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Absolute quantification of Bet v 1 in birch polymerized allergenic extracts via mass spectrometry-targeted analysis

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Abstract

Background

Quantifying major allergens is essential for evaluating the quality and efficacy of allergenic extracts. They are usually measured in non-polymerized extracts using immunoassays. However, the direct measurement of allergens in allergoids is currently not supported. This study set out to develop a method for quantifying Bet v 1 in polymerized birch extracts using mass spectrometry-based targeted analysis.

Methods

Three isotopically labelled peptide sequences of Bet v 1 were synthesized and used as internal standards for the development of a mass spectrometry-based targeted analysis. The calibration curves of the three peptides to assess the linearity and limit of detection, as well as reverse calibration curves with a constant amount of sample, were constructed. The Bet v 1 content was determined and measured in 18 batches of depigmented (native extracts purified by a mild acid treatment) and depigmented-polymerized extracts.

Results

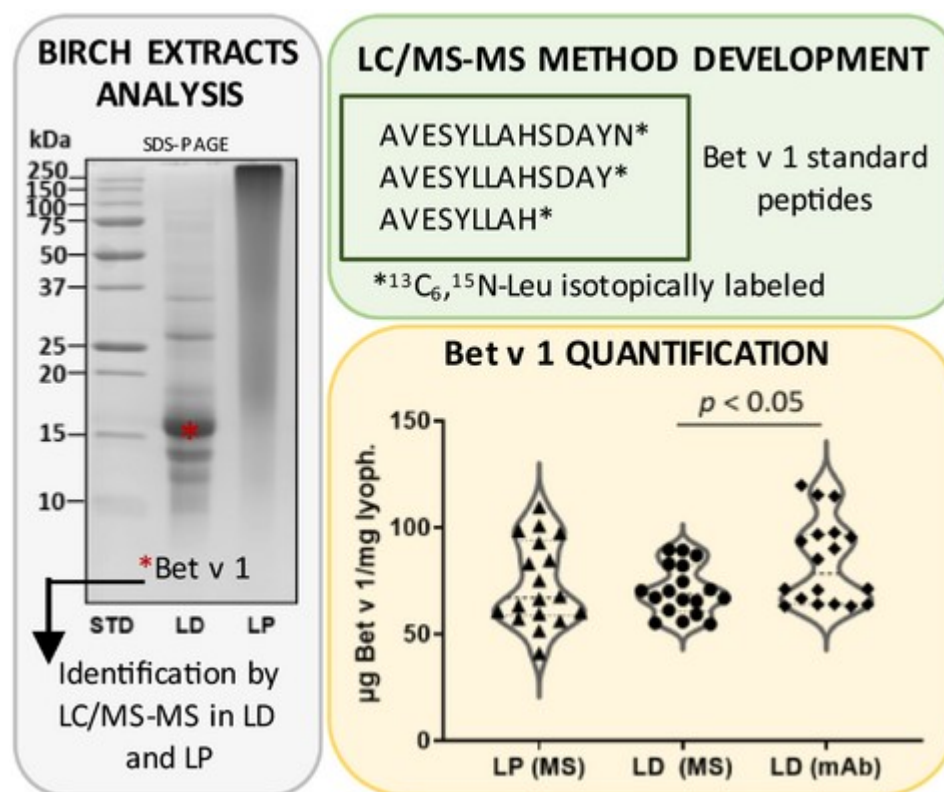
Bet v 1 isoforms were identified in both type of extracts by mass spectrometry. According to mass spectrometry-targeted analysis depigmented and depigmented-polymerized extracts exhibited mean values of 70.5 and 73.5 μg Bet v 1/mg of lyophilized extract, respectively. A statistically significant correlation between the allergen content of both extracts was identified. Statistically significant differences were observed when the Bet v 1 content in non-polymerized extracts was measured via mass spectrometry ($70.5 \pm 11.6 \mu\text{g}/\text{mg}$) or immunoassay ($83.7 \pm 19.8 \mu\text{g}/\text{mg}$).

Conclusions

These results represent the first direct quantification of Bet v 1 in allergoids using mass spectrometry-based targeted analysis. The proposed method demonstrates robustness and reliability and constitutes a promising alternative for detailed characterization of polymerized allergenic extracts.

Abstract

The aim of this study was to develop a method for quantifying Bet v 1 in polymerized birch extracts using mass spectrometry-based targeted analysis. Bet v 1 isoforms were identified in depigmented and depigmented-polymerized birch extracts by mass spectrometry. Three isotopically labelled peptide sequences of Bet v 1 were synthesized and used as internal standards. Depigmented and depigmented-polymerized extracts exhibited a Bet v 1 mean content around 70 $\mu\text{g}/\text{mg}$. Statistically significant differences were observed between mass spectrometry and immunoassay methods.



Key messages

A mass spectrometry-based targeted analysis was developed for Bet v 1 quantification in allergoids.

Depigmented and depigmented-polymerized extracts exhibited a similar mean content of Bet v 1.

Statistically significant differences were observed when the immunoassay- and mass spectrometry-obtained results were compared.

1 INTRODUCTION

Chemically modified allergens, also known as allergoids, are one of the foremost alternatives currently used for allergy treatment. The concept of allergoids¹ is used to describe high molecular weight allergen chains obtained by polymerization, mainly using glutaraldehyde or formaldehyde. The resulting product is a complex allergen extract that requires appropriate assays for its characterization, which involves allergen identification and quantification.

In 2002, the CREATE project² was established as a European initiative for improving the characterization and harmonization of the techniques used to standardize allergenic extracts according to their major allergen content.^{3, 4} The objective was to develop specific immunoassays for allergen quantification by using recombinant versions of the major allergens as standards.⁵⁻⁸ Reference standards for two major allergens, Bet v 1 and Phl p 5a (and their corresponding immunoassays), were validated and approved by the European Directorate for the Quality of Medicines (EDQM). As a consequence, major allergen quantification is currently considered a relevant parameter for evaluating the consistency and quality of allergenic extracts. In addition, there is a strong clinical demand for determining the major/minor allergen concentrations in the allergenic extracts.^{9, 10} In the case of allergoids, the complexity of their immune-physicochemical characteristics makes it impossible to quantify major allergens based on immunoassays because of their modified structure.

In 2009, Carnés et al.¹¹ published the first study demonstrating the presence of allergens in allergoids using a qualitative method based on mass spectrometry (MS). The reported method involved detecting and identifying specific peptide sequences obtained after the allergoids are subjected to enzymatic digestion. However, it did not yield information on allergen concentration. Nevertheless, MS is currently considered one of the most promising methods for protein quantification, not only for allergenic extracts¹²⁻¹⁶ but also for other proteins.^{17, 18}

The objective of this study was to develop a method for quantifying the major allergen of birch, Bet v 1, in depigmented and depigmented-polymerized allergenic extracts using MS. The proposed strategy consisted of selecting appropriate trypsin-digested peptides for quantifying Bet v 1 in both extracts, then using synthetic isotopically labelled versions of these peptides as standards in order to compare MS signals of the standards with those of Bet v 1 peptides obtained via the digestion of the depigmented and modified extracts.

2 MATERIALS AND METHODS

2.1 Allergen extracts

Eighteen depigmented extracts (LD) of *Betula verrucosa* were used to manufacture the corresponding depigmented-polymerized extracts (LP) via polymerization with glutaraldehyde in strict compliance with internal procedures (LETI Pharma S. L. U., Madrid, Spain) and GMP principles. Each batch was characterized in terms of its protein and allergenic profile following the European guidelines¹⁹ and the European Pharmacopoeia (<https://www.edqm.eu/en/european-pharmacopoeia-ph-eur-10th-edition>). Extract manufacturing and characterization are described in detail in the Supplementary Information (SuppInfo).

2.2 Protein digestion

Extract sample treatment and digestion are described in the SuppInfo. Briefly, proteins obtained from lyophilized LD and LP samples of birch were digested using Lys C, followed by further digestion using porcine trypsin. The peptides were cleaned up, dried and stored at −20°C until subsequent analysis.

2.3 LC-MS/MS data-dependent analysis for Bet v 1 identification

Digested peptides were analysed in data-dependent analysis (DDA) mode in an LC-MS system comprising a nanoAcquity liquid chromatograph (Waters, Mildford, MA, USA) coupled to an LTQ-Orbitrap Velos mass spectrometer (Thermo Scientific, Waltham, MA, USA). Peptides were analysed for 30 min at a flow rate of 250 nL/min in a C18 column using a gradient of 1 to 40% B (100% acetonitrile; 0.1% formic acid).

Raw data files were searched against a database containing the International Union of Immunological Societies (IUIS) protein entries for Bet v 1 isoforms using the Mascot search engine. Proteins were identified with at least two peptides with false discovery rate (FDR) 5%.

2.4 Development of an LC-MS/MS targeted analysis for the absolute quantification of Bet v 1

2.4.1 Analysis of trypsin-digested and heavy PEPotec peptides of Bet v 1

The following stable, isotopically heavy, labelled crude peptides ($^{13}\text{C}_6$, $^{15}\text{N}_2$ -Lys, $^{13}\text{C}_6$, $^{15}\text{N}_4$ -Arg, and $^{13}\text{C}_6$, ^{15}N -Leu, PEPotec SRM Custom Peptide, ThermoFisher Scientific) were synthesized: GVFNYESETTSVIPAAAR, GVFNYETETTSVIPAAAR, VAPQAISSVENIEGNGGGPGTIK, SVENIEGNGGGPGTIK, GVFNYEIETTSVIPAAAR, AVESYLLAHSDAYN and AVESYLLAHSDAY. These peptides were selected based on the feasibility of detecting them by MS in both depigmented and polymerized extracts and their presence in the highest possible number of Bet v 1 isoforms.

Each peptide was analysed to obtain the LC retention times and the MS and MS/MS spectra. Furthermore, 200 ng of digested LD and LP extracts with spiked-in PEPotec peptides were analysed in the same LC-MS system (Section 2.3), using a gradient of 1 to 40% B for 60 min. For each reference peptide, the amount of spiked-in peptide was chosen to have an area as close as possible to that obtained for the light Bet v 1 peptide.

Samples were acquired in triplicate using a targeted time-scheduled MS/MS method, where the seven peptides of interest (m/z values listed in the Table S1) were fragmented and analysed in the linear ion trap.

2.4.2 Calibration curves of AQUA peptide standards

The peptides AVESYLLAHSDAYN, AVESYLLAHSDAY and AVESYLLAH were ordered as stable isotopically labelled peptides ($^{13}\text{C}_6$, ^{15}N -Leu) with a minimum purity of 99% (HeavyPeptide™ AQUA Ultimate, ThermoFisher Scientific), for use as internal standards.

Different concentrations of the standard mixtures were analysed as described in Section 2.3. Time-scheduled targeted MS/MS was implemented, where the three peptides of interest (Table 1) were fragmented in the linear ion trap.

TABLE 1. Acquisition parameters for the time-scheduled MS/MS method for selected peptides

m/z ^a	RT min.	RT max.	NCE	M+H ⁺	Sequence
501.76640	28.2	29.1	35.0	1002.5255	AVESYLLAH
505.27500	28.2	29.1	35.0	1009.5426	AVESY(L)LAH
719.84608	30.1	31.1	35.0	1438.6849	AVESYLLAHSDAY
723.35466	30.1	31.1	35.0	1445.7021	AVESY(L)LAHSDAY
776.86754	29.1	30.1	35.0	1552.72784	AVESYLLAHSDAYN
780.37613	29.1	30.1	35.0	1559.7450	AVESY(L)LAHSDAYN

^a The isolation width was 2 Da. Fragment ions were detected using the Orbitrap (resolution of 7500 at $m/z = 400$). The FT Msn AGC target was 30000 and the FT maximum ion time was 300 ms.

Calibration curves were constructed using the peptide AVESYLLAHSDAYN (from 0.25 to 100 fmol) and the peptides AVESYLLAHSDAY and AVESYLLAH (from 0.25 to 25 fmol) (Table [S2](#)). To avoid carryover, the injections were performed in triplicate from the lowest to the highest concentration.

2.4.3 Analysis of different amounts of digested LD sample

The influence of sample concentration was evaluated by analysing different amounts (200, 100, 25, 4, 2 and 1 ng) of digested LD sample using the method described in Section [2.4.2](#).

2.4.4 Reverse calibration curves of AQUA peptide standards

Different concentrations of the AQUA peptides were mixed and added to a constant amount (4 ng total protein) of the digested LD and LP samples before being analysed as described in Section [2.4.2](#). Concentrations of 0.25, 0.5, 1, 2.5, 10, 25, 50, 100 and 250 fmol of the peptide AVESYLLAHSDAYN and 0.25, 0.5, 1, 2.5, 5 and 10 fmol of the peptides AVESYLLAHSDAY and AVESYLLAH were used. Each point was analysed in triplicate from the lowest to the highest concentration.

The lower limit of quantification (LLOQ) was determined as the lowest concentration at which the maximum variability of the assay is estimated to be 20% CV (coefficient of variation = standard deviation (H/L)/mean (H/L)). The lower limit of detection (LLOD) = LLOQ/3.

2.5 LC-MS/MS targeted analysis for the absolute quantification of Bet v 1 in LD and LP samples

Total protein quantification was performed before and after the acetone precipitation step using a PierceTM BCA Protein assay to calculate the precipitation factor, which was used to correct for the amount of Bet v 1. As described in Section [2.4.4](#), samples were acquired in triplicate.

2.6 LC-MS/MS data analysis

2.6.1 Relative quantification of LP against LD extracts and selection of peptides

Quantitative analysis of the targeted MS/MS data was performed using Skyline.²⁰ Peak area integration was conducted based on the extracted ion chromatograms of, up to, the five highest-ranked MS/MS fragment ion masses matching specific peptides in the spectral library. All transitions and peak areas were validated manually.

Samples were designated as “control” (LD) or “polymerized” (LP) groups and analysed using three replicates. The calculated peak areas were used for statistical analysis. Normalization was performed relative to heavy standard peptides.

2.6.2 Absolute quantitative analysis of the targeted MS/MS

The peptides AVESYLLAHSDAYN, AVESYLLAHSDAY and AVESYLLAH were imported into Skyline (v.19.1.0.193). Peak area integration was conducted based on extracted ion chromatograms of the six MS/MS fragment ion masses listed in the Table [S3](#). All transitions and peak areas were validated manually. Because 4 ng of total protein was injected for all points in the calibration curves, normalization was performed relative to the light versions of the peptides analysed. For each peptide, quantification was performed by adding the areas corresponding to all fragment ions for the heavy peptide and dividing the result by the sum of all fragment ion signals corresponding to the light peptide. This heavy/light ratio was used to calculate the amount of peptide present in the sample extract.

2.7 Immunoassay-based quantification of Bet v 1

The Bet v 1 content of the LD extracts was measured using an ELISA kit using the European Pharmacopoeia standard rBet v 1 (Ref. Y0001565). Briefly, microplates were coated with 0.1 µg/well of the monoclonal antibody (mAb) anti-Bet v 1 (4B10, Indoor Biotech). A dilution curve ranging from 250 to 7.8 ng/ml was prepared for each LD extract, while, in parallel, a standard dilution curve ranging from 500 to 0.49 ng/ml was also prepared. Next, the microplates were washed and blocked for 30 min with BSA 1%-PBS-Tween 0.05%. Then, different extract and standard dilutions were added to the microplates and incubated for 30 min. Afterwards, the microplates were washed and biotinylated mAb anti-Bet v 1 was added (BI-2E10, Indoor Biotech.). After washing, streptavidin-PO was added and the development of the reaction was recorded.

2.8 Statistical analysis

The MS-determined Bet v 1 content of the LP samples was compared against the LD samples using Student's t-test with 95% confidence at the peptide level.

The normality of the distribution of variables was investigated using the Shapiro-Wilk normality test, while the Pearson correlation test was used to identify linear relationships between the LD and LP Bet v 1 levels. Spearman's rank-order correlation was used to identify linear relationships between the immunoassay- and MS-determined Bet v 1 levels in the LD extracts. Differences were considered statistically significant if $p < 0.05$. The statistical analysis was performed using GraphPad Prism 9.1.1 software (GraphPad Software, San Diego, CA, USA).

3 RESULTS

3.1 *Betula verrucosa* extracts

The manufacturing process achieved a high degree of consistency between different batches of the same type of extract.

The LD extracts presented bands between 10 and 100 kDa, with a principal band, corresponding to Bet v 1, at approximately 17 kDa, upon SDS-PAGE analysis. In contrast, the LP extracts showed predominantly high molecular weight components (Figure 1). This difference in molecular weight distribution was confirmed by size exclusion chromatography (Figure S1).

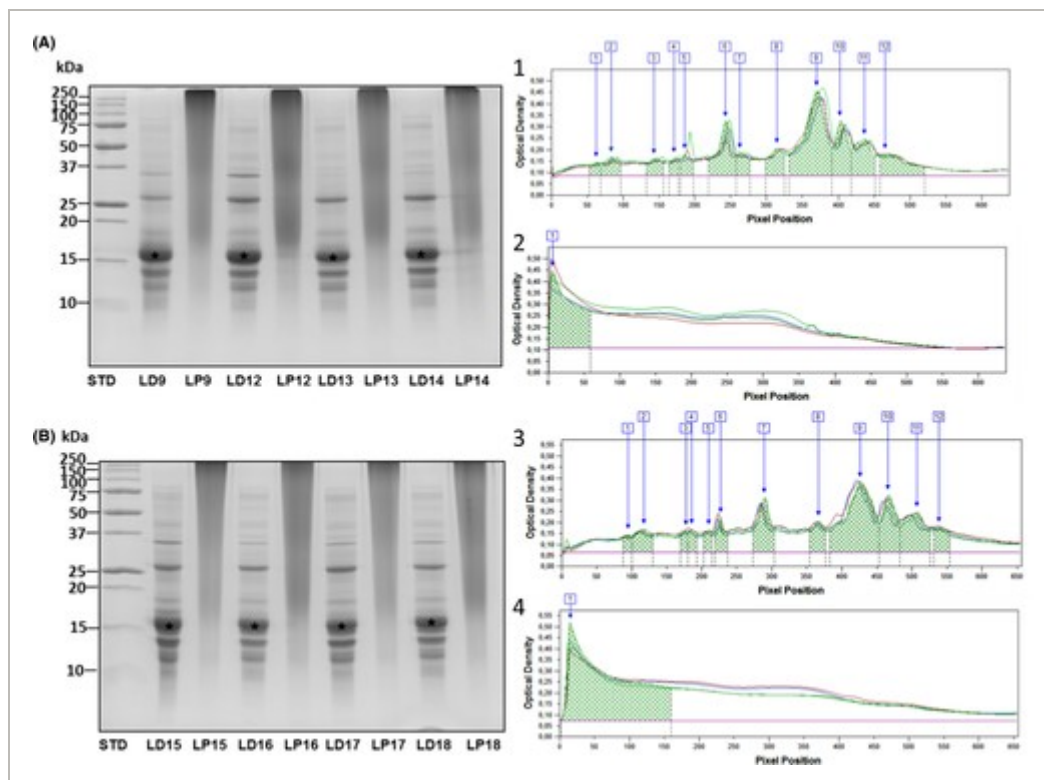


FIGURE 1

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Fifty µg of 8 different batches of LD extracts and their corresponding LP extracts analysed by SDS-PAGE under reducing conditions (A, B). STD: Molecular weight standard. Bet v 1 allergen is marked with an *. Right panel shows protein profiles obtained for each sample by scanning densitometry and grouped by type of extract (1, 3: depigmented extracts; 2, 4: polymerized extracts)

In addition, the allergenic profiles (Figure S2) were consistent for all LD batches. The presence of Bet v 1 (approximately 17 kDa) was recognized using the pool of sera. No IgE-binding protein was observed in the LP extracts.

3.2 Identification of Bet v 1 isoforms

Peptides corresponding to the 27 Bet v 1 isoforms described in the IUIS were identified in all

LD and LP batches. Due to the high homology of sequence between Bet v 1 isoforms, many identified peptides are shared by more than one isoform. Isoform.0301 (Q39415) was identified in all LD batches but in only three of the 18 LP samples. The mean sequence coverage was higher in LD samples than in the LP samples for all Bet v 1 isoforms (Figure S3).

3.3 Development of an LC-MS/MS targeted analysis for the absolute quantification of Bet v 1

3.3.1 Peptide selection

Nine LP samples were previously analysed by MS (data not shown) to identify trypsin-digested peptides candidates to be used as standards for Bet v 1 quantification. Figure 2 shows the alignment of the 27 Bet v 1 isoforms, the candidate peptides detected in these previous analyses and the selected peptides used to establish a targeted MS/MS method. As the same amount of total protein was used to analyse the LD and LP samples, a constant MS signal intensity was expected for every peptide analysed in both samples.

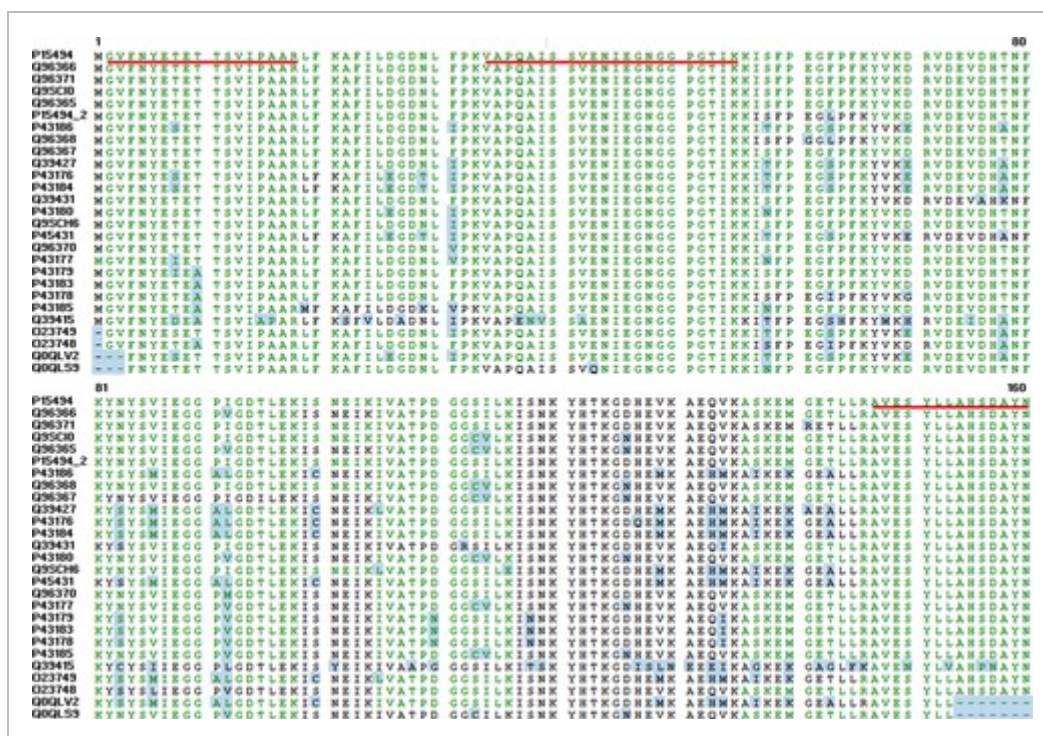


FIGURE 2

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Bet v 1 isoforms described by the IUIS. Amino acids highlighted in green correspond to peptides that were identified by mass spectrometry in previous analyses performed in 9 polymerized samples. Sequence differences are highlighted in blue. Underlined in red the three regions containing the eight peptides selected for methodology optimization: GVFNYESETTSVIPAAAR, GVFNYEITETTSVIPAAAR and GVFNYEITETTSVIPAAAR, which correspond to the N-terminal region, VAPQAISSVENIEGNNGPGTIK and its semitryptic version, SVENIEGNNGPGTIK, which correspond to a conserved internal region and the C-terminal peptide AVESYLLAHSDAYN, and its shorter versions AVESYLLAHSDAY and AVESYLLAH

Nevertheless, the results (Table 2) indicated that the peptides not located in the C-terminal of the protein, namely GVFNYESETTSVIPAAAR, GVFNYETETTSVIPAAAR, VAPQAISSVENIEGNGGPGTIK, SVENIEGNGGPGTIK and GVFNYEIETTSVIPAAAR, had significantly higher signals in the LD sample than in the LP sample. Conversely, peptide signals corresponding to the C-terminal of the protein (eg AVESYLLAHSDAYN and AVESYLLAHSDAY) had similar signal intensities (ie fold changes closer to 1).

TABLE 2. Comparison of mass spectrometry signals for the Bet v 1 selected peptides in the targeted MS/MS analysis of LD and LP samples of *Betula* extracts

Peptide	Fold Change ^a Result (LP/LD)	p-value ^b
GVFNYESETTSVIPAAAR	0.12 (95% CI:0.1 to 0.15)	.00
GVFNYETETTSVIPAAAR	0.23 (95% CI:0.19 to 0.27)	.00
VAPQAISSVENIEGNGGPGTIK	0.02 (95% CI:0.02 to 0.02)	.00
SVENIEGNGGPGTIK	0.06 (95% CI:0.06 to 0.07)	.00
GVFNYEIETTSVIPAAAR	0.1 (95% CI:0.08 to 0.13)	.00
AVESYLLAHSDAYN	0.99 (95% CI:0.85 to 1.156)	.82
AVESYLLAHSDAY	1.22 (95% CI:0.94 to 1.59)	.12

Note

The results represent the Skyline-calculated output for differential abundance test at the peptide level. Comparisons were performed by summing the selected transition peak areas for a specific peptide, normalizing to the standard, calculating the log2 value, averaging the technical replicates and performing a t-test on the resulting values.

^a Fold change (ratio between signals) at the peptide level for the LP extracts with respect to the LD extract sample.
^bStatistically significant changes are indicated by $p \leq .05$.

Therefore, only the C-terminal peptides AVESYLLAHSDAYN, AVESYLLAHSDAY and AVESYLLAH were validated.

3.3.2 Validation of the selected peptides

The AVESYLLAHSDAY and AVESYLLAH contents were 20–40 times lower than the AVESYLLAHSDAYN content. Thus, for these peptides, the ranges of the heavy, labelled internal standards used to construct the standard curves were lower. Each sample/point in the standard curves contained the three peptides diluted in water (see Table S2 and Figure S4).

The final calibration curves were generated by mixing different concentrations of the heavy standard peptides in a constant amount of sample to avoid matrix effects. When 200 ng of sample were used, overloading in the column resulted in retention time shifts and poor chromatography for the peptide AVESYLLAHSDAYN (see Figure S5) and therefore, the sample amount was reduced and tested at different concentrations, down to a minimum of 1 ng.

Finally, the amount of protein loaded on the system was set at 4 ng. A standard curve was constructed for each LD and LP sample, revealing, as shown in Figure 3, that linearity occurs from 0.25–100 fmol ($r^2 > .99$) for the peptide AVESYLLAHSDAYN, and from 0.25–10 fmol ($r^2 > .99$) for the peptides AVESYLLAHSDAY and AVESYLLAH.

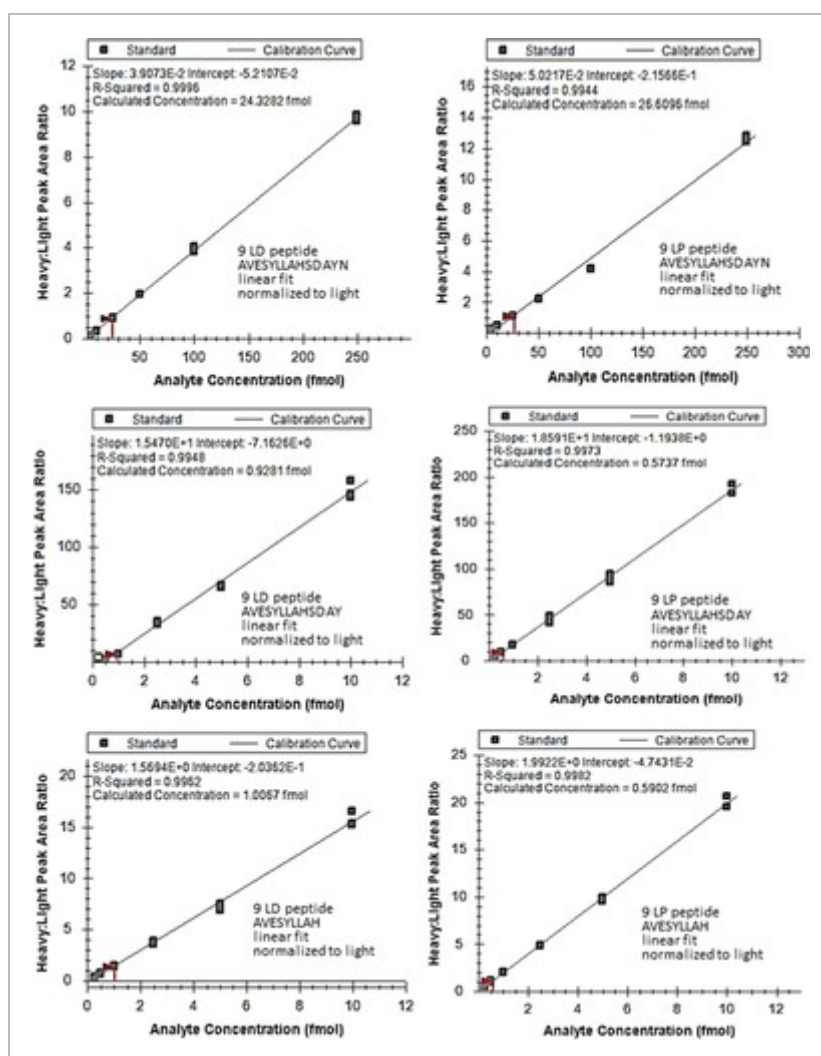


FIGURE 3

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Standard curves for the (A) LD and (B) LP samples. The peak area ratio of internal standard to analyte (H/L area ratio) is plotted against the heavy standard concentration at each point of the curve. The concentrations indicate the amount of heavy peptide on the column

Although the CV was less than 20% ($n = 3$) at 0.25 fmol, some of the signals for the fragment

ions were not present (not shown). Therefore, the LLOQ was set at 0.5 fmol on the column and, subsequently, an LOD of 0.166 fmol was calculated.

Based on the reverse calibration curves, the calibration points within the linear range, where the signal intensity and peak area of the AQUA peptide were closest to the naturally occurring peptide from the sample (ie heavy/light ratio of approximately 1) were used to quantify the Bet v 1 content.

3.4 Absolute quantification of Bet v 1 by LC-MS/MS targeted analysis

The mean Bet v 1 content measured in the 18 different LD and LP batches was $70.5 \pm 11.6 \mu\text{g}/\text{mg}$ for the LD extracts and $73.6 \pm 19.8 \mu\text{g}/\text{mg}$ for the LP extracts (Figure 4A, Table S4). The paired t-test results showed no significant differences in Bet v 1 content between the LD and LP extracts. An example of calculation of the absolute Bet v 1 content is provided in the SuppInfo (Table S5).

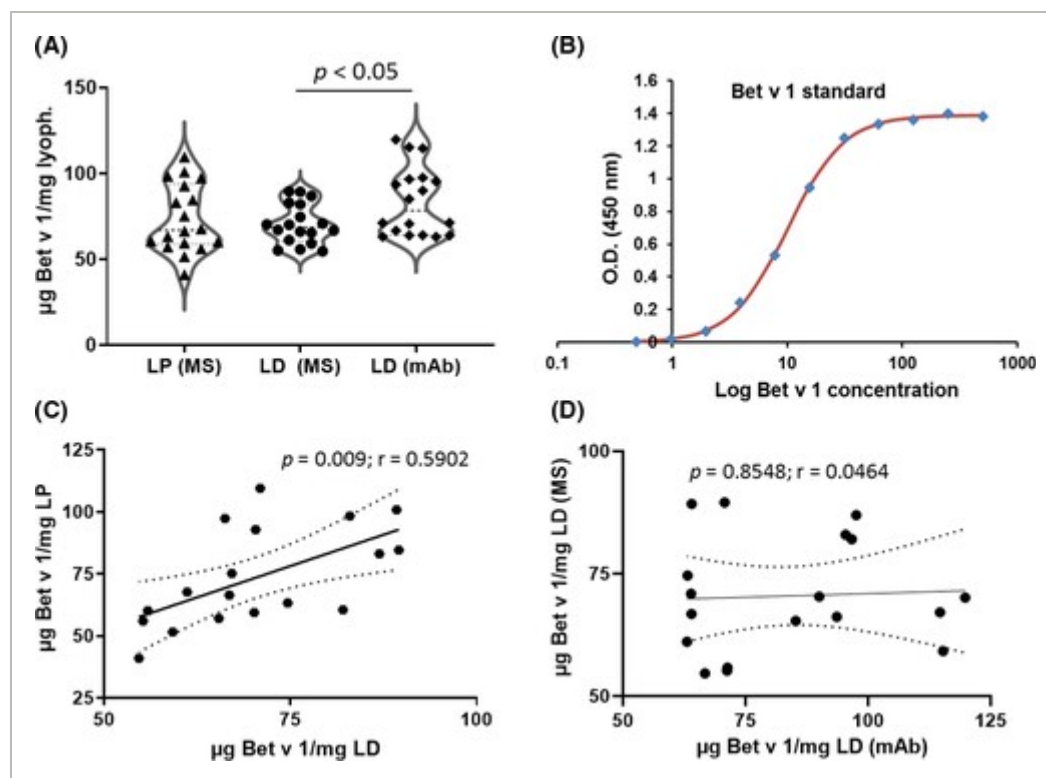


FIGURE 4

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(A) Bet v 1 content ($\mu\text{g}/\text{mg}$ lyophilized extract) determined in 18 batches of LD or LP extracts of *Betula verrucosa* using mass spectrometry analysis (MS) and immunoassays (mAb). (B) Bet v 1 standard curve of the ELISA assay (O.D.: Optical Density). Correlations between (C) the Bet v 1 levels in the LD and LP extracts determined using MS, and (D) the Bet v 1 levels in the LD extracts determined using mAb and MS

3.5 Immunoassay-based quantification of Bet v 1

The semi-log plot in Figure 4B shows that serial dilutions of the Bet v 1 standard produced a sigmoid curve. A four-parameter regression of $R^2 \geq .99$ was obtained for all assays.

The ELISA-measured Bet v 1 content in the LD extracts was $83.7 \pm 19.8 \mu\text{g}/\text{mg}$ (Figure 4A, Table S4).

3.6 Comparison of Bet v 1 quantification methods

A significant difference ($p < .05$, Wilcoxon test) was observed in the MS- and immunoassay-obtained Bet v 1 contents of the LD extracts.

A significant correlation (Pearson test: $p = .009$; $r = .5902$) was found between the Bet v 1 contents of the LD and LP extracts (Figure 4C) but not between the MS- and immunoassay-obtained Bet v 1 contents of the LD extracts (Spearman test: $p = .8548$; $r = .0464$, see Figure 4D).

4 DISCUSSION

Quantifying major allergens is a promising alternative for allergen extract standardization, for demonstrating batch-to-batch consistency and for correlating allergen concentration with the efficacy of immunotherapy.^{21, 22} Although some major allergens are being measured in native allergenic extracts, before being diluted and/or chemically or physically modified for human treatment, an effective allergen quantification method that can be applied to final products is required. Typically, the concentration of individual allergens in non-polymerized extracts is measured by an indirect method involving an immunoassay kit composed of allergen standards and specific antibodies. Although this is a consistent and useful method with the exception of Bet v 1 and Phl p 5a,⁵⁻⁷ there is a general lack of consensus regarding allergen standards. Therefore, in most cases, the use of different reagents or differences in the methodological conditions results in different values being obtained. Regarding standards, immunoassays require the production of recombinant or purified native allergens, in-depth characterized and with a preserved structure and immunological properties (especially the IgE-binding capacity). Furthermore, ELISA assays typically employ monoclonal antibodies, which can identify single epitopes and/or single isoforms of an allergen.^{2, 23} This method does not allow the allergen content of allergoids to be quantified, and the cross-reactivity between different allergens and isoforms within the same extract may also influence the final results. In fact, the concentration of major allergens in allergoids is estimated or calculated using non-polymerized extracts.

Recently, MS has become a potent tool for the identification and quantification of allergens in native allergenic extracts. This process involves the digestion of the allergenic extracts and recognition of specific allergen sequences. For quantification, specific amino acid sequences are synthesized and isotopically labelled for use as standards and their concentrations are compared with those of the sequences obtained from the digested extract. Thus, we developed a method for the direct quantification of Bet v 1 in chemically modified birch

extracts.

In Europe, owing to their excellent safety and efficacy, modified allergens have become the first option for the subcutaneous treatment of allergic patients.²⁴ In accordance with international guidelines, rigorous controls are applied during the manufacturing and characterization of these products to ensure batch-to-batch consistency.^{21, 25} By using allergen and protein profile analysis in combination with Bet v 1 identification, this consistency was demonstrated for the 18 batches of depigmented and depigmented-polymerized extracts included in this study. To date, 27 isoforms of Bet v 1 have been described (<http://www.allergen.org/>) and identified by LC-MS/MS in the extracts included in this study, although many peptides are shared by more than one isoform. One isoform was not detected in certain polymerized batches. This, and the generally lower sequence coverages obtained for polymerized samples, can be explained by the nature of this type of extract and the difficulty in accessing the cleavage sites using trypsin.

Three peptide sequences, corresponding to the C-terminal region and common to most of the Bet v 1 isoforms were synthesized, isotopically labelled and used as internal standards. They could be detected by MS with similar signal intensities in both the non-polymerized and polymerized extracts, thereby indicating minimal changes during the comparative analysis of the extracts. The other analysed peptides exhibited significantly higher signals in the LD sample than in the LP sample and were therefore discarded. This could be explained by lysine being one of the trypsin cleavage sites, given the involvement of this amino acid in the polymerization reaction. This leads to inefficient protein digestion in the polymerized samples, which results in a lower number of peptides as well as a lower concentration of digested lysine-containing peptides.

To create peptide calibration curves and assess the linearity and the detection limit, different ranges of peptide concentration were investigated. Nevertheless, for protein quantification, the susceptibility of MS signals to matrix effects must be considered. Therefore, for each sample, reverse calibration curves were generated by mixing different concentrations of the standard peptides in a constant amount of the sample. The resultant signals were linear within the concentration ranges used to construct the calibration curves. The LOQ was 0.5 fmol, and the corresponding LOD was set to 0.166 fmol.

The developed method was applied to quantify the Bet v 1 content in 18 commercial batches of LD and LP samples. Both types of extract exhibited a similar mean allergen content, approximately 70 µg/mg of lyophilized extract, and a statistically significant correlation was found for Bet v 1 levels between types of extracts. These results reflect the consistency of the manufacturing process and the effectiveness of the polymerization step, verifying that most of the proteins and allergens present in the depigmented extract are preserved in the corresponding allergoid. Moreover, the direct quantification of the major allergen content in both extracts constitutes a new alternative for confirming polymerization efficacy.

Nevertheless, statistically significant differences were observed when the immunoassay- and MS-obtained results were compared. These differences can be attributed to the inherent differences between the two methods. For example, whereas the immunoassay uses the whole protein as a standard, the calibration of which can interfere with the results, MS uses synthetic peptides. In addition, the detection of a protein via immunoassays is based on an antibody-allergen interaction, which implies a higher variability in the results. Moreover, this could explain the higher dispersion observed for the ELISA data compared with those obtained using MS. Similar studies on MS-based allergen quantification have been investigated native allergenic extracts from pollen,^{12, 13, 26} insects^{16, 27} and different food matrices.^{14, 28, 29} However, this study is the first to report the development of an MS method for quantifying the allergen content of allergoids. The results show that MS-based targeted analysis is a robust and reliable technique that can measure the allergen concentration in polymerized extracts, a service currently not offered by existing alternatives. The main limitation of this method is that the peptides used as standards must fulfil specific requirements, as previously described, and therefore not all polymerized allergens could generate peptides suitable for MS quantification. Nonetheless, this study lays the groundwork for validating this type of MS methods for quantifying new allergens in different types of modified extracts and even in the final product.

5 CONCLUSION

In summary, this study represents the first time that MS-based targeted analysis has been applied to quantify the allergen content of allergoids. Although additional data must be generated and validated, the results presented herein suggest a promising alternative for performing more detailed characterizations of polymerized allergenic extracts, including the possibility of measuring the isoforms and the major and minor allergens concurrently. As such, the proposed method could become a definitive tool for improving allergen extract standardization and, consequently, provide significant benefits for individuals undergoing immunotherapy.

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CONFLICT OF INTEREST

RM, MG and JC are/have been employees of LETI Pharma, S.L.U.

AUTHOR CONTRIBUTIONS

RM contributed to the acquisition, analysis and interpretation of data, drafting the manuscript and agrees to be accountable for all aspects of the work. MAO contributed to the design of the work, the acquisition, analysis and interpretation of data, drafting the

manuscript and agrees to be accountable for all aspects of the work. MG, EO and JC contributed to the design of the work, analysis and interpretation of data, revising the manuscript critically, final approval and agrees to be accountable for all aspects of the work.

Open Research



DATA AVAILABILITY STATEMENT

Data are available upon reasonable request from the corresponding author.

Supporting Information



Filename	Description
cea14067-sup-0001-FigS1-S5.docx Word 2007 document , 842.2 KB	Fig S1-S5
cea14067-sup-0002-TableS1-S5.docx Word 2007 document , 39.1 KB	Table S1-S5

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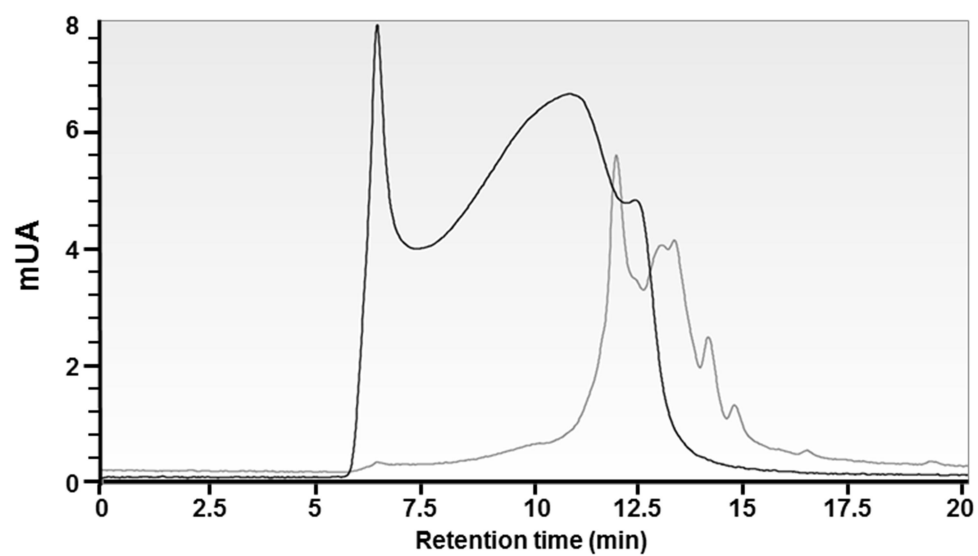
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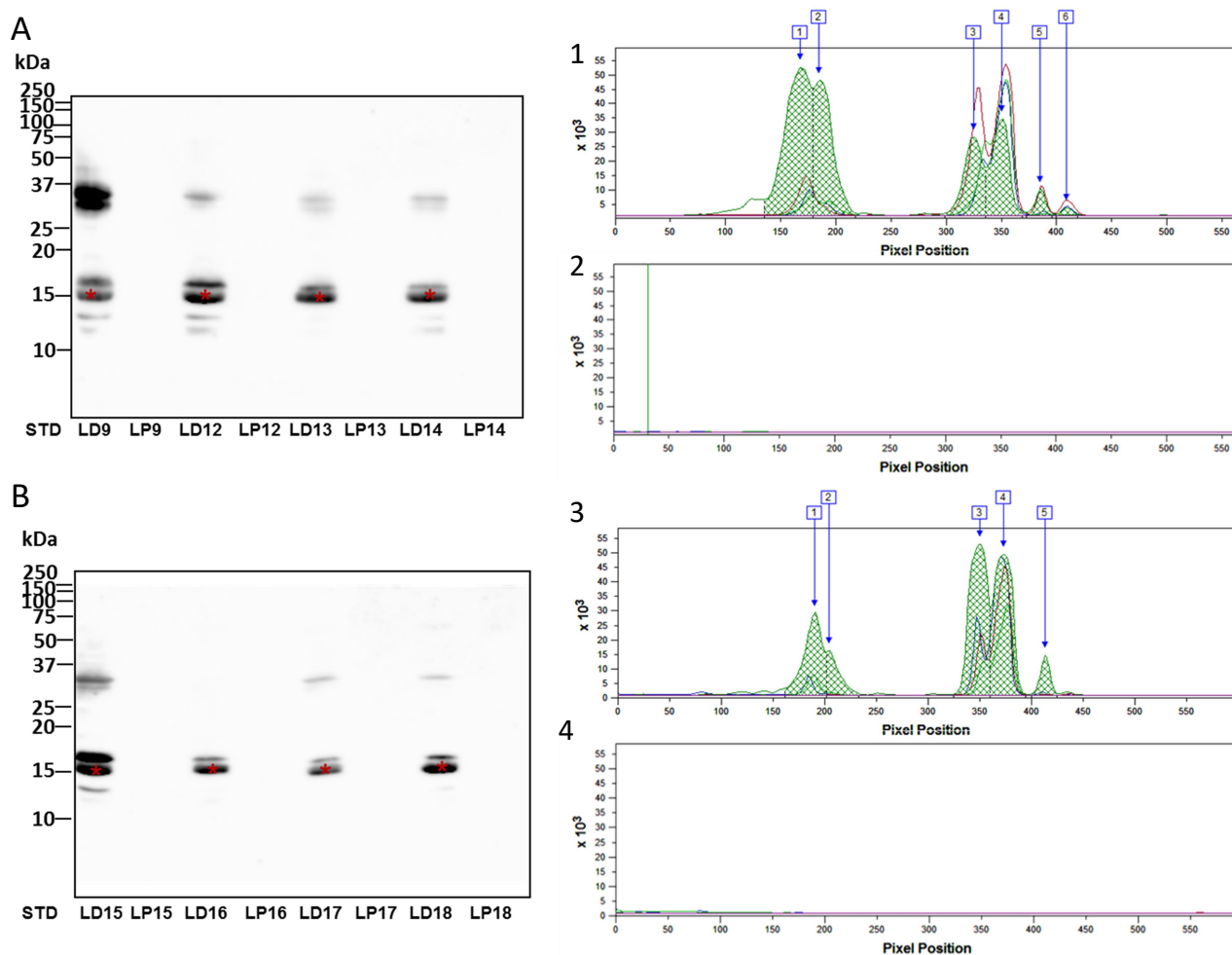
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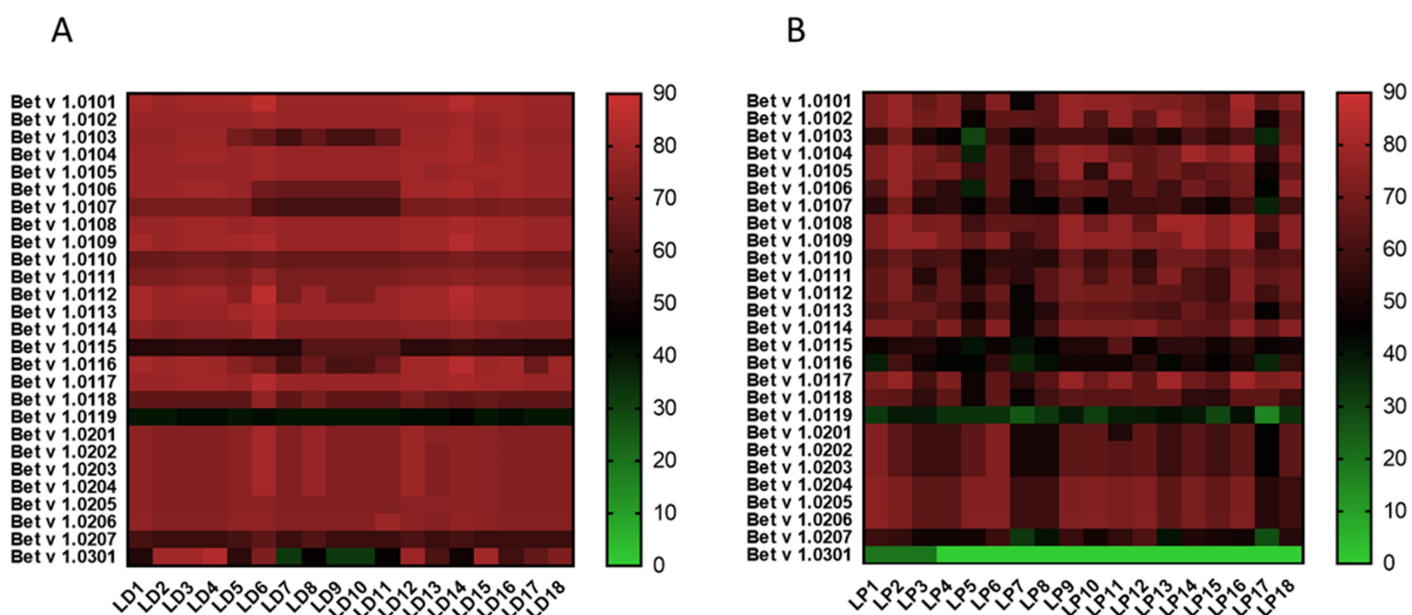
SUPPLEMENTARY INFORMATION FIGURES



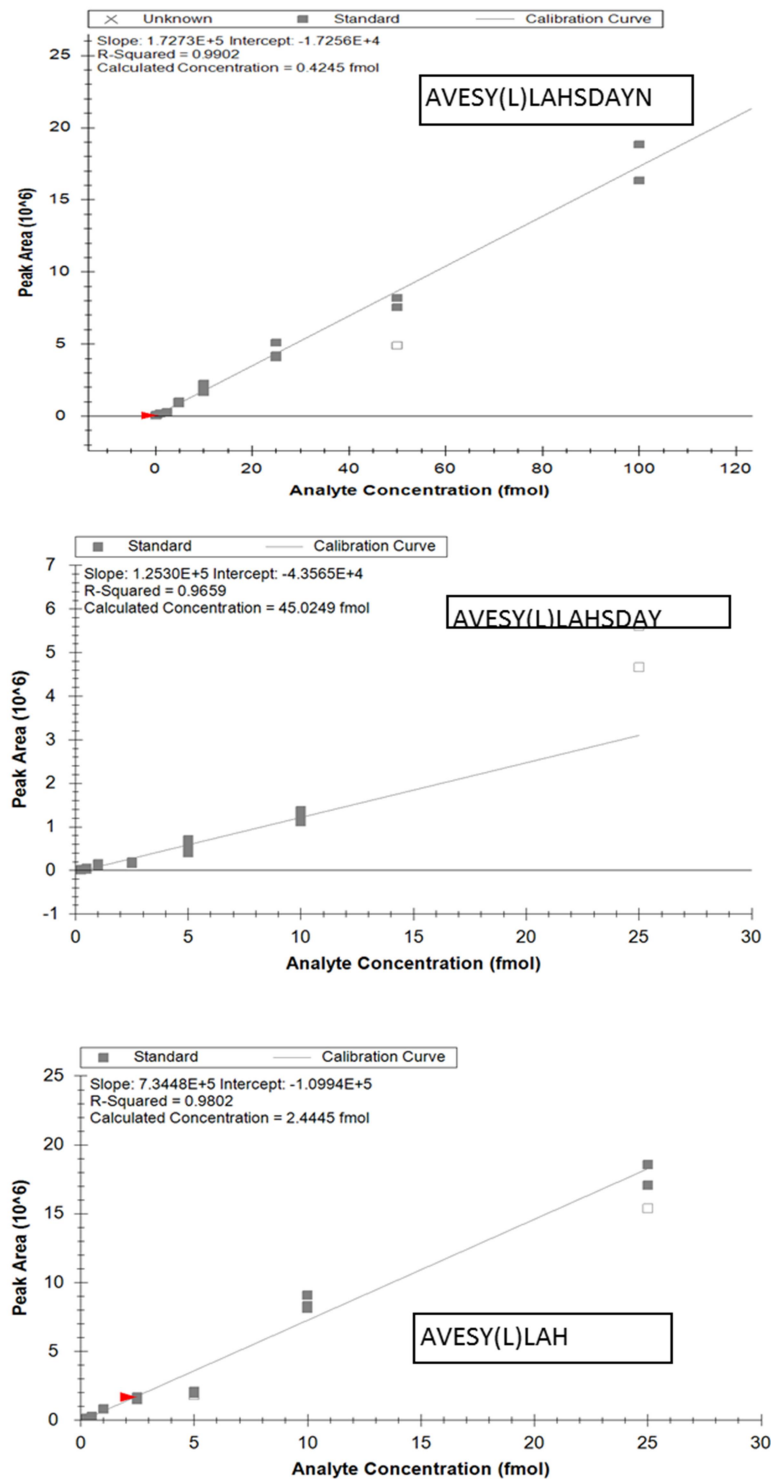
Supplementary Figure 1. Size exclusion chromatograms of one LD Betula batch (gray line) and its corresponding LP extract (black line) detected at 280 nm. Elution time in minutes.



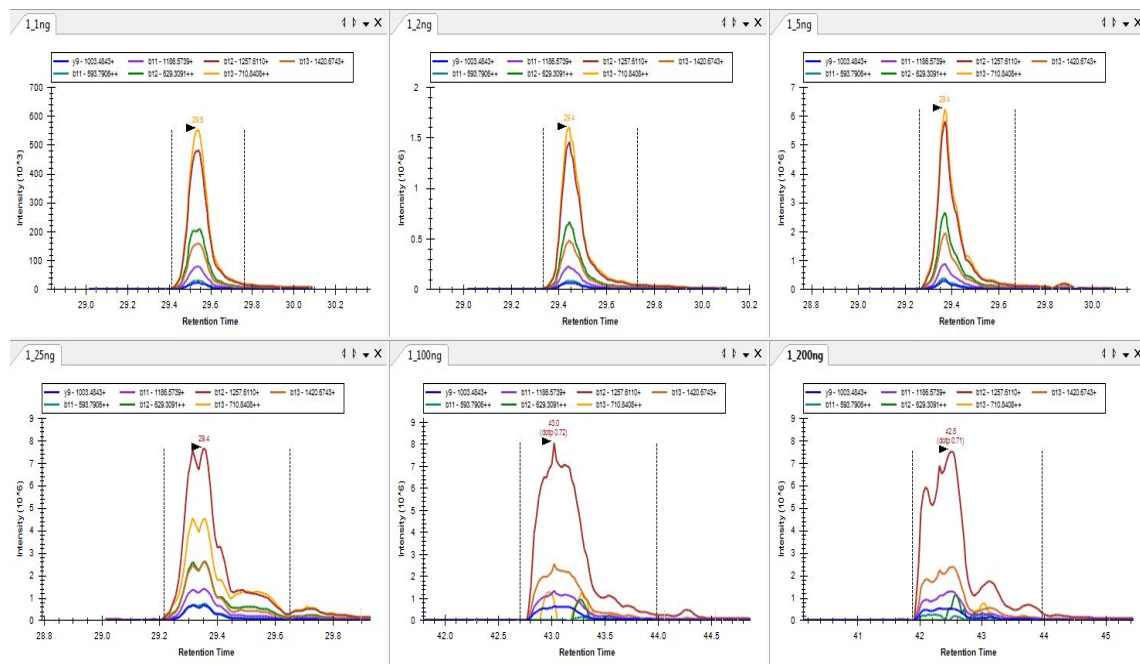
Supplementary Figure 2. Fifty μ g of 8 different batches of LD extracts and their corresponding LP extracts analyzed by immunoblot and incubated with a pool of sera from birch allergic patients (A, B). STD: Molecular weight standard. Bet v 1 allergen is marked with an *. Right panel shows allergenic profiles obtained for each sample by scanning densitometry and grouped by type of extract (1, 3: depigmented extracts; 2, 4: polymerized extracts).



Supplementary Figure 3. Bet v 1 identification using LC-MS/MS. A heat map illustrating the sequence coverage (%) was obtained for the different isoforms of Bet v 1 in the 18 batches of (A) depigmented and (B) depigmented-polymerized *Betula* extracts examined in the study.



Supplementary Figure 4. Standard curve tested with AQUA peptides diluted in water. Peak areas corresponding to sum of areas for the different fragment ions of each peptide. Concentrations as amounts of heavy peptide on column.



Supplementary Figure 5. Peak areas for the different fragment ions corresponding to peptide AVESYLLAHSDAYN when 1, 2, 5, 25, 100 and 200 ng of total protein were analyzed. Overloading of the column resulted in poor chromatography when higher amounts of sample were injected into the system.

SUPPLEMENTARY INFORMATION

Materials and Methods

1. Extract manufacturing

Eighteen different depigmented extracts and their corresponding depigmented-polymerized extracts of *Betula verrucosa* were manufactured in strict compliance with GMP principles following internal procedures (LETI Pharma S.L.U., Madrid, Spain). Briefly, different batches of *B. verrucosa* pollen, collected in different years, were individually homogenized and extracted (1:10 w/v) in phosphate-buffered saline buffer (PBS) 0.01 M pH 7.4. With the objective to purify the extracts, the pH was progressively reduced to 2 with 2 M HCl and kept under magnetic stirring for 15 minutes. Finally, the extract was extensively dialyzed against bi-distilled water with a cut-off membrane of 3.5 kDa (Millipore, Bedford, USA) for removing low molecular weight substances, pH adjusted to 7 with NaOH and sterile-filtered, frozen and lyophilized. This extract was termed depigmented extract (LD).

The depigmented extracts were reconstituted at 15 mg/ml in PBS 0.01M and polymerized with glutaraldehyde. The reaction was stopped with glycine, the extracts dialyzed in 100,000 kDa dialysis membranes (Millipore, Burlington, MA, USA) and freeze-dried. This extract was named depigmented-polymerized extract (LP).

2. Protein and allergenic profile

The protein profile of LD and LP extracts was investigated by SDS-PAGE under reducing conditions (50 µg of freeze-dried material of each extract). In addition, molecular weight distribution was analyzed by Size Exclusion Chromatography (SEC) using a Bio SEC-3 Column (Agilent Technologies, Santa Clara, CA, USA) in a 1260 series HPLC system (Agilent), at 1ml/min in 150 mM phosphate buffer, pH 7.

Detection was performed at UV–215 nm and Gel Filtration Standard (Bio-Rad Laboratories, Hercules, CA, USA) was used as molecular weight marker.

The allergenic profile of the extracts was analyzed by immunoblot. Electrophoretically separated bands were transferred onto PVDF membranes and incubated overnight with a pool of sera composed of equal volumes of 20 serum samples from birch allergic individuals (Plasmalab International; Everett, WA, USA) and prepared following specifications described in the European guidelines. After washing, membranes were incubated with monoclonal a-human-IgE-PO (Ingenasa, Madrid, Spain), developed with luminol solution (Clarity™ Western ECL Substrate, Bio-Rad) and detected by

chemiluminescence (Amersham Imager 600 RGB system; GE Healthcare, Uppsala, Sweden).

Scanning densitometry of SDS-PAGE and immunoblot detected bands was performed with ImageQuant TL 8.1 software (GE Healthcare).

3. Proteins digestion

Birch lyophilized samples of LD and LP were dissolved in ammonium bicarbonate (50 mM NH_4HCO_3 , pH 8.5) and proteins were precipitated in acetone overnight at -20°C . The pellets were dissolved in 8 M urea/ 50 mM NH_4HCO_3 and proteins quantified using PierceTM BCA Protein assay (ThermoFisher Scientific, Waltham, MA, USA). Proteins were reduced (dithiothreitol 20 mM; 1.5 h, 32°C) and alkylated (iodoacetamide 35 mM; at RT for 30 min). Samples were diluted down to 1.5 M urea and digested with Lys C 1/10 (w/w) for 2h (Sequence grade Lys-C, Wako; pH 8, 35°C) followed by porcine trypsin (Promega, Madison, WI, USA) digestion 1/25 (w/w) (pH 8, 35°C , 16h overnight). Peptides were cleaned-up with a C18 Top Tip (Poly LC), dried-down and stored at -20°C until the subsequent MS analysis.

4. LC-MS/MS Data-Dependent Analysis (DDA) for Bet v 1 identification

Digested peptides were analyzed in DDA mode in an LC-MS system comprising a nanoAcquity liquid chromatograph (Waters, Mildford, MA, USA) coupled to an LTQ-Orbitrap Velos mass spectrometer (ThermoFisher Scientific). Digested peptides were analyzed for 30 min at a flow rate of 250 nL/min in a C18 column using a gradient of 1 to 40% B (Symmetry C18TM trap column ($5\ \mu\text{m} \times 180\ \mu\text{m} \times 20\ \text{mm}$); $75\ \mu\text{m}\ \text{Ø}$, 25 cm, 1.7 μm BEH column, Waters), (A: 0.1% formic acid; B: 100% acetonitrile; 0.1% formic acid).

Raw data files were searched against a database containing the International Union of Immunological Societies (IUIS) protein entries for Bet v 1 isoforms using the Mascot search engine.

Supplementary Table 1. m/z and retention times for the light and heavy versions of the peptides included in the targeted acquisition method for relative quantification of Bet v 1.

m/z	Start time (min.)	End time (min.)	Sequence
927.965248	43.53	49.53	GVFNYESSETTSVIPAAAR (light)
932.969382	43.53	49.53	GVFNYESSETTSVIPAAAR (heavy)
618.979257	43.53	49.53	GVFNYESSETTSVIPAAAR (light)
622.315347	43.53	49.53	GVFNYESSETTSVIPAAAR (heavy)
920.957423	43.33	49.33	GVFNYESSETTSVIPAAAR (light)
925.961557	43.33	49.33	GVFNYESSETTSVIPAAAR (heavy)
614.307374	43.33	49.33	GVFNYESSETTSVIPAAAR (light)
617.643463	43.33	49.33	GVFNYESSETTSVIPAAAR (heavy)
933.98344	47.25	53.25	GVFNYESSETTSVIPAAAR (light)
938.987575	47.25	53.25	GVFNYESSETTSVIPAAAR (heavy)
622.991386	47.25	53.25	GVFNYESSETTSVIPAAAR (light)
626.327475	47.25	53.25	GVFNYESSETTSVIPAAAR (heavy)
1069.558032	40.12	46.12	VAPQAISSVENIEGNGGPGTIK (light)
1073.565132	40.12	46.12	VAPQAISSVENIEGNGGPGTIK (heavy)
713.374447	40.12	46.12	VAPQAISSVENIEGNGGPGTIK (light)
716.045846	40.12	46.12	VAPQAISSVENIEGNGGPGTIK (heavy)
736.372994	31.8	37.8	SVENIEGNGGPGTIK (light)
740.380094	31.8	37.8	SVENIEGNGGPGTIK (heavy)
776.867545	40.09	46.09	AVESYLLAHSDAYN (light)
780.376127	40.09	46.09	AVESYLLAHSDAYN (heavy)
518.247455	40.09	46.09	AVESYLLAHSDAYN (light)
520.58651	40.09	46.09	AVESYLLAHSDAYN (heavy)
719.846081	41.76	47.76	AVESYLLAHSDAY (light)
723.354663	41.76	47.76	AVESYLLAHSDAY (heavy)

Supplementary Table 2. Concentrations of the AQUA peptides diluted in water in the different points of the standard curve tested. Concentrations as amounts of peptide on column.

	AVESY(L)LAHSDAYN*	AVESY(L)LAHSDAY*	AVESY(L)LAH*
Standard curve	fmol on column	fmol on column	fmol on column
AQUA_250amol	0.25	0.25	0.25
AQUA_500amol	0.5	0.5	0.5
AQUA_1fmol	1	1	1
AQUA_2_5fmol	2.5	2.5	2.5
AQUA_5fmol_1	5	5	5
AQUA_10fmol_1	10	10	10
AQUA_25fmol_1	25	25	25
AQUA_50fmol_1	50		
AQUA_100fmol_1	100		

*(L) corresponds to $^{13}\text{C}_6$, ^{15}N -Leu.

Supplementary Table 3. Fragment ions/transitions analyzed and used for the absolute quantification of the selected peptides.

<i>AVESYLLAHSDAYN</i> 776.8675++	<i>AVESYLLAHSDAYN</i> 780.3761++ (heavy)
L[y9] - 1003.4843+	L [y9] - 1010.5015+
D[b11]-1186.5739+	D [b11] - 1193.5910+
A[b12]-1257.6110+	A [b12] - 1264.6282+
Y[b13]-1420.6743+	Y [b13] - 1427.6915+
D[b11]-593.7906++	D [b11] - 597.2992++
A[b12]-629.3091++	A [b12] - 632.8177++
Y[b13]-710.8408++	Y [b13] - 714.3494++
<i>AVESYLLAHSDAY</i> 719.8461++	<i>AVESYLLAHSDAY</i> 723.3547++ (heavy)
A[b8]-847.4560+	A[b8]-854.4732+
H[b9]-984.5149+	H[b9]-991.5321+
D[b11]-1186.5739+	D[b11]-1193.5910+
A[b12]-1257.6110+	A[b12]-1264.6282+
D[b11]-593.7906++	D[b11]-597.2992++
A[b12]-629.3091++	A[b12]-632.8177++
<i>AVESYLLAH</i> 501.7664++	<i>AVESYLLAH</i> 505.2750++ (heavy)
E[y7]-832.4199+	E[y7]-839.4371+
S[y6]-703.3774+	S[y6]-710.3945+
Y[y5]-616.3453+	Y[y5]-623.3625+
L[y4]-453.2820+	L[y4]-460.2992+
E[y7]-416.7136++	E[y7]-420.2222++
L[b6]-663.3348+	L[b6]-670.3520+
L[b7]-776.4189+	L[b7]-783.4360+

Supplementary Table 4. Bet v 1 content ($\mu\text{g}/\text{mg}$ lyophilized extract) determined in 18 batches of depigmented (LD) and depigmented-polymerized (LP) extracts of *B. verrucosa* by immune-assays (mAb) or mass-spectrometry analysis (MS).

Extract family	Bet v 1 content ($\mu\text{g}/\text{mg}$ lyophilized extract)		
	LD extracts (MS)	LP extracts (MS)	LD extracts (mAb)
1	70.36	92.86	90.03
2	66.23	97.36	93.60
3	55.87	60.23	71.31
4	70.95	109.5	63.88
5	70.15	59.46	119.77
6	66.81	66.46	63.96
7	54.67	41.06	66.72
8	59.22	51.59	115.29
9	67.16	75.24	114.64
10	86.97	83.12	97.55
11	65.41	57.15	85.21
12	82.99	98.39	95.38
13	74.64	63.33	63.19
14	89.56	84.59	70.72
15	82.07	60.53	96.64
16	55.23	56.07	71.23
17	61.16	67.69	63.06
18	89.27	100.86	64.04

Supplementary Table 5. Bet v 1 content ($\mu\text{g}/\text{mg}$ lyophilized extract) determined in the depigmented (LD) and depigmented-polymerized (LP) extracts of *B. verrucosa* by mass spectrometry analysis. Example of calculations for sample 12. For the three peptides in each sample, the point in the calibration curve with H/L ratio closest to one was used to extrapolate the amount of the peptide in the sample, and the amount of the three peptides were summed for the final Bet v 1 quantification in fmol. The molecular mass of Bet v 1 (17571 Da) was used to calculate μg of the protein in the sample. The amount of Bet v 1 in the extracts was corrected with the precipitation factor, which was calculated by dividing the total protein concentration before and after the precipitation step.

Sample	Peptide Sequence	Replicate Name	Heavy to Light Ratio	Quantification skyline (heavy) (fmol)	Light peptide on column (fmol)	Lyophilized sample on column (ng)	fmol peptide / ng lyophilized extract	ng peptide/ ng lyophilized extract	μg Bet v 1 / μg lyophilized extract	precipitation factor ^a	μg Bet v 1 protein / mg lyophilized extract	Mean μg Bet v 1 protein/ mg lyophilized extract	SD	% CV
Extract 12	AVESYLLAHSDAYN	12_LD_4ng_50fmol_1	1	46.4	38.4	12	3.3	0.06						
	AVESYLLAHSDAY	12_LD_4ng_250amol_1	1	0.3	0.3	12	0.0	0.0						
	AVESYLLAH	12_LD_4ng_500amol_1	1	0.4	0.5	12	0.0	0.00	0.06	0.715	83.1			
	AVESYLLAHSDAYN	12_LD_4ng_50fmol_2	1	55.1	38.3	12	3.3	0.06						
	AVESYLLAHSDAY	12_LD_4ng_250amol_2	1	0.3	0.3	12	0.0	0.0						
	AVESYLLAH	12_LD_4ng_500amol_2	1	0.4	0.5	12	0.0	0.00	0.06	0.7	82.8			
	AVESYLLAHSDAYN	12_LD_4ng_50fmol_3	1	51.3	38.3	12	3.3	0.06						
	AVESYLLAHSDAY	12_LD_4ng_250amol_3	1	0.3	0.3	12	0.0	0.0						
	AVESYLLAH	12_LD_4ng_500amol_3	1	0.4	0.5	12	0.0	0.00	0.06	0.7	83.1	83.0	0.19	0.23
Extract 12	AVESYLLAHSDAYN	12_LP_4ng_25fmol_1	1	22.1	30.2	6	4.8	0.08						
	AVESYLLAHSDAY	12_LP_4ng_250amol_1	1	0.1	0.6	6	0.1	0.0						
	AVESYLLAH	12_LP_4ng_500amol_1	1	0.4	0.2	6	0.0	0.00	0.09	0.9	98.6			
	AVESYLLAHSDAYN	12_LP_4ng_25fmol_2	1	22.6	30.2	6	4.8	0.08						
	AVESYLLAHSDAY	12_LP_4ng_250amol_2	1	0.1	0.2	6	0.0	0.0						
	AVESYLLAH	12_LP_4ng_500amol_2	1	0.4	0.5	6	0.1	0.00	0.09	0.9	98.3			
	AVESYLLAHSDAYN	12_LP_4ng_25fmol_3	1	21.5	30.2	6	4.8	0.08						
	AVESYLLAHSDAY	12_LP_4ng_250amol_3	1	0.2	0.2	6	0.0	0.0						
	AVESYLLAH	12_LP_4ng_500amol_3	1	0.4	0.5	6	0.1	0.00	0.09	0.9	98.2	98.4	0.22	0.22

^a Precipitation factor was calculated by dividing the total protein concentration measured by colorimetric assay before and after the precipitation step. SD: standard deviation; CV: coefficient of variation