeutic purposes. Currently, each manufacturer uses its own analytical method, its own standards (IHR), and, in the case of biological activity, its own units. This makes it difficult to establish appropriate equivalence between different products. This is reflected in our results, where the quantification of Group 5 major allergens differs from the published values (Table 1). In this context, our study takes on particular relevance, as all extracts were analysed under the same experimental conditions and validated techniques, allowing for an objective and reliable comparison between the different products. The need for a common unit to evaluate different extracts is also emphasised by the FDA.[18]

As early as the CREATE project,[19] attention was drawn to the differences in both the standards and analytical techniques used, as well as in the variation of products when subjected to the same technique and the same standard. The absolute results obtained may not be "universal." Thus, it is essential to have comparable data, which is only possible if products are analysed under the same

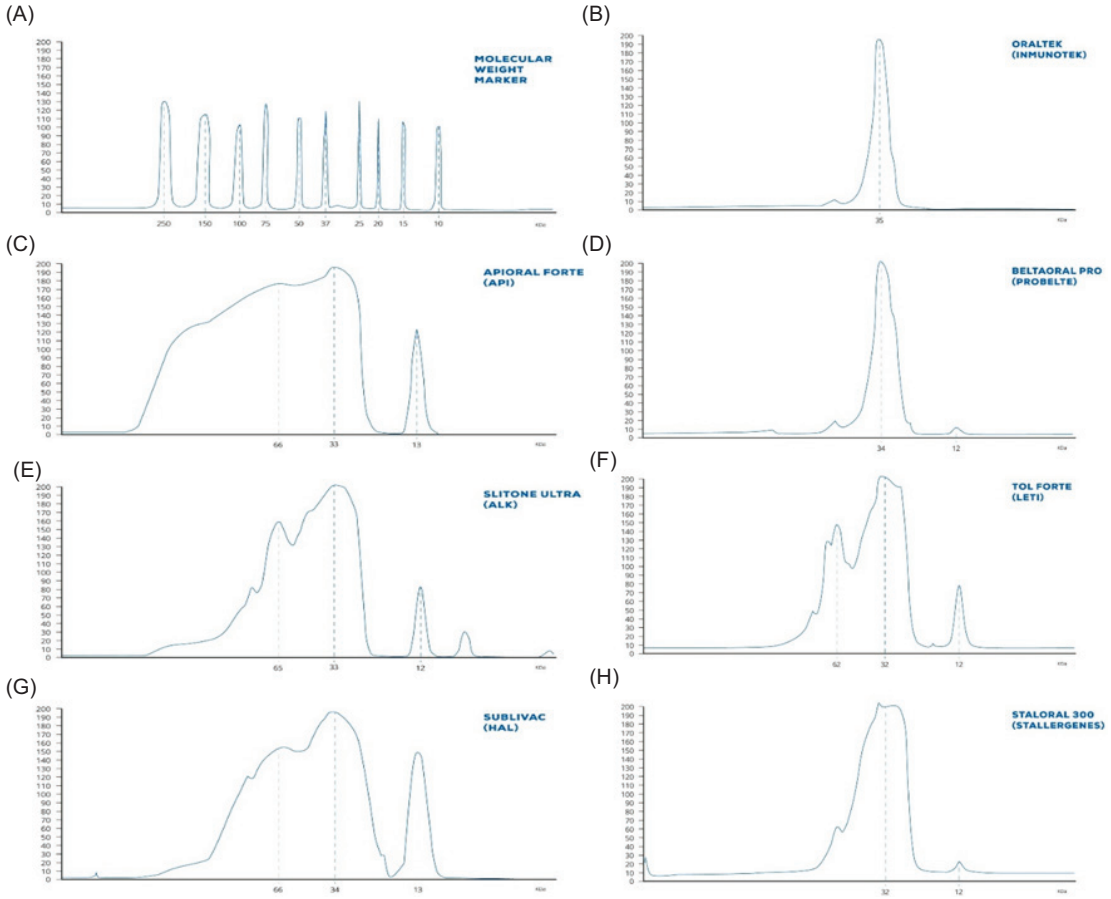

Figure 4 Densitometry of the allergenic profile of the products. (A) Molecular weight marker; (B) Apioral Forte; (C) SlitOne Ultra; (D) Sublivac; (E) Oraltek; (F) Beltaoral Pro; (G) Tol Forte; (H) Staloral 300.

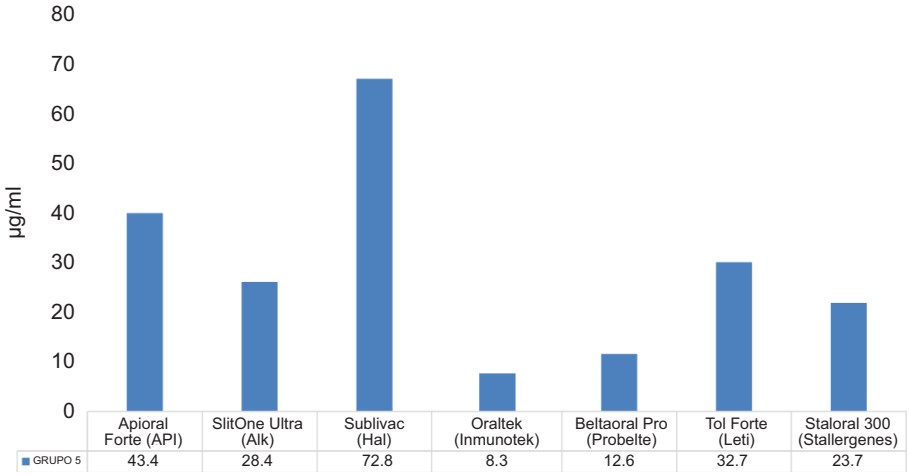

Figure 5 Quantitative determination of major allergens (Group 5) in the products.

conditions. For this reason, our study stands out from a methodological standpoint due to the strength of its experimental design, having subjected each extract to the same validated analytical techniques and ensuring compliance with the established suitability criteria. This allowed for correlational results to be obtained both across different techniques and within each extract analysed.

However, the study does have some limitations, notably that only a single batch of each product was analysed, making it impossible to assess batch-to-batch variability. Additionally, the absence of a formally documented chain of custody may have limited the reliability of comparisons between samples. Nonetheless, all extracts were obtained from the same community pharmacy and transported to the analysis centre under identical transport conditions. Furthermore, all samples were stored in the same location under controlled conditions with temperature monitoring, thereby ensuring cold chain maintenance up to the time of analysis.

Conclusion

Our study highlights the significant variability between marketed therapeutic allergenic extracts, both in allergenic composition and biological activity. Given the particular mechanism of action of SLIT, these differences are clinically relevant and should be considered in clinical practice. We believe it is necessary to have objective information, standardised analytical methods and universal standards, and strongly recommend resuming initiatives such as the CREATE project, starting with allergens of greatest geographical prevalence and therapeutic need.

Data Availability Statement

Raw data were generated at ASAC Pharmaceutical Immunology. Derived data supporting the findings of this study are available from the corresponding author, Jenaro José Hernández-Peña, on request.

Mandatory Disclosure on Use of Artificial Intelligence

The authors declare that no AI-assisted tools were used in the preparation of this manuscript. All references have been manually verified for accuracy and relevance.

Author Contributions

Clara Flores Infante and Andrea Vera Flores contributed to the design and conceptualisation of the study. Jenaro José Hernández-Peña coordinated the project and provided resources. Beatriz Brotons Silvar was responsible for the methodology and results analysis. Mónica Escorial and Flavia T. Hernández contributed to the data interpretation and manuscript writing. All authors participated in the supervision and critical review of the manuscript and approved the final version.

Conflicts of Interest

Mónica Escorial, Beatriz Brotons, and Flavia Tamara Hernández are employees of ASAC Pharmaceutical Immunology. Additionally, Jenaro Hernández serves as an external advisor to the company, which may be affected by the research reported in the enclosed paper.

Funding

None.

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Supplementary

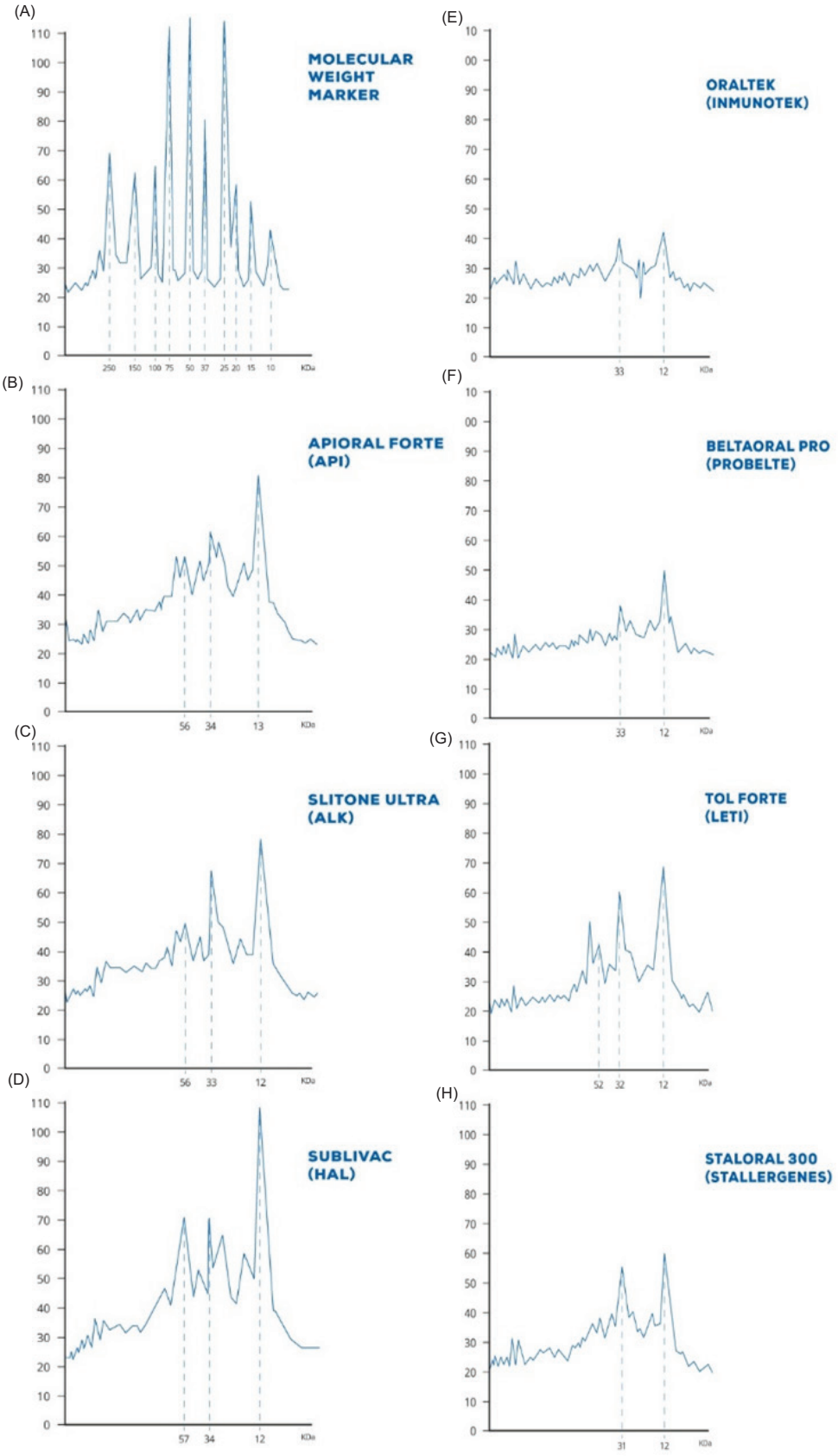

Figure S1 Results of the densitometric analysis for each product. (A) Molecular weight marker; (B) Apioral Forte; (C) SlitOne Ultra; (D) Sublivac; (E) Oraltek; (F) Beltaoral Pro; (G) Tol Forte; (H) Staloral 300.

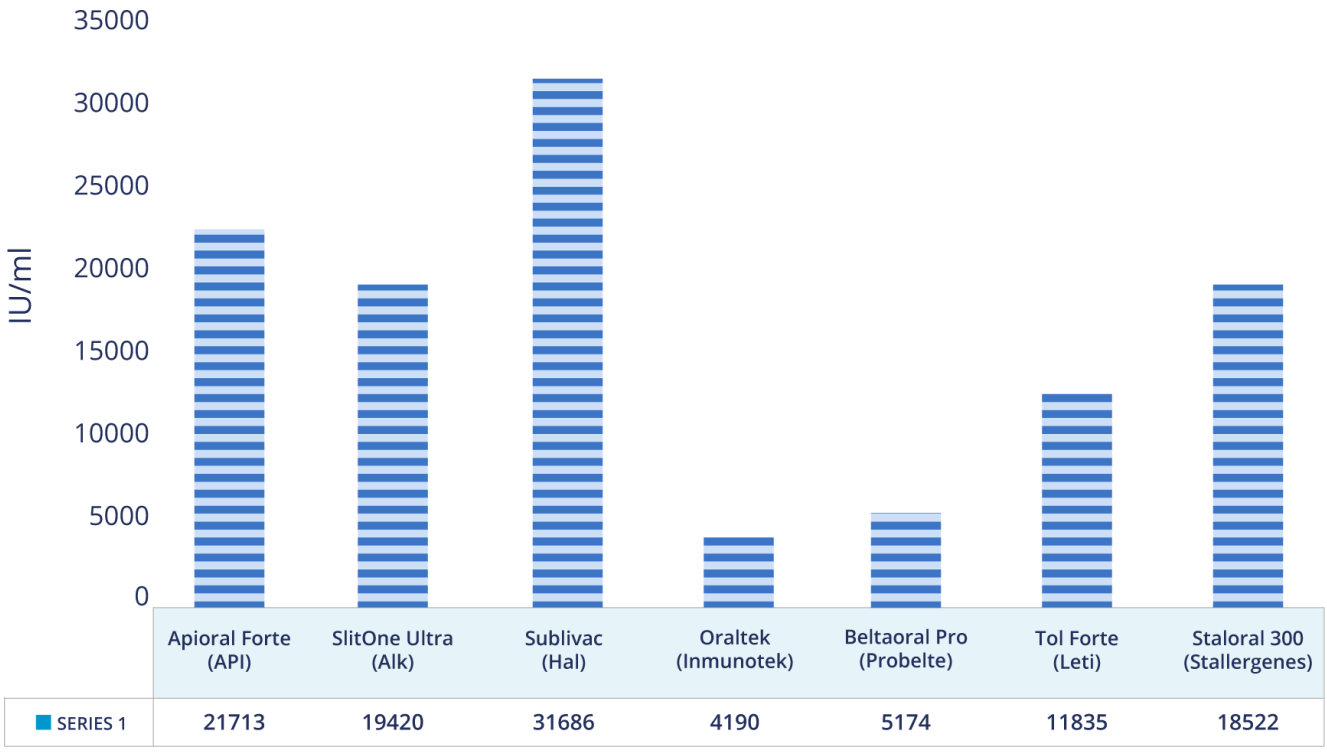

Figure S2 Total biological activity of the product in IU/mL.