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Proteomic profiling of commercial dust mite skin prick test solutions and allergy vaccines from India

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ABSTRACT

Background: Skin prick test (SPT) solutions and allergy vaccines (AVs) are crucial tools for diagnosis and therapy of allergies. It was the aim of this study to corroborate the content of products for diagnosis and treatment of dust mite allergies that are produced and sold in India.

Methods: SDS-PAGE, immunoblots and high-resolution mass spectrometric analysis was performed with 16 house dust mite (HDM) SPT solutions and AVs from 3 Indian manufacturers. Authority-approved European SPT solutions and in-house extracts were used as references.

Results: From the 5 Indian *Dermatophagoides pteronyssinus* products, none contained proteins from this source. Instead, 1 sample contained *Dermatophagoides farinae* and human serum proteins, 4 products contained allergens from the storage mite *Suidasia medanensis*, allergens from the legume *Cicer arietinum* (chickpea), and proteins from baker's yeast. From 4 Indian *D. farinae*-labeled products, 2 contained human serum proteins and a limited number of *D. farinae* allergens. Two contained only *Suidasia*, *Cicer*, and yeast proteins. In contrast, the European authority-approved *D. pteronyssinus* and *D. farinae* SPT solutions that were used as reference in this study, contained exclusively proteins of the respective species and covered the expected allergen spectra. The *Blomia tropicalis* sample contained no *Blomia* allergens at all, but consisted exclusively of *Suidasia*, *Cicer*, and yeast proteins. All 6 HDM samples consisted of human serum proteins and limited amounts of *D. farinae* allergens.

Conclusions: All commercial Indian SPT solutions and AVs analyzed in this study are not suitable for dust mite allergy diagnosis and therapy, as they contain either no, or only a limited number of, HDM allergens. In addition, their use could lead to misdiagnosis since some of them contain allergens from other sources, including the storage mite *Suidasia*, chickpea, as well as baker's yeast. Further, their application might be harmful to patients, as some products contain large amounts of proteins of human origin. Analysis of European SPT solutions, on the other hand, confirmed their suitability for dust mite allergy diagnosis.

Keywords: Skin prick test solutions, Allergy vaccines, House dust mites, Mass spectrometry, Proteomics

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INTRODUCTION

The "allergy epidemic" has become a major burden to public health, affecting more than 150 million people in Europe and approximately 25–30% of the world's population.[1-3] Particularly, in developing countries they are suspected to rise even further over the next few years. Skin prick testing (SPT) currently represents the most commonly used method applied by clinicians to verify sensitization of IgE-mediated allergic diseases. SPT is generally considered a sensitive, relatively specific, and minimally invasive procedure, in which patients are tested against diverse allergenic sources through the introduction of natural allergen extracts into the skin using a standard skin prick test lancet. A resulting wheal-and-flare reaction provides a visual and objective indication of sensitivity.[4,5] However, the accuracy of such tests is highly dependent on the quality of the employed extract solutions and thus on the underlying production processes, handling, and storage conditions. To guarantee batch-to-batch consistency and reproducibility of diagnostic tests, standardization of allergen extracts is essential. In this respect, initiatives such as the Global Allergy and Asthma European Network (GA_2LEN)[4,6] and the European Union CREATE project network[7,8] have significantly contributed for standardization of allergenic products in Europe and the United States, which are controlled by their respective authorities such as, the Paul Ehrlich Institute in Germany, and the US Food and Drug Administration (FDA) via its Center for Biologics Evaluation and Research (CBER).[9,10] Their protocols and guidelines regulate standardization procedures including the selection of source materials and the quality control of extracts according to: i) their major allergen contents, ii) allergen references/standards, and iii) their allergenic potency. Furthermore, the guidelines clearly state that extracts have to be free of other allergenic source materials, as it could lead to false-positive results.[11]

In other countries, allergen extracts do not necessarily follow all the above-mentioned guidelines and special requirements. For example in India, Drug Controller General of India (DCGI) and their associated agencies are responsible for the manufacture and approval of allergen preparations, which generally relies only on Good Manufacture Practice (GMP) and allergen units.[10]

House dust mites (HDMs) and related allergens are major allergen sources worldwide. They potently trigger innate and adaptive immune responses, which typically manifest as allergic rhinitis and bronchial asthma. The most dominant genera of house dust mites are *Dermatophagoides* (species: *D. pteronyssinus and D. farinae*) followed by *Blomia* (species: *B. tropicalis*), which globally affect 65 to 130 million allergic individuals.[12-14] Reported HDM sensitization rates differ widely in various regions reaching values higher than 20% in Europe, 12% in India, and up to 40% in North America.[15,16] This variability has been attributed to diverse factors such as geographical locations, quality of raw material and diagnostic tests used, socio-economic conditions, and ethnicities. *Dermatophagoides* not only represents the most common but also the best studied HDM genus. The WHO/IUIS Allergen Nomenclature Subcommittee database has currently 30 *D. pteronyssinus* (European HDM) and 36 *D. farinae* (American HDM) allergens acknowledged. Among them, cysteine protease allergens are classified as Group 1 allergens and comprise the major allergens Der p 1 and Der f 1. Group 2 includes MD-2 like lipid-binding proteins, such as the major allergens Der p 2 and Der f 2. Group 1 and Group 2 allergens account for 50–80% of the total IgE binding measured for HDM extracts.[17] For *B. tropicalis,* which is abundant mostly in tropical and subtropical regions, 14 allergens have been identified and listed in the WHO/IUIS Allergen database.[18] Among them, Blo t 5 is considered an important major allergen responsible for 60% of the IgE binding measured in the whole extract.[19,20]

In India, dust mites are among the most important sources of inhalant allergens.[13,21,22] However, reliable sensitization rates for HDM in India have been hampered by the use of different allergen extracts and different testing protocols. In fact, a recent review by Dey et al,[23] focusing on HDM allergy in India, pointed out the difficulties in comparing SPT results from different centers and

studies and highlighted the urgent need for standardization of reagents and procedures in allergy diagnosis and treatment within the country. In this respect, a study evaluating the potency of SPT allergen solutions used for diagnosis from 2 different Indian manufacturers revealed significantly lower mean wheal sizes compared to FDA-approved HDM extracts.[24] Taken together, these observations strongly suggest that there might be a high variability in the allergen content of extracts manufactured under Indian guidelines and regulations implemented in other countries. As variability of SPT solutions was shown to significantly impact the outcome of diagnostic tests, standardization of allergenic products from different manufacturers around the world is of utmost importance to avoid misdiagnosis of allergic conditions.

To evaluate the quality of SPT mite solutions available on the Indian markets, we characterized HDM solutions on a molecular basis. SPT solutions and allergen vaccines (AVs) from 3 Indian manufacturers that were labeled with "*D. pteronyssinus*", "*D. farinae*", "*B. tropicalis*", *and*"*H*" were analyzed by mass spectrometry, gel electrophoresis, and immunoblots using patients' sera and allergen-specific monoclonal antibodies. Detailed knowledge on the allergen content and thus potency of SPT solutions and AVs is essential for reliable diagnostics and therapeutic approaches.

MATERIALS AND METHODS

Samples

All samples were purchased by collaborating physicians in India from local vendors and shipped to Europe under temperature-controlled conditions at 4 °C. Upon arrival at the mass spectrometric facilities of the University of Salzburg, all sample vials were originally sealed and intact. Samples were stored at 4 °C according to the

Allergen source (according to label)	Type	Manufacturer	Batch Number on vial	Sample code
D. pteronyssinus	SPT	M1	AD0677	M1-DP
D. pteronyssinus	SPT	M2	084143[a]	M2-DPa
D. pteronyssinus	SPT	M2	084143[a]	M2-DPb
D. pteronyssinus	SPT	M3	A-080225	M3-DP
D. farinae	SPT	M1	AD0682	M1-DF
D. farinae	SPT	M2	084143[a]	M2-DF
D. farinae	SPT	M3	080225	M3-DF
House Dust Mite	SPT	M2	042743	M2-HDM
B. tropicalis	SPT	M1	AD0679	M1-BT
D. farinae	AV Dose 4	M1	T0783/0435/2018	M1-DF-AV4
D. pteronyssinus	AV Dose 4	M1	T0784/0435/2018	M1-DP-AV4
House Dust Mite	AV Dose 1	M2	Not available[b]	M2-HDM-AV1
House Dust Mite	AV Dose 2	M2	Not available[b]	M2-HDM-AV2
House Dust Mite	AV Dose 3	M2	Not available[b]	M2-HDM-AV3
House Dust Mite	AV Dose 2	M3	Not available[b]	M3-HDM-AV2
House Dust Mite	AV Dose 3	M3	Not available[b]	M3-HDM-AV3

Table 1. List of Skin prick test (SPT) solutions and allergy vaccines (AV) analyzed in this study. *a. These* D. farinae *and* D. pteronyssinus *products were labeled by manufacturer 2 with identical batch numbers. b. Batch numbers were not given on the vials.*

manufacturers' recommendations. The manufacturers were coded as follows:

M1 - Creative Diagnostic Medicare Pvt. Ltd. (Navi Mumbai, India).

M2 - All Cure Pharma Pvt. Ltd. (Bahadurgarh, India).

M3 - Alcit Pvt. Ltd (Delhi, India).

All samples were "ready-to-use" for physicians and contained glycerol and phenol. The samples were coded to indicate the manufacturer and the corresponding allergenic extract (*eg*, M1-DP, M2-DP, etc.). A full description of the samples is given in Table 1.

As reference for *D. pteronyssinus* and *D. farinae*, authority-approved SPT solutions from Allergopharma (Reinbek, Germany) were used (ref-DP, ref-DF). As reference for *B. tropicalis*, an in-house produced *B. tropicalis* extract was used (ref-BT).

Sample preparation

Protein concentrations of SPT solutions were determined with the Pierce 660 nm Protein Assay Reagent (Thermo Fisher Scientific, Rockford, USA). For SDS-PAGE, proteins were precipitated with trichloroacetic acid (TCA). To 100 µL SPT solution, 10 µL 6.1 N TCA were added. After 3 h at −20 °C, precipitated material was centrifuged and washed twice with cold acetone.

For mass spectrometric analyses, SPT solutions were prepared and digested with trypsin using 2 different approaches. TCA-precipitated material from 100 µL SPT solution was resuspended in Extraction buffer 1 of the ProteoExtract All-in-One Trypsin Digestion Kit (Merck Millipore, Billerica, USA) and proteins were reduced, alkylated, and digested using this kit following the manufacturer's instructions. Resulting peptides were desalted with 10 µl ZipTips C18 (Merck Millipore). In a second approach, samples were digested without prior precipitation. 50 µL SPT solution was mixed with 200 µl Extraction buffer 2 of the ProteoExtract All-in-One Trypsin Digestion Kit and 200 µL 100 mM ammonium bicarbonate. Samples were then reduced with dithiothreitol (DTT) at 60 °C for 1 h, alkylated with iodoacetamide for 1 h at room temperature (RT) and digested with MS-grade trypsin at 37 °C (all reagents from Pierce/Thermo Fisher Scientific). After 20 h, detergents present in Extraction buffer 2 were precipitated by adding 500 µL 0.5% trifluoroacetic acid (TFA). After centrifugation, peptides were enriched and desalted using 100 µl Pierce C18 Tips (Thermo Fisher Scientific). Allergy vaccines were digested similarly, except that volumes were adapted to compensate for their low protein concentrations: 500 µL sample, 450 µl Extraction buffer 2, 450 µL 100 mM ammonium bicarbonate.

SDS-PAGE

Dried TCA pellets were resuspended in SDS sample buffer (200 mM Tris, 8% SDS, 0.4% bromophenol blue, 40% glycerol, 400 mM β-mercaptoethanol, pH 6.75) and proteins were separated using a 15% polyacrylamide gel. Sample volumes were adjusted to compensate for the different protein concentrations of the samples. Proteins were visualized with the SilverXpress staining kit (Thermo Fisher Scientific, Waltham, MA, USA).

Immunoblots

Following SDS PAGE and blotting, nitrocellulose membranes (Whatman, Maidstone, UK) were placed into blocking buffer containing 25 mM Tris/Cl, pH 7.5, 150 mM NaCl, 0.5% (v/v) Tween, 0.5% (w/v) BSA, 0.05% (w/v) NaN_3 and left shaking for 1 h at RT. Blots were then either incubated with 1:10 diluted serum pools of house dust mite allergic individuals, with different dilutions of mouse monoclonal IgG antibodies (anti-Der f 1 1:1000, anti-Der p 1 1:1000, non-commercial products provided by Allergopharma, Germany) or with blocking buffer alone (for secondary antibody controls) and left shaking overnight at 4 °C. After several washing steps with blocking buffer, bound allergen-specific human IgE or mouse IgG antibodies were detected using AP-conjugated monoclonal anti-human IgE (BD Biosciences, Franklin Lakes, NJ, USA; diluted 1:4000 in blocking buffer) or monoclonal anti-mouse IgG + IgM antibodies (Jackson ImmunoResearch Europe Ltd, Cambridgeshire, United Kingdom; diluted 1:5000 in blocking buffer), respectively. Following 2 h of incubation and additional washing steps with blocking buffer, membranes were equilibrated for 5 min with AP buffer containing 100 mM Tris/Cl, pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$. For detection, a staining solution consisting of 10 mL AP buffer,

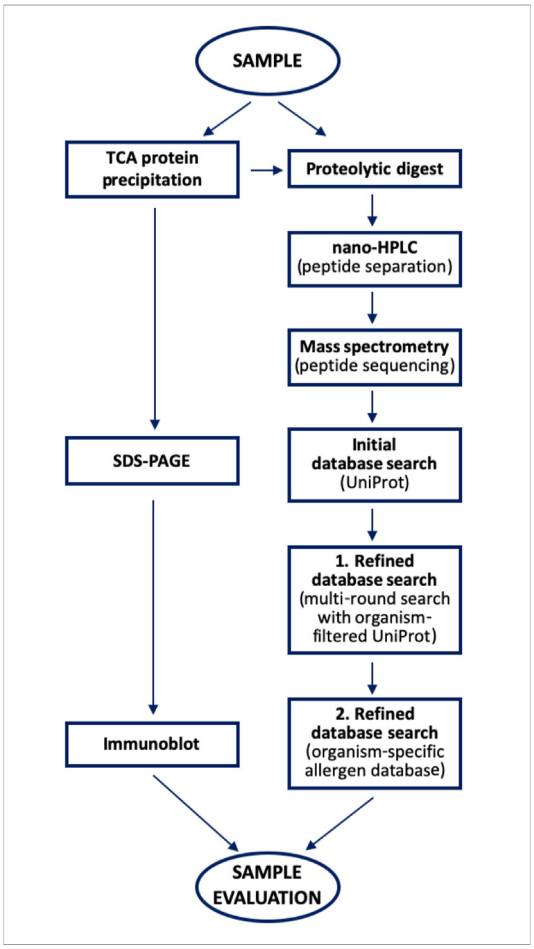

Fig. 1 Overview of sample preparation and analysis by mass spectrometry (LC-MS/MS) and gel electrophoresis/immunoblot.

30 µL BCIP (stock: 50 mg/ml in dimethylformamide) and 30 µL NBT (stock: 100 mg/ml in 70% dimethylformamide, 30% sterile water) was used. Membranes were washed with distilled water and air-dried.

Mass spectrometry

Desalted peptides resulting from the tryptic digests were separated by reverse-phase nano-HPLC (Dionex Ultimate 3000, Thermo Fisher Scientific, Bremen, Germany), directly coupled via nano electrospray to a Q Exactive Orbitrap mass spectrometer (Thermo Fisher Scientific). The column (Acclaim PepMap RSLC C18, 75 µm × 15 cm, Dionex, Thermo Fisher Scientific) was developed with an acetonitrile gradient (Solvent A: 0.1% (v/v) formic acid; solvent B: 0.1% (v/v) formic acid/90% (v/v) acetonitrile; 5–45% B in 120 min) at a flow rate of 300 nl/min at 55 °C. Capillary voltage of the nano spray was 2.5 kV. Lock mass calibration was used for highest accuracy. Peptide fragmentation/identification was done with a top 12 method and a normalized fragmentation energy at 27%.

Data analysis and protein identification

Survey and fragment spectra were analyzed/sequenced with PEAKS Studio 8.5 and X+, respectively (Bioinformatics Solutions, Waterloo, Canada). Primary searches were done against the complete UniProt database (release April 2019).[25] Based on the results obtained in the primary search, searches were repeated with specific subsets of this database in a multi-round approach. Settings for searches: trypsin (semi-specific), 0.1% FDR for UniProt, $-10\lg P \geq 35$ for refined searches. Fixed modification: carbamidomethylation (C), variable modifications: deamidation (NQ), oxidation (M).

Data presented here were based on 4 to 6 independent experiments for each sample. In order to compare the results of the different MS analyses, the data were normalized by calculating the sum of the ion count of all fragment spectra with a *de novo* sequencing score higher than 50% and using this value as normalization factor for individual peptides. Normalized values were then used to determine means and standard errors with Prism 5 (Graphpad, USA).

A flow chart of sample treatment and analysis is shown in Fig. 1.

RESULTS

Protein concentrations

In general, complex and glycerinated samples with very low protein content prepared from natural sources, such as allergy vaccines (AVs) and skin prick test (SPT) solutions, are difficult to analyze by standard photometric protein determination methods because of the low protein content and the possible interference of non-proteinaceous compounds found in natural extracts. We therefore aimed to determine, whether mass spectrometry (MS) could be used as a primary tool to estimate the protein concentration of such samples. For this purpose, an appropriate amount of sample (up to 1 mL) was digested with trypsin. Resulting peptides were enriched with C18

tips, fragmented by LC-MS/MS and analyzed with a *de novo* sequence analysis program. Signal intensities of all *de novo* sequenced peptides with a quality score ≥50 were summarized and compared to values obtained by a classical photometric assay (Supplementary Fig. 1A and B). Although mass spectrometry cannot provide absolute quantification under the chosen experimental conditions, it produced data comparable to the photometric assay, thus providing a useful tool for quality control of the samples. As shown in Supplementary Fig. 1, the Indian SPT solutions contained between 40 and 80 ng/µL total protein, which was comparable to the authority-approved references. Due to their low content, protein concentrations in the AVs could not be determined using photometric assays. Nevertheless, from the intensity values of the *de novo* sequenced peptides, a protein concentration between 0.05 and 5 ng/µL could be estimated. Determined protein concentrations were in accordance to the doses given by the manufacturers for each AV vial (Supplementary Fig. 1C, Table 1).

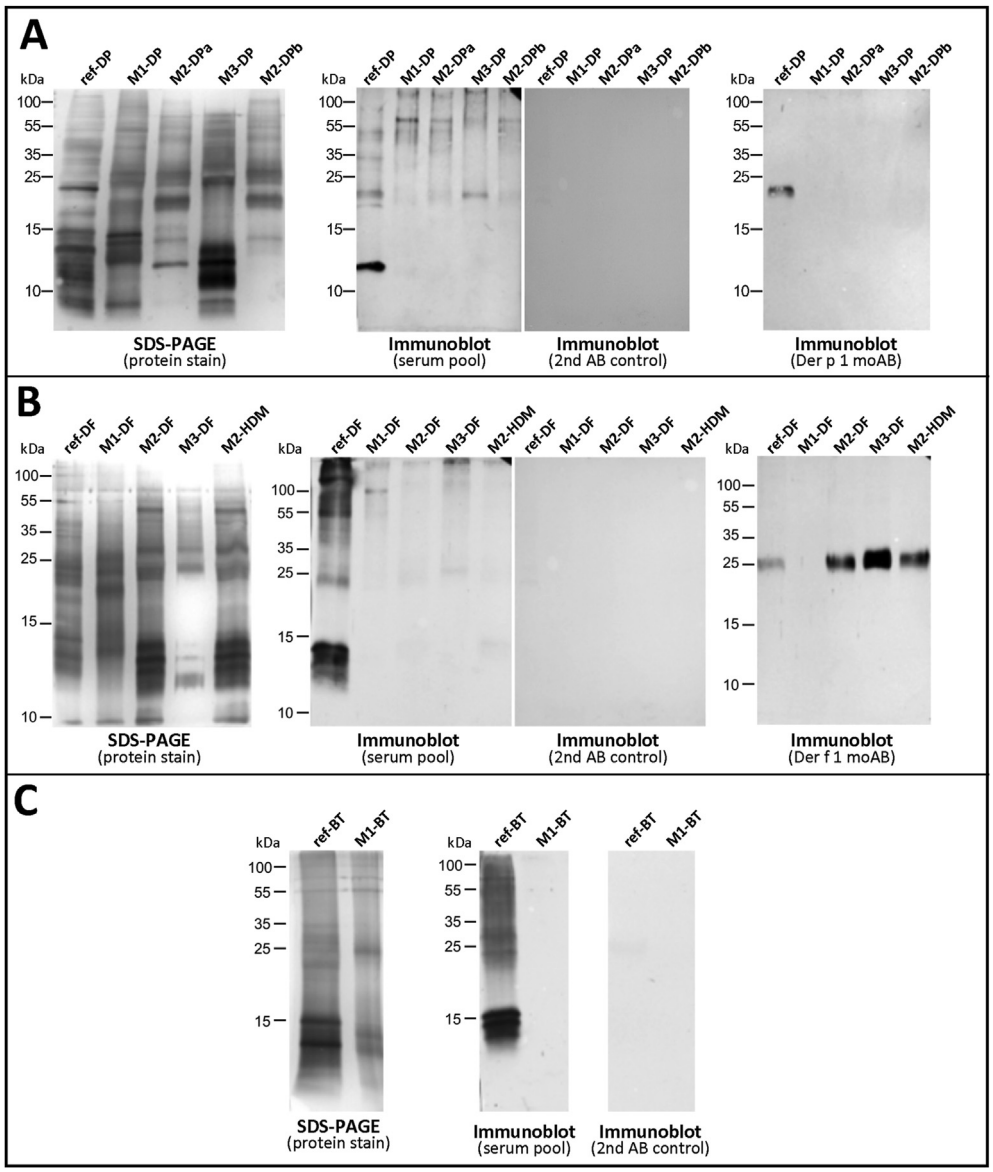

Fig. 2 SDS-PAGE and immunoblotting of SPT solutions from *D. pteronyssinus* (A), *D. farinae* and house dust mite (HDM) (B) and *B. tropicalis* (C). For optimal detection of allergens in immunoblots, different total protein concentrations of the references and SPT solutions were loaded onto the gels. Therefore, the immunoblots depicted in this figure represent qualitative and not quantitative comparisons.

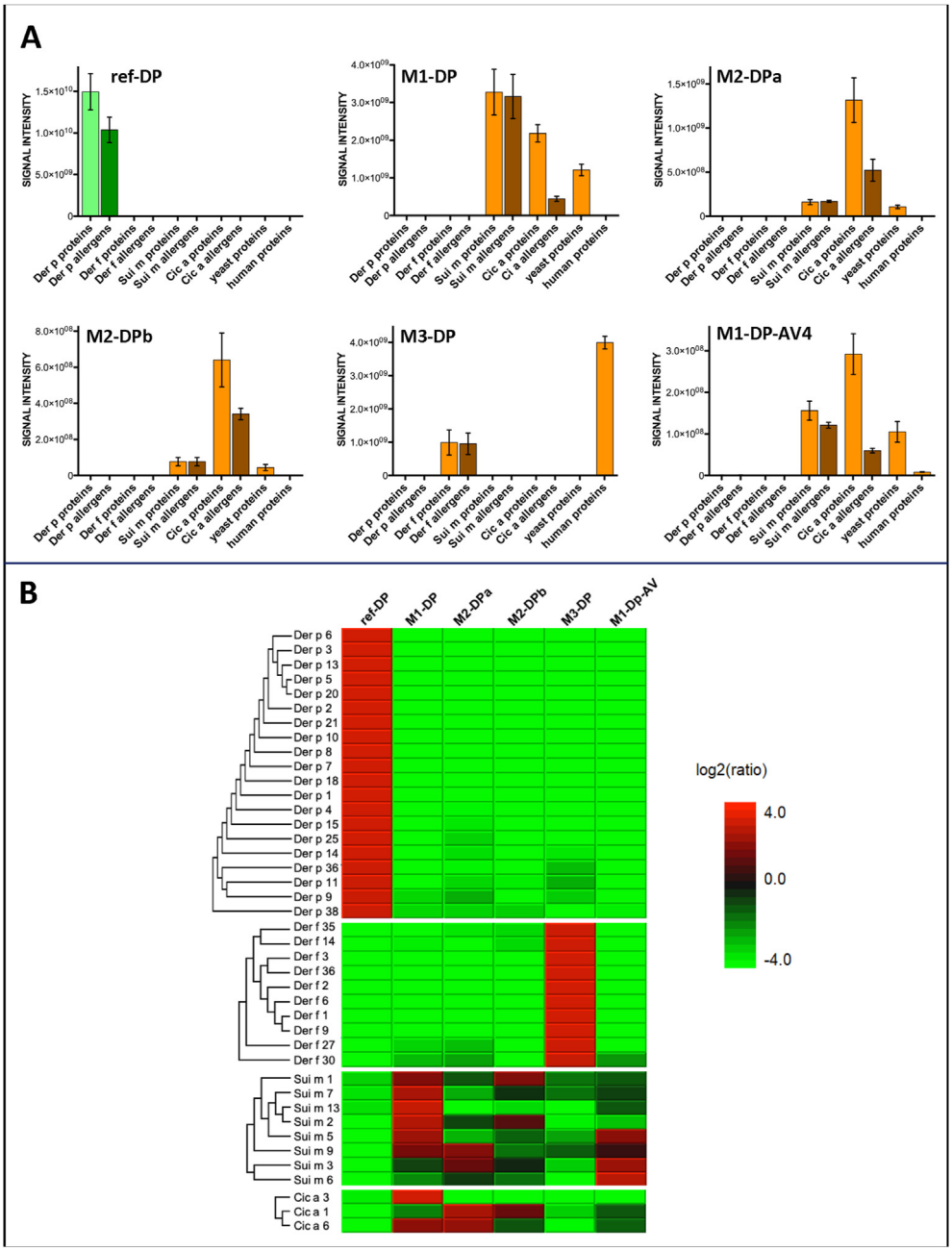

Fig. 3 Allergen and total protein content of the *D. pteronyssinus* products compared to the reference. Panel A shows signal intensities of peptides obtained in multi-round database searches. Panel B shows the relative quantitative comparison of the allergen content as heat map. Red indicates high allergen content, green indicated low/no content of the indicated allergen. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

SDS-PAGE and immunoblotting

The comparison of the Indian SPT solutions with authority-approved references (*D. pteronyssinus*, *D. farinae*) and the in-house reference (*B. tropicalis*) by SDS-PAGE indicated significant differences between the samples. As shown in Fig. 2, the pattern of protein bands showed no similarity between the 3 manufacturers and the reference samples. These differences were further confirmed by immunoblot analyses using a serum pool obtained from European mite allergic patients and specific monoclonal antibodies

(moAB) raised against Der p 1 and Der f 1. Compared to the references, positive immune reactions were weak (*D. pteronyssinus*) or missing altogether (*D. farinae*, *B. tropicalis* and HDM). No signals were observed with the *D. pteronyssinus* SPT solutions, while the reference showed clear reactivity with anti-Der p 1 antibody. Interestingly, Der f 1 moAB produced a strong signal in DF SPT solutions of manufacturers M2 and M3, although no significant IgE reactivity was observed with patients' serum pool. A possible explanation for this result could be that conformational IgE-binding epitopes identified in Der p 1 and Der f 1[26] are partially destroyed under SDS-PAGE/blotting conditions, while binding of moABs may not be affected by such denaturing conditions. To compensate for the low amounts of allergens in the SPT solutions (as determined by mass spectrometry, see below), higher protein amounts of these samples were used for the moAB immunoblots. Therefore, the signals of the Der f 1 moAB were significantly stronger with M2-DF, M3-DT and M2-HDM than with the reference sample. For this reason, gels and immunoblots depicted in Fig. 2 provide only qualitative but not quantitative comparisons.

Mass spectrometry-based proteomics

To further investigate the composition of the Indian SPT and AV products, we performed extensive MS analyses of these samples in comparison to European reference products. SPT solutions were trypsin-digested both after TCA precipitation and in solution without prior precipitation. Due to their low protein content, AVs were digested in solution only. Resulting peptides were analyzed by LC-MS/MS. Quantitative data presented here were based on signal intensities of identified peptides. The mean of 4–6 independent experiments is shown. Special care was taken to unequivocally distinguish between proteins of closely related species, like *D. pteronyssinus* and *D. farinae*. To achieve this goal and avoid false-positive hits, database searches were performed with the multi-round option of PEAKS: for the initial search, the complete UniProt library was used. This first round provided an overview of proteins present and gave a first hint on possible contaminations. The following rounds were based on the preliminary results from the first pass, using sequence subsets of single organisms only. After each search round, peptides with positive matches were excluded from the following searches. The remaining non-matched peptides were then searched against a new sequence set, and so forth. Applying this search method, the identification of unique/diagnostic peptides was greatly facilitated, and hence the identification of false-positive hits was avoided. As example, the differentiation of Der p 2 and Der f 2 in the samples ref-DP and M3-DP is shown (Supplementary Fig. 2). Both proteins share 128 of 146 amino acids (88% identity). In the initial search with the complete database, both Der p 2 and Der f 2 were listed as positive hits. After the multi-round search with *D. farinae* sequences in the first and *D. pteronyssinus* sequences in the second round, on the other hand, it became evident that sample M3-DP did not contain Der p 2, but only Der f 2, as 5 peptides were matched to Der f 2, but no peptides to Der p 2. In the reference sample, on the other hand, only Der p 2, but no Der f 2 specific peptides were identified, indicating the exclusive presence of Der p 2. These results are summarized in Supplemental Fig. 2. Detailed results for all samples obtained with this method are described below.

D. pteronyssinus samples

Mass spectrometric analyses showed that the content of all commercial *D. pteronyssinus*-labeled samples did not match their description and confirmed the results obtained by SDS-PAGE and immunoblot experiments. No *D. pteronyssinus* proteins were detected in the SPT solutions of manufacturers M1, M2 and M3, and in the AV of manufacturer M1 (Fig. 3). Instead, samples M1-DP, M2-DPa, M2-DPb and M1-DP-AV4 contained proteins from the storage mite *Suidasia medanensis* (*S. medanensis*), the legume *Cicer arietinum* (*C. arietinum*, chickpea) and the baker's yeast *Saccharomyces cerevisiae (S. cerevisiae)*. For instance, 255 *S. medanensis* peptides (assigned 12 proteins out of 16 UniProt entries) were detected in sample M1-DP. Also, 2177 *C. arietinum* peptides (assigned to 330 proteins) and 1497 yeast peptides (assigned to 486 proteins) were identified with high confidence in this sample. In this context, it should be mentioned that both *S. medanensis* and *C. arietinum* are allergen sources. Allergome[18] lists 10 potential allergens from

S. medanensis, 8 of which were identified in M1-DP. Similarly, 6 potential allergens are listed for *C. arietinum*, 3 of which were found in this *D. pteronyssinus* sample. Results from M2-DPa, M2-DPb and M1-DP-AV4 were similar. In M3-DP, no *S. medanensis*, *C. arietinum* and yeast proteins were detected. Instead, this sample contained *D. farinae* proteins (226 peptides assigned to 73 proteins) and a substantial amount of human serum proteins (2391 peptides, 641 proteins). In contrast, the authority-approved SPT solution (ref-DP) contained exclusively *D. pteronyssinus* proteins (1254 peptides assigned to 57 proteins). Fig. 3 shows the quantitative comparison of proteins/allergens identified in the samples and reference. A detailed analysis of all allergens

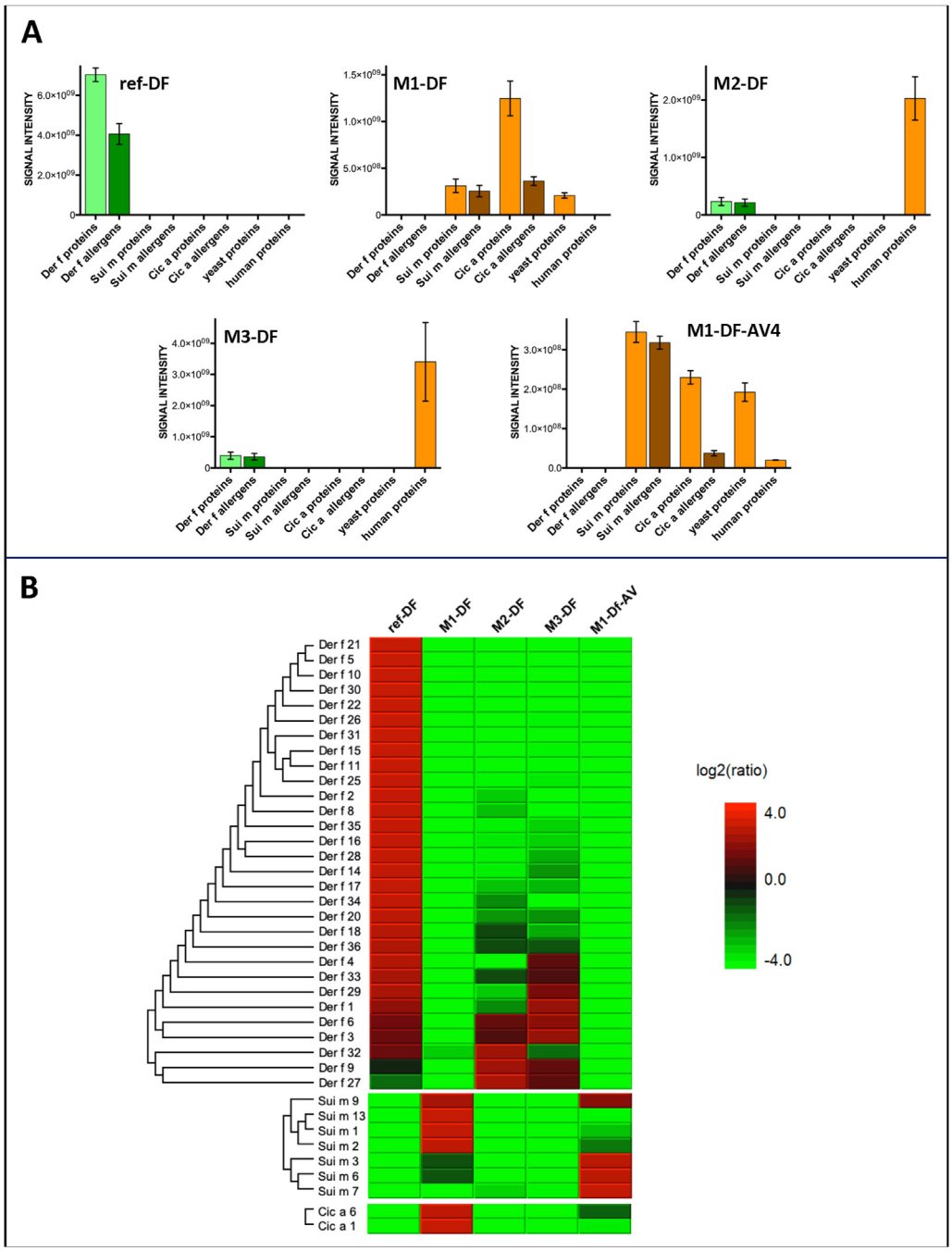

Fig. 4 Allergen and total protein content of the *D. farinae* products compared to the reference. For details, see the legend of Fig. 3.

detected in these samples is given in Supplemental Fig. 3. Allergens Der p 26, 28, 29, 30, 31, and 37 were also identified in the reference, but not included in this figure, as their protein sequences are not listed in public databases.

D. farinae samples

Similar to the *D. pteronyssinus* samples of manufacturer M1, the SPT solution M1-DF and allergy vaccine M1-DF-AV4 contained *S. medanensis*, *C. arietinum* and yeast proteins/allergens No *D. farinae* proteins could be detected (Fig. 4). For manufacturers M2 and M3, both M2-DF and M3-DF samples contained mostly human serum proteins and a limited number of *D. farinae* proteins (Fig. 4). In sample M2-DF, 251 *D. farinae* peptides (39 proteins) and in M3-DF, 224 *D. farinae* peptides (43 proteins) were identified. This is significantly less than in the reference ref-DF, where 1302 *D. farinae* peptides (assigned to 83 proteins) were identified. The number and relative amounts of *D. farinae* allergens was also reduced in these products, when compared to the reference (Supplementary Fig. 4). Whereas the reference contained a total of 30 *D. farinae* allergens, in M2-DF and M3-DF only 15 allergens could be identified. In M2-DF and M3-DF, both Der f 1 and Der f 2 were present, but only in reduced amounts (Der f 1: 16% and 41% of the reference, respectively, Der f 2: 1% and 0.7% of the reference, respectively) (see summarizing Fig. 7). The following allergens were not detectable in M2-DF and M3-DF, although present in the reference: Der f 4, 5, 7, 8, 15, 16, 20, 26, 28, 31, 32 and 33. Der f 18 and 21 were present in M3-DF, but missing in M2-DF. On the other hand, Der f 25, 29 and 34 were present in M2-DF, but missing in M3-DF. It is interesting to note that the reference sample contained a significant amount of non-allergenic *D. farina* proteins, mostly common household enzymes. As a result, the intensity ratio of total Der f proteins to Der f allergens was close to 2:1. In samples M2-DF and M3-DF, on the other hand, non-allergenic proteins are nearly completely absent (data not shown), resulting in a total Protein to allergen ratio of 1:1 (Fig. 4A). Human serum proteins constituted the majority of proteins in samples M2-DF (1542 peptides, 479 proteins/protein families) and M3-DF (1775 peptides, 668 proteins/protein families). Abundant human proteins identified in the *D. farinae* samples are listed in Supplementary Table 1.

B. tropicalis sample

Only 1 *B. tropicalis* SPT solution was available for this study. As shown in Fig. 5 and Supplementary Fig. 5, M1-BT did not contain any identifiable *B. tropicalis* proteins/allergens. The constituents of this sample, similar to the

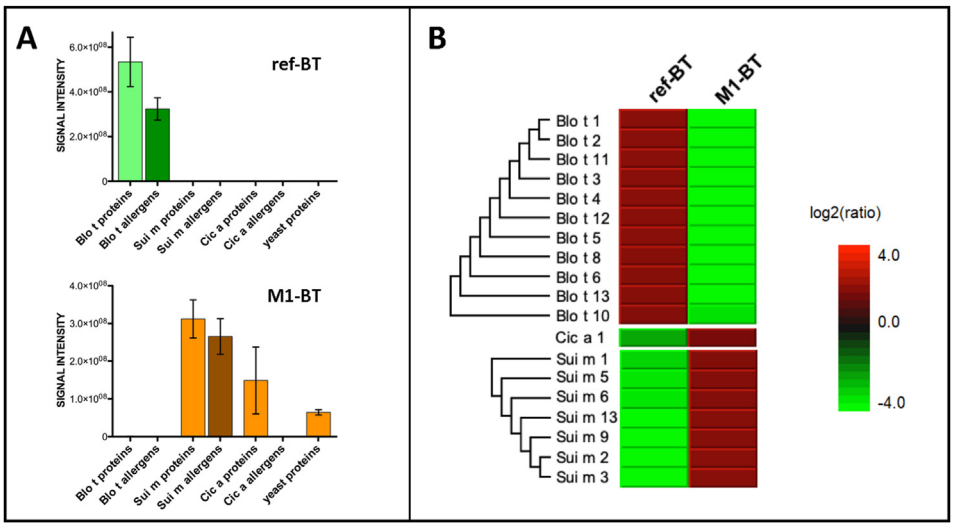

Fig. 5 Allergen and total protein content of the *B. tropicalis* sample compared to the reference. For details, see the legend of Fig. 3.

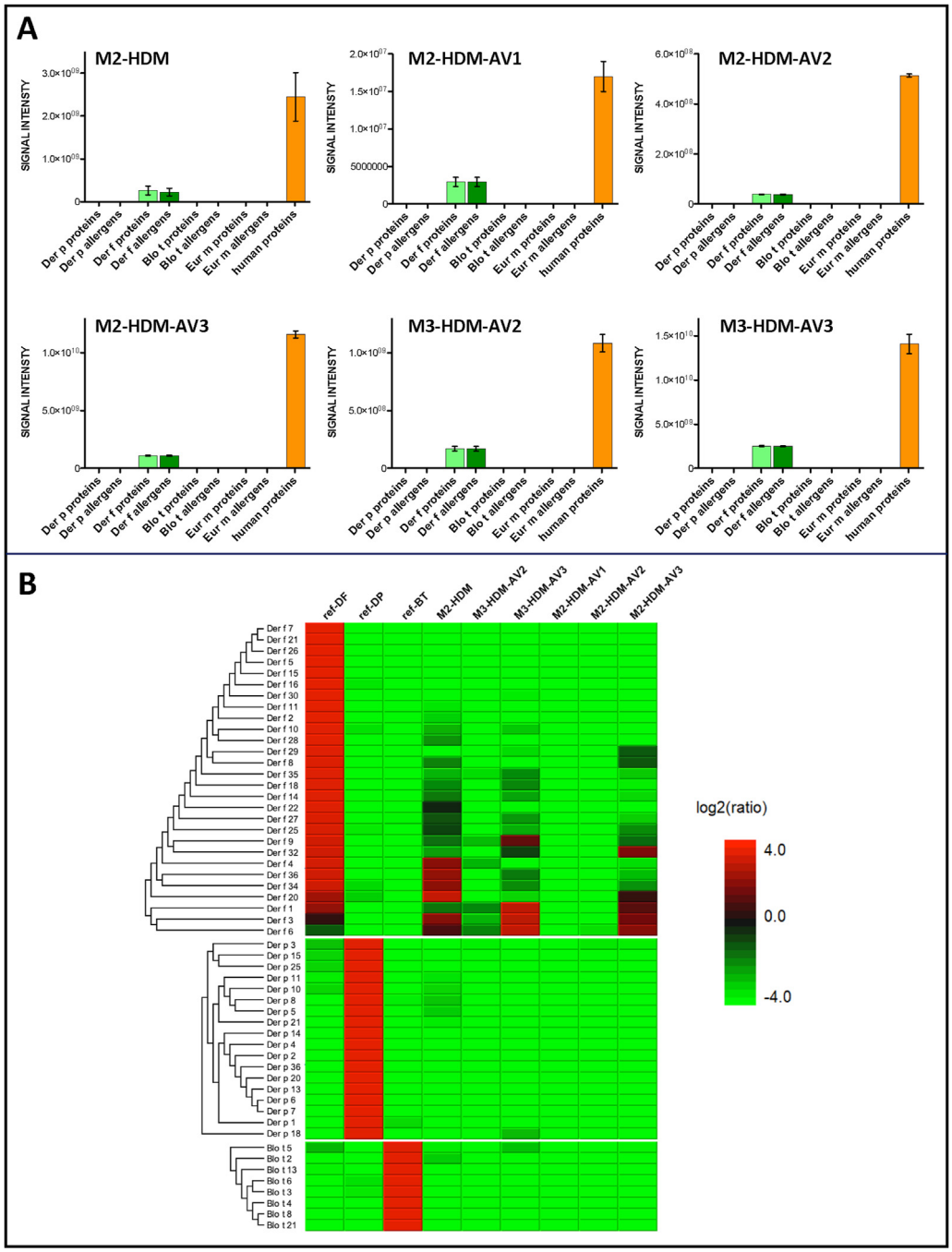

Fig. 6 Protein and allergen content of the house dust mite (HDM) products (A) and comparison to the *D. pteronyssinus*, *D. farinae* and *B. tropicalis* references (B). For details, see the legend of Fig. 3.

D. pteronyssinus and *D. farinae* products of this manufacturer, were proteins from *S. medanensis* (236 peptides, assigned to 13 proteins), *C. arietinum* (358 peptides, 124 proteins) and baker's yeast (287 peptides, 106 proteins). In addition, nearly 8000 peptides without a sequence equivalent in UniProt were detected in M1-BT. This might be due to the fact that only a limited number of sequences of *S. medanensis* and *C. arietinum* proteins are presently available. A total of 7 *S. medanensis* allergens were identified. Cic a 1 was the only identified chickpea allergen, albeit in minute amounts. By contrast, 44 *B. tropicalis* proteins (1401 peptides) and 11 *B. tropicalis* allergens (988 peptides) were identified in ref-BT.

House dust mites (HDM) samples

"House dust mites" generally refers to mites of the species *D. pteronyssinus*, *D. farinae*, *B. tropicalis,* and *Euroglyphus maynei*.[27] Consequently, refined database searches concentrated on proteins of these mites. In all 6 samples labeled "House Dust Mites" (M2-HDM, M2-HDM-AV1, M2-HDM-AV2, M2-HDM-AV3, M3-HDM-AV2, M3-HDM-AV3), no *D. pteronyssinus*, *B. tropicalis* or *E. maynei* proteins were identified. The only mite proteins detected were from *D. farinae* (Fig. 6). As with the *D. farinae* samples, the allergen composition of all samples significantly differed from the reference, Der f 1 and Der f 3 being the only allergens present in all samples. Other allergens (*e.g.* Der f 4, 5, 7, 8) were not detectable (Fig. 6B and Supplementary Fig. 6). The major constituents of all commercial HDM SPT solutions and AVs were human serum proteins (Fig. 6A).

Fig. 7 summarizes the main results of this study for selected mite allergens. The amounts of 3 important *D. pteronyssinus*, *D. farinae,* and *B. tropicalis* allergens detected in the corresponding samples are shown and compared to the references. Der p 1, Der p 2 and Der p 5 were not detected in any of the samples, just as Blo t 1, Blo t 2, Blo t 5 and Der f 5. All these allergens were present in the references. Der f 2 was present, albeit in very small amounts, in 2 *D. farinae* and 1 HDM SPT solution, as well as in 3 HDM AVs. Although in lower amounts than in the reference, Der f 1 was present in 2 *D. farinae* SPT solutions (M2-DF, M3-DF) and in 2 HDM AVs (M2-HDM-AV3, M3-HDM-AV3).

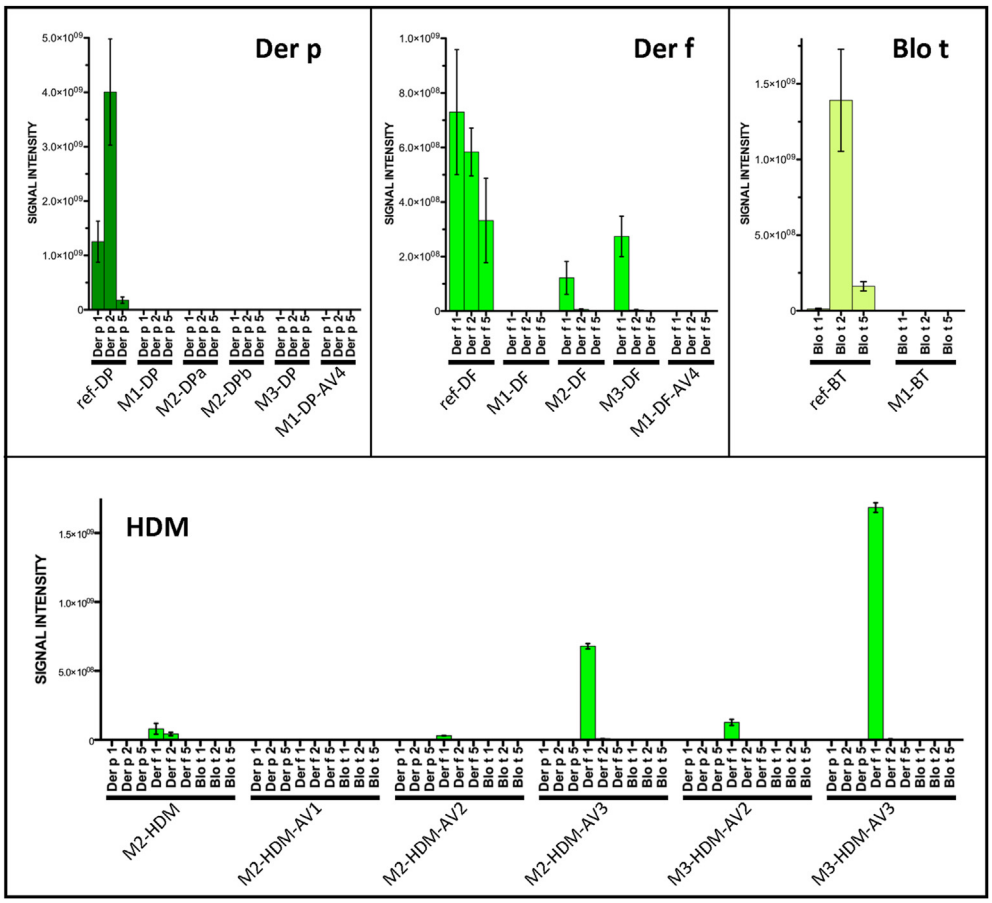

Fig. 7 Direct quantitative comparison of selected important allergens in SPT-solutions and allergy vaccines with the corresponding references. Signal intensities are shown in linear scale.

DISCUSSION

Allergic sensitization to HDM allergens is a major risk factor for allergic rhinitis and asthma,[20,28] with more than 50% of asthmatic patients being allergic to either *D. pteronyssinus* or *D. farinae*, or to both species. Thus, accurate diagnosis based on information about exposure to allergen sources combined with appropriate tests to confirm sensitization[29] is critical for the management and therapy of HDM allergy. However, studies focusing on HDM prevalence and quality of diagnostic tools reported wide variability on the allergen composition of commercial HDM extracts used for in vivo diagnosis through SPT.[24,30,31] The lack of comparability between allergen extracts, particularly for HDM, is a well-known problem. Variability of these diagnostic/therapeutic products seems to be linked to: (i) cultivation conditions employed for different mite species, and (ii) materials used for the extract preparation (*eg*, mite bodies, fecal particles, mite bodies plus fecal particles) and their degree of purity.[30–32] Together, these 2 factors tremendously impact the protein composition and concentrations of the extracts. Further, insufficient standardization of these extracts, which in most cases is based solely on "in-house assays", likely accounts for the large differences among allergenic products from various manufacturers.[11]

In our study, we uncovered additional problems with HDM SPT solutions and AVs commercialized by 3 different Indian manufacturers, namely a discrepancy between the products' labels and their contents. None of the 5 *D. pteronyssinus*-labeled products contained *D. pteronyssinus* proteins. Instead, 1 sample contained *D. farinae* and human serum proteins, and the remaining 4 products contained allergens from the storage mite *S. medanensis* and from chickpea, as well as proteins from baker's yeast. From 4 *D. farinae*-labeled products, 2 contained human serum proteins plus a limited number of *D. farinae* allergens and the other 2 contained only *S. medanensis,* chickpea and yeast proteins. The *B. tropicalis* sample contained no *B. tropicalis* allergens at all, but consisted exclusively of *S. medanensis*, chickpea and yeast proteins. All 6 HDM-labeled samples consisted of human serum proteins and limited amounts of *D. farinae* allergens. These alarming findings demonstrate that the challenge in standardization and quality control of allergenic products should not be overlooked and underestimated.

Although it is certainly difficult to explain these results, it could be speculated that the divergent contents of these products could have originated from the production of the mite source material. In general, the production of HDM extracts involves cultivation and growth of mites in large quantities under specified and controlled conditions using appropriate culture media.[32] In the past, HDM were cultured using human skin scales, which are their natural food source.[33] Later on, alternative culture media consisting of animal dander, dog food, rodent chow, or fungal cultures were developed.[34] Presently, manufacturers use a mix of different food sources (eg, soybean powder, wheat germ) or yeast supplemented with vitamins[35] or amino acids[36] to culture mites. Thus, it is conceivable that yeast and chickpeas were used in the cultivation media for the HDM products analyzed here. Independent of the type of culture media used for the mite cultivation, the FDA sets that foreign material should not exceed 1% of the raw preparation,[37] whereas the European Pharmacopoeia indicates that foreign species should not be present in the raw material.[38] For approval of allergen products, the Indian Pharmacopoeia Commission (IPC) indicates that: (i) contamination by foreign species/strains should be avoided, (ii) the composition of culture media must be justified, and (iii) purification procedures should be designed to minimize the presence of potential irritants and non-allergenic components. However, the IPC Guidelines are very general and do not give precise specifications for allergen products.[39] The complete absence of *D. pteronyssinus* proteins (allergenic and non-allergenic) indicates that the respective SPT and AV products contained only culture media components contaminated by other mite species such as *D. farinae* and *Suidasia medanensis*. Because mites belonging to the genus *Suidasia* are common in house dust and extensive IgE cross reactivity with *D. farinae* and *B. tropicalis* have been reported,[40] the *D. pteronyssinus*-labeled products would not identify genuinely *D. pteronyssinus*-sensitized

patients. Similarly, the *B. tropicalis*-labeled products would fail to diagnose *B. tropicalis*-sensitization and would give false positive tests for patients sensitized to chickpeas and/or *S. medanensis*.

In the process of manufacturing allergen extracts from raw materials, it is considered appropriate to include measures to minimize allergen degradation (eg, extraction time, temperature, pH) and to maximize stability (eg, aqueous formulation containing 50% glycerol or 0.01–0.05% human serum albumin; inclusion of preservatives such as phenol).[32] Of note, the inclusion of human or animal products in the culture medium or extracts needs to be justified and appropriate measures (eg, γ-radiation) should be employed to ensure that they do not contain any potential pathogens.[32] Our data revealed that both *D. pteronyssinus*- and *D. farinae*-labeled products contained either no mite proteins or very low amounts of *D. farinae* allergens. Instead, large amounts of human serum proteins made up the bulk of the proteinaceous material in these samples. It remains unclear whether human proteins were used in the culture media or as stabilizer in the extract formulation. In addition to serum albumin, we detected high amounts of several other proteins found in human serum (eg, immunoglobulins, S100 proteins, transferrin, lactoferrin, complement, macroglobulin) supporting the notion that human serum was included in the manufacturing process. In contrast, European SPT products used as reference in this study covered the respective allergen spectra and lacked contaminations.

CONCLUSIONS

The value of extract-based mite products for diagnosis and therapy depends on several factors, including the selection of the mite species, appropriate cultivation, and the manufacturing procedures used to process the raw material and to prepare the mite extracts. Thus, characterization and standardization of such extracts is the difficult but inescapable task for diagnosis and therapy of allergic diseases caused by mites. Our investigation demonstrated the value of mass spectrometry to fingerprint complex allergen extracts. The use of non-standardized diagnostic and therapeutic products, such as the ones analyzed here, could lead not only to misdiagnosis of HDM allergy, due to contamination by allergens from other sources, but also their application may be even harmful to patients, as some of these products contain large amounts of proteins of human origin.

Ethics approval

The use of dust mite patients' sera was approved by the ethics committee of the University of Vienna (Ethics permission number EK1263_2014 to BB).

Authors contributions

SH: Made substantial contributions to design of experiments, acquisition of data and drafting the manuscript.

GG: Made substantial contributions to the design of experiments and drafting the manuscript.

BB: Made substantial contributions to the design of experiments.

FF: Made substantial contributions to the design of experiments and drafting the manuscript.

PB: Made substantial contributions to the design of experiments, acquisition and analysis of proteomic data and drafting the manuscript.

Availability of data

Mass spectrometry proteomics data of one representative dataset per sample have been deposited to the ProteomeXchange Consortium via the PRIDE[41] partner repository with the dataset identifier PXD021383 and 10.6019/PXD021383.

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Confirmation of unpublished work

The authors declare that this manuscript is original, has not been published before, is not currently being considered for publication elsewhere, and has not been posted to a preprint server.

Consent for publication

All authors agreed to the publication of the work.

Declaration of competing interest

S. Huber reports personal fees from Allergopharma GmbH & Co. KG during the conduct of the study. F. Ferreira is a member of Scientific Advisory Boards (HAL Allergy, NL;

AllergenOnline, USA) and has been supported by the Austrian Science Fund (FWF). The rest of the authors declare that they have no relevant conflict of interest in relation to the methods or materials employed in this study.

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Appendix ASupplementary data
Supplementary data to this article can be found online at https://doi.org/10.1016/j.waojou.2021.100516.

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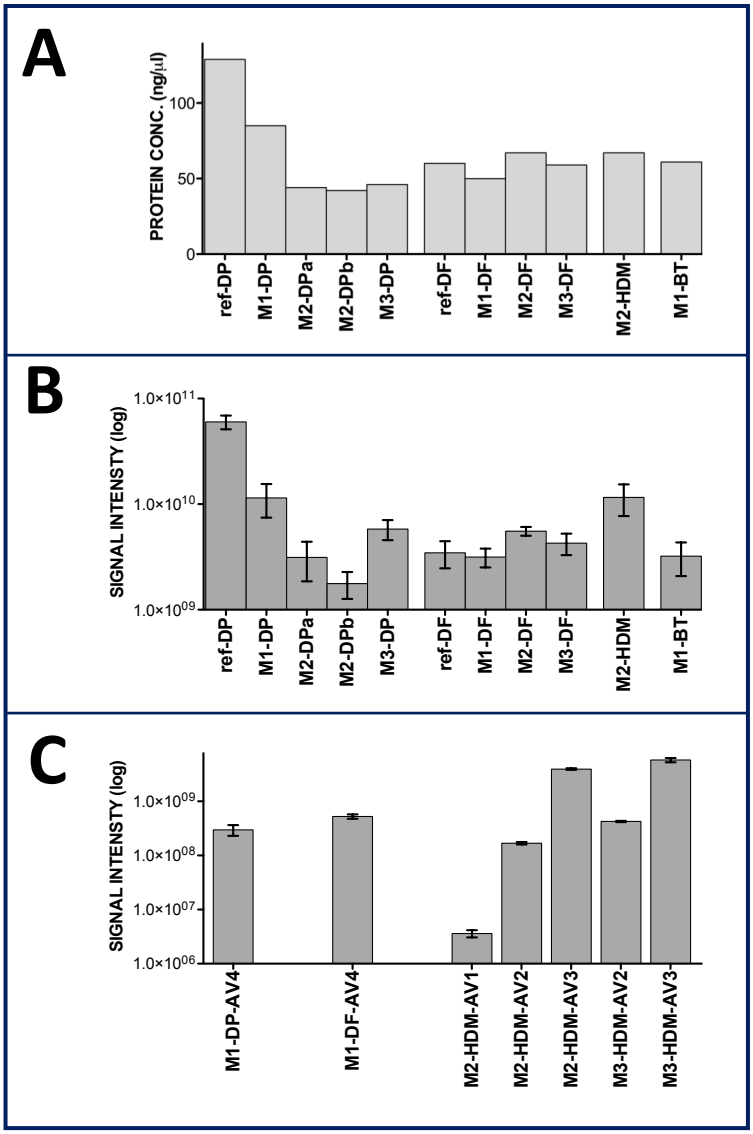


Supplementary Figure 1. Comparative protein quantification using a photometric assay (Pierce 660nm) (A) and summarized signal intensities obtained by mass spectrometry (B, C). A and B show data from the SPT solutions, C from AVs. Total signal intensities were determined by calculating the sum of the intensities of all *de novo* sequenced peptides. It should be noted that values of SPT solutions (B) were obtained from 50 µl sample, while values of AVs (C) were obtained from 500 µl sample.

ref-DP

Initial search
(complete database)

# peptides assigned to	
Der p 2	Der f 2
8	3

Refined search
(multi-round with Der p and Der f sequence sets)

	# peptide matches
1. round: Der p	8
2. round: Der f	no match
control:	
1. round: Der f	3
2. round: Der p	5

```
Der p 2   MMYKILCLSLLVAAVARDQVDVKDCANHEIKKVLVPGCHGSEPCIIHRGKPFQLEAVFEA 60
Der f 2   MISKILCLSLLVAAVVADQVDVKDCANNEIKKVMVDGCHGSDPCIIHRGKPFTLEALFDA 60

Der p 2   NQNTKTAKIEIKASIDGLEVDVPGIDPNACHYMKCPLVKGQQYDIKYTWNVPKIAPKSEN 120
Der f 2   NQNTKTAKIEIKASLDGLEIDVPGIDTNACHFMKCPLVKGQQYDIKYTWNVPKIAPKSEN 120

Der p 2   VVVTVKVMGDDGVLACAIATHAKIRD 146
Der f 2   VVVTVKLIGDNGVLACAIATHGKIRD 146
```

M3-DP

Initial search
(complete database)

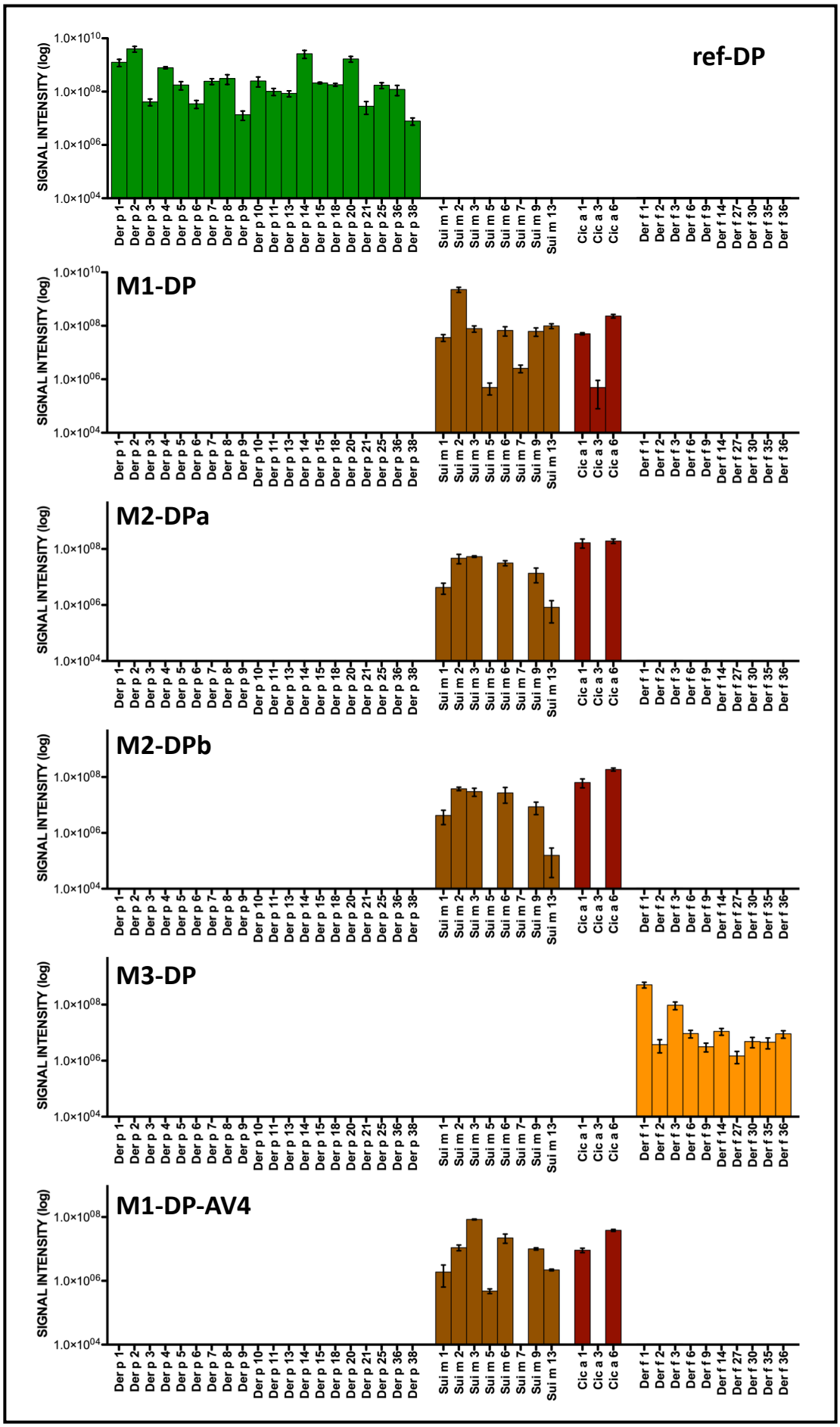
# peptides assigned to	
Der p 2	Der f 2
2	5

Refined search
(multi-round with Der p and Der f sequence sets)

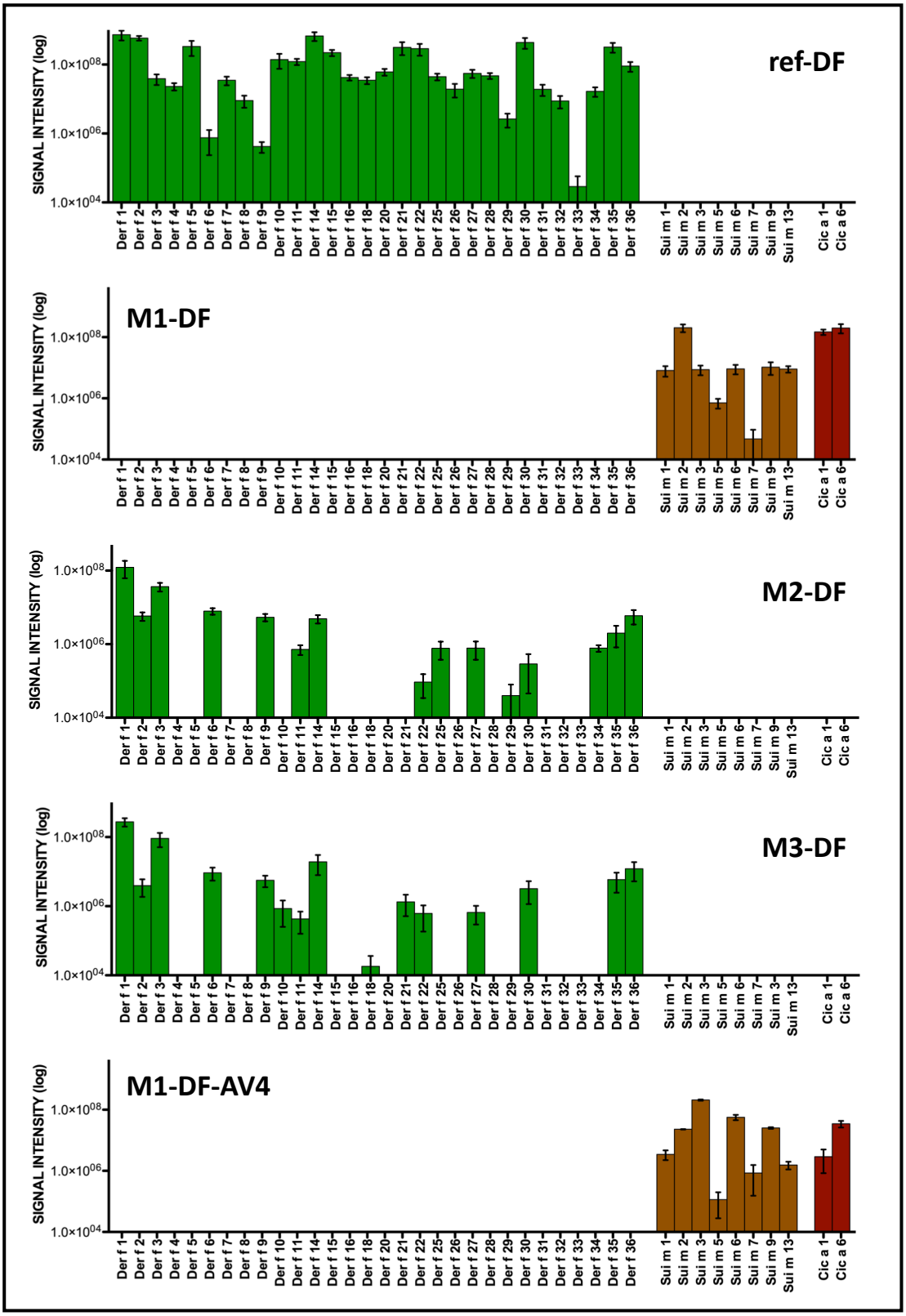
	# peptide matches
1. round: Der f	5
2. round: Der p	no match
control:	
1. round: Der p	2
2. round: Der f	3

```
Der p 2   MMYKILCLSLLVAAVARDQVDVKDCANHEIKKVLVPGCHGSEPCIIHRGKPFQLEAVFEA 60
Der f 2   MISKILCLSLLVAAVVADQVDVKDCANNEIKKVMVDGCHGSDPCIIHRGKPFTLEALFDA 60

Der p 2   NQNTKTAKIEIKASIDGLEVDVPGIDPNACHYMKCPLVKGQQYDIKYTWNVPKIAPKSEN 120
Der f 2   NQNTKTAKIEIKASLDGLEIDVPGIDTNACHFMKCPLVKGQQYDIKYTWNVPKIAPKSEN 120

Der p 2   VVVTVKVMGDDGVLACAIATHAKIRD 146
Der f 2   VVVTVKLIGDNGVLACAIATHGKIRD 146
```

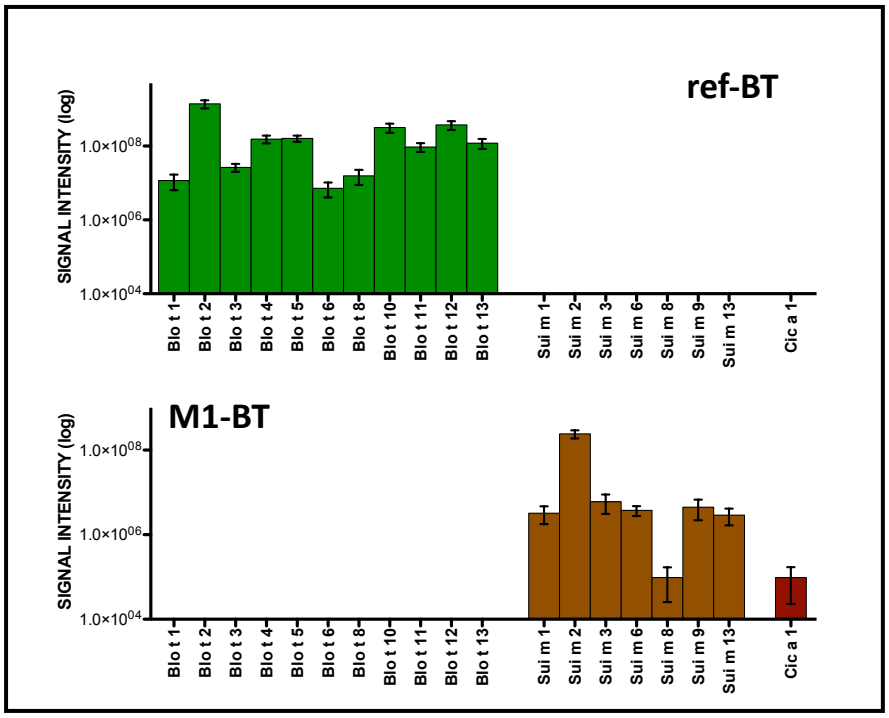
Supplemental Figure 2. Distinction between closely related allergens using multi-round searches and diagnostic peptides. As example, the discrimination between Der p 2 and Der f 2 in samples ref-DP (upper panel) and M3-DP (lower panel) is shown. The tables show the number of peptides identified when the search order was changed. In the sequence panels, the sequence coverage is indicated. Identified tryptic peptides matching exclusively Der p 2 sequences are indicated in green, peptides matching exclusively Der f 2 are indicated in blue. Shared peptides are shown in yellow. Amino acid residues differing in both allergens are shown in red.



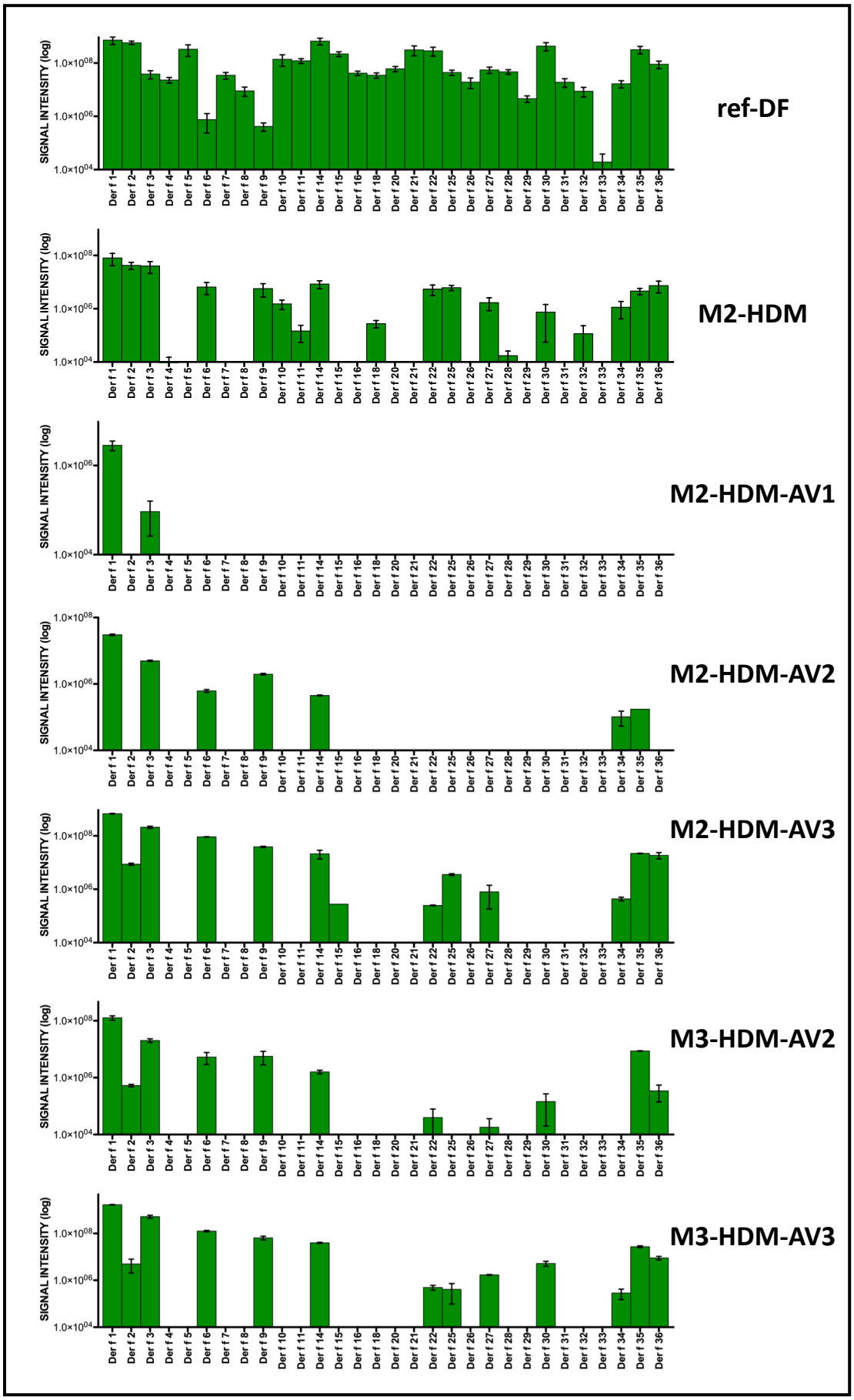
Supplementary Figure 3. Comparison of allergens detected in *D. pteronyssinus* SPT-solutions, AV and reference. Note that signal intensities are plotted in logarithmic scale. No allergens with intensities below 10^4 could be detected.



Supplementary Figure 4. Comparison of allergens detected in the *D. farinae* SPT solutions, AV and reference. Note that signal intensities are plotted in logarithmic scale. No allergens with intensities below 10^4 could be detected.



Supplementary Figure 5. Comparison of allergens detected in the B. tropicalis skin prick test solution and reference. Note that signal intensities are plotted in logarithmic scale. No allergens with intensities below 10^4 could be detected.



Supplementary Figure 6. Comparison of allergens detected in house dust mite (HDM) skin prick test (SPT) solutions and allergy vaccines (AV). Note that signal intensities are plotted in logarithmic scale. No allergens with intensities below 10^4 could be detected.

Accession UniProt	Score (-10lgP)	Sequence Coverage %					Peptides Identified					Description
		ref-DF	M1-DF	M2-DF	M3-DF	M2-HDM	ref-DF	M1-DF	M2-DF	M3-DF	M2-HDM	
F6KPG5	424,64	0	0	68	64	68	0	0	93	88	74	Albumin (Fragment)
F6KPG5	411,15	0	0	70	70	68	0	0	72	60	52	Albumin isoform CRA_k
Q6GMX6	316,54	0	0	40	34	49	0	0	30	18	42	IGH@ protein
Q6MZV7	308,94	0	0	39	32	47	0	0	26	17	38	Uncharacterized protein DKFZp686C11235
P06702	306,74	0	0	73	78	75	0	0	45	78	82	Protein S100-A9
Q86W05	295,76	0	0	41	58	75	0	0	25	36	55	Squamous cell carcinoma antigen 2
Q6GMX0	290,13	0	0	37	37	52	0	0	15	9	17	Uncharacterized protein
Q6P5S8	288,86	0	0	37	37	48	0	0	15	9	17	IGK@ protein
P0DOX7	286,82	0	0	41	41	50	0	0	14	9	16	Immunoglobulin kappa light chain
Q0KKI6	286,02	0	0	40	40	47	0	0	15	9	16	Immunoblobulin light chain (Fragment)
P31151	285,43	0	0	79	75	78	0	0	33	23	40	Protein S100-A7
B3VMW0	278,56	0	0	20	30	32	0	0	13	23	23	Lactoferrin
E9KL23	270,98	0	0	29	22	32	0	0	20	15	22	Epididymis secretory sperm binding protein
P30740	269,7	0	0	34	39	32	0	0	15	19	17	Leukocyte elastase inhibitor
Q06AH7	269,54	0	0	16	28	29	0	0	9	20	20	Transferrin
A0A384MDQ7	268,1	0	0	29	18	32	0	0	19	13	21	Epididymis secretory sperm binding protein
P05109	265,73	0	0	83	83	83	0	0	27	53	67	Protein S100-A8
B4E1B2	265,25	0	0	14	26	28	0	0	8	18	19	cDNA FLJ53691 highly similar to Serotransferrin
Q6N030	264,7	0	0	21	16	26	0	0	21	10	26	Uncharacterized protein
A0A024R6P0	256,48	0	0	13	25	31	0	0	5	12	14	Serpin peptidase inhibitor clade A
P01024	248,9	0	0	2	4	15	0	0	2	5	19	Complement C3
C9JZ65	245,51	0	0	32	61	65	0	0	12	18	26	Serpin B4 (Fragment)
S6C4R6	236,15	0	0	34	23	34	0	0	11	6	11	IgG L chain
P02675	226,14	0	0	18	9	14	0	0	6	3	4	Fibrinogen beta chain
S6B294	224,43	0	0	19	18	34	0	0	9	5	10	IgG L chain
S6AWF4	219,65	0	0	19	19	24	0	0	9	5	9	IgG L chain
C9JC84	216,82	0	0	19	13	20	0	0	8	5	10	Fibrinogen gamma chain
Q3KRA7	211,09	0	0	19	17	16	0	0	7	8	6	FGA protein (Fragment)
P01023	210,08	0	0	2	1	12	0	0	2	1	14	Alpha-2-macroglobulin
A6XGL1	196,91	0	0	27	40	33	0	0	8	5	6	Transthyretin
Q96K68	195,38	0	0	10	10	20	0	0	4	4	8	cDNA highly similar to Homo sapiens SNC73
Q86SG5	194,65	0	0	42	39	72	0	0	15	12	23	Protein S100-A7A
P00738	193,71	0	0	0	21	31	0	0	0	8	14	Haptoglobin
A0A0U4BW16	191,83	0	0	2	2	2	0	0	2	3	4	Non-muscle myosin heavy chain 9
H0Y7A7	189,02	0	0	14	20	34	0	0	2	4	7	Calmodulin-2 (Fragment)
P02751	189,01	0	0	3	1	4	0	0	4	1	6	Fibronectin
P25311	188,88	0	0	13	13	22	0	0	3	3	6	Zinc-alpha-2-glycoprotein
A2KBC0	187,41	0	0	15	24	40	0	0	2	5	10	Anti-(ED-B) scFV (Fragment)
Q9NZT1	185,32	0	0	16	23	59	0	0	1	2	6	Calmodulin-like protein 5
Q8WY24	180,47	0	0	4	6	22	0	0	2	2	8	SNC66 protein
P01008	179,55	0	0	12	18	13	0	0	4	7	5	Antithrombin-III
V9HWF6	179,11	0	0	22	27	22	0	0	7	11	5	Alpha-1-acid glycoprotein
P02790	176,62	0	0	5	0	19	0	0	2	0	5	Hemopexin
Q8NEJ1	173,04	0	0	31	6	40	0	0	5	1	8	Uncharacterized protein
P19652	172,3	0	0	27	27	36	0	0	6	7	6	Alpha-1-acid glycoprotein 2
Q60FE6	170,95	0	0	3	2	4	0	0	4	3	6	Filamin A
P01040	169,4	0	0	31	62	77	0	0	2	4	7	Cystatin-A
Q96P63	167,57	0	0	15	0	8	0	0	3	0	2	Serpin B12
P0DOX8	165,82	0	0	22	9	29	0	0	4	1	6	Immunoglobulin lambda-1 light chain
Q0VAS5	161,54	0	0	0	26	39	0	0	0	11	8	Histone H4
Q86TT1	157,39	0	0	12	3	19	0	0	3	1	5	Full-length cDNA clone of Neuroblastoma
P00739	153,33	0	0	0	13	19	0	0	0	4	9	Haptoglobin-related protein
A0A024QZX5	149,26	0	0	8	3	16	0	0	3	1	5	Serpin B6
A2IPI3	139,26	0	0	14	14	54	0	0	1	1	5	HRV Fab N28-VL (Fragment)
sp|Q08188	139,07	0	0	3	0	12	0	0	1	0	7	Protein-glutamine gamma-glutamyltransferase E
sp|P08779	136,67	0	0	7	7	3	0	0	2	4	1	Keratin type I cytoskeletal 16
B2MUD5	130,1	0	7	0	18	5	0	0	0	4	1	Neutrophil elastase (Fragment)
A0A125QYY9	129,06	0	0	13	6	30	0	0	1	1	4	IBM-B2 heavy chain variable region (Fragment)
A0A024R321	128,79	0	0	1	0	1	0	0	2	0	2	Filamin B beta (Actin binding protein 278) isoform CRA_a
A8K4B1	127,8	0	0	5	0	9	0	0	2	0	4	cDNA FLJ77984 highly similar to serpin peptidase inhibitor
P02753	127,08	0	0	15	0	15	0	0	3	0	3	Retinol-binding protein 4
Q6N091	126,15	0	0	9	3	6	0	0	3	1	2	Uncharacterized protein DKFZp686C02220 (Fragment)
P2016	126,05	0	0	8	23	8	0	0	2	4	2	Azurocidin
A5PL27	126,02	0	0	1	0	6	0	0	1	0	4	CP protein
O75594	124,1	0	0	10	18	26	0	0	1	2	3	Peptidoglycan recognition protein 1
P12273	123,23	0	0	18	0	16	0	0	3	0	2	Prolactin-inducible protei
P07108	123,06	0	0	0	47	57	0	0	0	6	6	Acyl-CoA-binding protein
P0DP02N	122,65	0	0	0	19	36	0	0	0	3	3	Immunoglobulin heavy variable 3-30-3
P31997	121,77	0	0	10	0	8	0	0	2	0	2	Carcinoembryonic antigen-related cell adhesion molecule 8
Q9UL90	116,75	0	0	0	19	31	0	0	0	3	3	Myosin-reactive immunoglobulin heavy chain variable region
A0A140VJQ2	114,05	0	0	3	19	11	0	0	1	2	2	Testicular tissue protein Li 128
P04080	113,68	0	0	34	0	31	0	0	2	0	3	Cystatin-B
A0A0B4J1X5	112,98	0	0	16	32	38	0	0	1	3	3	Immunoglobulin heavy variable 3-74
P01762	112,9	0	0	0	15	38	0	0	0	2	3	Immunoglobulin heavy variable 3-11
P01594	108,12	0	0	14	14	29	0	0	1	1	2	Immunoglobulin kappa variable 1-33
A0A0C4DH38	107,48	0	0	0	0	48	0	0	0	0	4	Immunoglobulin heavy variable 5-51
P80511	106,38	0	0	0	23	22	0	0	0	5	3	Protein S100-A12
A0A0B4J2H0	105,03	0	0	9	0	39	0	0	1	0	3	Immunoglobulin heavy variable 1-69D
P01833	105,02	0	0	0	0	5	0	0	0	0	3	Polymeric immunoglobulin receptor
A2JA14	101,62	0	0	0	18	19	0	0	0	3	2	Anti-mucin1 heavy chain variable region (Fragment)
B3GN61	101,09	0	0	0	0	4	0	0	0	0	2	Truncated E-cadherin
A0A3B3ISA6	99,2	0	0	0	1	2	0	0	0	1	2	Complement C4-B (Fragment)
Q9UL85	97,14	0	0	17	0	25	0	0	1	0	2	Myosin-reactive immunoglobulin kappa chain variable region

Supplementary Table 1. Human proteins in *D. farinae* and house dust mite (HDM) skin prick test solutions. The table shows the top hits identified in a search of *de novo* sequenced peptides against the human UniProt database.