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Minor allergen patterns in birch pollen allergen products – A question of pollen?

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ABSTRACT

Background:

Contrary to the scientific differentiation between major and minor allergens, the regulatory framework controlling allergen products in the EU distinguishes relevant and non-relevant allergens. Given the lack of knowledge on their clinical relevance, minor allergens are usually not controlled by allergen product specifications. Especially in birch pollen (BP) allergen products, minor allergens are commonly disregarded.

Objectives:

To quantify three minor allergens in BP allergen products from different manufacturers and to assess the influence of the utilized BP on minor allergen patterns.

Methods:

Besides common quality parameters like Bet v 1 content, Bet v 4, Bet v 6 and Bet v 7 were quantified in 70 BP allergen product batches from six manufacturers, using ELISA systems developed in-house. Batch-to-batch variability was checked for agreement with a variability margin of 50-200% from mean of the given batches for individual allergen content. Subsequently, minor allergen patterns were generated via multidimensional scaling and related to information on the pollen lots used in production of the respective product batches.

Results:

Like the already established Bet v 4 ELISA, the ELISA systems for quantification of Bet v 6 and Bet v 7 were successfully validated. Differences in minor allergen content between products as well as batch-to-batch consistency were observed. Correlations between minor and major allergen content were low to moderate. 20% of batches exceeded the variability margin for at least one minor allergen. Interestingly, these fluctuations could not in all cases be linked to the use of certain BP lots.

Conclusions & Clinical Relevance:

The impact of the observed minor allergen variability on safety and efficacy of BP allergen products can currently not be estimated. As the described differences could only in few cases be related to the used pollen lots, it is evident that additional factors influence minor allergens in BP allergen products.

KEYWORDS

Bet v 6, Bet v 7, minor birch pollen allergen, allergen immunotherapy

ABBREVIATIONS

AIT	allergen immunotherapy
BP	birch pollen
BPE	birch pollen extract
Bet v	birch allergen (<i>Betula verrucosa</i> , nomenclature according to the <i>International Union of Immunological Societies</i>)
CI	95% confidence interval
CV	coefficient of variation
HRP	horseradish peroxidase
Ig	immunoglobulin
mAb	monoclonal antibody
MDS	multidimensional scaling
MTP	microtiter plate
nBet v	native birch allergen
OD	optical density
Ph. Eur.	European Pharmacopoeia
PTS	prick test solution
rBet v	recombinant birch allergen
SD	standard deviation

SLIT	sublingual immunotherapy
SCIT	subcutaneous immunotherapy
TMB	3,3',5,5'-Tetramethylbenzidine

INTRODUCTION

All biological allergen products currently marketed in the European Union are based on natural source materials. Hence they contain a complex mixture of allergenic and non-allergenic molecules and are variable in content and concentration ratios of different ingredients. Several factors have been described to influence the allergen content of e.g. pollen, including the year of the harvest, the location of the plant as well as the maturity grade of the pollen [1–3]. Due to this inherent bio-variability, allergen products and their regulation differ from most other pharmaceutical products. This is exemplarily reflected by the requirements for potency determination in the *Monograph on Allergen Products* in the European Pharmacopoeia (Ph. Eur.) [4]: The potency of products for allergen-specific immunotherapy (AIT) is to be determined either via inhibition of the binding capacity of specific immunoglobulin (Ig) E antibodies or via quantification of single relevant allergen molecules. Reflecting the anticipated bio-variability, total allergenic activity may vary from 50-150% of a stated amount between product batches, while an even higher variability of 50-200% is permitted in the control of individual relevant allergen molecules.

Given that the requirements of the *Monograph on Allergen Products* relate to all relevant allergen components, there is no regulatory distinction between so-called major and minor allergens. However, both potency tests mainly focus on major allergens, to which more than 50% of allergic individuals are sensitized [5]. In case of total allergenic activity testing, IgEs specific to minor allergens are usually present in some sera, but the content of specific IgE to major allergens is generally much higher. In addition, the sera are commonly not tested for IgEs specific to minor allergens and therefore these IgEs are further diluted when preparing the sera pool according to the *Guideline on Allergen Products: Production and Quality Issues* [6]. Thus, changes in minor allergen content

between product batches will usually be hardly detectable in standard tests for total allergenic activity. In comparison, quantification of individual allergen molecules could include the control of single minor allergen molecules, provided a central question in the field of allergology is answered: Which allergen molecules have to be contained in an allergen product? By definition, major allergens are believed to have a great impact, although not all of them show a high IgE binding capacity [7] or are as dominant as Bet v 1, to which virtually every birch pollen (BP) allergic patient is sensitized [8]. In comparison, minor allergens with sensitization rates below 50% are commonly regarded as irrelevant. However, the distinction between major and minor allergens is solely based on the prevalence of specific IgE, a parameter which should not be mistaken for clinical relevance. Indeed, the definition of relevant allergens as required by the *Monograph on Allergen Products* relates to the capacity of an allergen to influence safety and/or efficacy of an allergen product. Notably, there exist no pre-set criteria for determination of allergen relevance. Consequently, not only findings from clinical studies but also results from *in vitro* experiments like basophil activation tests can be considered. Still, minor allergens have rarely been included in allergen product specifications, although deliberately not excluded in the *Monograph on Allergen Products*.

Nonetheless, there indeed exist some indications for minor allergen relevance, at least in certain patient populations. For instance, it was shown that sensitization to the olive tree (*Olea europaea*) pollen minor allergens Ole e 2 and/or Ole e 10 is an asthma risk factor [9]. Likewise, sensitization to profilin was identified as a marker for more severe symptoms in grass pollen allergic patients [10]. In addition it has been reported that high IgE levels to minor allergens are often associated with extensive cross-reactivities [11, 12]. Nevertheless, there exists only one published example of a minor allergen specification: After discovering that an elevated level of the glucanase Ole e 9 in some product batches was associated with an increased rate of adverse events during AIT, the respective manufacturer introduced a specification limiting the content of this minor allergen [13, 14]. Although this example indicates that variability in minor allergen content may pose a safety risk for sensitized patients, there exists no information on the impact of minor allergens on allergen product efficacy in AIT so far. By contrast, the risk of false-negative results in allergy diagnosis due to variable (minor)

allergen content in allergen extracts is widely recognized and has induced a progressive shift from extract-based to molecule-based allergy diagnosis [15–17].

Competent authorities do not request the control of minor allergens in allergen products due to the obvious lack of studies on their relevance in AIT. Consequently, allergen product manufacturers do currently not consider minor allergens in product specifications, not to mention manufacturing process validation or stability studies. On this background, we were interested in the minor allergen content of BP allergen products available on the German market. As already mentioned, standardization of BP allergen products is particularly biased towards a single major allergen (Bet v 1), because sensitization rates to other BP allergens like the pan-allergen polcalcin (Bet v 4 [18]), phenyl coumaran benzylic ether reductase (Bet v 6 [19]) and cyclophilin (Bet v 7 [20]) are approximately at 20%. However, the prevalence of minor allergen sensitization depends significantly on the patient population examined and the geographic location. For example, reported prevalence of Bet v 4-specific IgE in BP allergic subjects ranges from 2.5% in a Japanese population [21], 3.7% in an North Italian and 14.4% in a South Italian population [22] to 17.8% in a pediatric population in the Czech Republic [23] and 27% in a small group of Italian patients [24]. An effect of minor allergen content on safety and efficacy has been postulated for patients sensitized to minor BP allergens, especially in the approx. 5% of BP allergic patients without detectable sensitization to Bet v 1 [23]. However, studies linking AIT outcome with the sensitization pattern of single patients and the minor allergen content of the product used are currently not available, and likewise the clinical relevance of sensitization to minor allergens remains a matter of debate [25, 26]. Hence it cannot be universally predicted to which degree BP allergic patients would benefit from products additionally standardized in regard to minor allergens. In order to provide a basis for a better understanding of minor BP allergens, we made use of three sandwich ELISA systems specific for Bet v 4, Bet v 6 and Bet v 7 developed in our lab. The results from 70 BP allergen products were tested for agreement with variability margins of 50-200% from mean of the given batches for the three minor BP allergens, following the requirements for individual allergens in the Ph. Eur. Afterwards we combined our results on minor allergen variability with information on the pollen source material lots used in production of the respective products batches.

Thereby, we verified the magnitude of influence of the pollen raw material on the minor allergen pattern in BP allergen products.

MATERIAL&METHODS

Recombinant allergens and BP extract

Three recombinant allergens (rBet v 4, rBet v 6 and rBet v 7) were used as reference standards in the respective ELISA systems, for preparation of standard addition samples during assay validation and for immunizations. rBet v 6 and rBet v 7 were produced in-house as previously described [27], whereas rBet v 4 was produced and kindly provided by Paul Rösch (University of Bayreuth, Bayreuth, Germany) [28]. Birch pollen extract (BPE) purchased from Stallergenes Greer (Antony Cedex, France) containing the three minor allergens in their native form (nBet v 4, nBet v 6 and nBet v 7), was used for sample preparation during assay validation. The amount of the native minor allergens in the BPE was determined relative to the respective recombinant allergen used as reference in allergen-specific sandwich ELISA systems (see below).

Antibodies and antisera

The primary capture antibodies rat anti-mouse IgG1 and rat anti-mouse IgG2a were purchased from Becton Dickinson GmbH (Heidelberg, Germany). The secondary detection antibody (horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG) was purchased from Jackson ImmunoResearch Europe Ltd (Newmarket, United Kingdom).

Bet v 4-specific monoclonal antibody and antiserum

The production of the Bet v 4-specific monoclonal antibody (mAb) in female C3H/HeJ mice (Charles River, Sulzfeld, Germany) and the Bet v 4-specific rabbit antiserum has been described previously [27].

Bet v 6-specific monoclonal antibodies

Female BALB/c mice were immunized repeatedly with 5 µg rBet v 6 adsorbed to aluminium hydroxide (Sigma-Aldrich, Taufkirchen, Germany) via intraperitoneal injection in 2 week intervals. Hybridoma cell lines were generated as previously described [27]. Resulting clones were screened for specificity towards rBet v 6 and nBet v 6 in BPE by indirect ELISA. After clonal expansion in serum-free media and isotype determination via the IsoStrip mouse mAb Isotyping Kit (Roche Diagnostics GmbH, Mannheim, Germany), the Bet v 6-specific IgG1 mP80 and mP83 were purified using a Hi-Trap protein-G column (GE Healthcare, Freiburg, Germany). mAb mP83 was biotinylated with the EZ-Link Sulfo-NHS-LC-Biotinylation Kit (Thermo Fisher Scientific, Waltham, USA). The mAbs were quantified relative to an IgG1 standard (Becton Dickinson) using rat anti-mouse-IgG1 (Becton Dickinson)-coated microtiter plates (MTP), a biotinylated rat anti-mouse IgG1 for detection (Becton Dickinson), streptavidin-HRP (Sigma-Aldrich) and 3,3',5,5'-Tetramethylbenzidine (TMB, Sigma-Aldrich) for visualization at 450 nm with 630 nm as reference.

Bet v 7-specific monoclonal antibody and antiserum

Female BALB/c mice were immunized repeatedly with 10 µg BPE in combination with 5 µg rBet v 7 and aluminium hydroxide (Sigma-Aldrich). Hybridoma cell lines were generated as previously described [27]. Resulting clones were screened for specificity towards rBet v 7 and nBet v 7 in BPE by indirect ELISA. Isotype determination, purification and quantification of the resulting mP74 were performed as described for mP80 and mP83 in the previous paragraph.

Bet v 7-specific rabbit antiserum was produced following a standard procedure. A New Zealand white rabbit was subcutaneously immunized four times with a mixture of 100 µg rBet v 7 and 400 µg BPE, in combination with aluminium hydroxide. IgG titers in the serum were assessed by ELISA titration.

Total protein quantification

The total protein content in cell culture supernatants, extract preparations, recombinant proteins and allergen extract batches was determined as previously described using the method according to Bradford [27].

Allergen-specific ELISAs

Development and validation of all in-house produced minor allergen-specific ELISAs has been performed in accordance with the quality assurance system of the Paul-Ehrlich-Institut (PEI) and general guidelines [29–31].

Bet v 4-specific sandwich ELISA

Development, design and procedure of the Bet v 4-specific ELISA have been described previously [27]. In short, a Bet v 4-specific mAb is immobilized on a rat anti-mouse IgG2-coated MTP. After incubation of samples, an anti-Bet v 4 rabbit serum is added, followed by detection via an HRP-conjugated anti-rabbit IgG and subsequent oxidation of the substrate TMB.

Bet v 6-specific sandwich ELISA

MTPs (Nunc MaxiSorp, Thermo Fisher Scientific) are coated with 200 ng/well of the primary antibody (anti-Bet v 6 mP80) by incubation overnight at 4 °C. The plate is blocked with 5% milk powder (Carl Roth, Karlsruhe, Germany) in PBS for 30 min at RT. Samples are incubated for 1 hour under agitation at RT. The biotinylated secondary antibody (anti-Bet v 6 mP83bio) is added in a concentration of 100 ng/well and incubated for 1 hour at RT under agitation. 100 µl/well of a 1:10,000 dilution of streptavidin-HRP (Sigma-Aldrich) in PBS is incubated for 1 hour at RT under agitation. The substrate solution consisting of 2.5% TMB (21 mM stock, Sigma Aldrich) and 0.034% H₂O₂ in a potassium citrate buffer, pH4, is incubated for 10 min at RT. Substrate oxidation is stopped by adding 50 µl/well of a 20% sulphuric acid solution. Colorimetric analysis was performed at OD 450 nm with 630 nm as reference in a SpectraMax 240 reader using the SoftMax Pro 5.2 rev C software (Molecular Devices Corp, Sunnyvale, USA).

Bet v 7-specific sandwich ELISA

MTPs (Nunc MaxiSorp, Thermo Fisher Scientific) are coated with 200 ng/well of an anti-mouse IgG1 antibody (Beckton-Dickinson) by incubation over night at 4°C. The plate is blocked with 5% milk powder (Carl Roth) in PBS for 30 min at RT. 100 ng/well of the primary antibody (anti-Bet v 7 mP74) are immobilized on the coated MTP via incubation for 1 hour at RT under agitation. Samples are likewise incubated overnight at 4 °C covered with an adhesive film. 100 µl/well of an anti-Bet v 7 rabbit serum are added at a dilution of 1:5,000 and incubated for 1 hour at RT under agitation. 100 µl/well of a 1:15,000 dilution of an anti-rabbit IgG-HRPO (Jackson) are incubated for 1 hour at RT under agitation. Color reaction and colorimetric analysis were performed as described for the Bet v 6 ELISA.

Bet v 1-specific sandwich ELISA

The major birch allergen Bet v 1 was quantified in BP allergen products using the commercially available kit (EL-BV1) from INDOOR Biotechnologies Inc. (Charlottesville, USA) according to the manufacturer's instructions.

BP allergen products

24 Prick test solutions (PTS), 22 product batches for sublingual immunotherapy (SLIT) and 24 intermediates of products for subcutaneous immunotherapy (SCIT) have been included in the study. All analyzed product batches had successfully passed official batch release testing at PEI. Consent was obtained from all allergen product manufacturers concerned (ALK-Abelló Arzneimittel GmbH, Hamburg, Germany; Allergopharma GmbH&Co KG, Reinbek, Germany; Bencard Allergie GmbH, Munich, Germany; HAL Allergy GmbH, Düsseldorf, Germany; LETI Pharma GmbH, Ismaning, Germany; Stallergenes Greer, Kamp-Lintfort, Germany; listed in alphabetical order). Five out of six allergen product manufacturers provided information on the BP lots used in the production of the BP allergen product batches. All product-related results are presented blinded and manufacturers are referred to as M1 to M6 in arbitrary order. Each product batch was tested in at least two independent experiments in each ELISA system, with inter-assay variability controlled by a limit for the

coefficient of variation (CV) of 15%. The batches were diluted to fit the measurement range of the respective system.

Total allergenic activity (potency)

The IgE inhibition assay for determination of total allergenic activity was performed as previously described [27].

Statistics

Statistical analysis was performed as previously described [27], using SoftMax Pro 5.2 rev C software (Molecular Devices) for processing of ELISA data, CombiStats 5.0 [32] for assessment of parallelism and linearity of curves as well as potency assessment, a Mixed ANOVA Model with the SAS/STAT Software, Version 9.3, MIXED Procedure (SAS Institute Inc., Heidelberg, Germany) for estimation of precision, the software R version 3.0.3 [33] for calculation of Pearson's correlation coefficient (r), the 95%-confidence intervals (CI) as well as the respective p-values controlled with the Holm-Bonferroni method [34] and GraphPad Prism Version 6.07 (GraphPad Software, La Jolla, USA) for generation of graphs. Multidimensional scaling (MDS) was used to visualize the minor allergen patterns of the BP allergen products. MDS projects objects with known pairwise distance into a low-dimensional space. The projection was chosen in such a way that distances measured in the projection are as close as possible to the real distances. The geodesic distance is used since it does not depend on the absolute minor allergen 'measurements'/concentration but rather measures the difference in composition between products.

RESULTS

Validation of Bet v 6- and Bet v 7-specific ELISA systems

Recombinant and native allergen are equally recognized by the two highly specific ELISA systems

The standard curve courses of the Bet v 6- and Bet v 7-specific ELISA systems are shown in Figure 1 A/B. Dilutions of the recombinant allergen as well as BPE resulted in parallel typical sigmoid curves in a semi-log plot. The linear section of the curves, allowing for most accurate measurements, ranges from 0.2 to 1.9 ng/ml for the Bet v 6-ELISA and from 7.8 to 31.3 ng/ml for the Bet v 7-ELISA, representing the respective quantitation range framed by lower and upper limit of quantification. The CV of eight replicates of rBet v 6 or rBet v 7 and six replicates of BPE did not exceed 15% in the quantitation range in both ELISA systems (Figure 1 C/D). The limit of detection, defined as $2.6 \times SD + mean$ in relation to a confidence limit of 99% [35], has been calculated on the basis of eight blank values, respectively, to be at OD 0.017 for the Bet v 6-ELISA and at OD 0.083 for the Bet v 7-ELISA, corresponding to approximately 6 pg/ml Bet v 6 and 280 pg/ml Bet v 7.

Specificity of the Bet v 6- and Bet v 7-specific ELISA systems was verified using high concentrations of recombinant birch allergens (rBet v 1, rBet v 2, rBet v 4, rBet v 6, rBet v 7) as well as a set of allergen extracts (BP, soy bean, peanut, hazelnut) tested in total protein concentrations up to 10 µg/ml (Table 1). Only the respective recombinant protein as well as their native counterparts in BPE resulted in signals above the cross-reactivity limit defined as the quadruple of the mean blank value of the two ELISA systems.

Confirmation of high accuracy and precision in standard addition experiments

Accuracy and precision of the two ELISA systems were assessed in standard addition experiments. For each ELISA, two matrices composed of BPE were selected and spiked with four different concentrations of either the recombinant allergen or BPE. Concentrations were selected in order to span the quantitation range of the respective ELISA: In the Bet v 6-ELISA, the two matrices containing 0.3 and 0.5 ng/ml nBet v 6, spiked with 0.125, 0.25, 0.5 and 1 ng/ml rBet v 6 were

assessed in fourteen independent experiments, performed by two operators on eight different days. In comparison, matrices and spikes tested in thirteen experiments in the Bet v 7-ELISA were 3 and 5 ng/ml nBet v 7, spiked with 2.5, 5, 10 and 12.5 ng/ml nBet v 7. In both ELISAs, a linear relationship between the expected versus the measured allergen content could be observed in both matrices, indicating a very high degree of accuracy (Figure 2).

The same dataset was used for determination of precision. Based on the intra-assay variability of six replicates per sample on the one hand and inter-assay variability between at least thirteen experiments on the other hand, the variance was assessed via the SAS Mixed Procedure. For the Bet v 6-ELISA 84 replicates were evaluated, whereas 78 replicates were included for the Bet v 7-ELISA. In both ELISA systems, the resulting total variability did not exceed the predefined precision limit of a CV of 25%, neither for unspiked nor for spiked matrices (Table 2).

Comparative analysis of BP allergen product composition

Interdependency of major and minor birch pollen allergen content differs between product groups

In total, 70 BP allergen product batches produced by six manufacturers were assessed. These had been selected from 14 different birch allergen product lines available in Germany. All batches had been tested experimentally in official batch release for their total allergenic activity, expressed relative to an internal standard in PEI-U/ml. In addition, total protein as well as Bet v 1, Bet v 4, Bet v 6 and Bet v 7 content of each batch were determined. The three main quality parameters of BP allergen products (total allergenic activity, total protein and Bet v 1 content) showed highly significant correlations to each other, irrespective of the analyzed product group (Fig. 3A/B/C). The correlations with the total protein content of PTS were the only exceptions as these were found to be only moderate. In comparison, correlations between the Bet v 1 content and the three minor allergens Bet v 4, Bet v 6 and Bet v 7 were far more diverse (Fig. 3D/E/F). Clear differences between the product groups and allergens could be observed. For example, correlations between Bet v 1 and Bet v 6 concentrations were comparatively weak in all product groups. Furthermore, all three minor allergens showed low or no interdependency with Bet v 1 in PTS.

Minor allergen content is commonly within a 50-200% variability margin from mean of the given batches

The *Monograph on Allergen Products* requests that neither total allergenic activity nor total protein content of an allergen product batch should exceed the limits of 50 to 150% of a stated amount, whereas the content of individual relevant allergens in different batches may vary between 50 and 200% [4]. We addressed conformity with these requirements for all products for which at least three batches had been included in our analysis. To make this possible, we defined the respective mean value of the tested batches as stated amount (100%) for each parameter and subsequently calculated a theoretical specification for total allergenic activity, protein content and Bet v 1 content and variability margins for the three minor allergens for each product.

As expected, all tested batches complied with the theoretical specifications set for Bet v 1 content (Fig. 4A) as well as for total allergenic activity and total protein content (data not shown). In contrast, one out of five batches did exceed the variability margin for at least one of the minor allergens (Fig. 4B/C/D). Nevertheless, in all tested batches the three BP minor allergens included in this study could be detected and many products showed surprisingly good consistency in Bet v 4, Bet v 6 and Bet v 7 estimates, for instance all products of M2 and M3. Most results exceeding the variability margin were observed for Bet v 7 in PTS of M1, M4 and M5 as well as in SLIT products of M5. In contrast, all but three Bet v 4 and Bet v 6 estimates were found within the range of 50-200% around the respective mean.

Comparison between manufacturers reveals substantial differences between products

It is also apparent from Figure 4 that there exist clear differences between BP allergen products from different manufacturers, both in major and minor allergen content. The mean Bet v 1 concentration in PTS products was relatively similar but still the lowest and highest Bet v 1 value detected in a PTS were 5 and 50 µg/ml. In comparison, Bet v 1 concentrations in SCIT products were highly similar as the greatest difference between single batches was less than 4-fold. However, given that only SCIT intermediates could be included in the analysis, one cannot extrapolate the observed similarity to the

finished products. In contrast, great differences were observed between SLIT products. Here, mean Bet v 1 content ranged from 14 µg/ml in the SLIT product of M3 to 235 µg/ml for M1. The respective lowest and highest Bet v 1 content determined in a SLIT product batch were 10 versus 280 µg/ml.

Similarly, BP allergen products differed greatly in their minor allergen content. For example, mean Bet v 4 content in SLIT products of M1 and M3 differed up to 15-fold (Fig. 4B), while mean Bet v 7 content differed 11-fold (Fig. 4D). Likewise, the highest and lowest Bet v 6 content determined in a SLIT batch were 0.7 µg/ml (M1) versus 6.1 µg/ml (M5, Fig. 4C). In comparison, a 20-fold difference could be observed in mean Bet v 6 content between the PTS of M1 and M3 (Fig. 4C). Notably, Bet v 7 levels in different intermediates of SCIT products even ranged from less than 1 µg/ml to more than 120 µg/ml, despite the clear similarity in Bet v 1 content (Fig. 4A/D).

Minor allergen patterns of BP allergen products differ between manufacturers

In order to compare the minor allergen patterns of the different products within the three product groups, the combined results for Bet v 4, Bet v 6 and Bet v 7 were subjected to MDS. This technique visualizes the degree of similarity in minor allergen composition by placing each product batch in a two-dimensional scatterplot (Fig. 5). Note that the smaller the distance between batches in the plot, the higher the similarity in minor allergen composition. Consequently, batches exceeding the variability margin for at least one of the minor allergens (diamond symbols) are only observed for products with greater distribution in the plot. In general, the analyzed PTS show a wide distribution, both among batches as well as between products of different manufacturers. Interestingly, PTS and SCIT products from M1 and M4 differ markedly in their minor allergen pattern compared to the respective products from other manufacturers. However, for SLIT products, only one batch of M5 is significantly separated from the other products and therefore dissimilar in minor allergen pattern.

Pollen source material: Main determinant of minor allergen content?

Our results indicate clear differences in minor allergen concentrations and patterns between BP allergen products of different manufacturers on the one hand, but also variability between batches of the same product on the other hand. These observations raise the question which factors determine the

content of Bet v 4, Bet v 6 and Bet v 7. Especially for inter-batch variability, the most obvious candidate is the BP source material. Therefore, we tested the hypothesis that batches produced from the same source material lot are more similar in their minor allergen pattern. In order to verify this hypothesis, the information on minor allergen content was subjected to MDS per manufacturer and, where available, combined with information on the BP lots used in production of each batch (Fig. 6).

Interestingly, an overlap of the product groups PTS, SLIT and/or SCIT can be observed for most manufacturers, indicating similarity across product group borders. In addition, the plots of M2, M3 and M6 show a comparatively low scaling. Both observations indicate that the minor allergen patterns of the BP allergen products are similar despite differences in production and pharmaceutical form. A clear exception to this rule is M1. The product groups of M1 form distinct groups in a plot with wide scaling, implying distinct differences in minor allergen patterns between PTS, SLIT and SCIT product. Furthermore, one of the PTS batches of M1 with a result exceeding the variability margin for one minor allergen differs significantly in its minor allergen composition although the same source material (lot A) has been used for two other batches. Hence, the utilized source material lot does not seem to be the cause for the observed variation in minor allergen composition in the PTS of M1. This trend is further confirmed by the fact that M1 utilized the pollen lots F and A for single batches of several product groups, but the resulting minor allergen patterns are very different.

In comparison, batches and products from M2 and M3 appear to be very similar. It is therefore difficult to tell whether repeated use of the same source material lot does reproducibly increase similarity of batches of the same product. In contrast, the differences between product batches of M5 and M6 may be linked to the use of different source material lots. Both PTS and SCIT product batches produced by M6 seem to be more similar when the same source material lot had been used. This is especially highlighted by the two SCIT batches produced from source material lot D. Those two batches are highly similar in their minor allergen composition, exceed the variability margin for at least one minor allergen and differ significantly from the other SCIT batches. Importantly, another batch based on a mixture of the pollen lots D and F is within the minor allergen variability margin but located between the two groups.

DISCUSSION

Up to now, minor allergens are usually not controlled during the production of allergen products. Although some observations imply a link between more severe allergy symptoms and sensitization to minor allergens [9–11], their relevance for safety and efficacy of allergen products is virtually unknown and data from controlled clinical trials are lacking. While standardization of allergen product manufacturing and composition has generally advanced in the past decade [36, 37], neither raw materials nor production processes or storage conditions are standardized with regard to minor allergens [38]. If at all, methods for minor allergen quantification are developed in scientific contexts and are not commercially available.

Although mass spectrometry-based techniques present a promising alternative to antibody-based immunoassays [39–41], ELISA is still the method of choice for allergen quantification since it is easy to perform and cost effective, and transfer to other laboratories is simple. In addition to the already published Bet v 4 ELISA [27], we developed mAb-based ELISA systems for quantification of the minor BP allergens Bet v 6 and Bet v 7. Validation studies of the two new ELISA systems confirmed high specificity and precision (Tab. 1, Tab. 2), allowing for accurate and reproducible quantification of Bet v 6 and Bet v 7 in BP extracts (Fig. 2). The three ELISA systems enabled the first parallel assessment of three minor BP allergens in a large set of BP allergen products taking into consideration Ph. Eur. quality requirements.

Given the lack of knowledge on the clinical relevance of minor BP allergens, their regulation is not requested by competent authorities and they are currently not specifically controlled during BP allergen product manufacturing. Nevertheless, we could confirm that all allergen products for diagnosis and therapy of BP allergy included in our study contained Bet v 4, Bet v 6 and Bet v 7. However, the minor allergen content of products and batches was in part highly variable. These observed differences, especially between products from different manufacturers, are not limited to minor allergens though. As already described in the literature, BP allergen products also differ greatly with regard to their main quality parameters total allergenic activity, total protein and Bet v 1 content

[27, 42–45]. Regarding SCIT products, these parameters could only be determined in intermediates, prior to chemical modification and/or absorption. Therefore, the transferability of our findings to the finished products administered to the patients is limited. In contrast, PTS and SLIT products represent the drug product directly used in diagnosis and therapy respectively, but it has to be taken into account that the amounts applied during SLIT differ largely between products. Indeed, the extreme differences shown in Figure 4A are compensated to a certain extent when considering the daily Bet v 1 dose actually applied to the patient. Taking into account the instructions provided in the respective Summary of Product Characteristics, the greatest difference observed in mean Bet v 1 daily dose between the tested SLIT products is reduced from 17-fold to 6-fold. The effects on the observed differences in minor allergen concentration (Fig. 4B/C/D) differ when relating the content to the SLIT daily dose. For example, the 5-fold difference between highest and lowest mean Bet v 6 content was only marginally reduced to 4-fold, while the 15-fold difference in Bet v 4 content was reduced to 5-fold.

Surprisingly, the variability in minor allergen content between batches of the same product was smaller than expected. Although 20% of batches surpassed the calculated variability margins of 50–200% from mean of the given batches for at least one minor allergen, this variability seemed to be restricted to single products and manufacturers. For example, all products of M3 showed high homogeneity in minor allergen content, while the products of M5 seem to be more prone to variability in Bet v 4, Bet v 6 and Bet v 7. It is further apparent that most results exceeding the variability margin were found in PTS (Fig. 4), which is reflected by the poor correlation observed between Bet v 1 and the three minor allergens in this product group (Fig. 3).

Our findings underline that marketed allergen products for diagnosis and therapy of BP allergy are significantly different also in their minor allergen content. Thus, safety and efficacy of BP allergen products might differ for patients sensitized to minor BP allergens. Analyses of patient sensitization patterns in conjunction with therapy outcomes on the one hand and information on minor allergen content on the other hand are at need to clarify the clinical relevance of the observed differences. Furthermore, we aim to expand our panel of BP allergen-specific ELISAs to the quantification of the

pan-allergen profilin (Bet v 2 [46]) to complete the picture of minor allergen patterns in BP allergen products.

In view of the presented results, we tried to detect the determining factor for variability in minor allergen content. Therefore, we combined our results on Bet v 4, Bet v 6 and Bet v 7 with information on the BP lots used in production of the respective product batches. Given the limited number of batches included in the analysis, the influence of the source material was only assessable for some products. In some cases, analysis was further complicated, e.g. due to strong general similarity in minor allergen composition, use of mixtures of BP lots or incomplete information provided by the manufacturers. Nevertheless, it was possible to observe clear trends for some manufacturers and products. For example, the BP lot does not seem to be the main determinant of the minor allergens in the products of M1. Here, the three manufacturing processes lead to three distinct minor allergen patterns and inter-batch variability can be observed irrespective the use of the same BP lot. In turn, the pollen lot utilized seems to be a main determinant of the minor allergen content in the products of M6. Analyses of more products as well as of BP will be necessary to fully clarify the influence of the utilized pollen material on the patterns of minor allergens of BP allergen products.

In conclusion, the three minor allergen-specific ELISA systems established and validated in-house provided the basis for the first comparative assessment of minor allergen patterns in various BP allergen products. Our findings confirm that minor allergen composition and variability differ between manufacturers. Due to the lack of data from controlled clinical trials it is unfortunately not possible to deduce the effect of such differences on safety and efficacy for certain patient populations. Interestingly, differences between batches and products of the same manufacturer are not in all cases explained by the BP lots utilized, but may also be related to manufacturing processes or other product characteristics. It is therefore likely that several influencing factors will present a challenge for some manufacturers if future clinical investigations would reveal a need for control of minor allergen content in BP allergen products.

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CONFLICT OF INTEREST

All authors declare to have no conflict of interest in regard to this publication.

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FIGURE LEGENDS

Figure 1: Standard curve progression and precision profile of Bet v 6- and Bet v 7-ELISA

A/B) Standard curves were prepared using the respective recombinant allergen and BPE. Four replicates each were tested in A) the Bet v 6 ELISA and B) the Bet v 7 ELISA, in concentrations as indicated. The measured OD is depicted as the mean value with standard deviation (SD). Note that the SD is smaller than size of data points in most cases. The curves follow a four-parametric nonlinear curve fitting. The grey striped section highlights the concentration range in which curves are parallel. In the blank grey section, curves are parallel and linear. **C/D)** The coefficient of variation (CV) was determined based on the calculated allergen concentrations of eight replicates of C) rBet v 6 or D) rBet v 7, in combination with six replicates of BPE respectively, in concentrations as indicated.

Figure 2: Standard addition experiments for estimation of accuracy

A) 14 tests were performed with six internal replicates each in the Bet v 6-ELISA. Two matrix samples consisting of BPE were spiked with four different concentrations of rBet v 6 (S1 - S4: 0.125, 0.25, 0.5 and 1 ng/ml). **B)** 13 tests were performed with six internal replicates each using the Bet v 7-ELISA. Spikes S1 - S4 consisted of BPE, containing 2.5, 5, 10 and 12.5 ng/ml nBet v 7. Mean and standard deviation (SD) of all measurements were plotted against the respective expected Bet v 6 or Bet v 7 content. Note that in some cases the SD is smaller than the size of the data points. For the resulting linear regression lines, slope and determinant of correlation (R^2) are given.

Figure 3: Interdependency of birch pollen allergen product quality parameters and minor allergens

In total, 70 BP allergen product batches were included in the analysis. Mean values of Bet v 1, Bet v 4, Bet v 6, Bet v 7, total protein content and total allergenic activity were plotted against each other. For each pairing and product group (PTS, SLIT, SCIT), the Pearson correlation coefficient (r) as well as the respective 95% confidence interval (CI) are presented. Statistical significance of correlations is marked as follows: ^{ns} $p \leq 0.05$, * $p > 0.05$, ** $p > 0.01$, *** $p > 0.001$.

Figure 4: Control of product batches for agreement with variability margins of 50 to 200% from mean of the given batches

The **A)** Bet v 1, **B)** Bet v 4, **C)** Bet v 6 and **D)** Bet v 7 content of each product batch is depicted per manufacturer (M1-M6) and product group (PTS, SLIT, SCIT). For each product with at least three batches included in the analysis, the mean value as well as the 50-200% range around this mean value is given. Batches exceeding the respective variability margin are marked with diamond symbols.

Figure 5: Minor allergen patterns of birch pollen allergen product batches per product group

The product batches of six manufacturers were subjected to multidimensional scaling (MDS) in the three product groups PTS, SLIT and SCIT on the basis of the individual Bet v 4, Bet v 6 and Bet v 7 content of each batch. Batches exceeding the respective variability margin for at least one minor allergen according to Figure 4 are marked with diamond symbols.

Figure 6: Minor allergen patterns per manufacturer in combination with pollen lot information

The product batches of six manufacturers were subjected to multidimensional scaling (MDS) on the basis of their individual Bet v 4, Bet v 6 and Bet v 7 content. Where available, the BP lot used for production of the respective batch is indicated by capital letters. Batches exceeding the respective variability margin for at least one minor allergen according to Figure 4 are marked as diamonds.

TABLES**Table 1: Lack of cross-reactivity to other birch pollen allergens and selected allergen extracts**

sample	Bet v 6 ELISA			Bet v 7 ELISA		
	protein [µg/ml]	OD	> OD 0.050 (4x blank)	protein [µg/ml]	OD	> OD 0.288 (4x blank)
rBet v 1	1.000	0.016	-	1.250	0.073	-
rBet v 2	1.000	0.015	-	1.250	0.079	-
rBet v 4	1.000	0.020	-	1.250	0.072	-
rBet v 6	0.015	2.980	+++	1.250	0.201	-
rBet v 7	1.000	0.016	-	0.015	1.566	+++
birch pollen extract	0.160	2.879	+++	1.100	1.768	+++
soy bean extract	10.000	0.034	-	10.000	0.107	-
peanut extract	10.000	0.034	-	10.000	0.126	-
hazelnut extract	10.000	0.017	-	10.000	0.235	-

Table 2: Adequate assay precision estimated based on standard addition experiments

sample	total variability: CV [%]			
	Bet v 6-ELISA		Bet v 7-ELISA	
	matrix 0.3 ng/ml	matrix 0.5 ng/ml	matrix 3 ng/ml	matrix 5 ng/ml
unspiked matrix	13.7	13.2	14.9	16.7
matrix + S1	9.4	9.1	15.2	16.9
matrix + S2	9.3	9.8	15.3	16.5
matrix + S3	8.0	9.7	15.0	16.8
matrix + S4	8.7	9.8	14.0	15.9







