

Original article

Characterization and comparison of commercially available mite extracts for *in vivo* diagnosis

Background: Assessment of sensitization by allergen-specific IgE testing and skin prick testing (SPT) are primary tools in routine clinical diagnosis of allergies. To perform a correct diagnosis, it is critical that the allergen reagent used contains an adequate amount of all relevant components. This study aimed at evaluating commercially available mite extracts for *in vivo* diagnosis from eight manufacturers.

Methods: Eight extracts from *Dermatophagoides pteronyssinus* and eight from *Dermatophagoides farinae* were analysed for total protein content by Bradford and for major allergen content by ELISA. SDS-PAGE, immunoblotting and SPT were also carried out.

Results: The protein amount ranged from 27.7 µg/ml extract to 361.1 µg/ml (*D. pteronyssinus*) and from 20.3 to 353.0 µg/ml (*D. farinae*). In regards major allergen concentration, Der p 1 ranged from 9.6 to 36.2 µg/ml, Der f 1 26.5–196.1 µg/ml, mite group 2 0.7–31.7 µg/ml in *D. pteronyssinus* and 1.3–10.4 µg/ml in *D. farinae*. SDS-PAGE experiments showed that some components are poorly represented or absent in extracts from most manufacturers. Similar results were obtained by IgE-immunoblotting and SPT with 10 mite allergic patients confirmed a broad spectrum of reactivity of the extracts in the same subject.

Conclusions: Immunochemical analysis showed a heterogeneous amount of component/s among mite extracts from different manufacturers. These data were confirmed by *in vivo* testing, suggesting that, for some of the patient tested, the absence of relevant allergens could strongly affect the diagnosis.

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Assessment of sensitization by specific IgE testing (*in vitro*) and skin prick testing (SPT) are primary tools in routine clinical diagnosis of allergies (1). To perform an appropriate diagnosis, it is critical that the extract applied contains an adequate amount of all relevant allergen components. Furthermore, despite the fact that the quality of allergen extracts should be similar among different manufacturers, it is recognized that this is a difficult task, although the real level of heterogeneity of the commercially available products and its clinical impact are not well understood. A few studies have shown great variation in allergens content among different manufacturers' extracts and, in some cases, severe shortage or lack of important components, which is likely to affect the outcome of diagnosis (2, 3), was reported. Moreover, over the past decades, pressure to set an allergen standardization process has increased, and regulatory agencies in several European countries have started asking allergen manufacturers for improvement of major allergen standardization of their products (4).

Nowadays, considerable variability still exists in how allergen extract potency is measured and reported

worldwide. In Europe, manufacturers report allergen extract potency as units based on an in-house reference preparation (IHRP), making it difficult to understand the exact doses used (5). On the other hand, an allergen extract could be defined as fully standardized only when, as a condition of release to the public, the allergen content of each manufactured lot of the product is compared to an international reference preparation. However, because this is not currently feasible, and although the quality of extracts for diagnosis has been improved, a lot of variability problems still exist among different lots of the same product from a manufacturer as well as among the same product from different manufacturers (2–4, 6).

Since sensitization to house dust mites (HDMs) is regarded as very common, and this source of allergens is one of the most important for respiratory allergies (7–9), the poor quality of the allergen extracts could affect the outcome of the disease in a large number of patients. Therefore, the aim of this study was to compare mite (*Dermatophagoides pteronyssinus* and *Dermatophagoides farinae*) extracts for SPT available on the Italian market from eight manufacturers. This

research project was funded by the Italian Agency of Medicines (AIFA).

Methods

Manufacturers

All the manufacturers present on Italian market were officially requested to participate in the project. They were informed about the purpose of the project and it was underlined that the participation was on a voluntary base. Each manufacturer which participated in the project was requested material for *in vivo* diagnosis. All companies which had products distributed on the Italian market accepted to participate in the study. These companies were (in alphabetical order) Allergopharma, Allergy Therapeutics, Alk-Abello, Anallergo, Hal Allergy, Lofarma, Sarm, Stallergenes. The companies Laboratories Leti and Bio-Allergologica, were also contacted but they answered that, at the time of request, they were not selling diagnostic products in Italy. As stated in the material transfer agreement, all products were codified. Throughout the present study, the results will be shown with a code without the name of the company in a random order (i.e. manufacture 1–8).

Protein analysis

Bradford. Total protein content was analysed by Bradford (Bio-Rad, Hercules, CA, USA) (10). The standard curve ranged from 1 to 25 µg/ml of BSA (Bio-Rad). The method was validated according to the guidelines from the European Agency for the Evaluation of Medicinal Products (EMA, CPMP/ICH/381/95 and CPMP/ICH/281/95). Each experiment was carried out at least three times and each point was tested in double. Extracts from some manufactures were analysed by adding only the maximum dilution possible, due to very small amount of protein. The standard deviations (SD) were calculated by GRAPH PAD PRISM, version 4 (GraphPad Software, Inc., La Jolla, CA, USA).

SDS-PAGE. The extracts were analysed by 15% SDS-PAGE under reducing condition (Bio-Rad). SDS-PAGE experiments were carried out as previously described (11, 12). The amount of extract loaded on the gel was 15 µl extract + 15 µl reducing buffer for each lane (4 mm × 7 cm). This method was validated according to the guidelines CPMP/ICH/381/95 and CPMP/ICH/281/95.

ELISA. Analysis of major allergen content in the extracts was carried out by using quantitative ELISA kits (Indoor Biotechnologies, Cardiff, UK) according to the manufacturer's instructions. Allergen content was measured for Der p 1, Der f 1 and Mite group 2. Each extract was analysed with the following dilutions: 1 : 500, 1 : 1000, 1 : 2000, 1 : 4000, 1 : 8000, 1 : 16000 in phosphate buffered saline plus tween 0.05% v/v. The results were calculated by interpolation on the standard curve by CombiStats version 3.1, EDQM – Council of Europe, <http://www.combistats.eu>. For each extract, the three points interpolated in the linear range of the curve were selected and a medium value was calculated. Each experiment was repeated at least three times. The SD of the three experiments was calculated by GraphPad PRISM, version 4.

Allergen analysis

Human sera. Human sera were obtained from 10 HDM allergic patients. The inclusion criteria were specific IgE *in vitro* detection

(ImmunoCAP Phadia, Uppsala, Sweden) and a consistent clinical history. The subjects were free from allergen-specific immunotherapy, corticosteroid and antihistaminic therapy. Their informed consent to participate in the study was obtained. Sera were collected, aliquoted and stored at –20°C until used. Two sera from non-atopic subjects were used to define the negative cut-off.

Skin testing. The 10 patients were SPT using a standard method (1) with *D. pteronyssinus* (five patients) or *D. farinae* (five patients) extracts from the 8 manufacturers. Extracts from manufacturers 1–4 were tested on the right forearm and extracts from manufacturers 5–8 on the left forearm. In order to reduce the effect of the skin reactivity, in each forearm the same panel of extracts was applied twice, with inverted order from the elbow to the wrist and *vice versa*.

Histamine was used as a positive control and extract diluent as a negative control. Each test was carried out in a single-blinded way, as the product identities were unknown to the allergist. Reactions were recorded 20 min after testing by transferring the ballpoint pen-surrounded wheal area with a scotch tape to paper. Data analysis was performed as previously reported (13, 14). Wheal areas were analysed by a densitometer (GS 700; Bio-Rad). Each result was the mean of two values. Values ≥ 7 mm² were regarded as positive (13).

Immunoblotting. Sera from the 10 patients were tested in immunoblotting with eight *D. pteronyssinus* and eight *D. farinae* extracts. For each experiment, one previously tested serum was used as a positive control and two sera from non-atopic subjects were used as negative controls to define the negative pattern. Procedures for immunoblotting analysis were essentially those suggested by manufacturer instructions (Amersham ECL Plus Western Blotting Detection Reagent; GE Healthcare, Uppsala, Sweden).

Results

Protein analysis

To evaluate the level of heterogeneity of the extracts from different manufactures, a quantitative analysis of total proteins was set up. The protein concentration of *D. pteronyssinus* ranged from 27.7 µg/ml of extract to 361.1 µg/ml, whereas the protein concentration of *D. farinae* ranged from 20.3 µg/ml to 353.0 µg/ml (Table 1). To obtain more information, we evaluated the major allergen content in the individual extracts. Der p 1 ranged from 9.6 µg/ml of extract to 36.2 µg/ml and Der f 1 from 26.5 µg allergen/ml to 196.1 µg/ml (Table 2). It is noteworthy that the analysis of *D. farinae* indicated an amount of Der f 1 in some extracts (i.e. manufacturers 3 and 7) higher than the total protein amount as detected by the Bradford method.

The concentration of Mite group 2 in the individual extracts was in general lower than group 1 (Table 2). In fact, Mite group 2 ranged from 0.7 to 31.7 µg/ml in *D. pteronyssinus* and from 1.3 to 10.4 µg/ml in *D. farinae*.

Each extract was analysed by means of SDS-PAGE. Typical stained gels for the extracts are shown in Fig. 1. The separated proteins of some extracts were at or below the detection limit of staining with Coomassie Brilliant Blue. As shown in Fig. 1A,B, intensities for specific

Table 1. Protein analysis by Bradford's method in *Dermatophagoides pteronyssinus* and *D. farinae* extracts for *in vivo* diagnosis from various manufacturers

Manufacturers	<i>D. pteronyssinus</i> Mean* (±SD)	<i>D. farinae</i> Mean* (±SD)
1	258.5 (±4.1)	326.0 (±2.2)
2	253.0 (±3.4)	84.9 (±6.3)
3	63.8 (±3.9)	28.4 (±0.5)
4	180.5 (±0.5)	191.6 (±2.3)
5	361.1 (±6.4)	353.0 (±10.5)
6	99.8 (±9.0)	73.2 (±1.1)
7	27.7 (±5.7)	20.3 (±1.4)
8	154.8 (±6.1)	167.7 (±2.8)

*Results are the mean of three different determinations. Values are expressed as µg protein/ml extract.

components varied among extracts from different manufacturers. In addition, some components are absent in a few *D. pteronyssinus* and *D. farinae* extracts. Specifically, *D. pteronyssinus* extracts from manufacturers 1, 2 and 5 have a comparable intensity. In the extract 8, bands have a lower intensity. The extracts 3, 6 and 7 have a comparable intensity but components belonging to groups 2 and 5 (14 kDa) are absent in the extracts 6 and 7. The protein profile of extract 4 is apparently unique due to the presence of a prevalent component at about 66–68 kDa without other prevalent components.

Dermatophagoides farinae extracts from manufacturers 1 and 5 have a comparable intensity, as well as the extracts 2 and 8. The extracts 3, 6 and 7 have a comparable intensity but components belonging to groups 2 and 5 are absent. Also in this case, the extract from manufacturer 4 has got a prevalent band at about 66–68 kDa.

Allergen analysis

Protein content and protein profile results were completed by information on allergenic properties of

Table 2. Der p 1, Der f 1 and Mite group 2 analysis by ELISA in *Dermatophagoides pteronyssinus* and *D. farinae* extracts for *in vivo* diagnosis from various manufacturers

Manufacturers	Der p 1 <i>D. pteronyssinus</i>	Der f 1 <i>D. farinae</i>	Mite group 2 <i>D. pteronyssinus</i>	Mite group 2 <i>D. farinae</i>
	Mean* (±SD)	Mean* (±SD)	Mean* (±SD)	Mean* (±SD)
1	36.2 (±5.7)	122.9 (±17.3)	31.7 (±7.6)	3.3 (±0.7)
2	9.6 (±1.7)	36.5 (±3.8)	8.5 (±1.0)	3.6 (±0.2)
3	n.a.	196.1 (±7.7)	6.1 (±0.01)	1.3 (±0.2)
4	11.1 (±1.5)	115.7 (±26.9)	1.3 (±0.1)	2.4 (±0.5)
5	21.7 (±1.6)	190.4 (±26.5)	23.4 (±1.0)	10.4 (±2.1)
6	20.4 (±2.8)	59.1 (±1.7)	0.7 (±0.1)	1.5 (±0.2)
7	12.8 (±2.2)	114.0 (±11.1)	2.4 (±0.7)	2.0 (±0.06)
8	15.7 (±2.1)	26.5 (±6.8)	2.6 (±0.3)	4.0 (±0.4)

n.a., not available.

*Results are the mean of three different determinations. Values are expressed as µg protein/ml extract.

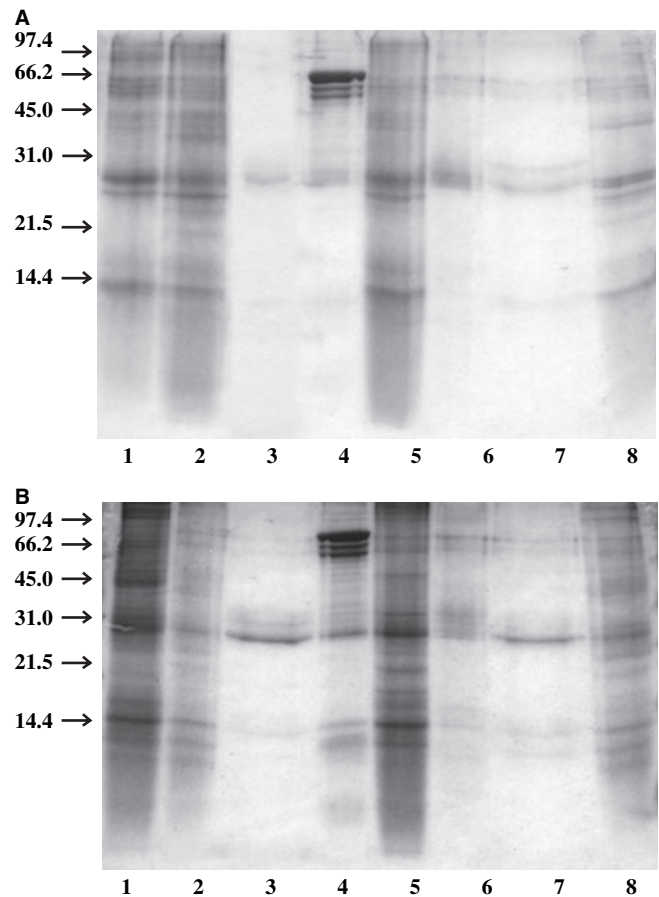


Figure 1. SDS-PAGE analysis of *Dermatophagoides pteronyssinus* (A) and *Dermatophagoides farinae* (B).

individual extracts. For this purpose SPT on 10 mite allergic patients and immunoblotting experiments with their sera were carried out.

As shown in Table 3A,B, mean wheal areas are different depending on extract. Sometimes data were < 7 mm² and were scored as negative. In order to correct results for variability due to SPT technique (14, 15), data were also calculated as a ratio between mean wheal area of the extract/histamine and also in this case the heterogeneity among the extracts was still present (data not shown).

Different patterns of reactivity could be observed by using individual sera in immunoblotting experiments against the HDM extracts. Specifically, the two major allergens belonging to group 1 (25 kDa) and group 2 (14 kDa) were differently recognized depending on serum and extract. In fact, for the same serum, major allergens belonging to groups 1 and 2 were both detected only on some extracts, sometimes only one group was detected and in some cases none of them. The group of allergens ranging from 55 to 177 kDa (16), were recognized in almost all extracts by the sera with different intensities. Immunoblotting experiments from two representative

Table 3. Skin prick test results mean wheal areas (mm²)

	Mean wheal area (mm ²)				
	Patient CE	Patient TM	Patient DS	Patient GL	Patient DG
(A) <i>Dermatophagoides farinae</i> extract					
Histamine right forearm	21.5	7.0	13.2	11.4	27.7
Manufacturer 1	68.1	47.6	53.6	10.4	80.6
Manufacturer 2	68.7	21.4	50.2	11.0	33.0
Manufacturer 3	63.2	7.0	23.1	9.9	31.0
Manufacturer 4	50.4	12.0	76.8	2.2	35.7
Histamine left forearm	19.5	7.0	14.9	7.3	36.6
Manufacturer 5	104.3	12.7	49.3	3.1	60.1
Manufacturer 6	48.9	0.7	37.7	4.2	52.5
Manufacturer 7	43.7	9.5	29.2	1.6	61.3
Manufacturer 8	77.2	18.0	47.9	6.0	39.1
	Patient FS	Patient MI	Patient CP	Patient GA	Patient PV
(B) <i>Dermatophagoides pteronyssinus</i> extract					
Histamine right forearm	11.5	7.0	9.5	23.3	12.6
Manufacturer 1	21.3	29.7	30.0	23.2	28.3
Manufacturer 2	11.7	36.9	44.1	2.2	26.7
Manufacturer 3	14.7	38.1	24.2	12.6	11.1
Manufacturer 4	6.4	13.7	23.6	1.3	3.5
Histamine left forearm	25.7	13.8	14.4	7.0	16.6
Manufacturer 5	16.5	41.8	25.9	15.6	29.2
Manufacturer 6	10.2	24.8	6.9	6.9	14.9
Manufacturer 7	17.3	36.9	0.0	5.0	6.6
Manufacturer 8	19.0	63.5	17.5	11.5	24.1

Ten mite allergic patients were subjected to skin prick test with *D. farinae* or *D. pteronyssinus* extracts from 8 manufacturers. Bold types are negative values (according to cut-off < 7 mm²).

sera are shown in Fig. 2A,B. In manufacturers 1, 2, 5, 8 almost all allergens previously characterized in *Dermatophagoides* spp. extracts are recognized by patient CP (Fig. 2A) although in manufacturer 1, the reactivity was stronger. Manufacturers 6 and 7, which elicit a negative result in SPT, did not show any reactivity of major allergens (groups 1 and 2). In Fig. 2B, it is shown the IgE reactivity of a serum (patient MI) that prevalently recognizes the major allergen belonging to group 2. This component is recognized in extracts from manufacturer 1, 2, 3, 5 and 8, although manufacturer 1 showed a stronger signal. For this patient, each extract elicited a positive SPT, although the reactivity was variable depending on extract.

Discussion

Allergen extracts for diagnosis and immunotherapy of allergies are complex mixtures of biological material. Although substantial progress has been made in the field of allergen extracts standardization and quality in the last 20 years (17), there are still problems of potentially large differences among suppliers. Despite the fact that recombinant allergen molecules closely resembling the properties of the natural allergens have been

produced, and could be used for the *in vivo* diagnosis and therapy of allergy, natural allergen extracts are currently in use for routine diagnostic and therapeutic purposes.

Hence, there is a crucial need for high-quality allergenic extracts to prepare *in vivo* diagnostic as well as therapeutic products. In regards diagnostic extracts, they should comprise the various allergens to which most patients are naturally exposed. Importantly, batch to batch consistency among the extracts of a manufacturer and homogeneity among a definite extract from different manufacturers should be assured (18).

For these reasons, we aimed to evaluate the quality of allergen extracts for SPT available on the Italian market and we chose HDMs, as they represent a major cause of allergies worldwide. At least 17 allergens have been described (19) for *Dermatophagoides* spp.: groups 1–11, 13 and 14 (*D. pteronyssinus* and *D. farinae*); groups 15–18 (*D. farinae*); group 20 (*D. pteronyssinus*). Furthermore, recent studies have demonstrated that a mixture of groups 1, 2, 5, 7, 8 and 10 allergens binds an average of 76% of *D. pteronyssinus*-specific IgE and a diagnostic test consisting of Der p1, Der p 2, Der p 5 and Der p 7 seems to be sufficient for the diagnosis of a genuine HDM sensitisation in Europe (20) and in Australia (21).

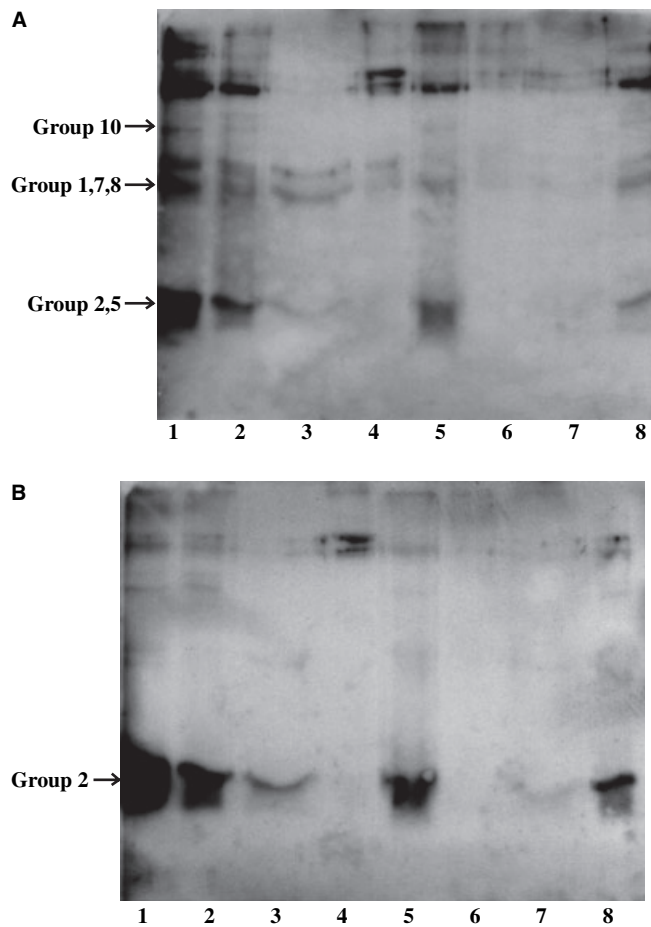


Figure 2. (A and B) Immunoblotting analysis with 2 (out of 10) representative sera. Sera were tested with both *Dermatophagoides farinae* and *Dermatophagoides pteronyssinus*.

Our analysis has revealed several substantial heterogeneous behaviours in the tested products that may have important implications for the appropriateness of the diagnosis of mite allergy. First, we found that the *D. pteronyssinus* extracts showed an almost 13-fold variation of the total protein content and the *D. farinae* extracts an 18-fold variation. Although protein content is not representing allergenic quality of the extract; nevertheless, the observed range is quite surprising. Secondly, a considerable variability regarding the major allergen content was also detected when testing the extracts by monoclonal antibody-ELISAs. However, this information needs further evaluation because the amount of major allergen measured was clearly overestimated in Der f 1 ELISAs, as it was higher than the total protein content in the sample. The issue of overestimating the allergen content by ELISAs methods has been already described (22) and a possible explanation is associated to an aggregation-state of the allergen in some extracts, which could cause an overestimation if epitopes are concentrated or an underestimation if epitopes are hidden. Therefore, a straight comparison of major allergens content does not neces-

sarily reflect or predict the real *in vivo* behaviour. However, the tremendous protein heterogeneity was confirmed by SDS-PAGE. In fact, we found that important molecules (i.e. group 2 and 5) were absent in some extracts. Even taking into account the detection limit of Coomassie staining, which could justify the lack of detection of some components, the results clearly confirm that a number of bands (i.e. group 10 tropomyosin, 37 kDa) seems to be under represented in most extracts and also molecules that ranged from 55 to 177 kDa presented different intensity depending on extract, thus confirming the need for further standardization of HDM extracts.

However, the heterogeneity observed could not necessarily have an impact at the level of the appropriateness of the diagnosis performed with such extracts, being the protein content, protein pattern and major allergen content important parameters which do not directly reflect the IgE potential binding. Therefore, to evaluate the impact of such heterogeneity directly at the *in vivo* diagnostic test level, we carried out *in vivo* SPT and IgE-immunoblotting. Skin prick test experiments were carried out in the same condition adopted by clinicians during they routinely activity, to reproduce what really happens in the common clinical practice. When the eight *D. pteronyssinus* extracts were tested on the first five patients, each extract was able to elicit a positive (although variable) result in one patient only. For the other four patients, some extracts were nonreactive, and therefore, were not able to diagnose *D. pteronyssinus* allergy. An analogous situation was evidenced for *D. farinae*, where three patients showed a positive result although a substantial variability of the intensity of the reaction was observed. For the other two patients, some extracts were not able to elicit a positive result.

Our data clearly demonstrated that allergenic extracts manufactured from various companies currently available for SPT are so heterogeneous that in some patients a few of them are incapable of showing a positive response. These results confirmed that the absence of important components can affect the outcome of diagnosis for a main source of allergy as HDMs. However, these components are not necessarily restricted to major allergens, as for some extracts, a positive result in SPT does not match with detectable levels of major allergens in immunoblotting, thus confirming that allergens other than group 1/2 can have a role in elicit the reaction.

The present study identifies a strong heterogeneity of mite extracts obtained from a number of companies distributing their products in Italy. Surprisingly, this heterogeneity is so extended that a patient can be scores either negative or positive accordingly to the extract used. A number of aspects can be taken into consideration to explain such a huge degree of heterogeneity. A critical point could be the production process which cannot be suitable for an optimal and complete recovery

of all components present in the material. Some major allergens are associated with mite faecal particles (i.e. Der p1, Der f1) and could be lost if mite bodies only are extracted (23). These aspects cannot be easily identified, as each manufacturer has his own IHRP, and the comparison of each lot will be only based on the IHRP reactivity; therefore, any weakness in the production/extraction process will be difficult to be highlighted.

In the present study, we presented data regarding SPT preparations, but our preliminary results confirm that analogous problems are present for immunotherapeutic vaccines (24). Therefore, this heterogeneity could strongly affect not only the appropriateness of the SPT performance but also immunotherapy, due to lack of correct identification of patient sensitisation profile and to the poor quality of the therapeutic preparations.

The goal for the next future should be to make uniform the potency of the extracts from different companies by

promoting the development of international reference preparation in order to have products inter-companies standardized. In the meantime, it would be important to improve the immunological characterization, purification or cloning of the most relevant allergens to be used as standards for the development of methods for allergens quantification in the extracts. These methods will improve the quality and the standardization of extracts as well as homogeneity between manufacturers. Some projects that aimed at this goal have already been funded by European Union (4, 17, 25), but a lot of work is still needed.

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