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STANDARDIZATION OF ALLERGEN PRODUCTS: 3. Validation of Candidate European Pharmacopoeia

Standard Methods for Quantification of Major Birch Allergen Bet v 1

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Short title: BSP090 - Validation of Bet v 1-specific ELISAs

ABSTRACT

Background: The BSP090 project aims at establishing European Pharmacopoeia Reference Substances in combination with the corresponding ELISA methods for the quantification of major allergens in allergen products. Two sandwich ELISAs proved suitable for quantification of Bet v 1, the major birch pollen allergen, in preceding phases of BSP090.

Methods: Two Bet v 1-specific ELISA systems were compared with respect to accuracy and precision in a ring trial including 13 laboratories. Model samples containing recombinant rBet v 1.0101 as well as native birch pollen extracts were measured independently at least three times in each facility. The assessment was completed with a comparative quantification of Bet v 1 in 30 marketed birch allergen products in one laboratory, simulating the future use as reference method.

Results: In the collaborative study, both candidate ELISAs confirmed their suitability to quantify recombinant and native Bet v 1. ELISA-A showed higher precision and lower inter-laboratory variability, yet ELISA-B exhibited slightly higher accuracy. Subsequent parallel measurement of Bet v 1 in a panel of “real-life” birch allergen products indicated better repeatability of ELISA-B. Both systems detected substantial differences in Bet v 1 content between allergen products, but the effect

was more pronounced using ELISA-B due to persistently higher values compared to ELISA-A.

Conclusions: In the collaborative study, no deciding differences were observed between the two candidate ELISAs. Further comparison under conditions simulating the intended use combined with the criterion of long-term availability enabled the selection of one Bet v 1-specific ELISA for proposal as European Pharmacopoeia standard method.

KEY WORDS

Bet v 1, biological standardisation, ELISA, European Pharmacopoeia, major allergen

ABBREVIATIONS

AIT	allergen immunotherapy
CRS	chemical reference standard
gcv	geometric coefficient of variation
GM	geometric mean
mAb	monoclonal antibody
Ph. Eur.	European Pharmacopoeia
PTS	prick test solution
RS	reference standard
SCIT	subcutaneous immunotherapy
SLIT	sublingual immunotherapy

INTRODUCTION

Legally binding requirements for the quality of allergen product are laid down in the European Pharmacopoeia (Ph. Eur.) general monograph *Allergen Products* (1063) (1). To further increase and harmonize the quality of products for allergen immunotherapy (AIT) and to enhance the validity of respective clinical trials, the European Medicines Agency (EMA) published guidelines on the quality and clinical development of AIT products (2–4). Despite this regulatory framework, a direct comparison of allergen extracts in terms of allergen content or potency is at present often impossible (5). This is due to the use of manufacturer-specific analytical methods in combination with proprietary units expressing the potency of allergen preparations in the European Union (EU) (6–8). Thus, thorough characterization methods and standardization of allergen products are at need. In contrast to the USA, the respective efforts in the EU focus on major allergen content (9, 10): The CREATE project was initiated in 2001 with the aim to develop and validate candidate reference standards (RS) for eight major allergens in combination with respective immunoassays (11, 12). The successor of CREATE is BSP090, a joint programme included in the Biological Standardisation Programme (BSP) of the European Directorate for the Quality of Medicines & Healthcare (EDQM), running under the aegis of the Council of Europe and the European Commission. The BSP090 consortium, consisting of researchers, allergen product manufacturers, biotechnology companies and medicines agencies, focuses on establishing reference preparations and *in vitro* assays for quantification of Phl p 5a, a major allergen of timothy grass (*Phleum pratense*) pollen, and Bet v 1, the major allergen of birch (*Betula verrucosa*) pollen (13, 14). Bet v 1 is one of the most prominent allergens in Europe and hence the superordinate target for AIT in birch pollen allergic patients (15–18). Under the aegis of the EDQM, two feasibility studies were successfully performed in the course of BSP090 (14, 19) resulting in the official adoption of recombinant (r)Bet v 1 and rPhl p 5a as Ph. Eur. Chemical Reference Substances (CRS). Subsequently, a multi-centre study to validate the corresponding immunoassays for their suitability to become a Ph. Eur. standard method was initiated. Here, we present the results for the two Bet v 1-specific ELISAs investigated in this

collaborative study in 13 laboratories. The methods were validated using model samples consisting of a native birch pollen extract spiked with the well-characterized, GMP-produced rBet v 1.0101 CRS (20). This study was complemented by a comparative assessment of Bet v 1 content in marketed birch pollen allergen products in order to simulate the future use of the ELISA systems in quality control.

MATERIAL AND METHODS

Participants: Fourteen laboratories were part of the collaborative study, whereof thirteen submitted results (Tab. S1). They are referred to by code numbers not necessarily corresponding to the order presented in Tab. S1. The subsequent evaluation of birch allergen products was performed in the laboratory of the project leader (Paul-Ehrlich-Institut (PEI), Langen, Germany).

Study materials: Two Bet v 1-specific sandwich ELISAs were compared (ELISA-A: ALK-Abelló A/S, Hørsholm, Denmark; ELISA-B: Stallergenes Greer, Antony Cedex, France) (13, 14, 19). For the collaborative study, lyophilised test samples and run controls were prepared (Tab. 1). The rBet v 1 CRS was provided by the EDQM to be used as reference standard in the assays and for preparation of run controls 1 and 2 with defined rBet v 1 concentrations. For preparation of the test samples 1-4, lyophilised birch pollen extract (Allergopharma, Reinbek, Germany) was reconstituted in ultra-pure water, spiked with pre-defined concentrations of the CRS and re-lyophilized elsewhere (Virion-Serion GmbH, Würzburg, Germany). Test and run control samples were to be reconstituted in ultra-pure water and diluted serially in the corresponding ELISA buffer.

ELISA reagents including monoclonal antibodies (mAbs) were provided by ALK-Abelló and Stallergenes (14). Some specific reagents, i.e. enzyme substrates, were procured by the EDQM to avoid batch-to-batch variability and provided to the participants. Additional reagents less susceptible to variability, such as buffer ingredients, were procured individually by the participants following specified recommendations regarding grade and purity. All specific reagents and samples were pre-

tested at PEI for suitability. Based on these pre-testing results, acceptance criteria for the two run controls were defined as 70-130% from the mean values determined in each of the two ELISAs (Tab. 2). All instruments used by participating laboratories in the course of the studies were appropriately calibrated and qualified.

For Bet v 1 determination in “real-life” samples, 30 birch pollen allergen products from six manufacturers were analysed (ALK-Abelló Arzneimittel GmbH, Hamburg, Germany; Allergopharma Reinbek, Germany; Bencard Allergie GmbH, München, Germany; HAL Allergie GmbH, Düsseldorf, Germany; Leti Pharma GmbH, Witten, Germany; Stallergenes Greer GmbH, Kamp-Lintfort, Germany). The randomly chosen samples covered the whole spectrum of birch pollen allergen products, with ten prick test solutions (PTS), eleven sublingual immunotherapy (SLIT) solutions and nine subcutaneous immunotherapy (SCIT) products. In case of products containing adsorbed and/or chemically modified allergen extracts, the non-modified, non-adsorbed intermediates were tested. All products were diluted to match the measurement range of the ELISAs (ELISA-A: 0.1 - 6.6 ng/ml; ELISA-B: 0.2 - 10.0 ng/ml) (14). In addition, total allergenic activity of the products was determined as previously described (21).

Study design: Generally, all samples were tested in duplicate relative to a reference curve prepared with the CRS. In the collaborative study, all samples were measured in the two ELISAs in at least three independent assays on different days by one operator. Birch allergen products were tested at PEI for their Bet v 1 content in at least three independent assays in each ELISA on three different days by two operators.

Statistical analysis: All results obtained were submitted to four-parameter logistic curve analysis in accordance with the general method described in Ph. Eur. general text 5.3 Statistical analysis of results of biological assays and tests with the CombiStats 4.0 software (22). Logistic curves were fitted through the data points of the reference and test samples, with the constraint that slope and asymptotes of all preparations were equal and the curves only differed from each other in horizontal

shift. Accordingly, the following equation was used:

$$y = d + (a - d)f(b(x - c_i)) + \varepsilon$$

where y = observed optical densities (ODs); d = common lower asymptote; a = common upper asymptote; b = common slope factor; x = $\ln(\text{dose})$; c_i = points of inflexion of preparations; ε = error term with constant variance and expectation 0; f = logistic function defined by $f(x) = (1 + e^{-x})^{-1}$

In the collaborative study, laboratory 2 submitted an insufficient number of data points for ELISA-B to estimate the upper asymptote. In this case the model was reduced to three parameters:

$$y = d + f(b(x - c_i)) + \varepsilon$$

where f = exponential function defined by $f(x) = e^x$; other parameters as described above.

The relative allergen contents were calculated as $\exp(c_i - c_S)$ where c_S and c_i are the points of inflexion of the standard and the respective test samples.

The calculations were carried out irrespective from the specific protocols provided by the project partners who developed the respective ELISA (14, 19). For each sample, the geometric mean (GM) of the assays per laboratory was calculated together with the geometric coefficient of variation (gcv), defined as:

$$gcv = \sqrt{(\exp(v) - 1)} * 100\%$$

where v = sample variance of the log-transformed activities.

Moreover, overall GM and gcv were determined in the collaborative study. Accuracy was determined as previously described (14):

$$Recovery = \left[\frac{S - U}{T} \right] * 100$$

where S = concentration of spiked sample; U = concentration of unspiked sample; T = theoretical spike concentration.

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An empirically defined acceptance range was set to $\pm 30\%$ of the theoretical spike concentration. The calculation was performed for individual estimates as well as based on the overall GM values to calculate overall spike recovery. For assessment of precision, a 20% and 30% range around the median of all estimates obtained for a sample in all laboratories was calculated. Differences in precision parameters as well as between estimates obtained with the two ELISAs were assessed for statistical significance using Student's t-test. Intra-assay variability was defined as the median width of the confidence intervals expressed as percentage of the estimate divided by 4, representing approximate the standard deviation of the estimated value. Inter-assay variability was calculated based on the median gcv of the estimated concentrations within laboratories. Inter-laboratory variability was defined as the gcv of the GM within laboratories. Pearson's correlation coefficient (r) was calculated using GraphPad Prism version 6.07 (Graph Pad Software, La Jolla, USA).

RESULTS

Collaborative study

On the basis of four model samples and two run controls with pre-defined concentrations (Tab. 1), the performance of the two candidate ELISAs was evaluated. Twelve laboratories submitted results of three, laboratory 13 of four assays per ELISA. Due to problems encountered by laboratory 12, results of this participant had to be excluded, resulting in evaluation of 37 assay runs per ELISA method. Though individual results from laboratory 12 could be calculated for ELISA-A, they were not part of the general evaluation. For ELISA-B, laboratory 12 had to be excluded entirely.

Validity

Validity of the assays was checked by quantification of rBet v 1 in the two run controls containing 2 and 6 ng/ml of CRS respectively (Fig. 1). 33 out of 37 assays performed with ELISA-A complied with the pre-defined acceptance criteria (Tab. 2). In comparison, only 26 out of 37 assays performed with

ELISA-B fulfilled the acceptance criteria, mainly due to overestimation of rBet v 1 content in run control 1, especially in laboratories 9 and 13. Comparison of the overall GM (Fig. 1, solid line) with the theoretical rBet v 1 content (dashed line) shows that the estimates of ELISA-B were on average slightly higher than expected. In contrast, the majority of estimates of ELISA-A were below 2 and 6 ng/ml Bet v 1 respectively as indicated by the comparatively low overall GM values.

Precision

Precision was assessed based on the total estimates of the unspiked extract Sample 1 (6 ng/ml Bet v 1) and three extract samples spiked with 60, 20 and 5 ng/ml of the CRS (Sample 2, 3 and 4 respectively). The percentage of concentrations deviating by more than 20% or 30% from the median of all estimates was assessed for each sample. Ninety-six percent of estimates of ELISA-A were within the range of 20% from median, while 99% of estimates fell within 30% from median (Fig. 2A). In comparison, only 70% and 85% of estimates from ELISA-B fell within the range of 20% and 30% from median respectively (Fig. 2B). Hence, the precision of ELISA-A was higher compared to ELISA-B because Bet v 1 concentrations determined with ELISA-B for a given sample are more scattered around the median compared to ELISA-A.

Accuracy

Accuracy was compared by assessing the recovery of the recombinant spikes from the extract matrix. The calculated spike estimates were compared to a pre-defined range of $\pm 30\%$ deviation from the theoretical spike concentrations. For ELISA-A, almost all spike estimates were found to comply with this range (Fig. 2C). All the same, it should be noted that the majority of results was below the respective theoretical spike concentration. This trend is clearly visible when comparing the overall GM for each spike (Fig. 2C, solid lines) with the theoretical spike concentration (dashed lines). In detail, the overall spike recovery in Samples 2, 3 and 4 was 88%, 92% and 88% of the theoretical spike concentration respectively.

In contrast, the spike estimates of ELISA-B were more equally distributed around the theoretical spike values (Fig. 2D). The small differences between overall GM for each spike (solid lines) with the theoretical spike concentration (dashed lines) are also reflected by the overall spike recovery: 89%, 103% and 103% of the theoretical spike content were recovered from Samples 2, 3 and 4 respectively. However, when assessing the individual results obtained with ELISA-B, 20 of 111 spike estimates did not meet the criterion of $\pm 30\%$ deviation from the respective theoretical value. Notably, as many estimates were found below this acceptance range as were above it.

The presented findings are summarized in the two-way plot in Figure 3A. Here, the median results per sample and laboratory of each ELISA are plotted against each other. The majority of values can be found above the line of equivalence, highlighting again that most results of ELISA-B were higher; a trend independent of the nature of the sample. Especially the estimates for Samples 1 and 4 were markedly lower in ELISA-A.

Repeatability, intermediate precision and reproducibility

Repeatability, intermediate precision and reproducibility were assessed pertaining to intra- and inter-assay as well as inter-laboratory variation (Fig. 3B) (23). Repeatability and intermediate precision were comparable for both systems: Intra-assay variability was consistently low for all samples and run controls and the median gcv was 2.2% with ELISA-A and 2.1% with ELISA-B. Inter-assay variability was well-below 15% in both systems for all samples, with a median gcv for ELISA-B of 8.6%, compared to 5.8% for ELISA-A. Concerning reproducibility, a clear difference between the ELISAs is already apparent in Figure 3A by the elliptic shape of most grey areas. Indeed, ELISA-B showed gcv results systematically above 15%, with a maximum of nearly 30% for Sample 1 and a median value of 18.7%. In contrast, ELISA-A exhibited significantly lower inter-laboratory variability, none of the samples or run control yielding a gcv value above 10%.

Quantification of Bet v 1 in birch pollen products

To simulate the future use as Ph. Eur. standard method, Bet v 1 was quantified in 30 birch allergen products in both ELISAs (Fig. 4A). The primary trend of systematically higher values in ELISA-B observed in the collaborative study persisted. For only 1 of the 30 products tested, the Bet v 1 content obtained with ELISA-A was higher than the respective value of ELISA-B. Regarding the different product groups, PTS expectedly contained rather low amounts of Bet v 1 (approximately 5 to 30 µg/ml). The two ELISAs yielded significantly different results in 4 of 10 PTS. Similarly, a significant deviation between ELISA-A and ELISA-B was observed for 4 of 9 SCIT products. Bet v 1 content in SCIT products ranged from approximately 30 to 160 or 220 µg/ml depending on the utilized ELISA. In SLIT solutions, an even higher variability of the major allergen content was observed between the products (approximately 6 to 170 or 350 µg/ml, according to ELISA-A and ELISA-B respectively). Especially for those products with a very high Bet v 1 content, highly significant differences between the estimates of the two ELISAs were identified.

Similarly to the collaborative study, intra-assay variability during product testing was generally very low, with a median gcv of 2.2% for ELISA-A versus 1.7% for ELISA-B (Fig. 4B). However, inter-assay variability was significantly increased for ELISA-A, with a median gcv of 13.1% and a maximal gcv of nearly 40% for one SCIT product. Of note, gcv values above 20% were observed for PTS, SLIT and SCIT products alike, independent of product manufacturers. In contrast, ELISA-B exhibited a mean gcv of only 9.1% for inter-assay variability, with a maximal overall gcv of 19.3%.

In addition, results for Bet v 1 content determined by ELISA-A and ELISA-B were plotted against the allergenic activity of the respective allergen products (Fig. 5). Correlation between major allergen content and allergenic activity was expectedly strong in all tested product groups, independent of the ELISA method used.

DISCUSSION

A reliable quantification of a major allergen in a given allergen product requires the combination of a well-characterized purified allergen RS and a corresponding method for its quantification (9, 24). The fact that the establishment of an RS alone is insufficient was again underlined in BSP090: Despite the use of the same RS, significant differences in Bet v 1 estimates could be observed between the two candidate ELISAs in various birch allergen products. This comparative testing also confirmed the great differences in major allergen content reported for allergen products (7, 25) and therefore the necessity to enable comparability by advancing allergen product standardization.

The rBet v 1.0101 preparation provided by Stallergenes had been extensively studied and found appropriate to be established as Ph. Eur. CRS (20). Consequently, one of the main objectives of BSP090 was to evaluate Bet v 1-specific sandwich ELISAs in combination with this CRS. After positive assessment during the two initial phases of the project (13, 14, 19), a collaborative study was launched for extensive comparison of two candidate ELISAs. In general, both systems proved suitable for quantification of recombinant as well as natural Bet v 1.

Precision was assessed based on four model samples containing birch pollen extract. Using ELISA-B, Bet v 1 estimates showed an increased spread around the median and some values even exceeded the “ $\pm 30\%$ from median” acceptance range. By comparison, precision of ELISA-A was higher. Given that intra- and inter-assay variability were well below a gcv of 15% for both assays in the collaborative study, the lower precision observed for ELISA-B seems to originate for the most part from increased inter-laboratory variability. This is predominantly related to consistently low estimates reported by laboratory 7 on the one hand and exceptionally high values submitted by laboratory 9 on the other hand. As all laboratories followed identical instructions, potential reasons for this discrepancy apart from the ELISA method itself have not been identified.

The high precision of ELISA-A was confirmed in the quantification of run controls and in spike recovery. However with respect to accuracy, ELISA-A tended to underestimate the theoretical rBet v 1 content in run controls and spikes. In comparison, the accuracy of ELISA-B was higher, despite slightly lower overall precision.

All in all, no pivotal difference between the two systems became apparent in the collaborative study that would have allowed for the definite selection of one of the ELISAs as candidate Ph. Eur. standard method. Hence, the ELISAs were further tested for their ability to quantify Bet v 1 in a panel of birch allergen products, consisting of PTS, SLIT and SCIT products alike. Again, both ELISAs showed a generally good performance. Interestingly, the inter-assay variability of ELISA-B was consistent with the observations in the collaborative study, while this parameter was markedly increased for ELISA-A regardless of the type of product considered. Inter-laboratory variability could not be assessed as these measurements were exclusively performed at PEI.

As already described, ELISA-A gave consistently lower estimates compared to ELISA-B in all sample types included in the collaborative study as well as in virtually all tested birch allergen products. Interestingly, this trend seemed to be especially pronounced in samples containing no or little recombinant Bet v 1. However, in contrast to run controls and spikes containing a definable amount of rBet v 1, the total Bet v 1 content in samples containing birch pollen extract cannot be compared to a fixed theoretical value due to the lack of an absolute method or a gold standard. Similarly, it is not possible to deduce which ELISA is closer to the “true” Bet v 1 content in a birch allergen product. Published data on Bet v 1 content in birch allergen products (7, 21, 26) are in a similar range compared to the estimates reported here, although the differences between e.g. SLIT products were found to be even greater (up to 50-fold) compared to previously reported differences of up to 10-fold (27).

Even though an absolute evaluation of the results is impossible, there are indications that the apparent discrepancy between the two ELISAs might relate to the high number of Bet v 1 isoforms present in birch pollen extract (28–30) and a potentially lower number of isoforms detected by ELISA-A. Investigations by the cooperation partner providing ELISA-A have demonstrated that the utilized mAbs recognize an isoallergen profile very similar to the profile obtained with specific IgE from birch pollen allergic patients (manuscript in preparation). However, further analysis using Biolayer Interferometry (Octet platform, ForteBio) revealed that the Bet v 1-specific mAbs showed a higher dissociation constant for natural Bet v 1 compared to rBet v 1. This implies that, due to a lower affinity of the antibodies to several natural isoforms in comparison to the recombinant protein, the content of Bet v 1 in birch pollen extract is underestimated if rBet v 1 is used to prepare the standard curve. Given the use of the Bet v 1 CRS as reference in the collaborative trial as well as in post-study testing, these results provide a plausible reason for the observed underestimation. Still, it needs to be noted that absolute quantification of an allergen with as many isoforms as Bet v 1 is probably impossible in any mAb-based ELISA system due to a limited number of isoforms recognized by mAbs. Consequently, there was still a “neck-and-neck race” of the two candidate ELISAs to become nominated as Ph. Eur. standard method. Also accessory information like the degree of correlation between Bet v 1 content and total allergenic activity did not facilitate the decision as correlation was found to be expectedly strong in all product classes and the results were virtually identical for the two ELISAs. Therefore other characteristics like the question of long-term availability had to be considered for recommending one system. ELISA-B is based on two mAbs. This setup guarantees a standardized supply of coating and detection reagents in the future. In comparison, the polyclonal rabbit serum used in ELISA-A is a more limited resource and the production of new batches depends on a new immunization of rabbits, conflicting with the principle of the 3Rs (replacement, reduction, refinement of animal experimentation (31)) and the framework of Directive 2010/63/EC on the use of animals for scientific purposes.

In summary, both ELISAs were found generally appropriate for valid quantification of Bet v 1. Subsequent parallel testing of a large panel of products, simulating the future application as reference method, demonstrated slight superiority of ELISA-B with respect to intermediate precision. Combined with the considerations concerning long-term mAb availability, ELISA-B was recommended to the members of the BSP090 consortium as candidate standard method and accepted as such. The necessary steps have been initiated to reference this method in the relevant texts of the Ph. Eur.

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CONFLICT OF INTEREST STATEMENT

Asturias JA, Barber D, Buchheit KH, Carn s J, Costanzo A, Daas A, Dayan-Kenigsberg J, Dehus O, D ring S, F hrer F, Hanschmann KM, Ledesma A, Sandberg E, Sander I, Strecker D, Valerio C and Zimmer J have nothing to disclose.

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AUTHORS CONTRIBUTIONS

Author (alphabetic order)	Contribution
Asturias JA	responsible for performance of experiments in laboratory participating in collaborative study; critical revision of article draft; final approval of the version to be published
Barber D	development and refinement of candidate ELISA system; responsible for performance of experiments in laboratory participating in collaborative study; interpretation of data; critical revision of article draft; final approval of the version to be published
Buchheit KH	interpretation of data; critical revision of article draft; final approval of the version to be published
Carnés J	responsible for performance of experiments in laboratory participating in collaborative study; critical revision of article draft; final approval of the version to be published
Chapman M	responsible for performance of experiments in laboratory participating in collaborative study; critical revision of article draft; final approval of the version to be published
Costanzo A	main coordinator BSP090; design, preparation and coordination of collaborative study; interpretation of data; critical revision of article draft; final approval of the version to be published

Daas A	statistical analysis and interpretation of data; critical revision of article draft; final approval of the version to be published
Dayan-Kenigsberg J	responsible for performance of experiments in laboratory participating in collaborative study; critical revision of article draft; final approval of the version to be published
Dehus O	design of collaborative study; responsible for performance of experiments during post-study testing phase; analysis and interpretation of data; critical revision of article draft; final approval of the version to be published
Döring S	performance of experiments during post-study testing phase and in preparation of the collaborative study; critical revision of article draft; final approval of the version to be published
Führer F	responsible for performance of experiments during post-study testing phase; critical revision of article draft; final approval of the version to be published
Hanschmann KM	design of collaborative study; statistical analysis of data; critical revision of article draft; final approval of the version to be published
Holzhauser T	responsible for performance of experiments in laboratory participating in collaborative study; critical revision of article draft; final approval of the version to be published
Kaul S	design of collaborative study and post-study testing; responsible for performance of experiments during post-study testing phase; analysis and interpretation of data; critical revision of article draft; final approval of the version to be published
Ledesma A	development and refinement of candidate ELISA system; responsible for performance of experiments in laboratory participating in collaborative study; interpretation of data; critical revision of article draft; final approval of the version to be published
Moingeon P	development and refinement of candidate ELISA system; responsible for performance of experiments in laboratory participating in collaborative study; interpretation of data; critical revision of article draft; final approval of the version to be published
Nony E	development and refinement of candidate ELISA system; responsible for performance of experiments in laboratory participating in collaborative study; interpretation of data; critical revision of article draft;

	final approval of the version to be published
Pini C	responsible for performance of experiments in laboratory participating in collaborative study; critical revision of article draft; final approval of the version to be published
Plunkett G	involved in assay testing and pre-phase of the collaborative study; critical revision of article draft; final approval of the version to be published
Reese G	responsible for performance of experiments in laboratory participating in collaborative study; critical revision of article draft; final approval of the version to be published
Sandberg E	responsible for performance of experiments in laboratory participating in collaborative study; critical revision of article draft; final approval of the version to be published
Sander I	responsible for performance of experiments in laboratory participating in collaborative study; critical revision of article draft; final approval of the version to be published
Strecker D	performance of experiments during post-study testing phase and in preparation of the collaborative study; critical revision of article draft; final approval of the version to be published
Valerio C	responsible for performance of experiments in laboratory participating in collaborative study; critical revision of article draft; final approval of the version to be published
van Ree R	responsible for performance of experiments in laboratory participating in collaborative study; critical revision of article draft; final approval of the version to be published
Vieths S	project leader BSP090; analysis and interpretation of data; critical revision of article draft; final approval of the version to be published
Zimmer J	responsible for performance of experiments during post-study testing phase; analysis and interpretation of data; drafting of the article; final approval of the version to be published

FIGURE LEGENDS

Fig. 1: Validity of Bet v 1- ELISAs in the collaborative study

Distribution of Bet v 1 estimates grouped by controls and laboratories is depicted for the two Bet v 1 ELISAs. Crosses show the measured values per test for each control sample, grouped by laboratories numbered 1 to 13. Note that the results obtained with ELISA-A in laboratory 12 are indicated by stars, but were not included in the calculation of the geometric mean (solid lines). Dashed lines specify the respective theoretical Bet v 1 concentration, while grey areas mark the pre-defined acceptance range specified for each ELISA for the respective run control (see Tab. 2).

Fig. 2: Accuracy of Bet v 1-ELISAs in the collaborative study

Distribution of Bet v 1 estimates grouped by samples and laboratories is depicted for the two Bet v 1 ELISAs respectively. Crosses show the measured value per test for each sample, with the exception of the results obtained in laboratory 12 with ELISA-A depicted as stars. Results are grouped by laboratories, numbered 1 to 13. **A)/B)** Estimates of total Bet v 1 content in unspiked and spiked extract samples are given. Dark and light grey areas identify the 20% and 30% range around the median (solid line) respectively. **C)/D)** Spike estimates are depicted, with solid lines indicating the respective geometric mean. Grey boxes mark the range of 70 – 130 % variation from the respective theoretical spike concentration (dashed lines).

Fig. 3: Comparison of ELISA performance in collaborative study

A) In the two-way plot, crosses depict the median of the total measured Bet v 1 content per sample and laboratory, marked by the respective laboratory numbers. Grey areas indicate the group of results for a specific sample preparation. Dashed lines specify the theoretical Bet v 1 content of the two run controls. **B)** Variability parameters (intra-assay, inter-assay and inter-laboratory) for

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measurements of all controls and samples in both ELISAs are compared in a box whisker plot, indicating the median gcv, the upper and lower quartiles as well as the minimum and maximum of the respective data set. The dashed line marks a geometric coefficient of variation of 15%. Based on Student's t-test, significant differences between the two ELISAs are indicated (** $p \leq 0.001$).

Fig. 4: Post-study comparison of ELISA methods in Bet v 1 quantification in birch allergen products

A) The two-way plot depicts the GM of Bet v 1 content for each birch allergen product with standard deviation (SD) based on at least three independent experiments. Results for PTS ($n = 10$) are marked in white, those for SCIT ($n = 9$) and SLIT products ($n = 11$) in grey and black respectively. Based on Student's t-test, significant differences between the values obtained with the two ELISAs are indicated as * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$. **B)** Intra- and inter-assay variability are depicted for the birch allergen product measurements in ELISA-A and ELISA-B in a box-whisker-plot, indicating the median gcv, the upper and lower quartiles as well as the minimum and maximum of the respective data set. The dashed line marks a geometric coefficient of variation of 15%. Based on Student's t-test, significant differences between the two ELISAs are indicated (* $p \leq 0.05$; ** $p \leq 0.01$).

Fig. 5: Correlation of Bet v 1 content and allergenic activity of birch allergen products

The Bet v 1 content of each birch allergen product determined in ELISA-A and ELISA-B was plotted against the respective allergenic activity determined in official batch release. Results for PTS ($n = 10$) are presented in white, SCIT products ($n = 7$) in grey and SLIT products ($n = 11$) in black. For each product group, the correlation coefficient (Pearson's r) was calculated.

TABLES

Tab. 1: Sample composition in collaborative study

identifier	EDQM code	rBet v 1 [ng/ml] [†]	extract [ng/ml] [*]	theoretical concentration [ng/ml]
Control 1	42540	2	-	2
Control 2	42541	6	-	6
Sample 1	42536	-	6	6
Sample 2	42537	60	6	66
Sample 3	42538	20	6	26
Sample 4	42539	5	6	11
rBet v 1 CRS	42335	n/a	n/a	n/a

[†]concentration determined prior to lyophilisation based on amino acid analysis

^{*}concentration determined prior to lyophilisation as the mean of several measurements in both candidate ELISAs
CRS: chemical reference standard

Tab. 2: Acceptance criteria for run controls in collaborative study

identifier	ELISA-A [ng/ml]	ELISA-B [ng/ml]
Control 1	1.3 – 2.4	1.4 – 2.6
Control 2	3.9 – 7.3	4.5 -8.3

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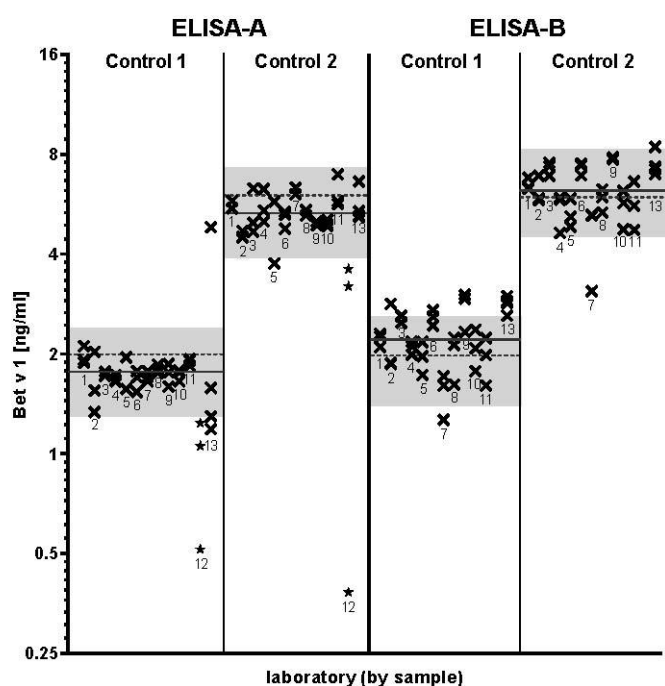
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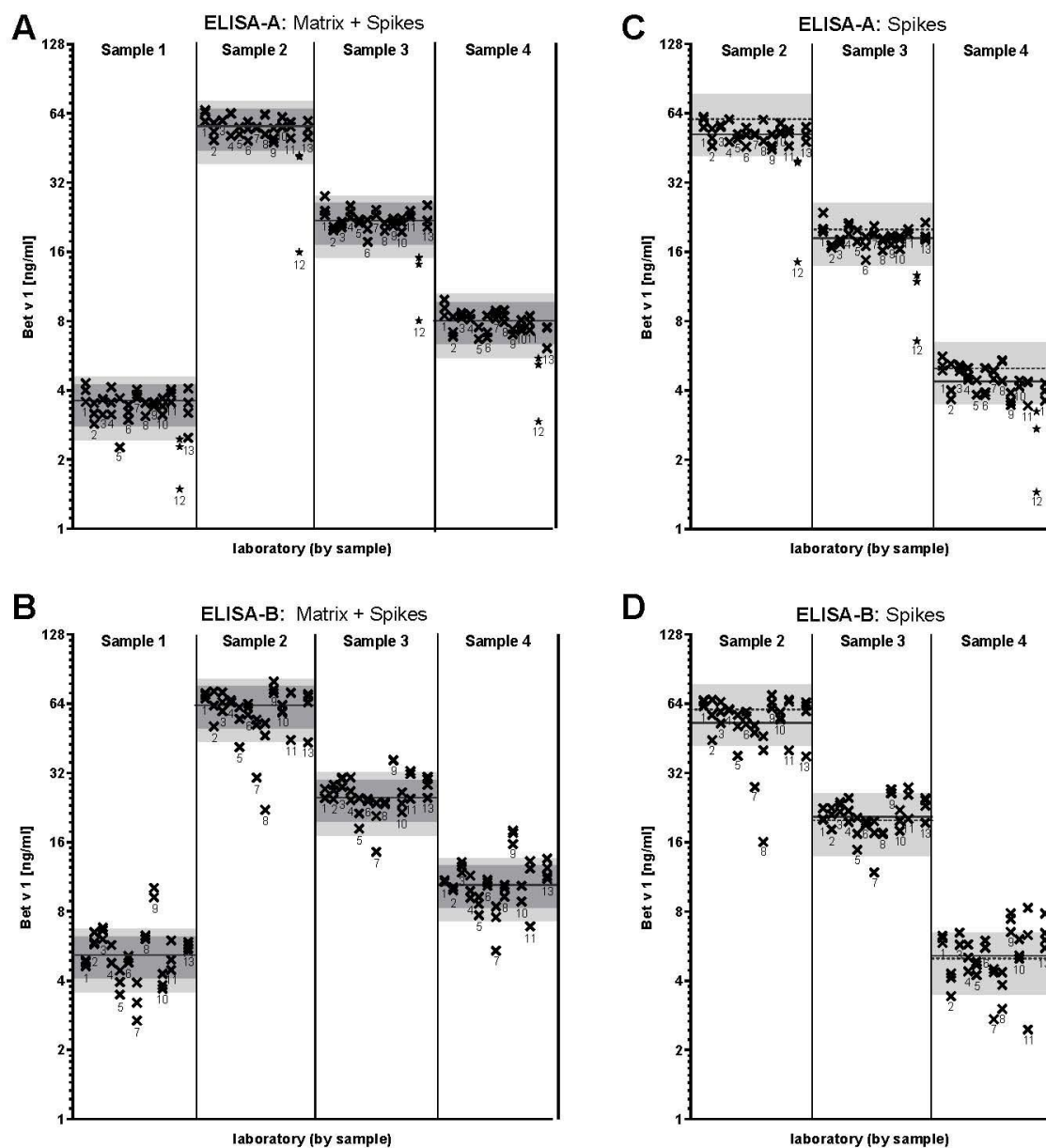
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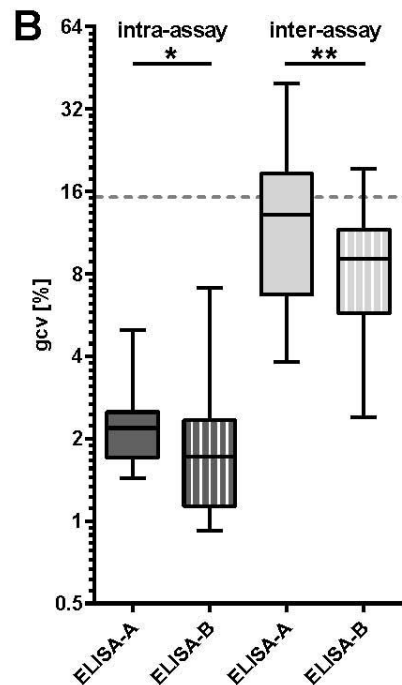
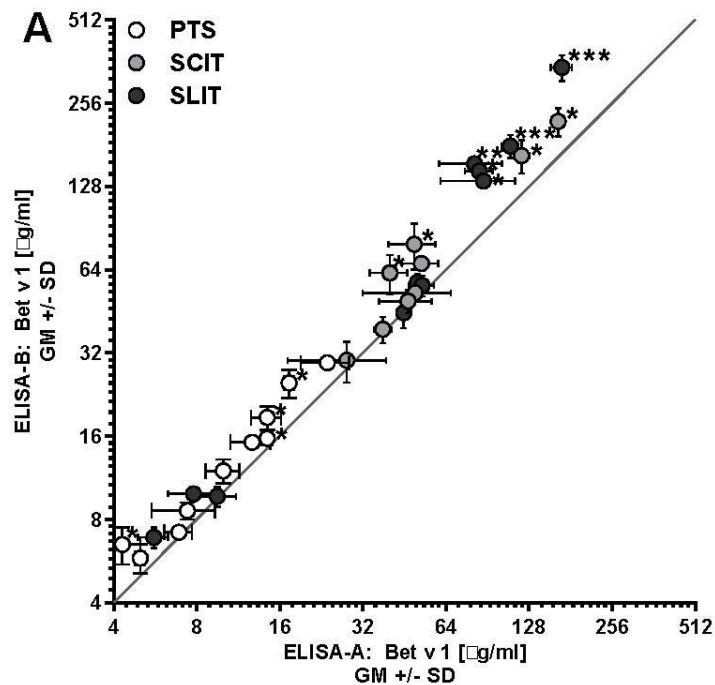
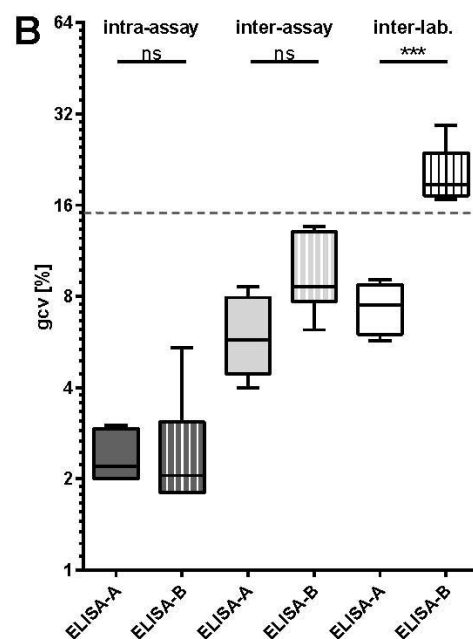
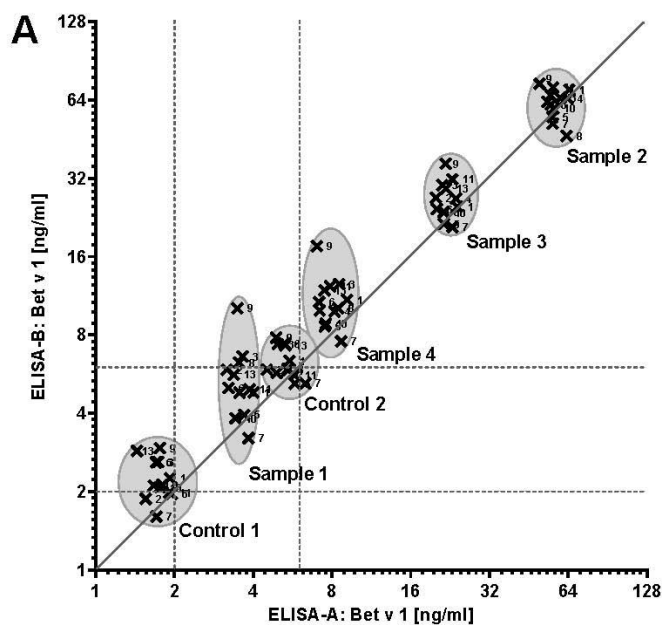
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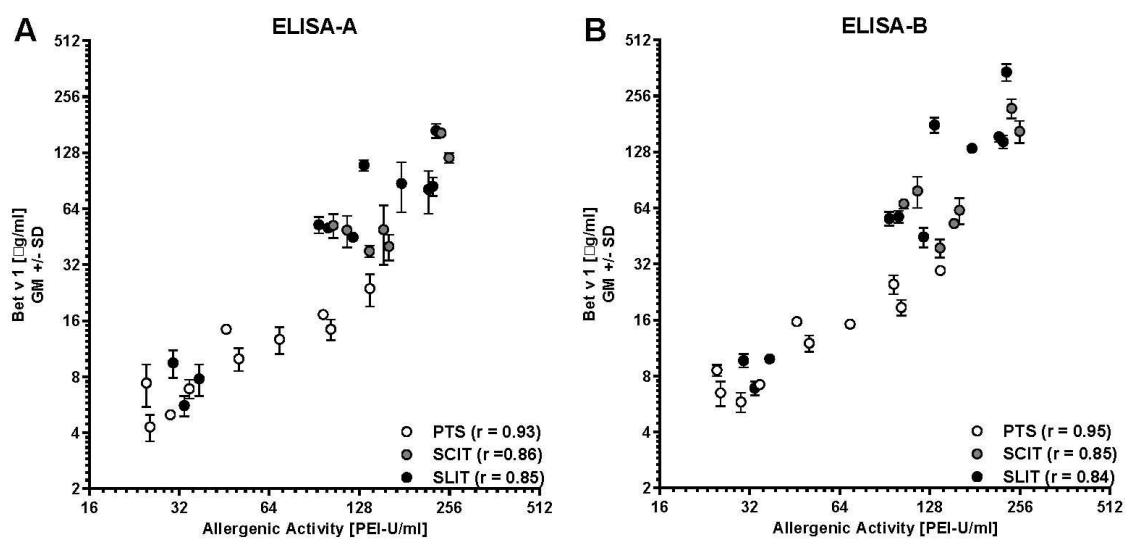
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Tab. S1: Participating laboratories in collaborative study

collaborator	institute/company/agency	country
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H. Fiebig G. Reese	Allergopharma	Germany
M. Raulf I. Sander	Institute for Prevention and Occupational Medicine of the German Social Accident Insurance (IPA)	Germany
T. Holzhauser	Paul-Ehrlich-Institut (PEI)	Germany
C. Pini P. Iacovacci B. Brunetto	Istituto Superiore di Sanità, Centro per la Ricerca e la Valutazione dei prodotti Immunologici (ISS CRIVIB)	Italy
J.A. Asturias M.C. Arilla	BIAL Industrial Farmacéutica	Spain
D. Barber A. Ledesma	ALK-Abelló	Spain
J. Carnés	Laboratorios Leti S.L.	Spain
R. Van Ree J. Akkerdaas	Academic Medical Centre	The Netherlands
M. Chapman	INDOOR Biotechnologies Inc.	United States of America
C. Valerio	Food and Drug Administration (FDA)	United States of America

