

Determination of Ole e 1 by enzyme immunoassay and scanning densitometry. Validation by skin-prick testing

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Abstract

Ole e 1 is an important allergen in *Olea europaea* pollen extracts. This study describes the development of two new methods that can be used to estimate the Ole e 1 content in olive tree pollen extracts. They are based on (1) an enzyme immunoassay that uses rabbit polyclonal, monospecific antibodies and purified Ole e 1, and (2) scanning densitometry of SDS-PAGE gels. Twelve extracts were evaluated by in vivo and in vitro methods. The in vivo biological potency was estimated by prick skin testing 17 allergic individuals; the in vitro allergenic potency by direct IgE and IgE inhibition assays. The enzyme immunoassay showed an operative range of 0.03–100 µg/ml and demonstrated to be specific for Ole e 1. The Ole e 1 content ranged from 1% to 5% of the total protein in the 12 extracts. The amount of Ole e 1, assessed by gel scanning densitometry significantly correlated with the Ole e 1 content obtained by the immunoassay ($r = 0.92$; $p < 0.001$). The Ole e 1 content showed a significant correlation with the total allergenic potency of the extracts, evaluated by direct IgE, specific IgE inhibition and skin-prick testing. These two methods can be used to determine the Ole e 1 content in olive pollen extracts. The content of Ole e 1 can vary from 1% to 5% of the total protein in the extracts. © 1999 Elsevier Science B.V. All rights reserved.

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1. Introduction

There is an increasing interest in the identification, purification, characterization and quantification of major allergens in allergen extracts (King et al., 1995; Liebers et al., 1996). Various investigators have estimated the concentration of major allergens using monoclonal antibodies alone (Chapman et al., 1987; Lombardero et al., 1988; Pollart et al., 1991;

Fahlbusch et al., 1994) or in combination with rabbit polyclonal antibodies (Ford et al., 1985; Schou et al., 1991).

The European Pharmacopoeia 3rd edition has recently devoted a section to allergenic products (Producta Allergenica, 1997). In this section, it is contemplated that the allergenic and antigenic profiles, the total allergenic activity and the individual allergen content, when possible, of the in house reference preparations should be stated. The individual allergen content, determined by a suitable method, when available, must be comprised between 50% and 200%

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of the declared value. The same concept applies to the total allergenic activity.

Ole e 1, an important allergen of *Olea europaea* pollen, has a molecular weight of approximately 20 kDa and an IgE binding prevalence greater than 80%. It has been suggested that Ole e 1 has a significant contribution to the total allergenicity of olive pollen extracts and that its concentration is closely related to the allergenic potency (Lombardero et al., 1992; Carreira et al., 1993). In addition to Ole e 1, other allergens have been identified and characterized. Ole e 2 is olive pollen profilin with a molecular weight of 15 kDa (Asturias et al., 1997; Ledesma et al., 1998). Ole e 3 is a calcium binding protein of 9.5 kDa that exhibits homology with pollen allergens from *Brassica* species (Batanero et al., 1996). Ole e 4 is a major allergen of 32 kDa with an unknown function. The Ole e 5 is a minor allergen of 16 kDa with a high degree of homology with plant's superoxide dismutases (Boluda et al., 1998a).

The standardization process of *O. europaea* pollen extracts is often hampered by the high degree of variability of these extracts (Barber et al., 1990; Waisel et al., 1996). Ole e 1 content can vary up to 20 times, depending on the olive pollen batch (Carreira et al., 1993).

Herein we describe the estimation of the Ole e 1 content in 12 batches of *O. europaea* pollen extracts, collected in different locations, by means of (1) a newly developed immunoassay that uses rabbit polyclonal, monospecific antibodies and (2) a gel scanning densitometry method. The Ole e 1 content were correlated with the allergenic potency of these extracts by in vivo and in vitro methods.

2. Material and methods

2.1. Allergenic extracts

O. europaea pollen was provided by different suppliers. The origin of the different batches is shown in Table 1.

Extracts were prepared from ether-defatted dry pollen grain. Pollen was extracted 1:20 w/v in PBS (pH 7.4) during 4 h at 4°C. The resulting suspension was centrifuged at 13,000 rpm for 30 min and fil-

Table 1

Suppliers of the different batches of *O. europaea* pollen used for the study

Lot	Supplier	Protein concentration (mg/ml)	Year
1	Mediterráneo Polen (Spain)	2.19	1996
2*	Mediterráneo Polen (Spain)	2.92	1996
3	J.A. Jiménez (Spain)	3.06	1989
4	Jaén (Spain)	2.00	1983
5	Univ. Aut. Barcelona (Spain)	0.33	1996
6	J.A. Jiménez (Spain)	4.18	1995
7	Artu Biologicals (Holland)	2.22	1989
8	Allergon (Sweden)	2.69	1991
9	Biopol (USA)	0.76	1990
10	Biopol (USA)	0.89	1996
11	Biopol (USA)	1.98	1994
12	Allergon (Sweden)	2.11	1994

*Wild olive tree (Acebuche).

tered through 0.22 µm. The aqueous extracts were dialyzed overnight against distilled water at 4°C in membranes with a nominal cut-off point of 3.5 kDa (Spectrum Medical Industries, TX, USA), filtered and lyophilized. The freeze-dried preparations were reconstituted just before use.

2.2. Human sera

Serum samples were collected from 30 Spanish individuals with allergic rhinitis and/or asthma and positive skin tests to a standardized olive pollen extract (100 HEP/ml; C.B.F. LETI). The presence of specific IgE was confirmed by CAP-FEIA system (Pharmacia Biotech, Uppsala, Sweden). All samples were stored in aliquots at -20°C and pooled just before use.

2.3. Total protein determinations

The protein concentrations of the *O. europaea* pollen extracts were estimated by the Biuret–Lowry method (Sigma, St. Louis, MO, USA) following the manufacturer's instructions.

2.4. Skin-prick test

Seventeen Spanish patients were skin tested with the 12 *O. europaea* extracts according to the recom-

recommendations of the Nordic Council on Medicines (Nordic Guidelines, 1989). The tests were performed at a concentration of 1 mg/ml in glycerinated saline solution. Histamine HCl at 10 mg/ml (54.3 mmol/l) and a physiological saline solution were used as positive and negative controls, respectively. Tests were conducted in duplicate using both arms and alternating sites. All the reactions were recorded after 15 min of the applications. The area of each wheal was calculated as a product of the 2 diameters, the longest (D) and the midpoint orthogonal (d). For statistical calculations, the geometric mean of the areas was employed.

2.5. Purification of Ole e 1

The *O. europaea* extract was fractionated by gel filtration chromatography using a $90 \times 3 \text{ cm}^2$ Sephadex G-75 column equilibrated in PBS 0.01 M pH 7.4. The column was previously calibrated with dextran blue and a mixture of proteins with known molecular weights such as peroxidase (44 kDa), soybean trypsin inhibitor (29 kDa) and lysozyme (14.3 kDa). In each run, 300 mg of lyophilized extract were applied at a flow rate of 50 ml/h; 8.9 ml fractions were collected. The elution profile was followed by continuous monitoring at 280 nm and divided into four pools of fractions in descending order of molecular weights. These pools were dialyzed overnight against distilled water and lyophilized. After measuring their protein concentrations, these fractions were subjected to SDS-PAGE according to the method of Laemmli (1970) with minor modifications.

We selected a pool of fractions enriched of Ole e 1 according to the SDS-PAGE analysis. This preparation was run in two separated gels following the same procedure that of SDS-PAGE. The allergens were located by staining a lateral of the gel with Coomassie blue during 10 min. The part of the gel that contained the allergen was cut into slices and eluted by diffusion in 5 mM ammonium bicarbonate and 0.05% SDS solution. The elution was conducted overnight at room temperature (RT). After removing the gel pieces, the solution was extensively dialyzed against elution buffer, followed by distilled water and lyophilization. The SDS of the sample was removed by acetone precipitation in the cold.

2.6. Production of rabbit polyclonal antibodies anti-Ole e 1

Gel slices containing purified Ole e 1 were emulsified in complete Freund adjuvant and injected into white New Zealand rabbits to produce polyclonal specific antibodies. The production of antibodies was monitored by measuring the antibody titers 1 week later after each injection.

2.7. IgG immunoblotting

The specificity of the anti-Ole e 1 antibody was tested by IgG immunoblotting. An *O. europaea* extract was run in SDS-PAGE gel and electroblotted to Immobilon-P membranes (Millipore, Bedford, USA) in a Tank Blot (Bio-Rad) apparatus. After transferring, the blots were allowed to dry completely and the sheets incubated with various dilutions of rabbit polyclonal anti-Ole e 1 in a blocking buffer consisting of 1% human serum albumin (HSA) in PBS 0.1% Tween 20 for 1.5 h at RT. After five washing steps with PBS 0.1% Tween 20, the sheets were incubated with horseradish peroxidase (HRP)-conjugated to goat anti-rabbit IgG (Cappel, Durham) at a 1/8000 dilution for 1.5 h. After five washing steps, the enzymatic reaction of the peroxidase was developed by adding a solution of 0.1 M citrate buffer (pH 5.0) containing 10 mM EDTA and 1% dextran sulfate (1 ml), tetramethylbenzidine (TMB; Boehringer Mannheim, Germany) in DMSO (Merck, Germany; 20 μ l) and 1 M H_2O_2 (2 μ l).

2.8. Determinations of Ole e 1

A total of 100 μ l aliquots of rabbit polyclonal anti-Ole e 1 antibodies, diluted 1/5000 in 0.2 M, pH 9.2 sodium bicarbonate buffer, was incubated overnight in flat-bottom microtiter plates. Ole e 1 was subsequently labeled with HRP according to the method of Nakane and Kawaoi (1974).

After washing with PBS 0.1% Tween, a competition between a fixed concentration of HRP labeled Ole e 1 (0.5 μ g/ml) and serial concentrations of purified Ole e 1 in a blocking solution containing PBS 0.1% Tween, 25% fetal calf serum (FCS) and 1% heparin was established. The starting Ole e 1 concentration was 125 μ g/ml. Serial dilutions of the

12 lots of *O. europaea* extract, starting at 500 µg/ml, were established to calculate their Ole e 1 content. The Ole e 1 standard and the extracts assayed were analyzed in duplicate. After an incubation period of 1 h at RT, other cycle of washings were done. Finally, the HRP the peroxidase was evaluated by incubation with a mixture of 0.16 M acetate buffer (pH 6; 1 ml), TMB in DMSO (20 µl) and 1 M H₂O₂ (2 µl). The enzymatic reaction was stopped by the addition of 100 µl of 2 N sulfuric acid. Optical densities were finally read at 450 nm in a microplate reader (Titertek Multiskan, Finland).

An extract of *Trisetum paniceum*, *Parietaria judaica* and *Platanus hybrida*, prepared following the same procedure as the *O. europaea* extracts, were used as negative controls. The starting concentration for *P. judaica* was 1000 µg/ml and for *T. paniceum* and *Pl. hybrida*, 500 µg/ml.

2.9. Gel scanning densitometry

The SDS-PAGE gel was analyzed with the SHARP Film Scanning Unit (Model JX-3F6) for the SHARP JX-330 Colour Scanner. Lanes were analyzed using the ImageMaster software from Pharmacia Biotech. Raw volume of the Ole e 1 bands were calculated as an indication of the Ole e 1 content. A linear correlation between the different amounts of Ole e 1 per lane and the raw volume of the bands was obtained (Fig. 1). This curve exhibits an operative range of 5–100 µg of Ole e 1.

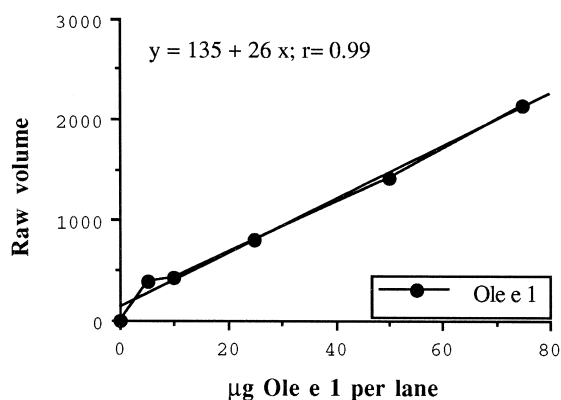


Fig. 1. Curve of the different amounts of purified Ole e 1 per lane in SDS-PAGE and the raw volume of the bands obtained by scanning densitometry.

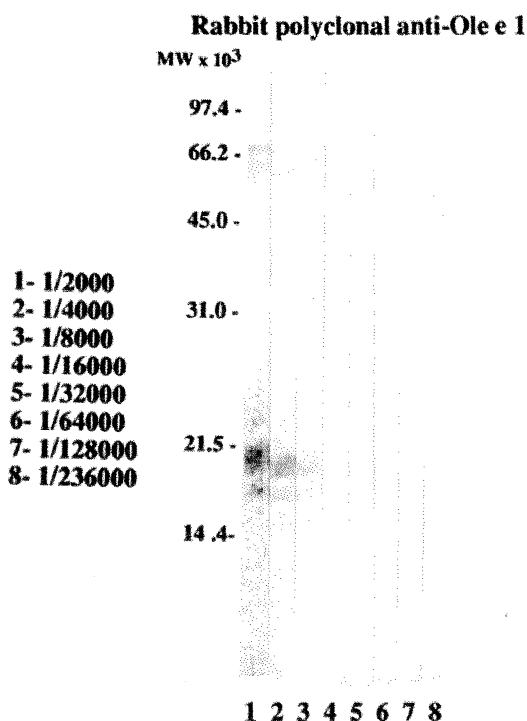


Fig. 2. IgG immunoblotting of the rabbit polyclonal anti-Ole e 1 using a whole *O. europaea* extract (lot no. 8) at different serum dilutions.

2.10. Solid-phase immunoassay for specific IgE and IgE inhibition experiments

Specific IgE binding and inhibition experiments were conducted as previously described (Boluda et al., 1998a,b). In brief, allergenic extracts were coated to the wells of flat-bottom microtiter plates (Immulon 4, Chantilly, USA). Allergen coating was done by dissolving allergenic preparations at 10 µg/ml in sodium bicarbonate buffer (0.2 M, pH 9.2) and incubating 100 µl aliquots per well, overnight, in a humid chamber. The coated wells were blocked with 1% HSA in PBS at RT for 30 min. After three washing steps with PBS 0.1% Tween, serum samples were incubated in this plates for overnight at RT. After other three washing steps, 100 µl of anti-IgE-HRP at 1 µg/ml diluted in blocking solution was then added and left for 2 h at RT. After washing, the peroxidase was evaluated as before. The value obtained at dilution 1/32 was taken as an estimate of relative specific IgE levels.

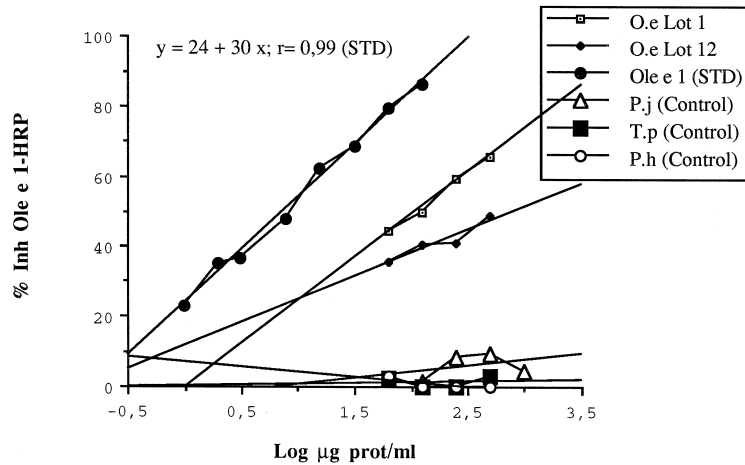


Fig. 3. Inhibition curves of Ole e 1-HRP versus the amount of total protein for the determination of Ole e 1 concentration. Purified Ole e 1 (standard curve), two representative lots of *O. europaea* and *P. judaica*, *T. paniceum*, *Pl. hybrida*, as negative controls, are shown.

Allergenic extracts were tested for allergic potency by EIA inhibition. In brief, 125 µl volumes of serial dilutions in PBS of allergenic extract starting at 100 µg/ml and following a factor 2 were established. Then, it was mixed with 125 µl of serum pool diluted at 1/15. These mixtures were left with shaking for 2 h at RT. After inhibition period, 100 µl of allergen *plus* serum was incubated for overnight in duplicate with a standard extract coated to the plates. Specific IgE bound to the wells was determined as before. Allergenic potencies were evaluated by establishing the point of 50% inhibition

from the regression lines obtained by plotting the percentage inhibition versus allergen protein in test system.

2.11. Statistic analysis

Linearity of the curves was tested by the *F*-test (analysis of variance) with upper and lower 95% confidence limits and linear regression analysis. The coefficient of regression was used to study the relationship among different variables. Calculations were

Table 2

Slope, upper and lower 95% confidence limits, correlation coefficient (*r*) and *F*-test results with the corresponding *p*-value of the Ole e 1 standard and *O. europaea* curves

Lot	Slope	95% Lower	95% Upper	<i>r</i>	<i>F</i> -test	<i>p</i> -value
Ole e 1 STD	29.4	26.0	32.8	0.99	452	0.0001
Lot 1	24.5	16.7	32.2	0.99	183	0.0054
Lot 2	25.7	13.1	38.3	0.98	77.5	0.0127
Lot 3	22.9	18.6	27.3	0.99	512	0.0019
Lot 4	21.4	16.3	26.5	0.99	327	0.0030
Lot 5	14.7	1.13	28.2	0.96	21.7	0.0430
Lot 6	13.9	6.11	21.7	0.98	59.1	0.0165
Lot 7	18.2	−10.9	47.3	0.88	7.23	0.1149
Lot 8	17.2	9.86	24.6	0.99	101	0.0097
Lot 9	15.6	15.3	16.1	1.00	28,829	0.0001
Lot 10	12.6	−1.42	26.6	0.94	14.9	0.0609
Lot 11	12.3	3.48	21.1	0.97	36.1	0.0266
Lot 12	12.1	−0.94	25.1	0.94	15.9	0.0574

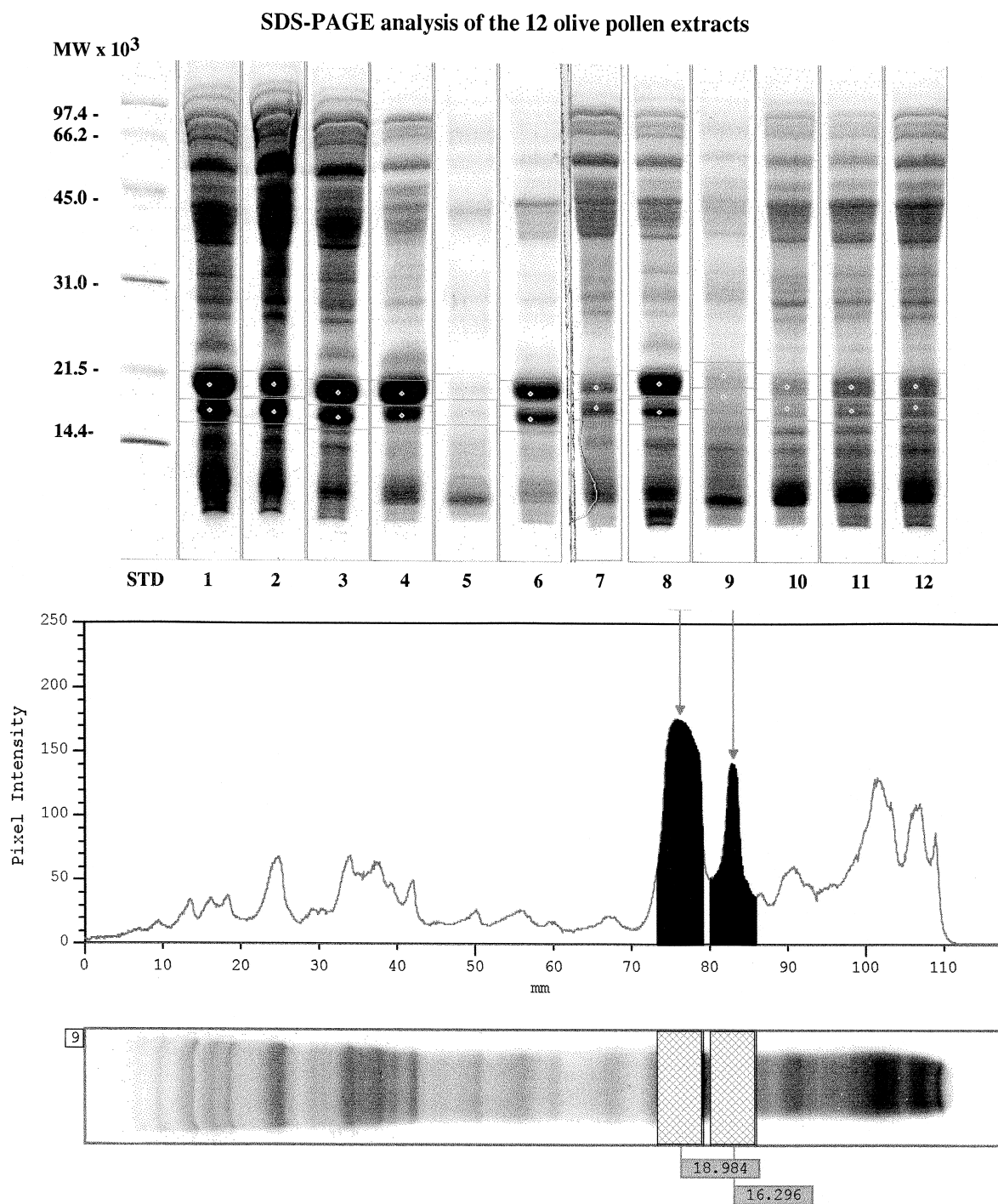


Fig. 4. SDS-PAGE analysis of the 12 *O. europaea* pollen extracts. An example of Ole e 1 bands estimation for the lot 8 is presented below.

performed with the StatView SE + Graphics™ program in a Power PC Macintosh computer.

3. Results

3.1. Specificity of Ole e 1 immunoassay

The rabbit polyclonal anti-Ole e 1 was tested at different serum dilutions by IgG immunoblotting using a whole *O. europaea* extract (Fig. 2). This experiment shows that this antiserum recognizes preferentially the two components of Ole e 1 molecule. Only a light staining in the high molecular weight range at a 1/2000 dilution was detected.

The purified Ole e 1 preparation, obtained by a combination of gel filtration and preparative SDS-PAGE, demonstrated to be homogeneous in analytical SDS-PAGE. Once the specificity of the antiserum and the purity of the Ole e 1 preparation were verified, the immunoassay was developed. Fig. 3 illustrates the Ole e 1-HRP inhibition curve with purified Ole e 1, 2 *O. europaea* batches and *P. judaica*, *T. paniceum*, *Pl. hybrida* as negative controls. The curves of *O. europaea* batches run parallel to the Ole e 1 standard curve whereas *P. judaica*, *T. paniceum* and *Pl. hybrida* extract curves were plane. In all cases, the regression coefficient for the Ole e 1 standard curve and *O. europaea* curves were higher than 0.9, except for lot 7 ($r = 0.88$; Table 2). The

p -values from the F -test ranged from 0.0001 in the standard to 0.11 in lot 7. The confidence intervals for the curves are included in Table 2. Inhibition points from olive pollen extract curves were extrapolated in the standard curve to calculate the Ole e 1 concentration.

3.2. SDS-PAGE, Ole e 1 content and allergenic potency of *O. europaea* batches

The Fig. 4 shows the electrophoresis image analysis of 12 olive pollen lots. This picture illustrates a great batch to batch variation in protein composition using the same amount of protein per lane.

The Ole e 1 content was evaluated in these 12 pollen extracts by two methods: immunoassay for Ole e 1 quantitation described above and scanning of Ole e 1 bands in SDS-PAGE gel. An example of Ole e 1 estimation by band scanning is shown in Fig. 4. The allergenic potency was also assessed in different manners: direct IgE binding, IgE inhibition and skin-prick testing.

The resulting data is presented in Table 3. The Ole e 1 content varied from 1% to 5% of the total protein of the extracts. The variation in Ole e 1 content by gel scanning was greater: ranging from 154 to 3419 raw volume units. These data are in concordance with the variations observed in SDS-PAGE gel.

Table 3

Ole e 1 concentrations, raw volume from gel scanning, direct IgE (OD 450 nm), nanograms of protein needed to produce 50% IgE inhibition and geometric means of the wheal size areas of the 17 patients

Lot	Ole e 1 EIA (ng/ μ g*)	Ole e 1 scan (raw volume)	Direct IgE (OD 1/32)	IgE inhibition (50% ng)	Skin test (mm ²)
1	51.4	3419	0.991	12.4	33.2
2	34.2	2753	0.915	11.8	27.7
3	33.9	3204	0.910	25.9	25.1
4	40.3	2753	1.039	13.8	22.5
5	11.0	154	0.527	203	5.9
6	38.3	1895	0.895	26.4	21.5
7	12.9	889	0.848	23.8	26.9
8	31.5	2142	0.938	22.1	22.3
9	14.4	261	0.687	41.0	17.1
10	16.4	476	0.896	22.2	16.5
11	21.9	955	0.838	15.1	13.8
12	23.0	1108	0.842	19.1	21.7

* Nanograms Ole e 1 per micrograms protein.

Table 4

Regression coefficients (r) among Ole e 1 content, the in vitro allergenic potency (direct IgE and IgE inhibition) and the biological activity by skin prick test of the allergenic extracts studied

	$r; p$				
	Ole e 1 EIA	Ole e 1 scan	Direct IgE	IgE inhibition	Skin test
Ole e 1 EIA	1				
Ole e 1 scan	0.92; 0.0001	1			
Direct IgE	0.76; 0.004	0.76; 0.004	1		
IgE inhibition	−0.47; 0.11	−0.47; 0.11	−0.83; 0.0008	1	
Skin test	0.69; 0.01	0.77; 0.003	0.76; 0.004	−0.70; 0.01	1

3.3. Correlation between the different determinations

The correlation between Ole e 1 content and allergenic potencies was analyzed by linear regression (Table 4). The regression coefficient between the Ole e 1 content estimated by the immunoassay and by gel densitometry was significant, $r = 0.92$; $p < 0.001$. The Ole e 1 contents were significantly correlated with the allergenic potencies evaluated by either direct IgE or IgE inhibition or skin-prick test. The regression coefficient between Ole e 1 content by gel scanning densitometry and skin tests ($r = 0.77$; $p < 0.01$) was higher than between Ole e 1 content by the immunoassay method and skin test ($r = 0.69$; $p < 0.05$).

4. Discussion

We describe two new methods to quantify Ole e 1 in olive pollen extracts. The immunoassay for the determination of Ole e 1 uses rabbit polyclonal, monospecific antibodies and is based on the competition between labeled and natural or purified Ole e 1. It has an operative range of 0.03–100 $\mu\text{g}/\text{ml}$. Monoclonal antibody based radioimmunoassays have a published detection range for Ole e 1 of 5 to 200 ng/ml (Lombardero et al., 1992). This newly developed assay shows good specificity and sensitivity. As expected, Ole e 1 was not detected in extracts of the grass *T. paniceum*, the weed *P. judaica* and the tree *Pl. hybrida*.

The gel scanning densitometry method is also a useful method to estimate the Ole e 1 content in olive pollen extracts. This method employs the Im-

ageMaster software, that permits the calculation of the raw volume of stained Ole e 1 bands in SDS-PAGE gels. The raw volume is the sum of the intensities of every pixel in the band and indicates the amount of material contained in such bands. The Ole e 1 estimation by gel scanning has a good correlation with the Ole e 1 content obtained by the immunoassay ($r = 0.92$; $p < 0.001$). This method relies on a previous measurement of the Ole e 1 content in a reference preparation, although arbitrary units could also be used. Once this measurement has been made, successive batches can be measured using a scanning densitometer. A standard curve can also be established as shown in Fig. 1. This method can also be applied (data not shown) to other stable allergens, such as Bet v 1, Par j 1, Amb a 5 and Der p 2, since these allergens are easily detectable in SDS-PAGE gels.

Both methods were used to evaluate the Ole e 1 content in different batches of olive pollen collected in several areas of Spain and the USA. The results demonstrate that Ole e 1 shows great variability, and that the concentration of Ole e 1 correlates well with both, in vitro and in vivo allergenic potencies.

The standardization based on the content of a major allergen, at least in the case of olive pollen, is hampered by the degree of batch to batch variability existing in this pollen. As shown in Table 3, extract 1 has an Ole e 1 content of 51.4 ng/ μg of protein and extract 7 of 12.9 ng/ μg , a concentration 4 times lower. Therefore, the concentration of extract 7 should have been increased 4 times to reach the equivalent concentration of Ole e 1 in extract 1. However, extract 7 produced a wheal size of 26.9 mm^2 , which was only 1.2 times smaller than that of extract 1 (33.2 mm^2). In this particular case, an

artificial adjustment of a major allergen concentration would have led to an unnecessary increase in the total biological potency, according to the skin test results, with a concomitant increase in the concentration of other allergens.

There are two possible explanations for the latter example. First, the recently described intra-species allergenic cross-reactivity between Ole e 1 and Ole e 4 indicates that both allergens share epitopes (Boluda et al., 1998b). These findings suggest that other Ole e 1-like epitopes, carried by other allergens in the extract, might contribute to the total allergenicity. Another possibility is that Ole e 1 is not the only important allergen in olive pollen extracts; Ole e 2 (olive profilin), Ole e 3, Ole e 4, Ole e 5 and probably others, also contribute to the overall allergenic potency of this pollen (Rodríguez et al., 1995; Batanero et al., 1996; Asturias et al., 1997; Boluda et al., 1998a,b; Ledesma et al., 1998). Thus, variations in the allergenic composition may produce situations in which, a low major allergen content does not reflect the overall allergenic potency. This phenomenon has also been observed by Waisel et al. (1996). Therefore, the assessment of the total potency of an extract should not only rely on the measurement of a single allergen; the contribution of other allergens, or cross-reactive epitopes, should always be contemplated.

Similar findings have also been reported for standardized *Dermatophagoides pteronyssinus* allergen extracts in the USA. The Der p 1 content in 6 standardized extracts varied from 12 to 30 µg/ml and Der p 2 from 3 to 15 µg/ml. However, all extracts had a biological standardized potency of 10,000 Allergy Units (AU) per ml (Meyer et al., 1994).

We acknowledge that the determination of Ole e 1 may be an important component of the standardization of olive pollen extracts, but always in combination with other methods such as IgE inhibition, skin-prick testing (Casanovas et al., 1994) and IgG inhibitions (Boluda et al., 1994). Furthermore, the knowledge of the Ole e 1 content allows establishing limits for the selection of raw materials for allergy vaccines (Van Ree, 1997). The specificity of the assays also allows the detection of possible contaminants, such as olive pollen in other extracts. It can also be used as a tool to verify the modification

process in the manufacturing of allergoids or similar products.

In summary, we have reported two new methods that can be used to quantitate Ole e 1 in *O. europaea* extracts. The concentrations of Ole e 1 detected ranged from 1% to 5% of the total protein. Skin test results demonstrated a good correlation between Ole e 1 content and in vivo allergenic activity.

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