

Original article

Rapid release of Ole e 1 from Olive pollen using different solvents

Background: Solubility is an important characteristic of allergenic molecules. The aim of this study was to investigate the solubility of Ole e 1, a major allergen of *Olea europaea*, using different solvents.

Material and methods: *Olea europaea* pollen was placed in a glass column and extracted using three different solvents: deionized water, phosphate buffer 0.01 M (PBS) and normal saline (NaCl 0.9%). Several fractions were collected after extraction with each solvent and pooled based on individual protein content. Each fraction corresponded to a different elution profile, as determined by linear regression analysis. After 130 min of extraction, the pollen that remained in the column was further extracted overnight. A control olive pollen extract was also prepared with each solvent. The antigenic and allergenic profiles of all the eluted and pooled fractions were analysed by SDS-PAGE and immunoblots. Protein and Ole e 1 content and the amount of protein needed to produce 50% inhibition were also calculated. Ten patients were skin prick tested with the fractions obtained with deionized water.

Results: Four elution profiles were obtained using deionized water as the extracting solution and three with the two other solvents. The three solvents produced different kinetics of allergen release. Ole e 1 was rapidly released when water was used, obtaining a total of 256 µg of Ole e 1/ml after only 7 min of extraction (fraction EC1). Using PBS, or NaCl 0.9%, the release of Ole e 1 started after 4 and 9 min of extraction, respectively. The highest amount of Ole e 1 was eluted after 44 and 26 min, with a total concentration of 162 and 203 µg of Ole e 1/ml, respectively. The presence of Ole e 1 in each phase was verified by SDS-PAGE and immunoblot analyses.

Conclusions: The extracting solution seems to determine the antigenic profile of olive pollen extracts. Ole e 1 is rapidly released from the pollen grain after extraction in deionized water. The solubility seems to be affected by the use of other solvents. These techniques could be used to manipulate the Ole e 1 content in *O. europaea* extracts.

**J. Carnés¹, E. Fernández-Caldas¹,
L. Boluda¹, M. Casanovas¹, J. Sastre²,
M. Lluch Bernal², M. Blanca³**

¹C.B.F. LETI, S.A., Research & Development Department, Tres Cantos, Madrid; ²Servicio de Alergia, Fundación Jiménez Díaz, Madrid; ³Servicio de Alergia, Hospital La Paz, Madrid, Spain

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Dr Enrique Fernández-Caldas, PhD
C.B.F. LETI S.A.
Calle del Sol, 5
28760 Tres Cantos
Madrid
Spain

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Several studies have shown that allergens are rapidly released during the extraction process. It has been shown that grass pollen allergens are completely extracted in the first 15 min (1). In 1978 (2), Loewenstein demonstrated that allergens from timothy pollen were eluted after approximately 20 min of extraction in normal saline. Viander (3) showed that more than 70% of the total allergenic activity of birch pollen was detected within the first 30 min of extraction. For short ragweed pollen, it has been shown that Amb a 1 and Amb a 3 are released slowly, whereas Amb a 5 is released rapidly (between 4 and 16 min) (4, 5). The observed differences in the release of the allergens could be caused by several factors, including the localization of the protein in the pollen grain. Differences may occur if the allergens are located in the intine, exine, or inner layers (6). The biochemical properties of the allergens, such as isoelectric points, amino acid composition, solubility, etc. (7), could also determine the speed of release. The physiological

condition of the plant, or the biological function of the allergens could also be responsible to the presence, or absence, in the pollen grain of a certain allergen at a specific moment (8, 9).

A rapid elution, or solubility, is an important prerequisite for a protein to behave as a major allergen (10) and provides an indication for which allergen(s) first comes in contact with the nasal mucosa. Moreover, it has been suggested that the allergenicity of proteins is more linked to their concentration and rapid elution from airborne particles, than to other intrinsic properties (11). The rapid elution of allergens and the fact that rhinitis symptoms are provoked within 30 s after an allergen challenge suggests that allergen release under natural conditions is also fast (12). However, natural pollen exposure cannot be considered equal to a nasal challenge with an allergen extract.

In most publications, the time of extraction used to prepare pollen extracts varies from a few hours to

overnight and, generally, saline solutions, such as PBS, borate buffers, ammonium bicarbonate, normal saline, etc. are used. In this study we have designed different experiments, based on a rapid release method described by Baraniuk et al. (7), to verify the extraction process of Ole e 1, the major allergen of *Olea europaea*, using different solutions and intervals of extraction.

Material and methods

Allergen release by pollen grains of *O. europaea*

Olea europaea pollen was collected in Jaen, Spain with a purity greater than 90%. Ten grams of defatted pollen was packed with fibreglass and introduced into a 20 × 1.6 cm column, following methods previously described, with minor modifications (7). Three different solvents were used: (a) deionized water (b) Phosphate Buffer Saline (PBS), containing NaCl, NaH₂PO₄ and K₂HPO₄, 0.01 M pH 7.4 and (c) NaCl 0.9% (normal saline). The solvents were pumped at a constant flux of 2 mL/min at room temperature and 3 mL fractions were collected. The fractions were pooled based on straight line segments, determined by linear regression analysis of the protein contents. Four pools of fraction, named as elution column (EC) and numbered according to the elution profile were prepared with the eluates obtained with deionized water (EC1, EC2, EC3 and EC4) and three pools (EC1, EC2 and EC3) with those obtained after extraction with PBS and normal saline. All these pooled fractions were dialyzed and lyophilized. After 130 min of column extraction, the pollen remaining in the column was further extracted 1 : 20 weight/volume in a continuous magnetic stirring conditions, in PBS for 16 h at 4°C. These extracts were named ER. Afterwards, these three extracts (ER) were centrifuged, dialyzed against bidistilled water in dialysis membranes with a 3.5-kDa cut-off (Spectra/Por Membrane), sterile filtered, frozen and lyophilized. A conventional extract (CE) was also prepared after extraction for 16 h, in a 1 : 20 weight/volume proportion dialyzed and freeze-dried using the same raw material and the three different solvents.

Total protein content

The protein content of the *O. europaea* pollen extract and fractions was estimated by the Lowry–Biuret method (Sigma Diagnostics, St. Louis, MO, USA) following the manufacturer's instructions. Fractions were pooled based on protein content.

Patient population

Prick test solutions were prepared with EC1, EC2, EC3, EC4, ER and the conventional extract (CE) used as a control, obtained after the extraction with deionized water. All these preparations, extracted with deionized water, were reconstituted at 1 mg of lyophilized/mL just before use.

Ten individuals with specific IgE mediated allergy (13) after the inhalation of *O. europaea* pollen and positive skin tests were included in the study. All patients were skin prick tested, in duplicate, in the forearm and the wheals were measured after 15 min. A serum sample was collected and stored at –20°C. A serum pool was prepared with the individual sera.

SDS-PAGE

SDS-PAGE analysis was used to determine the antigenic profile of the standardized *O. europaea* extract and the pools of each elution. The samples were run in SDS-PAGE gels with 2.67% C, 15% T

acrylamide as described by Laemmli (14). The samples were denatured at 100°C for 10 min and centrifuged for 1 min at 12 000 g. Reference markers with known molecular weights (Bio-Rad Laboratories, USA) were run in the same gel. After electrophoresis, the gel was stained with Coomassie Brilliant Blue R-250 (Bio-Rad Laboratories, Hercules, CA, USA).

Immunoblot

The separated proteins were electrophoretically transferred to P-Immobilon membranes (PVDF) (Millipore, Bedford, USA). After transfer, the membranes were dried at room temperature for 4 h. The membranes were incubated overnight with the serum pool in PBS 0.01 M Tween 2%. After washing with PBS 0.01 M and Tween 20, the specific IgE binding was detected with peroxidase-conjugated monoclonal antihuman IgE (Ingenasa, Madrid, Spain) for 2 h. The peroxidase was developed by adding a solution of 0.1 M citrate buffer (pH 5) containing 10 mM EDTA and 1% dextran sulphate (1 ml), TMB in DMSO (20 µl) and 1 M H₂O₂ (2 µl).

ELISA inhibition

The control *O. europaea* extracts and the pools were tested by ELISA inhibition to evaluate their allergenic potency *in vitro*. Plastic microtitre plates (Immulon IV, Dynex Technologies, Chantilly, USA) were coated (1 µg of protein/well) with the respective *O. europaea* conventional extract. A standard curve was prepared using several dilutions of the extracts. Each dilution was incubated with the serum pool for 2 h at room temperature. A positive and negative control were also included. Afterwards, the dilutions were transferred to the *O. europaea* coated plates and incubated overnight. After washing, 100 µL of antihuman IgE horseradish peroxidase labelled were added and let to stand for 30 min at room temperature. After washing, the plates were developed for 30 min and stopped with H₂SO₄ 1 N. Allergic activity was evaluated by establishing the 50% of inhibition using as control the pool of serum not inhibited and PBS as negative control.

Determinations of Ole e 1

Rabbit polyclonal anti-Ole e 1 antibodies, obtained after immunizing two rabbits with purified Ole e 1 following methods previously described (15), diluted 1/5000 in 0.2 M, pH 9.2 sodium bicarbonate buffer, were incubated overnight in flat-bottom microtitre plates (Immulon IV, Dynex Technologies, Chantilly, USA). Ole e 1 was subsequently labelled with horseradish peroxidase according to the method of Nakane and Kawaoi (16). After washing with PBS 0.1% Tween, a competition between a fixed concentration of HRP-labelled Ole e 1 (0.5 µg/mL) and serial concentrations of purified Ole e 1 in blocking solution was established. The starting Ole e 1 concentration was 125 µg/mL. Serial dilutions of fractions, starting at 500 µg/mL, were established to calculate their Ole e 1 content. After an incubation period of 1 h at room temperature, another cycle of washings was done. Finally, the HRP the peroxidase was evaluated as before.

Statistical analysis

A nonparametric statistic test (Kruskal–Wallis) was used to compare the mean wheal sizes obtained. Significant differences between the results were analysed using the Bonferroni test for multiple comparisons. The regression lines of the IgE inhibition curves and of the protein content in each fraction to establish linearity were assessed by regression analysis. The study was analysed using SPSS v.10 software.

Results

Kinetic of protein release

Graphs of protein concentration were plotted against elution time to establish the patterns of solute elution in the extraction with the three solvents. Four fractions were established according to the linearity of the protein extraction curve using deionized water. These phases were: EC1, from time 0–7 min; EC2, from 7 to 17; EC3, from 17 to 29; EC4 from 29 to 130; and ER after 16 h of extraction with continuous magnetic stirring. Using PBS 0.01 M the intervals were: EC1, from time 0–9 min; EC2, from 9 to 44; EC3, from 44 to 130; and ER after 16 h of extraction with continuous magnetic stirring. Finally, using normal saline as solvent, the intervals were: EC1, from time 0–4 min; EC2, from 4 to 26; EC3, from 26 to 130; and ER after 16 h of extraction with continuous magnetic stirring. The model of each solvent is shown in Fig. 1. No significant differences were obtained in the total protein content of the fractions.

Release of Ole e 1

The Ole e 1 content was established in each one of the conventional extracts (CE) prepared with each solvent. The results were: 24.8, 78.7 and 117.31 μg of Ole e 1/mg of freeze-dried material using deionized water, PBS and normal saline, respectively. The kinetic of Ole e 1 release was evaluated by determining the concentration of Ole e 1 in all the fractions. Ole e 1 was released faster in the extraction with deionized water (256 μg Ole e 1/ml in EC1) than in the extraction with PBS and normal saline (6.88 and 33 μg Ole e 1/mL in EC1). However, using PBS 0.01 M and normal saline as solvents, the highest concentrations of Ole e 1 were obtained at the end of the process (EC3 fractions) with 162 and 203 μg Ole e 1/ml, respectively. In the latest fractions, when water was used as solvent, 24.6 and 17.6 μg Ole e 1/ml were obtained in EC3 and EC4, respectively (Fig. 2). However, the total amount of Ole e 1 released after 130 min of extraction per gram of pollen was lower with deionized water (1.14 mg) than with PBS and normal saline (3.18 mg and 4.21 mg, respectively).

Skin prick test

The geometric mean of the wheal sizes induced by the skin prick test were: *O. europaea* control extract (CE): 45.16 mm^2 (69–7); EC1 26.96 mm^2 (97–6), EC2 12.59 mm^2 (65–7), EC3 25.07 mm^2 (117–3), EC4 11.52 mm^2 (72–5), and ER 13.37 mm^2 (45–6). The sizes of the wheals were analysed by the nonparametric Kruskal-Wallis test, confirming significant differences between the wheal sizes induced by the different extracts ($P = 0.012$). The Bonferroni test for multiple comparisons also confirmed significant differences between the control extract with pools EC2, EC4 and ER.

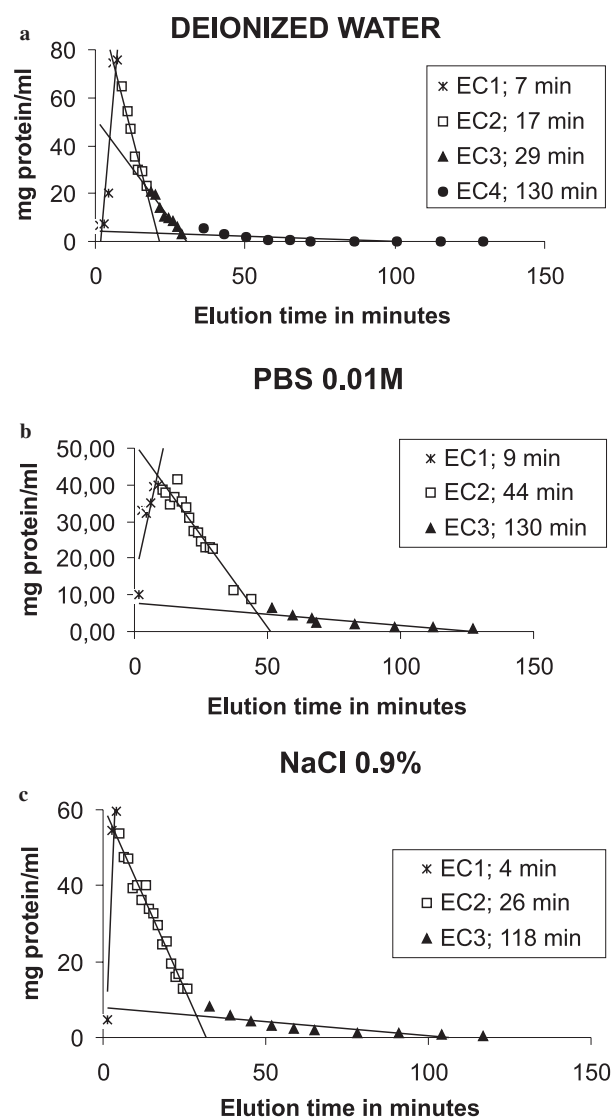


Figure 1. Kinetic of protein release by *Olea europaea* pollen using different solvents.

SDS-PAGE and immunoblots

SDS-PAGE and immunoblots of the extraction with different solvents are shown in Figs 3 and 4. In Fig. 3 (A), lane 1 corresponds to a conventional extract with deionized water, and lanes 2–5 to the fractions eluted at 7, 17, 29 and 130 min, respectively. As shown in Fig. 3 (A), Ole e 1 appears in the fraction eluted at 7 min and is barely visible in the rest of the fractions. However, in the extractions with PBS and normal saline, Ole e 1 appears with more intensity in ensuing fractions (Fig. 3B,C). The SDS-PAGE and immunoblots (Fig. 4) showed similar results to those obtained with the immunoassays.

Allergen potency by IgE inhibition

All fractions obtained were analysed by ELISA inhibition to determine the 50% inhibition point. All the curves had

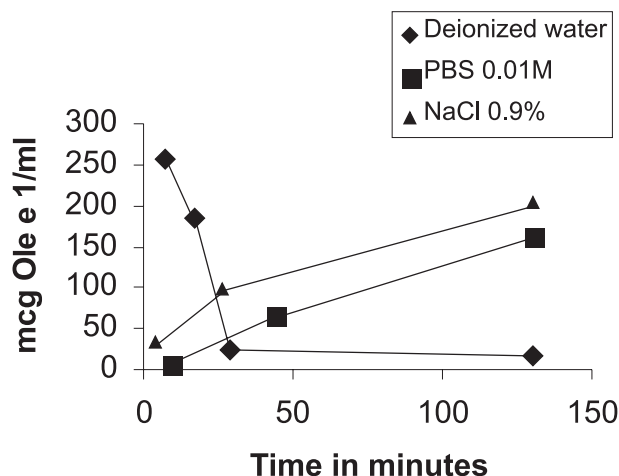


Figure 2. μg of Ole e 1 eluted per mL in each fraction.

a correlation coefficient (r -value) greater than 0.95. The amount of freeze-dried material needed to achieve 50% inhibition was calculated for each fraction; 0.59 and 2.05 μg were needed for the EC1 fractions when PBS and normal saline were used as solvent, respectively. However, only 0.15 μg were needed with deionized water. In the next fractions the amount needed to obtain 50% inhibition were lower; 0.17 (EC2) and 0.12 (EC3) with PBS, or 0.58 (EC2) and 0.47 (EC3) with saline. However, these values increased when deionized water was used with a 50% inhibition point of 0.74 (EC2), 0.36 (EC3) and 0.20 (EC4). Curves are represented on Fig. 5. The test of parallelism showed that the fractions with a greater protein content had a better correlation with their respective conventional extracts.

Discussion

Pollen grains produced by anemophilous flowers are dispersed into the air and can get in contact with the human nasal mucosa, causing an immediate release of allergens. This high local concentration of allergen sparks an immediate adverse response, producing symptoms and mediator release in allergic individuals (17, 18). The study of allergen release by pollen grains provides an indication on the clinical relevance of certain molecules. Ole e 1 is an allergen composed of two subunits, one glycosylated (20 kDa) and another non-glycosylated (18.5 kDa) and is a good model to study, because of its stability and solubility, its high frequency of recognition by human IgE and because it makes up a great proportion of the total protein in olive pollen.

We divided the extraction process into several phases according to the kinetic of protein release and designed the intervals of extraction following the linearity of the protein contents. When this linearity changed, as indicated by a significant decrease in the linearity coefficient, we established another fraction. The three solvents produced

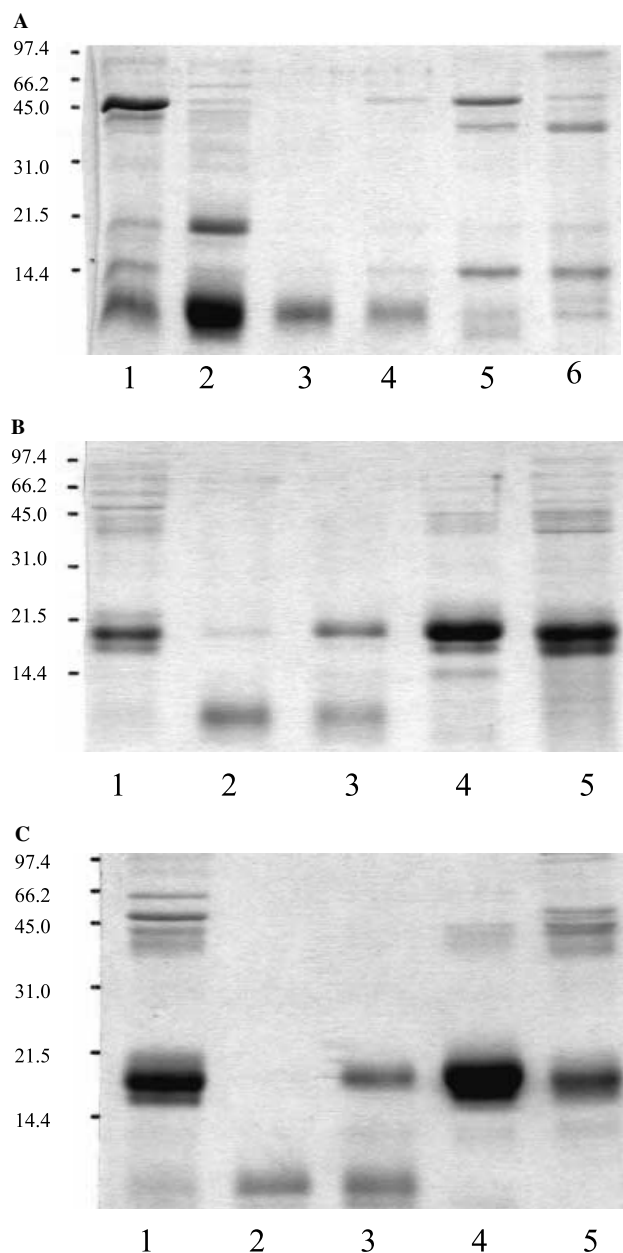


Figure 3. SDS-PAGE. (A) Elution with deionized water: Lane 1, *O. europaea* conventional extract (CE); Lane 2, EC1; Lane 3, EC2; Lane 4, EC3; Lane 5, EC4; Lane 6, ER. (B) Elution with PBS 0.01 M: Lane 1, *O. europaea* conventional extract (CE); Lane 2, EC1; Lane 3, EC2; Lane 4, EC3; Lane 5, ER. (C) Elution with NaCl 0.9%: Lane 1, *O. europaea* conventional extract (CE); Lane 2, EC1; Lane 3, EC2; Lane 4, EC3; Lane 5, ER.

different intervals, or kinetics of allergen release. Several important differences could be observed with the three solvents. Ole e 1 is rapidly released when water is used after only 7 min of extraction. A total concentration of 3.6 mg of Ole e 1 was obtained in EC1. However, using PBS 0.01 M or normal saline, the release of Ole e 1 started after 4 and 9 min of extraction, respectively. However, the highest concentration eluted after 44 and 26 min, with

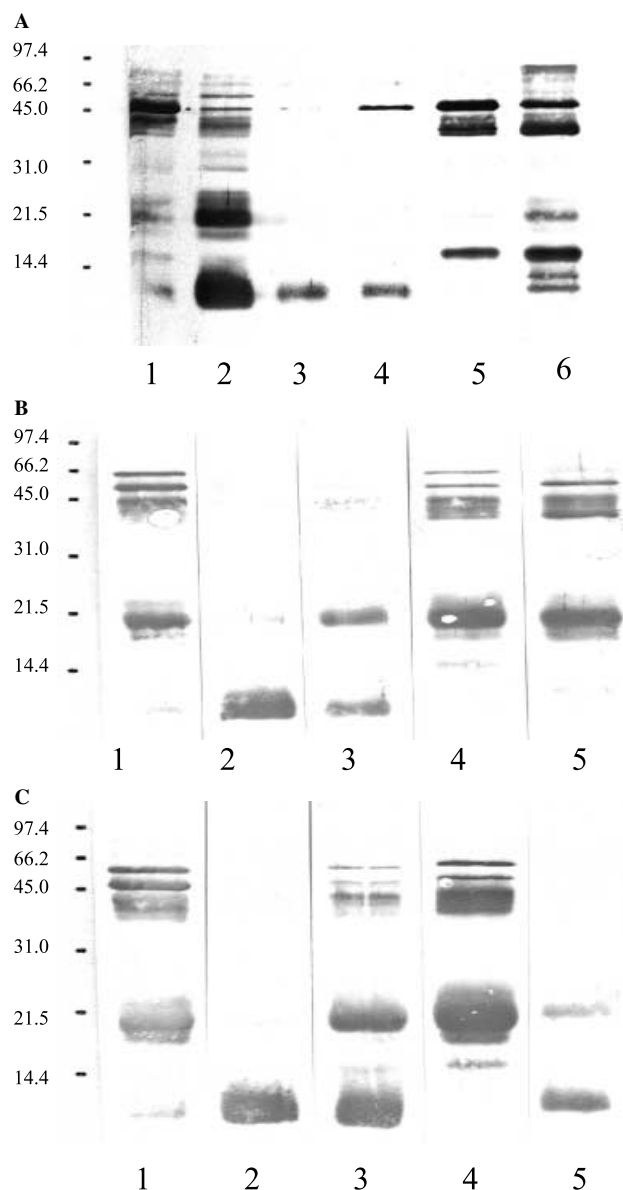


Figure 4. Immunoblot experiments. (A) Elution with deionized water: Lane 1, *O. europaea* conventional extract (CE); Lane 2, EC1; Lane 3, EC2; Lane 4, EC3; Lane 5, EC4; Lane 6, ER. (B) Elution with PBS 0.01 M: Lane 1, *O. europaea* conventional extract (CE); Lane 2, EC1; Lane 3, EC2; Lane 4, EC3; Lane 5, ER. (C) Elution with NaCl 0.9%: Lane 1, *O. europaea* conventional extract (CE); Lane 2, EC1; Lane 3, EC2; Lane 4, EC3; Lane 5 ER.

a total concentration of 27 and 37.4 mg of Ole e 1, respectively. The results of Ole e 1 content in each phase are consistent with those obtained with SDS-PAGE and immunoblot analyses.

We observed differences in the yield of the different extracts. Extractions with PBS 0.01 M or normal saline are more efficient and, in both cases, the final concentration of Ole e 1 was higher. Deionized water eluted Ole e 1 very rapidly, but was less effective in the final yield. Using PBS 0.01 M, the pollen started eluting Ole e 1 after 9 min;

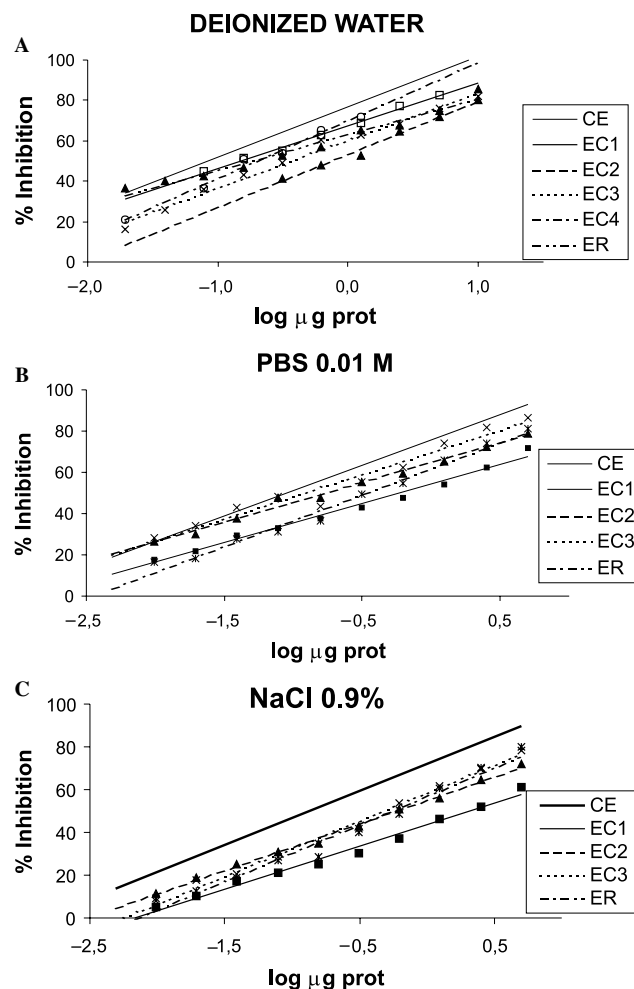


Figure 5. ELISA inhibition experiments using different solvents. The fractions obtained with each solvent were evaluated against their corresponding whole *O. europaea* conventional extract. (A) Solid phase: *O. europaea* extracted with deionized water. (B) Solid phase: *O. europaea* extracted with PBS. (C) Solid phase: *O. europaea* extracted with normal saline.

large quantities were obtained after between 44 and 130 min of column extraction. Using normal saline, the elution of Ole e 1 took place between minutes 4 and 130, peaking between minutes 26 and 130. Ole e 1 was also further extracted from ER preparations. Total amounts of 1.8, 28.9 and 8.3 mg of Ole e 1 were obtained from the ER extracts previously extracted with deionized water, PBS 0.01 M and normal saline, respectively.

Normal saline is the best extracting solution in a rapid release system, extracting the highest amount of the allergen in the early phases. On the other hand, deionized water is the worst solvent for total yield, although Ole e 1 was extracted rapidly. Overall, before the final extraction (ER), a total of 11.46 mg, 31.8 mg and 42.09 mg of Ole e 1 were obtained after extraction with deionized water, PBS 0.01 M and normal saline, respectively.

We have studied the kinetic of Ole e 1 because of the availability of an immunoassay. However, nine other

allergens have been described. It seems that the kinetic of the rest of the allergens is not influenced by the different solvents (see Figs 3 and 4). Several bands with low molecular weights, between 9 and 12 kDa are released in the first fractions using the three solvents. These allergens could correspond to Ole e 3, Ole e 6 and Ole e 7. Allergens with higher molecular weights are released in the last fractions and could represent Ole e 4 (32–36 kDa) and Ole e 9 (46.4 kDa). Allergens with a molecular weight of approximately 15 kDa are released in the last phases. These allergens could be Ole e 2 and Ole e 5. This pattern of allergen elution suggests the different degrees of solubility of the allergens in olive pollen and may be related to their different degrees of glycosylation. It seems that Ole e 1 is more affected by the nature of the solvent than the rest of allergens. Probably, this may be due to the presence of a glycosylated and a non-glycosylated chains in the Ole e 1 molecule. The presence of carbohydrate moieties on allergenic proteins clearly affects the solubility of the allergens (19).

In the presence of deionized water, an osmotic gradient between the interior of the grain and the medium is created. The pollen grain will capture water and eventually rupture, releasing allergens into the medium. However, the presence of certain salts, such as PBS or normal saline, equilibrates the medium, delaying the bursting of the grains because of a slower entrance of water. However, it seems that extraction with salts is more efficient and enduring. This would explain why deionized water induces a rapid release of Ole e 1, although it is less effective than salt-containing solvents, which may also confer a protective effect to the allergens.

We also prepared conventional extracts using the three different solvents and observed that the antigenic and allergenic profiles changed, based on the solvent used (Figs 3 and 4), mainly when water was used (Fig. 3, lane 1). This fact should be considered when manufacturing extracts for commercial purposes, or analysing the allergenic composition of pollen extracts.

The results obtained in the inhibition studies confirm the results obtained by SDS-PAGE. When water was used as solvent, EC1 was the fraction that needed less protein to achieve 50% inhibition, since the main allergens were released in the early phase. When PBS 0.01 M or normal saline were used, the early phases needed more protein than the latest phases to induce 50% inhibition. These results are more conclusive evaluating the parallelism of the inhibition curves by the *t*-test. Fractions with low protein contents and different

antigenic profiles needed a greater concentration to produce 50% of inhibition and failed the parallelism test. However the fractions with a higher protein content showed parallel curves.

Several studies have shown that the response of the nasal mucosa in contact with pollen is very quick. Therefore, the allergen has to be released within seconds of being in contact with the nasal mucosa and secretions (20). In this study we have tried to reproduce this effects by forcing different solvents to pass through a sample of *O. europaea* pollen. Using normal saline as solvent, we obtained the highest release of Ole e 1. Since normal saline is very similar to nasal secretions, the release pattern detected in this study may mimic natural conditions, although some important mediators and enzymes are not present in the elution process. The extraction of the pollen in the nasal mucosa could be even more efficient because of the presence of enzymes, such as lysozyme.

The structures which surround the pollen (intine and exine) contain numerous proteins, but these structures vary between plant species. Grass pollen has thinner walls than birch pollen grains and therefore the release is different (21). Suphioglu et al. (22) suggested that Lol p 9 is located in intracellular starch granules in the inside of the pollen grains and that when the pollen grain explodes, a great number of these starch granules containing the allergens are released. Recently Grote et al. (23) reported that grasses release allergen-bearing subcellular particles responsible for asthma attacks. The rapid release pattern of Ole e 1 is in contrast with the release of another important allergen such as Amb a 1, which is released later in the process (4, 5).

In conclusion, the extracting solution determines the antigenic and allergenic profile of olive pollen extracts, since allergens do not seem to be equally soluble in different solvents. Ole e 1 is rapidly released when extracted with deionized water. However, extraction with PBS and normal saline is more effective, but depends on the time of extraction. The total amount of Ole e 1 recovered varies and depends from the solvents used. The combination of solvent and extraction times allows the preparation of olive pollen extracts with a fixed range of allergen content. Ole e 1 depleted extracts can also be prepared using this technique. This methodology allows the purification of allergens according to their solubility and physicochemical properties. This information could be used to control the allergen composition of allergenic extracts used for diagnosis and immunotherapy.

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