
Monoclonal antibody-based method for measuring olive pollen major allergen Ole e 1

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Background: Olive tree pollen is an important cause of inhalant allergy in Mediterranean countries. The major allergen of this pollen, Ole e 1, has caused reactions in the sera of >80% of olive-sensitive patients. Accurate standardization of allergenic products for diagnosis and immunotherapy is essential to guarantee their quality, and measurement of the major allergen content is becoming an important aspect of standardization procedures.

Objective: To develop a two-site enzyme-linked immunoadsorbent assay (ELISA) for the quantification of Ole e 1.

Methods: BALB/c mice were immunized with purified natural Ole e 1. After fusion and screening by direct ELISA, one of the monoclonal antibodies (5A3) was selected as the capture antibody in an ELISA for Ole e 1 quantification. Bound allergens were detected by a combination of biotinylated Ole e 1-specific polyclonal rabbit antibody and peroxidase-conjugated streptavidin. This ELISA was subsequently evaluated and compared with other techniques.

Results: The developed ELISA was highly reproducible and sensitive, with a detection limit of 0.5 ng/mL and a practical range of 1 to 10 ng/mL. The Ole e 1 content ranged from 3 to 50% of the total protein among the nine *Olea europaea* pollen extracts studied. The assay also detected Ole e 1-like proteins in pollen from other Oleaceae. Correlation was good between the Ole e 1 content determined by ELISA and scanning densitometry and the immunoglobulin E-binding activity of the extracts.

Conclusion: The described Ole e 1 ELISA is sensitive, reproducible, specific, and reliable, and therefore, can be helpful for standardization of olive pollen extracts intended for clinical use.

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INTRODUCTION

Olive tree (*Olea europaea*) pollen has been reported to be the most important cause of type I allergic reactions in Mediterranean countries and in some areas of North America.¹ Although in northern and central Europe the olive tree is scarce and its pollinosis has no clinical significance, other members of the Oleaceae family, such as ash (*Fraxinus*), lilac (*Syringa*), and privet (*Ligustrum*), are increasingly being cultivated. Thus, they are able to cause symptoms of hay fever and asthma.² Several major and minor allergens from olive tree pollen have been identified and characterized during the past several years.³ The most relevant and abundant allergen in olive pollen is Ole e 1, a polymorphic protein that exists as two variants: glycosylated and nonglycosylated, with apparent molecular weights of 20.0 kD and 18.5 kD, respectively. Some investigators have suggested that Ole e 1 could have a role in the elongation of the pollen tube.⁴ Ole e 1-homologous proteins have been described as the main components of Oleaceae pollen extracts.^{5–7} Hence, the high degree of cross-reactivity found among pollen extracts of this family is explained.^{1,8,9}

Diagnosis and treatment of allergy are performed with whole extracts, which are prepared from natural source materials and contain complex mixtures of proteins, only a few of which are allergenic. One method for characterizing allergen extracts is to measure their major allergen content. Ole e 1 is the most important major allergen of olive pollen; serum samples from more than 80% of olive pollen-sensitive patients contained immunoglobulin (Ig)E that reacted with Ole e 1.^{10,11} Their content can vary, depending on the olive allergen source used in the production of the olive pollen extract.^{12–14} According to the *European Pharmacopoeia*, the individual allergen content in pollen extract, determined by a suitable method, should be between one-half and two times the declared value.¹⁵ For immunotherapy with ragweed, grass, cat, mite, and venom allergens, good evidence indicates that a maintenance dose of 5 to 20 μ g of major allergen per injection is associated with a substantial decrease in patient symptoms.¹⁶ Accordingly, the development of techniques to measure major allergen levels in diagnostic and therapeutic allergen products is of considerable interest. The development of hybridoma technology and the production of monoclonal antibodies (mAbs) provide new immunologic tools for management of allergy. Sandwich assays with enzymatic detection methods (enzyme-linked immunoadsorbent assay, or ELISA) can be particularly useful for allergen quantification because of

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the characteristics of accuracy, reproducibility, reliability, and specificity.

In this study, we describe a new ELISA, based on an mAb and a specific rabbit polyclonal antibody, to quantify Ole e 1 allergen.

METHODS

Allergenic Extracts and Ole e 1 Purification

Olive tree pollens were supplied by Mediterráneo Polen (Jaén, Spain), Biopol (Barcelona, Spain), New England Antigens (Portland, ME), and Allergon (Engelholm, Sweden), and their corresponding extracts were prepared in accordance with the methods described by Martínez et al.¹⁷ Ole e 1-depleted *O. europaea* extract was obtained by incubating the extract with rabbit polyclonal serum anti-Ole e 1 bound to protein G beads and removing the precipitate by centrifugation. Additional allergenic extracts for specificity studies were also prepared in our laboratory with use of the same methods. Natural Ole e 1 was purified from olive pollen extract by gel filtration and reverse-phase chromatography, as previously described.¹⁸ Recombinant Ole e 1 was expressed in *Escherichia coli* and purified in accordance with techniques previously reported by Asturias et al.¹⁹ The protein content was measured according to the method of Bradford.²⁰

Production and Purification of Ole e 1-Specific mAbs and Polyclonal Antiserum

Ole e 1-specific mAbs were prepared essentially as previously described.²¹ Briefly, spleen cells obtained from BALB/c mice immunized with purified Ole e 1 were fused with SP₂O myeloma cells in the presence of polyethylene glycol. Positive hybridomas were identified by ELISA with use of natural Ole e 1-coated plates and then subcloned by limiting dilution. Polyclonal serum was obtained by immunizing white New Zealand rabbits with purified Ole e 1. For antibody purification, hybridoma culture supernatants or rabbit anti-Ole e 1 serum was purified by affinity chromatography with protein G-Sepharose (Amersham Pharmacia Biotech, Buckinghamshire, UK). The purified rabbit IgG was labeled with biotin by using the enzymatic chemiluminescence protein biotinylation kit (Amersham Pharmacia Biotech). Isotype detection was done by IsoStrip kit (Boehringer Mannheim, Mannheim, Germany), according to the manufacturer's instructions.

Ole e 1 ELISA

Microtiter 96-well plates (Maxisorp, NUNC, Denmark) were coated overnight at 4° C with 100 μ L of mAb 5A3 at 2.5 μ g/mL in phosphate-buffered saline (PBS). The coated wells were blocked with 200 μ L of PBS containing 1% bovine serum albumin (BSA) and 0.05% Tween 20 (PBS-BSA-T). Afterward, they were sequentially incubated with 100 μ L/well of purified Ole e 1 or extracts, biotin-labeled rabbit antiserum (2.5 μ g/mL), and streptavidin-peroxidase (0.25 μ g/mL; Sigma Chemical, St. Louis, MO) in PBS-BSA-T. All incubations were performed at 37° C for 1 hour, with three

washes of 200 μ L/well of PBS-T between successive steps. Finally, wells were incubated at room temperature in the dark with a solution of *o*-phenylenediamine (Sigma). The reaction was stopped after 30 minutes by adding 50 μ L/well of 3 M H₂SO₄, and the absorbance was then measured at 492 nm. Crude extracts were assayed as dilution series in triplicate, and the concentrations were interpolated from the linear portion of the standard curve. The detection limit, accuracy, reproducibility, and specificity were calculated by use of the recommended methods.²² For evaluation of intraassay or interassay variations, three Ole e 1 concentrations (1, 5, and 10 μ g/mL) were incubated 20 times during the same day or during 20 days, respectively.

Electrophoresis and Gel Scanning Densitometry

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of whole extracts and purified Ole e 1 was performed with 12.5% acrylamide gel under reducing conditions.²³ The protein bands were detected with Coomassie brilliant blue R-250, and Ole e 1 was quantified with a GS-710 calibrated imaging densitometer (Bio-Rad, Hercules, CA) and use of the Bio-Rad Diversity software. This method allowed the quantification of stained Ole e 1 bands by comparing the contour density (optical density $\times$ mm²) obtained with known concentrations of purified allergen. A linear correlation between the different amounts of Ole e 1 per lane and the contour density of the bands was obtained (data not shown).

Immunoblotting

After SDS-PAGE, separated proteins were transferred electrophoretically to polyvinylene difluoride membranes (Immobilon-P membranes, Millipore, Bedford, MA) by the technique of Towbin et al.²⁴ Blocked membranes were incubated with 45 μ g/mL of purified mAb 5A3 or with 0.5 μ g/mL of rabbit antiserum for 1 hour at 37° C. Bound antibodies were detected with peroxidase-labeled anti-mouse IgG (Sigma) or anti-rabbit immunoglobulins (Dako, Copenhagen, Denmark) and developed with 4-chloro-1-naphthol.

Enzyme Allergosorbent Test (EAST) Inhibition

The IgE-binding activity of *O. europaea* pollen extracts was evaluated by EAST inhibition (Hytec specific IgE enzyme immunoassay; Hycor Biomedical, Kassel, Germany), as described by Yman et al.,²⁵ with use of paper disks coupled to an in-house reference *O. europaea* extract as solid phase and a serum pool from olive pollen-sensitive patients. The IgE-binding activity of the extracts, expressed in biologic units, was estimated on the basis of the 50% inhibition EAST values by comparison with an in-house *O. europaea* reference extract, previously calibrated by the skin prick test in accordance with the Nordic Guidelines and the recommendations from the European Academy of Allergology and Clinical Immunology,^{26,27} with use of 10 mg/mL of histamine as control.

Statistical Analysis

The nonparametric Spearman rank correlation test was applied to study the relationship between ELISA and either immunoblotting densitometry or EAST inhibition techniques.

RESULTS

Purification of Natural and Recombinant Ole e 1

Ole e 1 was purified from an extract of *O. europaea* pollen by gel permeation and reverse-phase chromatography. Purity was monitored by SDS-PAGE, as shown in Figure 1. Natural Ole e 1 consisted of two proteins with molecular weights of 20.0 kD and 17.5 kD, respectively, corresponding to the described molecular weights of glycosylated and nonglycosylated Ole e 1. *E. coli*-expressed recombinant Ole e 1 showed a molecular weight of 18.0 kD, slightly higher than nonglycosylated natural Ole e 1 because of an additional six amino acids included for cloning.¹⁹

Ole e 1 ELISA

Immunization of BALB/c mice with purified Ole e 1 resulted in 15 mAbs of IgG class with specificity for Ole e 1, as judged by ELISA. The most reactive mAbs (5A3, 5D9, 6A7, 6F9, and 6H5) and the specific polyclonal rabbit serum were purified and labeled with biotin. All possible pairs of mAbs and polyclonal rabbit sera were tested for the applicability in a two-site sandwich ELISA in which purified Ole e 1 (1 μ g/mL) was used (Table 1). The combination of mAb 5A3,

Table 1. Absorbance at 492 nm in a Two-Site Sandwich ELISA, with Use of mAbs to Capture Purified Ole e 1 (1 μ g/mL) in Combination with Labeled Antibodies as Detector

Detector antibody	Capture monoclonal antibody				
	5A3	5D9	6A7	6F9	6H5
Antiserum	2.602	1.309	1.378	0.514	1.820
5A3	...	0.567	0.724	0.432	0.735
5D9	0.096	...	1.070	0.418	0.448
6A7	0.083	0.812	...	0.307	0.777
6F9	0.061	0.738	0.530	...	0.448
6H5	0.063	0.288	0.743	0.323	...

typed as IgG1 subclass with κ light chains, as capture antibody and specific polyclonal rabbit serum as detector antibody rendered the highest optical density. The specificity of mAb 5A3 and specific anti-Ole e 1 serum was demonstrated by immunodetection after SDS-PAGE of *O. europaea* crude extract (Fig 1). Both of them recognized a 20.0-kD and a 17.5-kD band, consistent with the molecular weights of glycosylated and nonglycosylated Ole e 1. The standard dose-response curve (0.5 to 50 ng/mL) is shown in Figure 2. The detection limit was calculated as 0.5 ng/mL, with a practical working range of 1 to 10 ng/mL. A linear correlation, with a coefficient of regression of 0.997, was found in this range, where the lowest limit (1 ng/mL) can be considered the limit

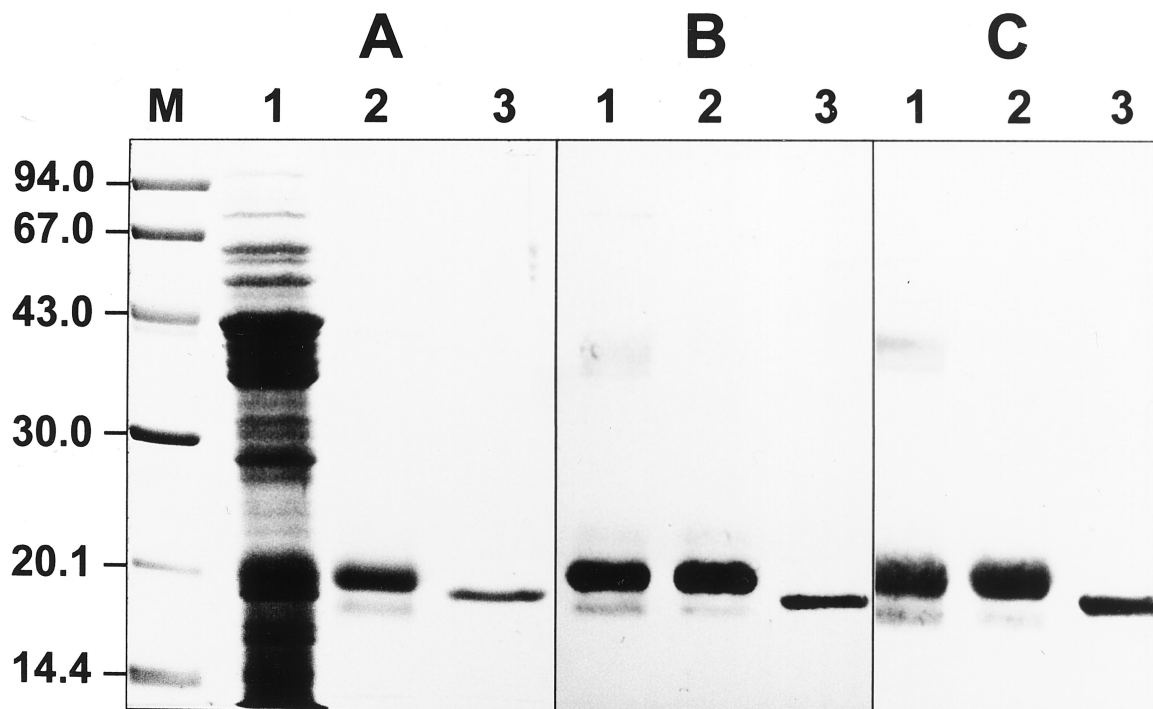


Figure 1. Coomassie brilliant blue-stained sodium dodecyl sulfate-polyacrylamide gel electrophoresis (A), immunoblots incubated with monoclonal antibody 5A3 (B), or rabbit anti-Ole e 1 monospecific serum (C) of *Olea europaea* pollen extract (column 1), natural Ole e 1 (column 2), and recombinant Ole e 1 (column 3).

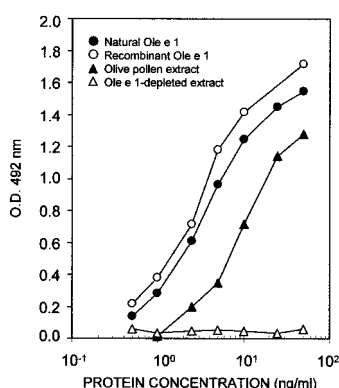


Figure 2. Dose-response curves in two-site sandwich ELISA of natural and recombinant Ole e 1, olive pollen extract, and Ole e 1-depleted olive pollen extract. O.D., optical density.

of quantitation. The interassay coefficient of variation was 14, 9, and 9% at concentrations of 1, 5, and 10 ng/mL ($N = 20$), respectively. At the same concentrations, the intraassay coefficient of variation was 11.5, 5, and 4%. Interference of the skin prick diagnostic solution components (glycerol and phenol) on the Ole e 1 ELISA was tested. No effect of these components was detected at 0.2% of glycerol and 0.002% of phenol. The dose-response curves obtained for both natural and recombinant purified Ole e 1 in the two-site ELISA were completely coincident (Fig 2); this finding suggests that both allergens have similar antigenic determinants. Further, purified Ole e 1 had a dose-response curve parallel to that of crude olive pollen extracts, an indication that the assay was measuring the same molecule in the different preparations and that the allergens had not been altered during the purification process.

The specificity of the method was evaluated against a broad battery of extracts from tree pollen (*Cupressus arizonica*, *Platanus acerifolia*, and *Betula verrucosa*), grass pollen (*Lolium perenne* and *Zea mays*), weed pollen (*Parietaria judaica*, *Plantago lanceolata*, and *Helianthus annuus*), and other nonplant extracts (*Alternaria alternata* and *Dermatophagoides pteronyssinus*). The assay showed no significant Ole e 1 content in these allergenic materials known to lack Ole e 1-like allergens.

Quantification of Ole e 1 in Olive Pollen Extracts

The Ole e 1 content of nine *O. europaea* pollen extracts was evaluated by using the currently developed two-site sandwich ELISA. The curves obtained showed good parallelism with the standard curve (data not shown). The Ole e 1 content of extracts D, E, F, G, H, and I, obtained from pollen batches of the same supplier company, ranged from 20 to 25% of total protein (Table 2). The other olive pollen extracts, however, which were produced from pollen batches supplied by other companies, had more variable Ole e 1 content (3, 7, and 50% of total protein). An Ole e 1-depleted olive pollen extract was obtained after incubation of the extract with the highest Ole

Table 2. Comparison of IgE-Binding Activity Values (Determined by EAST Inhibition) and Ole e 1 Content (Determined by Two-Site Sandwich ELISA and Scanning Densitometry) Among *O. europaea* Extracts from Different Pollen Suppliers

Extract	ELISA ($\mu\text{g/mL}$)	Densitometry ($\mu\text{g/mL}$)	EAST inhibition (UBE/mL)
A	70	110	21,979
B	500	248	143,958
C	33	77	23,644
D*	250	243	71,979
E*	230	287	40,763
F*	240	346	56,067
G*	200	163	68,876
H*	210	182	51,546
I*	230	156	49,623

* Olive pollen extracts produced from different pollen batches of the same supplier company.
UBE, biologic units.

e 1 content (extract B) with the rabbit polyclonal antibody bound to protein G beads. After three rounds of precipitation, the Ole e 1 content of the Ole e 1-depleted olive pollen extract was reduced more than 98% (Fig 2). The contribution of Ole e 1 to the IgE binding activity of this extract was determined from the effect of allergen depletion on EAST inhibition. The Ole e 1-depleted extract showed 1.1% of the IgE-binding activity present in the original extract.

The mAb 5A3 and rabbit monospecific anti-Ole e 1 serum detected Ole e 1-like proteins at 20 kD in pollen from different trees of the family Oleaceae (*Fraxinus*, *Ligustrum*, and *Syringa*) by both immunoblotting (data not shown) and ELISA. The levels in these extracts of proteins homologous to Ole e 1, calculated by the developed ELISA, were 3.7, 0.5, and 1.2% of total protein present in *Fraxinus*, *Ligustrum*, and *Syringa* extracts, respectively.

Comparison With Other Techniques

The Ole e 1 content of nine pollen extracts was also calculated by densitometry analysis of Coomassie brilliant blue-stained gels after SDS-PAGE, with use of known amounts of purified Ole e 1 as reference. Further, the total IgE-binding activity of the nine pollen extracts was analyzed by EAST inhibition with use of a biologic standardized in-house reference extract (Table 2). As shown in Figure 3, a significant relationship was demonstrated between ELISA and densitometry ($r = 0.778$; $P < 0.05$) as well as between ELISA and EAST inhibition ($r = 0.762$; $P < 0.05$).

DISCUSSION

The quantification of major allergens has become an important goal for the standardization of allergen products intended for clinical use. Ole e 1 is, in general, the most abundant and prevalent allergen of olive pollen extract.³ Therefore, Ole e 1 was a suitable candidate for standardization of *O. europaea* extracts in terms of major allergen content.

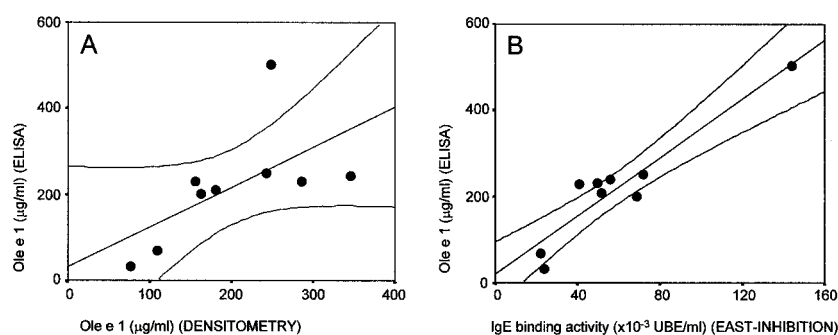


Figure 3. Correlation between Ole e 1 content measured by ELISA and densitometry on quantitative SDS-PAGE (A) and Ole e 1 content measured by ELISA and IgE-binding activity determined by EAST inhibition (B).

ELISA has the advantage of high sensitivity, reproducibility, and reliability, without the drawbacks of the earlier immunoassays (such as exposure to radioactivity). Because of these characteristics, ELISA is becoming the standard among the allergenic protein quantification techniques. In this study, we used a two-site sandwich ELISA to quantify Ole e 1. The developed assay has an adequate sensitivity to measure Ole e 1 in diluted olive pollen extracts, with sensitivity equivalent to other recently described immunoassays.^{28,29} Further, the assay is highly reproducible, as demonstrated by both intra-assay and interassay coefficients of variation, and specific for Oleaceae trees.

To investigate the practical application of the assay, we measured the concentration of Ole e 1 by ELISA in nine different *O. europaea* extracts. The Ole e 1 content varied from 3 to 50% of total protein. A narrower range of variation (20 to 25%) was found in the six extracts from pollen collected in the same geographic area. Therefore, this variability is attributable to the natural source material rather than to the extraction procedures. Allergen variability in *O. europaea* pollen has been previously described,^{12,13} and Ole e 1 content varied from 1 to 20% of the total protein present in the extract.^{14,28,29} Two possible factors have been proposed to explain the high variability of allergens found in *O. europaea* pollen: the existence of several cultivars of this tree with different protein composition¹³ and the effect of external factors on protein expression, including soil quality, hours of light, rainfall values, and maturation stage of the plant.¹² The mixing of representative cultivars from different geographic and climatologic regions should be a recommended practice for the preparation of extracts used for diagnosis and treatment.

The detection of Ole e 1-like allergens in other Oleaceae pollens, such as those from *Fraxinus*, *Ligustrum*, and *Syringa*, has been achieved by using the developed assay. The high homology in N-terminal amino acids or complementary DNA sequences of Ole e 1, Fra e 1, Lig v 1, and Syr v 1⁵⁻⁷ would explain the previously detected cross-reactivity among pollens of this family with frequently associated clinical manifestations.²⁸ In our current study, Ole e 1-like allergens from *Fraxinus*, *Ligustrum*, and *Syringa* pollens were detected by immunoblotting and ELISA with use of mAb 5A3 and rabbit monospecific anti-Ole e 1 serum. Nevertheless, to

design an exact quantification assay for Ole e 1-like allergens in Oleaceae pollen extracts, separate control curves should be established for each plant species by using the corresponding purified Ole e 1-like protein. Therefore, this assay can be used for closely related species that may be found in areas in which olive trees are not cultivated.

In our investigation, a good correlation was found between the content of Ole e 1 determined by ELISA and IgE-binding activity determined by EAST inhibition. Further, removal of 98% of Ole e 1 from the whole extract led to nearly total disappearance of the IgE-binding activity. Because the Ole e 1 concentration was representative of the overall IgE-binding activity in *O. europaea*, the two-site sandwich ELISA might be an alternative to EAST inhibition for quality control of the extracts. These results were in agreement with those reported by Lombardero et al.,²⁸ although other investigators have found no correlation between EAST inhibition and Ole e 1 ELISA.^{14,29} This fact could be explained if the serum pools used for the inhibition assays recognized other major allergens. For this reason, EAST inhibition is dependent on the serum pool used. The prevalence of individual allergens is closely related to the allergenic components of various cultivars of *O. europaea* and to the level of pollen in the atmosphere and, therefore, to the geographic area in which patients are sensitized.³

In our evaluation, the amount of Ole e 1 calculated by gel scanning significantly correlated with the Ole e 1 content determined by the immunoassay. Nevertheless, in extract B, the Ole e 1 content calculated by the two-site sandwich ELISA was twofold the Ole e 1 content determined by densitometry. This result could be attributable to the fact that Ole e 1 and Ole e 4 share some epitopes;³⁰ accordingly, the immunoassay could be detecting common epitopes present in other allergens. Alternatively, overestimations of Ole e 1 content calculated by gel scanning densitometry could occur because other olive pollen allergens, Ole e 5 and Ole e 8, display apparent molecular weights of 16 kD and 20 kD, respectively, similar to the molecular weight of Ole e 1. Currently, the trend in allergen standardization is the measurement of major allergens,³¹⁻³³ but the evaluation of the total allergenic potency of an extract should rely on the quantification of most representative allergens, as has been suggested for grasses, cats, and *Fagales* trees.³⁴⁻³⁶

CONCLUSION

We developed a sensitive and specific two-site sandwich ELISA for the quantification of Ole e 1. It will be an important tool for quality control of allergenic preparations from olive tree pollen intended for clinical use.

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