

A two-site monoclonal antibody ELISA for the quantification of group V allergens in grass extracts

B. FAHLBUSCH, G. SCHLENVOIGT, W.-D. MÜLLER, B. WEBER* and L. JÄGER

Department of Clinical Immunology, The University of Jena and *Allergopharma Joachim Ganzer KG, Reinbek, Germany

Summary

A two-site solid-phase enzymimmunoassay was used for the quantification of group V allergen in grass pollen extracts in mass units. The assay is based on the monoclonal antibodies (MoAbs), 1D11 and 3B2 which recognize different epitopes on the standard *Phl p V*. The MoAb-ELISA is very sensitive (15–100 ng/ml *Phl p V*) and highly specific for group V allergens. Six pollen extracts of different grasses demonstrated parallel binding curves. The group V content ranged between 73 and 673 µg/ml in the extracts. The International Standard of *Phleum pratense* (IS 82/520) contains 400 µg/ml *Phl p V*. A good correlation was observed between the group V content and RAST inhibition, with the exception of *Poa pratensis*.

Clinical and Experimental Allergy, Vol. 24, pp. 752–757. Submitted 21 May 1993; revised 15 November 1993; accepted 24 March 1994.

Introduction

In an age in which quality control of diagnostic and therapeutic agents is considered mandatory, the standardization of allergen preparations for the diagnosis and therapy of allergic disease is a priority. Extracts of grass pollen used for diagnostic and immunotherapeutic purposes are complex mixtures of proteins. Only some of these proteins are allergenically active. The predominate allergens in grasses are group I and V with a sensitivity index of > 90% [1,2] and few less important groups such as group II–IV. At the present time extracts are compared with the First International Standard for timothy grass (*Phleum pratense*) pollen extract (IS 82/520) established in 1984 [3,4]. This preparation was examined by radioallergosorbent test (RAST) inhibition, quantitative immunoelectrophoresis (CIE/CRIE and rocket immunoelectrophoresis), isoelectric focusing, histamine release and other methods. All these techniques involve the use of either serum or basophils from allergic patients or polyclonal antisera and are difficult to standardize. The exquisite specificity of monoclonal antibodies (MoAb) makes them ideal tools for analysis and quantification of allergen extracts.

Recently, we have produced MoAbs reacting specifically with group V allergens, which are highly cross-reactive between different grass species [5]. These MoAbs recognize different epitopes on group V allergens and have been used to develop an enzyme immunoassay (ELISA) [6]. This assay is highly sensitive, easy to perform, fast and suitable for testing large numbers of samples. In the present study we quantified group V allergens in mass units (µg/ml) in different grass extracts. The results show that the assay is specific and sensitive. The correlation with allergenic activity, as determined by RAST inhibition, is discussed.

Materials and methods

Extracts

The following lyophilized pollen extracts were obtained from Allergopharma, Reinbek, Germany: *Dactylis glomerata*, *Festuca pratensis*, *Holcus lanatus*, *Poa pratensis*, *Lolium perenne*, *Phleum pratense* and a 6-grass mixture prepared by extraction of a mixture of the six pollens in equal portions. The International Standard 82/520 was obtained from the National Institute for Biological Standards and Control (Potters Bar, Hertfordshire UK). For the investigations, the

Correspondence: Dr B. Fahlbusch, Institute of Clinical Immunology, Friedrich-Schiller-University, Humboldtstr. 3, 0-6900 JENA, Germany

lyophilized extracts were dissolved in 1 ml of double distilled water according to the recommendations.

Extracts were prepared as described previously [6]. Pollen grains were obtained from Sächsisches Serumwerk GmbH, Dresden, Germany. The protein content of extracts was measured by a modified Bradford method on microtitre plates as described by Vieths [7].

Purification of grass pollen allergens

Phl p V was isolated by affinity chromatography on MoAb 1D11 coupled Sepharose 4B-CL and characterized as described earlier [8]. *Phl p V* depleted *Phleum pratense* extract was prepared by passing the extract through the MoAb immunosorbent column. *Lol p I* was a gift from Dr Ansari, Baltimore, USA, and *Phl p I* a gift from Dr van Ree, Amsterdam, Netherlands. *Phl p IV* was prepared as described previously [5].

Two-site binding assay

Quantification of *Phl p V* was achieved by a two-site solid-phase ELISA. The purification and labeling of MoAbs have been described previously [6]. Briefly, microtitre plates were coated with 50 µl/well of purified MoAb 1D11 at 10 µg/ml in 20 mM sodium carbonate buffer pH 9.6 overnight at +4°C. All subsequent incubations were performed at room temperature for 2 h. After blocking with phosphate buffered saline, containing 0.5% v/v Tween-20, (TPBS) followed by a further two washes, 50 µl of *Phl p V* standard solution (concentration range 0.025–25.000 ng/ml) or dilutions of grass extracts (in most cases doubling dilutions) were added to the wells. The plates were washed three times and incubated with 50 µl of biotinylated MoAb 3B2 (dilution 10⁻³) for 2 h. After a further three washes, 50 µl of streptavidin-peroxidase was added. The ELISA was developed with *o*-phenylene diamine as substrate.

RAST-inhibition

The total allergenic activity of samples was determined by RAST inhibition [9,10] with allergen paper discs prepared with *Phleum pratense* extract [11], a serum pool prepared with sera from grass pollen allergic patients (*n* = 12) and *Phleum pratense* extract as reference. Bound IgE was detected by an ¹²⁵Iodine-labeled anti-human-IgE-antibody (Melja, Kassel, Germany). Relative potency (*p*_{rel}) was calculated on the basis of the 50% inhibition values and the slope of the regression curve according to the method described by US Food and Drug Administration, Bureau of Biologics [12].

SDS-PAGE and immunoblot

Extracts were applied to a discontinuous acrylamide gel according to Laemmli [13] (5% acrylamide stacking gel and 13% acrylamide separation gel) under reducing conditions in a vertical slab gel apparatus (PHASE, Lübeck, Germany). Following separation the proteins were transferred by electroblot (Pegasus semi-dry blot, PHASE, Lübeck, Germany; 0.8 mA/cm², 1 h, buffers: anodic I 30 mM Tris, 20% methanol; anodic II 300 mM Tris, 20% methanol; cathodic: 25 mM Tris, 40 mM ϵ -amino-capronic acid, 20% methanol) onto nitrocellulose-membrane (BA 83, 0.2 µm, Schleicher & Schüll, Dassel, Germany).

Membranes were washed with Tris-buffered saline with Tween-20 as blocking reagent. After incubation with human serum pool overnight at room temperature, membranes were washed, and bound IgE was detected by ¹²⁵Iodine-labeled anti-human-IgE-MoAb (Melja, Kassel, Germany; 5 h room temperature). Membranes were washed, dried and exposed to a Kodak-X-O-mat film, for 3 and then 7 days at -20°C.

Results

For quantification of group V allergen two specific MoAbs were used [5]. The purified *Phl p V* was bound to a MoAb coupled to a microtitre plate, and a second biotinylated MoAb, binding to a different, non-overlapping epitope was used to detect the bound allergen. The epitope mapping of MoAbs has been described previously [6]. The *Phl p V* assay was routinely carried out with MoAb 1D11 on the solid phase and biotinylated MoAb 3B2 as second antibody. Using affinity purified *Phl p V* as standard, it was possible to quantify the *Phl p V* content in the range from 15 to 100 ng/ml (Fig. 1) with an intra-assay coefficient of variation of approximately 10% in the middle zone of the curve. The binding curves for whole *Phleum pratense* extract (in-house extract, HE) and *Phl p* International Standard (IS 82/520) are also shown in Fig. 1. The binding curves were parallel to that obtained with the purified *Phl p V* preparation, suggesting that the antigenicity of the allergen had not been altered during the purification process [8], and that the assay was measuring the same molecule in different preparations. In subsequent investigations the in-house extract was defined as a in-house standard preparation. The specificity of the assay is shown in Table 1. There was no significant binding of labelled MoAb 3B2 to wells containing tree or weed pollen extracts known to lack group V allergen [14]. No crossreactive binding activity was

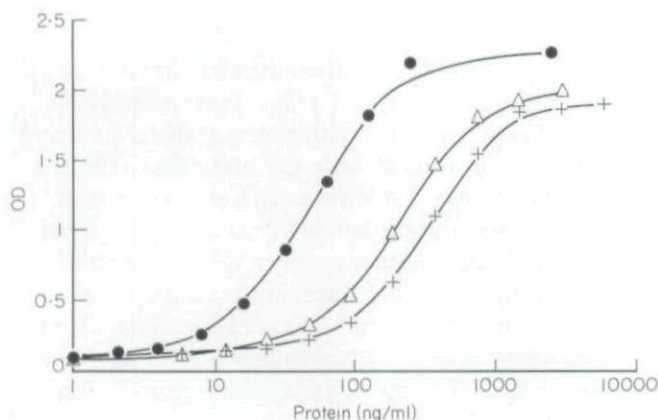


Fig. 1. Dose response curves in two-site MoAb ELISA for different *Phleum pratense* extracts and purified *Phl p V*. ●, affinity-purified *Phl p V*. △, in-house extract of *Phleum pratense*. +, International Standard IS 82/520 of timothy.

found with non-*Gramineae* pollen extracts. Furthermore, we investigated possible interactions with other major allergens contained in grass extracts. The results indicated that *Lol p I* or *Phl p I* and *Phl p IV* or *Phl p V*-depleted extracts do not interfere in the group V ELISA (Fig. 2).

The group V concentration was measured, using the MoAb ELISA, in several grass extracts (Table 2). The binding curves in the group V ELISA for different grass extracts were parallel (Fig. 3) and showed a shift on the x-axis in agreement with the different group V contents. The group V content ranged between 73 and 673 µg/ml, with the extracts from *Poa pratensis* and *Holcus lanatus* having the lowest concentrations (Table 2). The allergenic activity of these preparations was determined by RAST inhibition (Table 2). There was a significant correlation between the group V allergen content and the relative potencies of the various extracts (Spearman rank correlation $r_s = 0.893$, $P < 0.01$).

Although a good correlation exists between relative potency and group V content, these methods, either

Table 1. Specificity of the two-site ELISA for group V quantification

Sample	OD _{sample} - OD _{blank}	Group V content (µg/mg)
<i>Malus</i> (50 µg/ml)	0.18	< 1
<i>Betula verrucosa</i> (100 µg/ml)	0.17	< 1
<i>Alnus glutinosa</i> (50 µg/ml)	0.17	< 1
<i>Ulmus</i> spp (60 µg/ml)	0.17	< 1
<i>Acer negundo</i> (50 µg/ml)	0.5	< 1
<i>Salix alba</i> (100 µg/ml)	0.1	< 1
<i>Artemisia vulgaris</i> (6 µg/ml)	0.17	< 1
<i>Apium graveolens</i> (2.5 µg/ml)	0.2	< 1
<i>Rumex triangulivalvis</i> (50 µg/ml)	0.2	< 1
<i>Solidago canadensis</i> (250 µg/ml)	0.03	< 1
<i>Plantago lanceolata</i> (30 µg/ml)	0.05	< 1
<i>Carex</i> spp (35 µg/ml)	0.19	< 1
<i>Phragmites communis</i> (40 µg/ml)	0.97	5
<i>Bromus</i> spp (3.5 µg/ml)	1.3	25
<i>Molina coerulea</i> (40 µg/ml)	0.6	10
<i>Phleum pratense</i> (250 ng/ml)	1.65	210
<i>Lolium perenne</i> (250 ng/ml)	1.8	240
<i>Dactylis glomerata</i> (250 ng/ml)	1.5	165
<i>Festuca pratensis</i> (250 ng/ml)	1.6	230
<i>Holcus lanatus</i> (250 ng/ml)	0.87	70
IS (82/520) (750 ng/ml)	1.55	67

PVC wells were coated with MoAb 1D11 and then incubated with different concentrations of the samples indicated. After washing the wells were incubated with biotinylated MoAb 3B2 and streptavidin-POD as indicated in Materials and methods section.

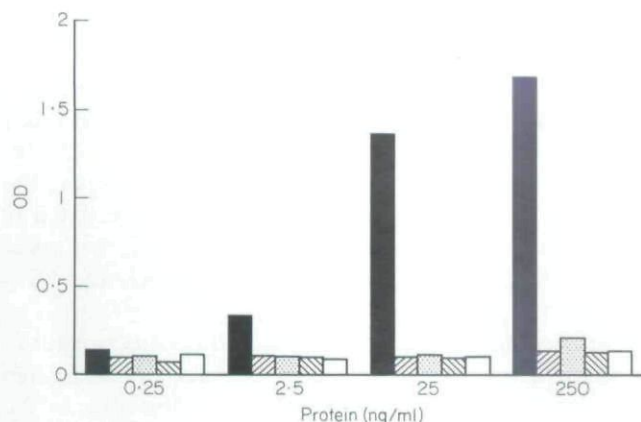


Fig. 2. Specificity of the ELISA for *Phl p V* quantification. PVC wells were coated with the MoAb 1D11 and then incubated with four different concentrations of the purified allergens and *Phl p V* depleted *Phleum pratense* extract. ■. *Phl p V*. ▨. *Phl p IV*. ▩. *Phl p I*. ▤. *Lol p I*. □. *Phl p V* depleted.

together or alone, may not be a sufficient basis for standardizing extracts. In order to obtain data to support this view we performed immunoblotting using equal amounts (25 µg) of protein and the same serum pool as in the RAST inhibition experiments (Fig. 4), including purified group I and group V allergen (4 µg each). Our results indicate, there are substantial differences in major allergen content, suggesting that different factors in various grass extracts may influence the relative potency measured by RAST inhibition. *Phl p V* depletion of *Phleum pratense* extract resulted in a three-fold reduction in the RAST inhibition capacity confirming the importance of *Phl p V* as a major allergen, but

also pointing to the contribution of other allergens (results not shown).

Discussion

At the present time, RAST inhibition is widely used to evaluate allergen extracts and compare them with international and national standards, and in-house reference preparations. However, the main drawbacks with RAST inhibition assays are that the results depend on the extracts used on the solid phase and that the serum pools depend on the spectrum of sensitivities exhibited by the donors, and consequently it is difficult to reproduce the assays between laboratories. Therefore, the expert committee of WHO has recently gone further in recommending that in future standards should be based on *in vitro* measurements of specific proteins using absolute units (µg/ml) [15]. Measurements of major allergens in mass units have previously been described for ragweed extracts (*Amb a I*) [16], cat extracts (*Fel d I*) [17], extracts of house dust mites (*Der p I* and *Der f I*) [18], and *Olea europaea* extract (*Ole e I*) [19]. Essential requirements are that the protein being measured has been purified and demonstrated to be an important allergen. Group V allergens are major allergens in grass pollen extracts with a sensitivity index of > 90% [1] and are highly crossreactive between different grass species [5]. In the present study we used two MoAbs (1D11 and 3B2) to develop a two-site binding ELISA to detect *Phl p V* in different *Phleum pratense* extracts (Fig. 1). The IS 82/520 preparation of timothy grass was used to compare an in-house extract. The absolute content of *Phl p V* was found to be in the range 350–400 µg/ml for the IS and the in-house extract.

Table 2. Comparison of group V content in different grass extracts and RAST inhibition

Extract	Total protein concentration (mg/ml)	Group V content (µg/ml)	Relative potency
<i>Dactylis glomerata</i>	1.99	324	1.26
<i>Festuca pratensis</i>	1.48	340	1.53
<i>Holcus lanatus</i>	1.04	73	0.32
<i>Poa pratensis</i>	1.59	79	0.77
<i>Lolium perenne</i>	2.84	673	4.39
<i>Phleum pratense</i>	1.63	344	1.00
IS (82/520)	6.0	400	1.67

Relative potency is calculated from 50% inhibition values of allergenic extract with respect to the reference extract (*Phleum pratense*) [11,12].

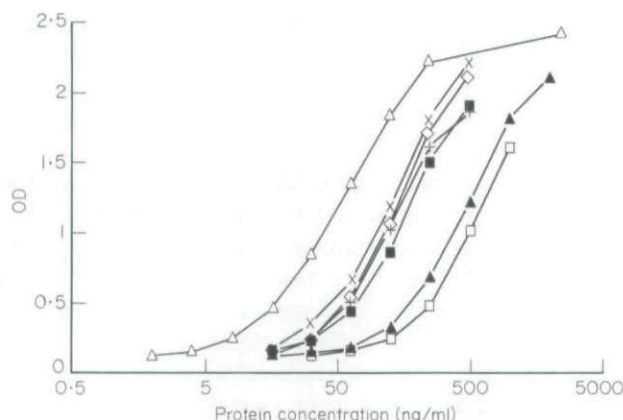


Fig. 3. Comparison of the dose response curves of various grass pollen extracts with purified *Phl p V*. —■—, *Dactylis*. —+—, *Festuca*. —▲—, *Holcus*. —□—, *Poa*. —×—, *Lolium*. —◇—, *Phleum*. —△—, *Phl p V*.

Similar concentrations of group V allergens were also found in extracts of different grass species (Table 2). The results confirm that group V allergens are important quantitative components of all grass extracts. No cross-reactive binding activity was found with non-Gramineae-pollen extracts in keeping with previous observations with dot-blot and EIA inhibition experiments [5]. Some distantly related grasses (e.g. *Phragmites* and *Molina*) contained only low or undetectable levels of group V (range < 1–5 µg/ml). This finding agrees with results of

Mathiessen *et al.* [20] for group V allergen in *Phragmites*. The assay has been found to be applicable for the routine measurement of group V allergen content in routine production batches of prick-test solutions (data not shown). Glycerol concentrations in excess of 0.2% (v/v) were found to have a considerable effect upon the reproducibility of the assay. However, the problem is easily overcome because the sensitivity of the assay allows dilutions < 1:500 (initial concentration: > 50% glycerol).

Interestingly, a correlation was obtained between the content of group V allergen in extracts and allergenic activity, determined by RAST inhibition (Table 2). Extracts with high group V concentrations also showed high allergenic potency and vice versa. These results confirm that group V allergens are of importance and indicate that the assay could be very useful for the standardization of whole extracts in terms of their group V allergen content. Furthermore, the RAST inhibition data support the view that the content of major allergens may be representative for the overall allergenic potency in selected extracts, including those of *Phleum pratense*, *Lolium perenne*, *Festuca pratensis*, *Dactylis glomerata* and *Holcus lanatus*. Similarly, correlations between the concentrations of a major allergen determined by monoclonal immunoassays and relative potency determined by RAST inhibition have been demonstrated in other systems [17,19].

Immunoblot analysis showed that there are differences in group I and group V concentrations between species (Fig. 4). The finding that group V-depleted *Phleum* extract gave 30% allergenic potency points to the importance of characterizing the RAST inhibition system. Furthermore it is known that group V allergens occur in different isoforms [21] and the reactive amounts of these isoforms and their ability to bind MoAbs or IgE may differ between species.

Although our experiments showed that the group V ELISA was able to detect the allergen in different species and that a correlation between allergenic potency and group V concentration is given, it is obvious that several important factors have to be considered when establishing standardization procedures. First, RAST inhibition experiments should be performed using allergen discs coupled with purified group V allergens; second, for grass extracts it is essential not only to determine group V allergens but also to quantify group I allergens; and third, it must be shown that the MoAbs bind to IgE-epitopes in order to insure that measurements are more comparable with RAST inhibition. In conclusion the determination of major allergens should prove to be useful to complement RAST inhibition data for standardization of allergen extracts and preparations.

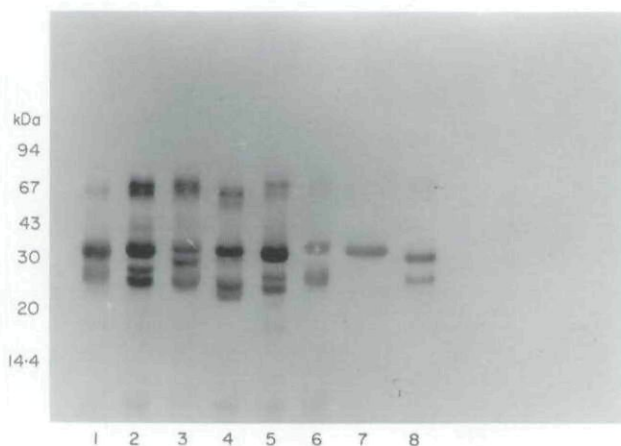


Fig. 4. Immunoblot from whole grass pollen extracts and purified allergens separated under reducing conditions by SDS-PAGE. Blots were developed using a serum pool from grass pollen allergic patients and ^{125} I-anti-IgE.

1. *Phleum pratense*. 2. *Festuca pratense*. 3. *Poa pratensis*. 4. *Dactylis glomerata*. 5. *Lolium perenne*. 6. *Holcus lanatus*. 7. *Lol p I*. 8. *Phl p V*.

References

- 1 Marsh DG. Allergens and the genetics of allergy In: Sela M. ed. *The Antigens*. Vol. 3. London: Academic Press, 1975: 271–359.
- 2 Matthiesen F, Löwenstein H. Group V allergens in grass pollens. I. Purification and characterization of the group V allergen from *Phleum pratense* pollen, Phl p V. *Clin Exp Allergy* 1991; 21:297–307.
- 3 Gjesing B, Jäger L, Marsh DG, Löwenstein H. The international collaborative study establishing the first international standard for timothy (*Phleum pratense*) grass pollen allergenic extract. *J Allergy Clin Immunol* 1985; 75:258–67.
- 4 Campbell PJ International Biological Standards and Reference Preparations. II Procedures used for the production of biological standards and reference preparations. *J Biol Stand* 1974; 2:259–67.
- 5 Fahlbusch B, Müller W.-D, Diener Ch, Jäger L. Detection of cross-reactive determinants in grass pollen extracts using monoclonal antibodies against group IV and group V allergens. *Clin Exp Allergy* 1992; 23:51–60.
- 6 Fahlbusch B, Müller W.-D, Schlenvoigt G, Jäger L, Wahl R, Weber B. Monoclonal antibody immunoassay for quantitative analysis of group V allergens in grass pollen extracts. *Clin Exp Allergy* 1993; 23:747–754.
- 7 Vieths S, Schöning B, Brockmann S, Aulepp H. Untersuchungen zur Allergie gegen Lebensmittel pflanzlicher Herkunft: Herstellung und Charakterisierung von Obst- und Gemüseextrakten für serologische Untersuchungen. *Deutsche Lebensmittel-Rundschau* 1992; 88:239.
- 8 Jung K, Fahlbusch B, Müller W.-D, Herrmann D, Diener C, Jäger L. Isolation of timothy (*Phleum pratense*) allergens using affinitychromatography with monoclonal antibodies. *Allerg. Immunol.* 1989; 35:287–94.
- 9 Yman L, Ponterius G, Brandt R. RAST-based allergen assay methods. *Develop. Biol. Standard.* 1975; 29:151–165, Karger-Verlag, Basel.
- 10 Wahl R, Maasch HJ, Geissler W. Comparison of three variations of the radioallergosorbent test-inhibition assay for measuring allergenic activity of grass pollen extracts. *Anal. Biochem.* 1983; 134:189–194.
- 11 Ceska M, Eriksson R, Varga JM. Radioimmunosorbent assay of allergens. *J Allergy Clin Immunol* 1972; 49:1–9.
- 12 Anderson MC, Baer H. RAST-inhibition procedure. Allergenic products Branch, Bureau of Biologics, Food and Drug Administration, Bethesda, Maryland.
- 13 Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970; 227:680–85.
- 14 Jäger L, Diener C, Schlenvoigt G, Jung K, Müller W.-D. Kreuzreaktion von Gräserpollen. *Mönchengladbacher Allergie-Seminare*, 1988, Band 1:32–49, Dustri-Verlag Dr. Karl Feistle, München-Deisenhofen.
- 15 Platts-Mills TAE, Chapman MD. Allergen standardization. *J Allergy Clin Immunol.* 1991; 87:621–625.
- 16 Krilis S, Baldo BA, Raison RL, Callard RE, Basten A. Standardization of antigen E in ragweed pollen extracts using a monoclonal antibody-based enzyme immunoassay. *J Allergy Clin Immunol.* 1983; 71:261–5.
- 17 Lombardero M, Carreira J, Duffort O. Monoclonal antibody based radioimmunoassay for the quantitation of the main cat allergen (*Fel d I* or *Cat-I*). *J Immunol Meth.* 1988; 108:71–76.
- 18 Luczynska Ch.M, Arruda LK, Platts-Mills TAE, Miller JD, Lopez M, Chapman MD. A two-site monoclonal antibody ELISA for quantification of the major Dermatophagoides spp. allergens, Der p I and Der f I. *J Immunol Meth.* 1989; 118:227–235.
- 19 Lombardero M, Quirce S, Duffort O, Barber D, Carpizo J, Chamorro MJ, Lezann A, Carreira J. Monoclonal antibodies against *Olea europaea* major allergen: Allergenic activity of affinity-purified allergen and depleted extract and development of a radioimmunoassay for the quantitation of the allergen. *J Allergy Clin Immunol.* 1992; 89:884–94.
- 20 Matthiesen F, Löwenstein H. Group V allergens in grass pollens. II. Investigation of group V allergens in pollens from 10 grasses. *Clin Exp Allergy.* 1991; 21:309–20.
- 21 Petersen A, Becker W-M, Schlaak M. Characterization of isoforms of the major allergen Phl p V by two-dimensional immunoblotting and microsequencing. *Int Arch Allergy Immunol.* 1992; 98:105–109.

This document is a scanned copy of a printed document. No warranty is given about the accuracy of the copy. Users should refer to the original published version of the material.