LETTER TO THE EDITOR

Detection of Ole e 7 in allergoid mixtures for enhanced immunotherapy efficacy

Carlos Pastor-Vargas, Mercedes Díaz-Mendoza, Mayte Villalba, Begoña Lavin

Department of Biochemistry and Molecular Biology, School of Chemistry, Complutense University of Madrid, Madrid, Spain

To the Editor,

Respiratory allergies are increasing worldwide, with approximately 30% of the European population affected. Among these individuals, over 20% experience allergic rhinitis, and 5−12% suffer from asthma (1). Olive (*Olea europaea*) pollen is a major cause of respiratory allergies in Mediterranean countries, primarily due to the widespread cultivation of olive trees. This trend is also impacting other regions globally, including the United States, South America, South Africa, Japan, and Australia (2). The symptoms caused by olive tree pollen are predominantly nasal and conjunctival. However, in areas with high antigenic loads, exposure to olive tree pollen can also trigger asthma exacerbation (3). To date, fifteen allergens have been identified in olive pollen (4). Ole e 1 is recognized as the major allergen, as nearly 80% of individuals with olive pollen allergies exhibit IgE specific to Ole e 1 (2, 3). Moreover, Ole e 7, a non-specific lipid transfer protein (nsLTP), has been associated with more severe clinical manifestations, particularly in areas of high pollen load such as Spain (2, 4).

The current treatment for olive pollen allergies involves allergen-specific immunotherapy (AIT) using whole-allergen extracts, administered either subcutaneously or sublingually (5, 6). These therapies offer significant clinical benefits by promoting immune tolerance to the aeroallergen through various mechanisms (6-9). However, their effectiveness varies significantly, influenced by factors such as the patient's sensitization profile, the severity of clinical symptoms, and the quality of the treatment (10, 11). A major challenge with AIT is the difficulty in standardizing allergenic extracts, which results in inconsistent therapeutic potential and an increased risk of sensitization to other extract components. Despite advancements in extract formulation, the lack of standardization remains a barrier to achieving reliable treatment outcomes. This study aimed to determine whether Ole e 7 is reliably present in olive pollen extract batches and vaccine production intermediates, thereby supporting its relevance in AIT preparations.

The investigated samples were sourced from Allergopharma, which developed a clinically approved vaccine, Allergovit Olive, for treating olive pollen allergy, now routinely used in clinical practice. The objective of this study was to detect and quantify Ole e 7, an allergen typically present in low concentrations, in two batches of lyophilized olive pollen extract (in-house reference preparations; E1 and E2), as well as in three lyophilized vaccine production intermediate samples (V_1-V_3), used for manufacturing of the hypoallergenic olive pollen drug product.

Manuscript accepted for publication

Bradford and ELISA assays were each performed twice. For the Bradford method, both, the standard curve and assayed samples were measured in duplicates. An initial ELISA was conducted to determine the appropriate Ole e 7 concentration range for the samples. A second ELISA was the performed to accurately quantify the samples. For normalization, the same protein concentration – previously determined by the Bradford assay – was used for all samples. The detection limit of the ELISA, considering the primary antibody dilution, was 0,5 ng of Ole e 7. The Ole e 7 used for the standard curve was purified in our laboratory (12)." An indirect ELISA method was used for these measurements. Ole e 7, purified in our laboratory, and the assayed simples were immobilized on the ELISA plate. Then, samples were incubated with a home-made rabbit anti-Ole e 7 primary antibody (dilution 1:20.000) followed by incubation with a goat anti-rabbit HRP-conjugated secondary antibody (1:5000; GAR/IgG(H+L)/PO, Nordic MUbio). To illustrate the variability between the extract batches, we used the Student's t-test. Data are presented as mean ± SD, and p values less than 0.05 were considered statistically significant.

Protein concentrations were quantified in five samples from each olive pollen protein extract batch using the Bradford assay (**Table I**). No significant differences were detected in total protein content either within individual samples or between batches (E_1: 0.378 ± 0.016 mg/mg lyophilizate *vs* E_2: 0.410 ± 0.020 mg/mg lyophilizate). Furthermore, the total protein content across the three vaccine production intermediates tested were consistent (0.348±0.033 mg/mg lyophilizate).

An Ole e 7 ELISA quantification was established using purified native Ole e 7 (nOle e 7) as standard, as previously described (12). The purified Ole e 7 (**Figure 1**) was used to establish a standard curve ranging from 0 to 1000 ng, enabling precise quantification of this allergen across all analyzed samples using an ELISA method. The ELISA results demonstrated the presence of Ole e 7 in the two representative olive pollen protein lyophilized extract batches (E_1: 171.8 ± 13.4 ng/mg lyophilizate *vs* E_2: 137.3 ± 5.5 ng/mg lyophilizate; **Table I**). Importantly, Ole e 7 was detected in the three distinct lyophilized vaccine production intermediate samples within the nanogram range (210.677±61.418 ng/mg lyophilizate; **Table I**).

Our findings provide the first quantification of Ole e 7 in vaccine intermediates, confirming its consistent presence in Allergopharma's in-house reference preparations of lyophilized olive pollen protein extract and vaccine production intermediates. The batches analysed in this study originate from different production batches and storage durations yet exhibit comparable concentrations of Ole e 7. This consistency is encouraging from a manufacturing standpoint, as it supports the reliability and reproducibility of future production processes.

The consistent presence of Ole e 7 in vaccine intermediates may enhance clinical efficacy in sensitized individuals, supporting a more tailored AIT approach. Including Ole e 7 in extract batches is particularly important, given that its concentration is typically low and

Manuscript accepted for publication

may fall below clinically effective levels in certain formulations, potentially reducing immunotherapy efficacy. However, the threshold concentration of Ole e 7 required to elicit an allergic response has not yet been clearly established. Moreover, the concentrations of Ole e 9 and Ole e 7 in extract batches standardized solely on Ole e 1 demonstrate substantial variability, highlighting the need for broader standardization criteria in extract manufacturing. These findings support the growing emphasis on molecular standardization in allergen immunotherapy and underscore the importance of component-resolved quality control in vaccine manufacturing.

Funding
This research was supported by financial funding from Allergopharma SL.

Contributions of each author
BL and CPV: Formal Analysis, Funding acquisition, Supervision, Validation, Visualization, Writing, review and editing. CPV: Methodology; MDM and MV: Writing, review and editing.

Conflict of interests
This research was supported by financial funding from Allergopharma SL.

References
1. Montoro J, Antolín-Amérigo D, Artés M, Izquierdo-Domínguez A, Zapata JJ, Mur P, et al. Impact of Climate Change–Related Environmental Factors on Allergen Production and the Epidemiology and Severity of Allergic Diseases. J Investig Allergol Clin Immunol. 2024; 34(6):358-366. doi: 10.18176/jiaci.0988.
2. Calzada D, Cremades-Jimeno L, López-Ramos M, Cárdaba B. Peptide Allergen Immunotherapy: A New Perspective in Olive-Pollen Allergy. Pharmaceutics. 2021; 13(7): 1007. doi: 10.3390/pharmaceutics13071007.
3. Benedé S, Ramos-Soriano J, Palomares F, Losada J, Mascaraque A, López Rodríguez JC, Rojo J, et al. Peptide Glycodendrimers as Potential Vaccines for Olive Pollen Allergy. Mol Pharm. 2020; 17(3):827-836. doi: 10.1021/acs.molpharmaceut.9b01082.
4. San-Segundo Acosta P, Oeo-Santos C, Navas A, Jurado A, Villalba M, Barderas R. Ole e 15 and its human counterpart -PPIA- chimeras reveal an heterogeneous IgE response in olive pollen allergic patients. Sci Rep. 2019; 9(1):15027. doi: 10.1038/s41598-019-51005-2
5. Mahler V, Esch RE, Kleine-Tebbe J, Lavery WJ, Plunkett G, Vieths S, et al. Understanding differences in allergen immunotherapy products and practices in North America and Europe. J. Allergy Clin Immunol. 2019; 143(3):813-828. doi: 10.1016/j.jaci.2019.01.024.
6. Komlósi ZI, Kovács N, Sokolowska M, van de Veen W, Akdis M, Akdis CA. Mechanism of subcutaneous and sublingual aeroallergen immunotherapy: what is new? Immunol Allergy Clin North Am. 2020; 40(1):1-14. doi: 10.1016/j.iac.2019.09.009.
7. Passalacqua G, Bagnasco D, Canonica GW. 30 years of sublingual immunotherapy. Allergy. 2019; 75(5):1107-1120. doi: 10.1111/all.14113.

Manuscript accepted for publication

8. Gómez E, Díaz-Perales A, Tordesillas L, Doña I, Torres MJ, Blázquez AB, et al. Effect of Pru p 3 on dendritic cell maturation and T-lymphocyte proliferation in peach allergic patients. Ann Allergy Asthma Immunol. 2012; 109(1):52-8. doi: 10.1016/j.anai.2012.05.017.
9. Palomares F, Gomez F, Bogas, G, Campo P, Perkins JR, Diaz-Perales A, et al. Immunological changes induced in peach allergy patients with systemic reactions by Pru p 3 sublingual immunotherapy. Mol Nutr Food Res. 2018; 62(3). doi: 10.1002/mnfr.201700669.
10. Vovolis V, Kalogiros L, Mitsias D, Sifnaios E. Severe repeated anaphylactic reactions to sublingual immunotherapy. Allergol Immunopathol (Madr). 2013; 41(4):279-81. doi: 10.1016/j.aller.2012.05.012.
11. Patronov, A.; Doytchinova, I. T-cell epitope vaccine design by immunoinformatics. Open Biol. 2013; 3(1):120139. doi: 10.1098/rsob.120139.
12. Tejera ML, Villalba M, Batanero E, Rodríguez R. Identification, isolation, and characterization of Ole e 7, a new allergen of olive tree pollen. J Allergy Clin Immunol. 1999; 104(4 Pt 1):797-802. doi: 10.1016/s0091-6749(99)70290-3.

Manuscript accepted for publication

Figure 1. Purified Ole e 7 isolated from olive pollen extract detected by Western blot staining.

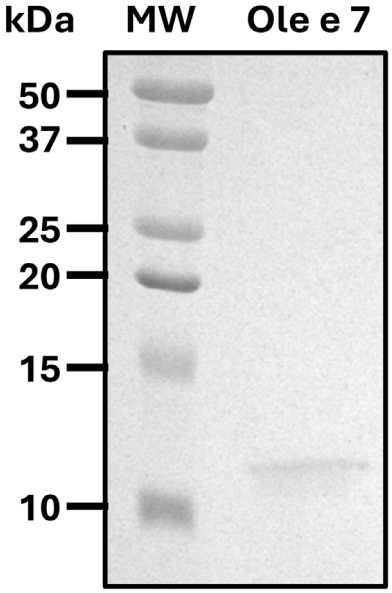

Figure 2. Comparative protein levels and Ole e 7 across batches and intermediates.

Manuscript accepted for publication

Table I. *Quantification of total protein and Ole e 7 content in all samples assayed*

Sample	Protein concentration in Lyophilizate (mg/ mg)	Mean protein content (mg/mg Lyophilizate)	Ole e 7 content (ng)/ lyophilizate (mg)	Mean Ole e 7 content (ng/mg Lyophylizate)
E_1	0.370	0.378 ± 0.016	155.770	171.760 ± 13.388
	0.400		181.600	
	0.360		162.360	
	0.390		188.370	
	0.370		172.790	
E_2	0.420	0.410 ± 0.020	141.120	137.344 ± 5.482
	0.380		128.060	
	0.420		141.540	
	0.400		138.800	
	0.430		137.600	
V_1	0.315	0.348 ± 0.033	213.445	210.677 ± 61.418
V_2	0.351		156.756	
V_3	0.381		279.473	

Manuscript accepted for publication