

Absolute Quantification of Allergens from Complex Mixtures: A New Sensitive Tool for Standardization of Allergen Extracts for Specific Immunotherapy

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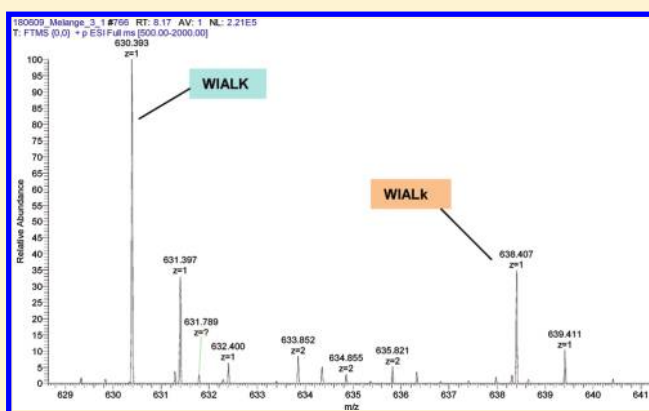
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ABSTRACT: Products for specific diagnosis and immunotherapy of IgE-mediated allergies are currently based on natural extracts. Quantification of major allergen content is an important aspect of standardization as important allergens particularly impact vaccine potency. The aim of the study was to develop a mass spectrometry (MS) based assay for absolute quantification of Timothy (*Phleum pratense*) pollen allergens Phl p 1 and Phl p 5 in *P. pratense* extract. High-resolution and accurate mass (HRAM) MS was selected for its ability to detect peptides with high selectivity and mass accuracy (<3 ppm). Isotope labeled heavy peptides were used for absolute quantification of specific isoallergens of Phl p 1 and Phl p 5 at low femtomole level in *P. pratense* extract. Robustness and linearity of the method was demonstrated with intra day precision $\leq 5\%$ ($n = 3$). Phl p 1b was shown to be 5 times less abundant than its variant Phl p 1a and Phl p 5b was shown to be 9 times more abundant than the Phl p 5a. The present study shows that allergen, and/or isoallergen specific, surrogate signature peptides analyzed with HRAM MS is a sensitive and accurate tool for identification and quantification of allergens from complex allergen sources.

KEYWORDS: quantification, allergen extract, mass spectrometry, standardization, *Phleum pratense*, peptide



INTRODUCTION

Allergic symptoms in humans are caused by exposure to specific allergens, which interact with the human immune system. Optimal management of allergic patients, therefore, includes identification of the offending allergen and treatment of the patient by specific immunotherapy using that particular allergen. Specific diagnosis and conventional specific immunotherapy are currently performed by use of allergen extracts produced by aqueous extraction of natural source materials, such as tree and grass pollen, dust mite cultures and bee or wasp venoms. The composition of natural materials is known to vary considerably, and therefore, knowledge of the composition and essential allergens is a prerequisite for the manufacturing of consistent products ensuring reproducibility, safety, efficacy and convenience in clinical use.^{1,2} Standardization of allergen extracts is a key element to control inherent variation in the natural source material. Standardization requires the application of scientifically documented methods for routine batch release, and it can be improved by introducing new and better methods as they are developed. Currently used methods for major allergen determination include single radial immuno diffusion,³ rocket immuno electrophoresis⁴ ELISA (enzyme-linked immunosorbent assay)⁵ and techniques using mono- and/or polyclonal

antibodies.⁶ All quantitative aspects of these techniques are dependent on antibodies as reagents and as such vulnerable to change over time. In addition, immunoassays are difficult to multiplex for concomitant quantification of several allergens.⁷

Allergens occur naturally as complex mixtures of major and minor allergens. The dynamic range is further enhanced as most allergens are expressed as mixtures of isoallergens and/or variants. In spite of their heterogeneous nature, each isoform contains signature sequence(s), which are characteristic within the allergen and/or the species, and can thus be employed for the purpose of quantification. The detection of these signature peptide sequences acts as a surrogate for the detection of the protein itself.

Advances in the field of biological mass spectrometry have resulted in techniques which can be used for direct quantification of proteins from biological samples. Relative quantification strategies include the use of labeling techniques such as iTRAQ (isobaric tag for relative and absolute quantification), TMT (tandem mass tags), or SILAC (stable isotope labeling by amino acids in cell culture),^{8–10} These methodologies are mainly

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applied to differential expression analysis in order to compare proteomes and for discovery of potential biomarkers.

MS based absolute quantification can be performed in different ways. The classic analytical strategy includes the creation of a standard curve based upon one or more peptides from the proteins of interest.¹¹ However, in this study, the lack of blank matrix sample which does not contain the protein(s) of interest renders difficult the use of this approach. The most commonly used strategy for peptide quantification is the isotope dilution approach which involves the use of stable isotope labeled homologues.^{12,13} In short, the method relies on the addition of a defined amount of isotope labeled peptide homologues to the peptide of interest. "Heavy" versions (e.g., ¹³C, ¹⁵N, etc.) of lysine or arginine amino acid residues are incorporated during the peptide synthesis producing a mass difference of 8 and 10 Da, respectively, with the endogenous form of the peptide. "Heavy" and "light" versions of the peptides co-elute when separated by reversed phase liquid chromatography allowing a concomitant detection and quantification with limited bias due to ionization or interferences from the sample matrix. The "heavy" to "light" peptide peak area ratio is then measured to calculate the peptide concentration in the sample. This method is the simplest approach to calculate the peptide concentration but does not provide any information about the precision of measurement over the concentration range of the assay and the linearity of the detector response. In this work, we generated a reverse calibration curve, which consists of building a calibration curve based on isotope labeled peptide homologues. The "heavy" peptide is incorporated at different concentrations into the matrix containing the endogenous form of the peptide to be quantified. Lower limits of detection can be assessed as well as incertitude of the measurement for each of the surrogate peptides and the corresponding proteins.

Reference methods for absolute quantification usually involve the use of a triple quadrupole mass spectrometer instrument operated in selective reaction monitoring (SRM) mode. SRM has the benefit of being sensitive, highly selective and easily multiplexed. The methodology has been extensively developed to quantify small molecules^{14,15} and has more recently been extended to the measurement of numerous peptides and proteins in complex matrixes.^{16,17} In the present study, however, we made use of HRAM provided by Orbitrap mass analyzer technology^{18,19} which enabled us to measure several *Phleum pratense* allergens and their isoforms in the same analytical run.

MATERIAL AND METHODS

Proteolytic Digestions

The nPhl p 1 and nPhl p 5 were purified from *P. pratense* extract as previously described.^{20,21} One nanomole of freeze-dried nPhl p 1 and nPhl p 5 proteins was dissolved into 25 mM Tris-HCl, 1 M urea (Sigma), pH 7.8. Freeze-dried *P. pratense* extract (8.0 µg/µL, w/v) (ALK-Abelló, Hørsholm, Denmark) was dissolved into 8 M Urea in 0.4 M (NH₄)HCO₃ buffer. Samples were reduced by 45 mM dithiothreitol (Sigma) at 56 °C for 30 min and alkylated by 100 mM iodoacetamide (Sigma) for 30 min at room temperature in the dark. Reduced and alkylated samples were cleaved into peptides at the final volume of 200 µL using 3–10% (w/w) trypsin (Seq. Grade Modified Trypsin, Promega, Madison, WI) at 37 °C for 18 h. The amount of trypsin used per sample was determined based on the previous experimental data (data not shown).^{22,23} The digestion was stopped by adding 5 µL of 5% formic acid. The samples were frozen and stored at –20 °C.

LC–MS/MS Analysis

Five picomoles of nPhl p 1 and nPhl p 5 digest samples was injected onto reversed-phase analytical Hypersil Gold column (1 mm × 5 cm 1.9 µm, Thermo Fisher Scientific). Separation was performed using a Thermo Scientific Accela MS pump (Thermo Fisher Scientific, San Jose, CA) at a flow rate of 150 µL/min. Peptides were eluted with a 5–40% acetonitrile in 0.1% formic acid gradient lasting 18 min (including column equilibration). A Thermo Scientific Exactive benchtop Orbitrap (ThermoFisher Scientific, Bremen, Germany) mass spectrometer was operated in positive mode with an Ion Max API electrospray source (Thermo Fisher Scientific, San Jose, CA). An electrospray ionization (ESI) spray voltage of 3.5 kV was applied. Sheath gas and auxiliary gas rates of 40 and 5 were used, respectively. The ion transfer tube temperature was set to 350 °C. The mass spectrometry method consisted of alternating full scan MS and full scan tandem mass spectrometry (MS/MS) without precursor selection. Full-scan mass spectra were acquired with the Orbitrap mass spectrometer at 50 000 resolving power with a mass range from 500 to 2000 *m/z*. Tandem mass spectrometry was performed in an external collision cell, termed the higher energy collision dissociation (HCD) cell, and MS/MS spectra were acquired with a resolving power of 10 000 and a mass range from 150 to 2000 *m/z*. HCD collision energy was set to 30 eV. A target value of 3E6 charges was set for MS and MS/MS scans and maximum injection time was set to 500 ms in both modes.

Selection of Allergen Specific Peptides

Protein sequence alignment was performed with ClustalW (EBI). Pinpoint software (Thermo Fisher Scientific, San Jose, CA) was used to generate lists of theoretical tryptic peptides of Phl p 1 and Phl p 5 isoforms appearing in Uniprot database. No missed cleavage was allowed for the creation of the peptide lists and peptides containing cysteine or methionine, which can potentially be modified, were excluded. An extracted ion chromatogram of each of the theoretical peptides was generated with QualBrowser software (Thermo Fisher Scientific, San Jose, CA) with 5 ppm mass tolerance and 5 points smoothing. Peptides sequences were confirmed by submitting tandem mass spectra to database search using Sequest algorithm. Peak lists were created in mgf format and precursor mass information was manually specified. MS/MS spectra were search against a Uniprot protein database containing only protein sequences from *P. pratense*. No missed cleavage was allowed. Mass tolerance for the precursor was set to 5 ppm and 10 mmu for MS/MS fragments. Searches were performed with no post-translational modifications since candidate peptides were selected for not containing any cysteine or methionine. An Xcorr threshold of 0.7 and 0.9 was applied for singly and doubly charged peptides, respectively.

Five micrograms of *P. pratense* extract was analyzed by LC–MS to confirm the presence of the putative allergen specific peptides in the complex sample and check for possible interferences. The chromatographic and ionization parameters were as specified above and the mass spectrometer was set to only acquire full scan spectra at 50 000 resolving power with a mass range from 500 to 2000 *m/z*. A target value of 3E6 charges was used and maximum injection time was set to 500 ms.

Reverse Calibration Curve

Stable isotope labeled synthetic peptides ThermoHeavy AQUA peptides were obtained from ThermoFisher Scientific (Ulm, Germany) at a concentration of 5 pmol/µL. A calibration

Table 1. Summary of the Allergens Identified by LC–MS/MS from Phl p 1 and Phl p 5 Purified Proteins

peptide sequence	protein accession number	protein name	theo mass (amu)	exp mass (amu)	delta mass (ppm)	Xcorr
WIALK	Q40967	Phl p 1a	629.390	629.390	0.51	1.68
IDTPEVLK	Q40967	Phl p 1a	913.512	913.511	1.58	2.04
DVIPEGWK	Q40967	Phl p 1a	942.482	942.480	1.65	1.47
FVAGDGDVVAVDIK	Q40967	Phl p 1a	1403.730	1403.729	0.81	2.13
WLDKAK	Q40967, P43213	Phl p 1a, Phl p 1b	631.334	631.33	1.89	1.46
VTFHVEK	Q40967, P43213	Phl p 1a, Phl p 1b	858.460	858.460	−0.30	1.90
ESWGAIWR	Q40967, P43213	Phl p 1a, Phl p 1b	1003.488	1003.488	−0.16	1.52
GSNPNYLALLVK	Q40967, P43213	Phl p 1a, Phl p 1b	1287.720	1287.719	0.79	1.42
WIELK	P43213	Phl p 1b	687.396	687.395	1.72	1.45
TEAEDVIPEGWK	P43213	Phl p 1b	1372.652	1372.652	−0.23	2.00
VDAAFK	Q40962; O81341; Q40960; Q84UI2; Q9AT26 Q40962; O65318; O65319; O65320; O65321; O81341; P93467;	Phl p 5a	649.344	649.344	0.28	0.92
FIPALEAAVK	Q40960; Q84UI2; Q9AT26 Q40962; O65318; O65319; O65320; O65321; O81341; P93467;	Phl p 5a	1057.618	1057.617	0.71	1.55
YTVFETALK	Q40960; Q84UI2; Q9AT26	Phl p 5a	1070.566	1070.566	0.09	1.09
AFAEGLSGEPK	Q40962; O81341; Q40960; Q84UI2; Q9AT26 Q40962; O65319; O65320; O65321; P93467; Q40960; Q84UI2;	Phl p 5a	1104.546	1104.545	0.68	0.93
YDAYVATLSEALR	Q9AT26	Phl p 5a	1470.736	1470.736	−0.26	2.69
AALAAAAGVPPADK	O65318; O65319; O65320; O65321; O81341; P93467; Q84UI2	Phl p 5	1221.672	1221.672	0.26	2.42
IDAAFK	Q40963; O81342; O81343; O81344; P93466; Q9SBE0	Phl p 5b	663.360	663.359	1.22	1.70
APGLVPK	Q40963; O81342; O81343; O81344; P93466; Q9SBE0	Phl p 5b	680.422	680.422	0.40	0.91
AITAMSEVQK	Q40963; O81342; O81343; O81344; P93466; Q9SBE0	Phl p 5b	1076.554	1076.553	0.97	0.95
LDAAYSVAYK	Q40963; O81342; O81343; P93466; Q9SBE0	Phl p 5b	1099.556	1099.555	0.99	1.83
YAVFEAALTK	Q40963; O81342; O81343; O81344; P93466; Q9SBE0	Phl p 5b	1111.592	1111.592	0.05	1.72
FTVFEEAFNK	Q40963; O81342; O81343; P93466; Q9SBE0	Phl p 5b	1172.588	1172.587	0.70	1.22
TFEAAFTSSSK	Q40963; O81342; O81343; O81344; P93466; Q9SBE0	Phl p 5b	1174.552	1174.551	0.74	1.21
IPAGELQIIDK	Q40963; O81342; O81343; O81344; P93466; Q9SBE0	Phl p 5b	1195.682	1195.682	0.09	2.29
VAATAAATAPADDK	Q40963; O81342; O81343; P93466; Q9SBE0	Phl p 5b	1271.636	1271.637	−0.52	1.94
FDSFVASLTEALR	Q40963; O81342; O81343; O81344; P93466; Q9SBE0	Phl p 5b	1454.742	1454.743	−0.49	2.55
VIAGALEVHAVKPVTEEPGMAK	Q40963; O81342; O81344; P93466; Q9SBE0	Phl p 5b	2245.214	2245.214	0.13	1.32

curve was generated based on varying the amounts of isotope labeled heavy peptides while using the constant endogenous amount of light peptides as an internal standard (also called reversed calibration curve). Heavy peptides were mixed together and spiked into the allergen extract at concentrations of 620, 62, 6.2, 0.62, and 0.062 fmol/ μ L. Five microliters (1.25 μ g) of the spiked solutions was analyzed in triplicate by LC–MS using the chromatographic and ionization parameters specified in the above LC–MS/MS Analysis and Selection of Allergen Specific Peptides section. The mass spectrometer was set to only acquire full scan spectra at 50 000 resolving power with a mass range from 500 to 2000 m/z . A target value of 3E6 charges was used and

maximum injection time was set to 500 ms. Samples were run from low to high concentration with no blank interspersed. QuanBrowser software was used for quantitative data analyses. Peaks were integrated with the Genesis algorithm with a mass tolerance of 5 ppm on the extracted ion chromatogram after smoothing (5 points). The constant endogenous light version of each peptide was used as an internal standard for building the reverse calibration curves with a $1/x$ weighting. Relative standard deviations were calculated for each concentration of isotope labeled heavy peptide solution and lower limits of detection were estimated for each of the surrogate peptides and the corresponding proteins.

A						
Q40967	1	IPKVPPGPNI	TATYGDKWLD	AKSTWYGKPT	AAGPKDNGGA	CGYKDVDKPP
P43213	1	IPKVPPGPNI	TATYGDKWLD	AKSTWYGKPT	GAGPKDNGGA	CGYKDVDKPP
		*****	*****	*****	*****	*****
Q40967	51	FSGMTGCGNT	PIFKSGRGCG	SCFEIKCTKP	EACSGEPVVV	HITDDNEEPI
P43213	51	FSGMTGCGNT	PIFKSGRGCG	SCFEIKCTKP	EACSGEPVVV	HITDDNEEPI
		*****	*****	*****	*****	*****
Q40967	101	AAYHFDLSGI	AFGSMACKGD	EQKLRSAGEV	EIQFRRVKCK	YPEGTKVTFH
P43213	101	APYHFDLSGH	AFGAMACKGD	EQKLRSAGEL	ELQFRRVKCK	YPEGTKVTFH
		* *****	*** *****	*****	* *****	*****
Q40967	161	VEKGSNPNYL	ALLVKFVAGD	GDVVAVDIKE	KGKDKWIALK	ESWGAIWRID
P43213	161	VEKGSNPNYL	ALLVKYVNGD	GDVVAVDIKE	KGKDKWIELK	ESWGAIWRID
		*****	*****	*****	*****	*****
Q40967	201	TPEVLKGPFT	VRYTTEGGTK	GEAKDVIPEG	WKADTAYESK	
P43213	201	TPDKLTGPFT	VRYTTEGGTK	TEAEDVIPEG	WKADTSYESK	
		*** ** *	*****	** *****	*****	***
B						
Q40962	1	-----ADL	GYGPATPAAP	AAGYTPATPA	APAGADAAGK	ATTEEQKLIE
Q40963	1	AAAVPRRGP	RGGPGRSYTA	DAGYAPATPA	AAG--AAAGK	ATTEEQKLIE
		.	** . . .	***:*****	* . . ****	*****
Q40962	44	KINAGFKAAL	AGAG-VQPAD	KYRTFVATFG	PASNKAFAEG	LSGEPKGAAE
Q40963	49	DINVGFKAAV	AAAASVPAAD	KFKTFEAAFT	SSS-----	-----KAAAA
		.**.*****:	*. . * .**	*::** *:*	.:*	*. **
Q40962	93	SSSKAALTSK	LDAAYKLAYK	TAEGATPEAK	YDAYVATLSE	ALRIIAGTLE
Q40963	87	KAP--GLVPK	LDAAYSVAYK	AAVGATPEAK	FDSFVASLTE	ALRVIAGALE
		.: . * . *	*****:***	:* *****	:*:*:*:*	***:***:*
Q40962	143	VHAVKPAAEE	--VKVIPAGE	LQVIEKVDAA	FKVAATAANA	APANDKFTVF
Q40963	135	VHAVKPVTEE	PGMAKIPAGE	LQIIDKIDAA	FKVAATAAAT	APADDKFTVF
		*****:***	: *****	**::*:*:*	*****	: ***:*****
Q40962	191	EAAFNDEIKA	STGGAYESYK	FIPALEAAVK	QAYAATVATA	PEVKYTVFET
Q40963	185	EAAFNKAIKE	STGGAYDTYK	CIPSLEAAVK	QAYAATVAAA	PQVKYAVFEA
		***** *	*****:***	**::*****	*****:*	*:***:***:
Q40962	241	ALKKAITAMS	EAQKAAPAA	----AATATA	TAAVGAATGA	ATAATGGYKV
Q40963	235	ALTKAITAMS	EVQKVSQPAT	GAATVAAGAA	TTAAGAASGA	ATVAAGGYKV
		.****	*.***:***:	.: .:*	*:*.***:***	**.*:*****

Figure 1. Amino acid sequence alignment of (A) Phl p 1 and (B) Phl p 5. The Phl p 1 isoallergens P43213 and Q40967 show 93.5% identity in 263 residues overlap. Phl p 5 is composed of two isoallergens and number of variants. The sequence alignment of Q40962 and Q40963 shows 64% identity in 286 and 284 residues overlap, respectively. Gray marked peptides were selected for absolute quantification.

Single-Point Quantification

Single-point quantification was performed by measuring the peak area ratio of light to heavy peptide in the allergen extract solutions spiked with 62 fmol/ μ L of heavy peptide for WIELK, WIALK, ESWGAIWR, and FIPALEAAVK and with 620 fmol/ μ L for FDSFVASLTEALR.

RESULTS

Identification and Selection of Allergen and Isoallergen Specific Peptides

Preliminary experiments were performed to characterize the putative native light peptides and to select the best peptides for quantification purposes. Purified nPhl p 1 and nPhl p 5 were digested with trypsin and analyzed by LC-MS/MS. Several peptides from each protein were identified based on exact mass information and confirmed by database search (Table 1). Two isoallergens of Phl p 1 were identified based on sequence

information from Uniprot, Swiss-Prot, and TrEMBL. For the convenience of this work, we named the proteins with accession number Q40967 and P43213, Phl p 1a and Phl p 1b, respectively. A sequence alignment was performed with the Clustal algorithm resulting in 93.5% amino acid sequence homology for the two isoallergens (Figure 1A). Phl p 5 exists in two major forms which are known as Phl p 5a and Phl p 5b with respective accession number Q40962 and Q40963. Both were identified with several peptides. Another sequence alignment revealed a sequence identity of 64% between the two forms (Figure 1B). Note that the peptide sequence AALAAAAGVPPADK which was identified with Xcorr of 2.42 is present in Phl p 5 variants with identification numbers O65318, O65319, O65320, O65321, O81341, P93467, Q84UI2.

P. pratense extract was analyzed under the same conditions in order to verify the presence of the putative allergen specific peptides and to identify peptides giving the highest signal to be used for quantification. An extracted ion chromatogram was

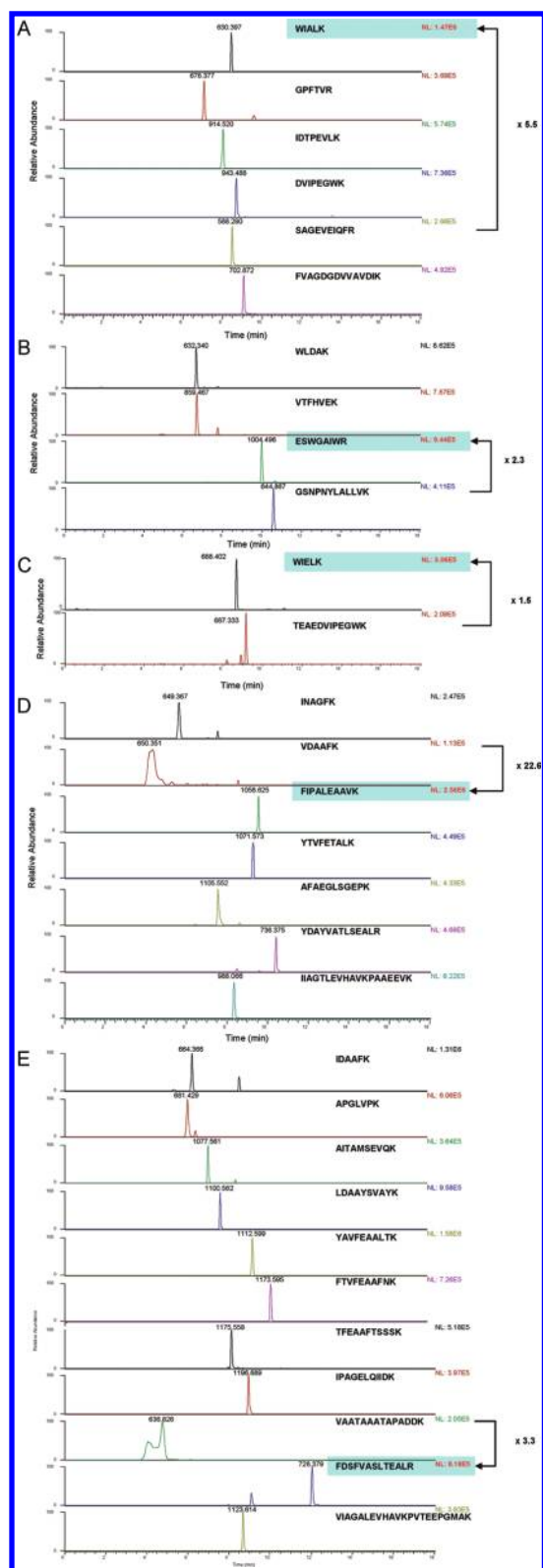


Figure 2. Signal intensity of Phl p 1 and 5 peptides A) - C) Phl p 1, D) - E) Phl p 5. The trypsin digested Phl p allergen extract was analyzed to verify the signal strength of the peptides identified from the purified Phl p 1 and Phl p 5 samples and check for possible interferences which could bias the quantification. These experiments demonstrated that the signal intensity used for the quantification can be improved 1.5 to 22.6 times by selecting the right peptide.

generated for each of the identified surrogate peptides corresponding to the proteins of interest (Phl p 1a, Phl p 1b, Phl p 5a and Phl p 5b). Peptides with high signal intensity were selected to be chemically synthesized as heavy homologues (Figure 2 selected peptides are highlighted in light blue). One specific peptide was chosen for each of the two variants of Phl p 1 (Figure 2A,C) as well as one common peptide (Figure 2B). Similarly, isoallergen specific peptides for Phl p 5a and Phl p 5b were identified and selected, each bearing an intense signal (Figure 2D,E). Note that the sequence selected for the Phl p 5b was not the most intense peptide. In this case, 3 peptides had intensity near or above 10^6 counts. The sequence FDSFVASLTEALR was subsequently selected because it is the biggest peptide which confers it more specificity and has a resulting low interfering signal in this region of the mass spectrum (Figure 3). This peptide was also identified with the best Xcorr among the 3 peptides (Xcorr = 2.55); a good ion match considering that there was no precursor selection prior to fragmentation. *In silico* analyses of Phl p 5a and 5b sequences revealed a common tryptic peptide (ATTEEQK) which was not detected by LC-MS/MS analyses, preventing the simultaneous quantification of both Phl p 5 isoallergens. There are several possible reasons for this. For example, poor ionization under electrospray conditions and/or ionization suppression is possible. It could also be that the peptide was either not retained by or not eluted from the C18 column. In addition, if the peptide was ionized preferentially as a doubly charged $[M + 2H]^{2+}$ ion, it would be detectable with an m/z of 403.6982 which would not have been detected in these experiments as the minimum m/z to be detected was set at 500. This issue could be resolved by further development(s) of the LC method.

Absolute Quantification of Phl p 1, Phl p 5a, and Phl p 5b from Allergen Extract

A total of five heavy isotope labeled standard peptides were synthesized. These heavy peptides were shipped in solution in order to alleviate possible pipetting error or bias due to incomplete solubilization (Table 2). The isotope labeled heavy peptides were spiked into the tryptic *P. pratense* extract digest at concentrations spanning 5 orders of magnitude and the samples were subjected to LC-MS analyses in triplicate injections. A method consisting of a total LC-MS acquisition duration of 18 min was developed and used for all experiments. The full scan MS trace acquired at 50 000 resolving power was used for peptide quantification, with an average scan time of 500 ms. Extracted ion chromatograms were generated for each heavy and light form of the peptide in all LC-MS files. Figure 3 shows an example for the peptide WIALK. The light and heavy versions were detected with high resolution and high mass accuracy (Figure 4A) which allows to accurately extract the signal of each respective peptide with a very narrow mass range (5 ppm) (Figure 4B). Peak areas were integrated and a reverse calibration curve was built after calculating the heavy/light signal ratio for each of the isotope labeled heavy peptide concentrations using QuanBrowser software from Thermo Fisher Scientific (Figure 5). The endogenous light forms of the peptides were used as internal standards for their isotope labeled heavy counterparts to account for ionization and matrix effects.²⁴ Linearity was observed with a coefficient of correlation greater than 0.99 for 4 peptides and 0.97 for 1 peptide. Limits of quantification were calculated for each peptide based on the lowest concentration exhibiting a relative standard deviation of the calculated concentration of less than 20%.²⁴ Observed limits of quantification were 3–30 fmol depending on

Table 2. Design of Stable Isotope Labeled Heavy Peptides for Phl p 1 and Phl p 5

peptide sequence	protein name	<i>m/z</i> (light)	<i>m/z</i> (heavy)	modification	RT
WIALK	Phl p 1a	630.397	638.412	Lys ¹³ C ¹⁵ N	8.17
ESWGAIWR	Phl p 1a, Phl p 1b	502.751	507.755	Arg ¹³ C ¹⁵ N	9.70
WIELK	Phl p 1b	688.403	696.417	Lys ¹³ C ¹⁵ N	8.42
FIPALEAAVK	Phl p 5a	529.816	533.823	Lys ¹³ C ¹⁵ N	9.32
FDSFVASLTEALR	Phl p 5b	728.378	733.382	Arg ¹³ C ¹⁵ N	11.82

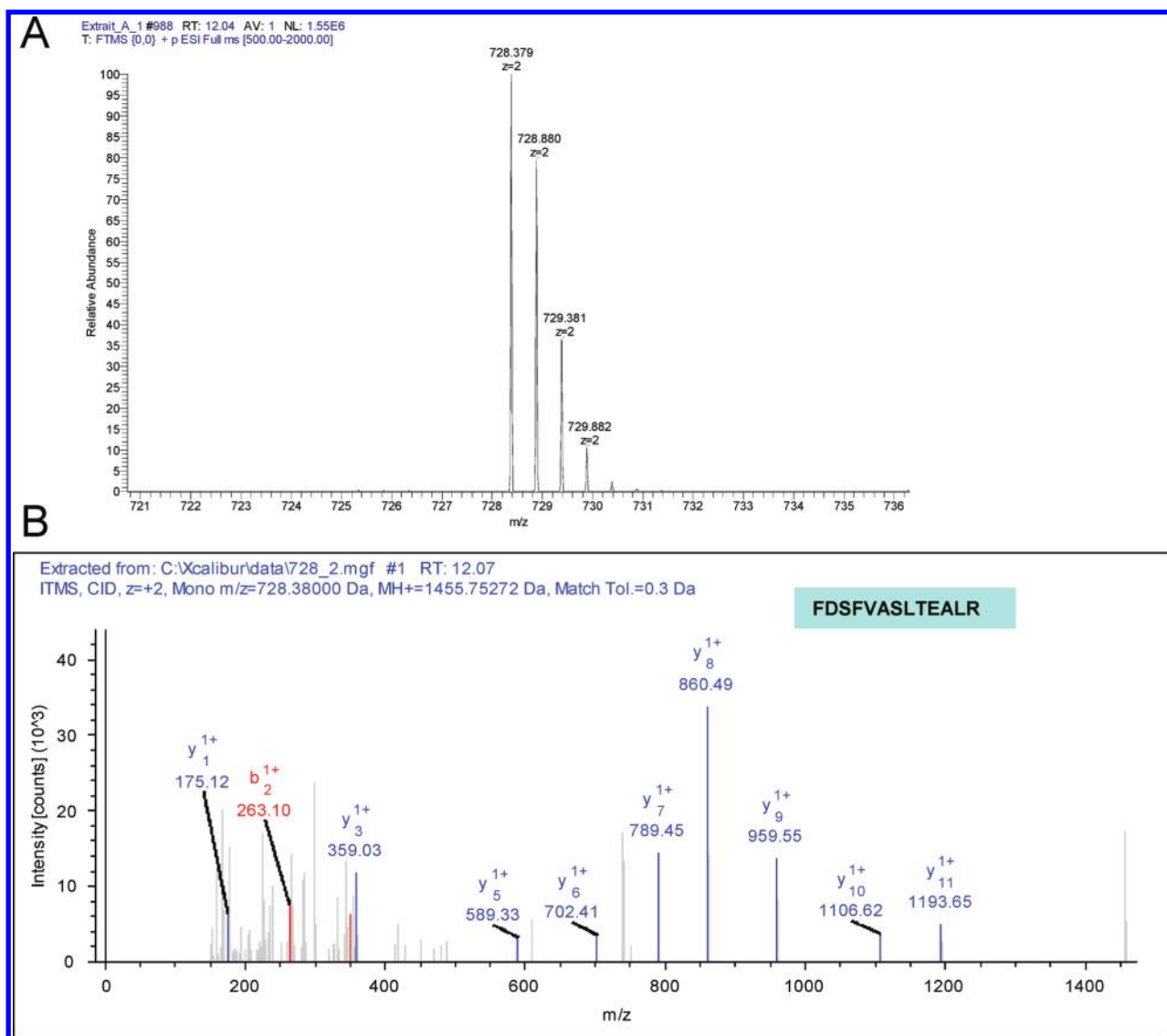


Figure 3. Selection of FDSFVASLTEALR (*m/z* = 728.378) as signature peptide for protein Phl p 5b. (A) Full scan MS spectrum from crude Phl p extract showing low interfering signal in this region of the mass spectrum. (B) Annotated MS/MS spectrum from Sequest database search. FDSFVASLTEALR peptide was identified with a Xcorr of 2.55.

the peptide (Table 3). The peak area ratio of light to heavy peptide was used to determine the absolute concentration of the endogenous peptide in the Phl p extract. The concentration of isotope labeled peptide which was the closest to the concentration of the endogenous form was selected for the calculation (ratio closest to 1).²⁵ Good precision was obtained with RSDs ≤ 5% (*n* = 3). The results showed that Phl p 1b is 5 times less abundant than Phl p 1a with respective concentrations of 150 and

724 fmol/μL. Furthermore, Phl p 5b was shown to be 9 times more abundant than Phl p 5a in the *P. pratense* extract (Table 3).

DISCUSSION

Quantitative proteomics is a subfield of proteomics which seeks to provide information about both protein and modified protein expression levels.¹¹ In the present study, a quantitative proteomics approach was applied to monitor active ingredients

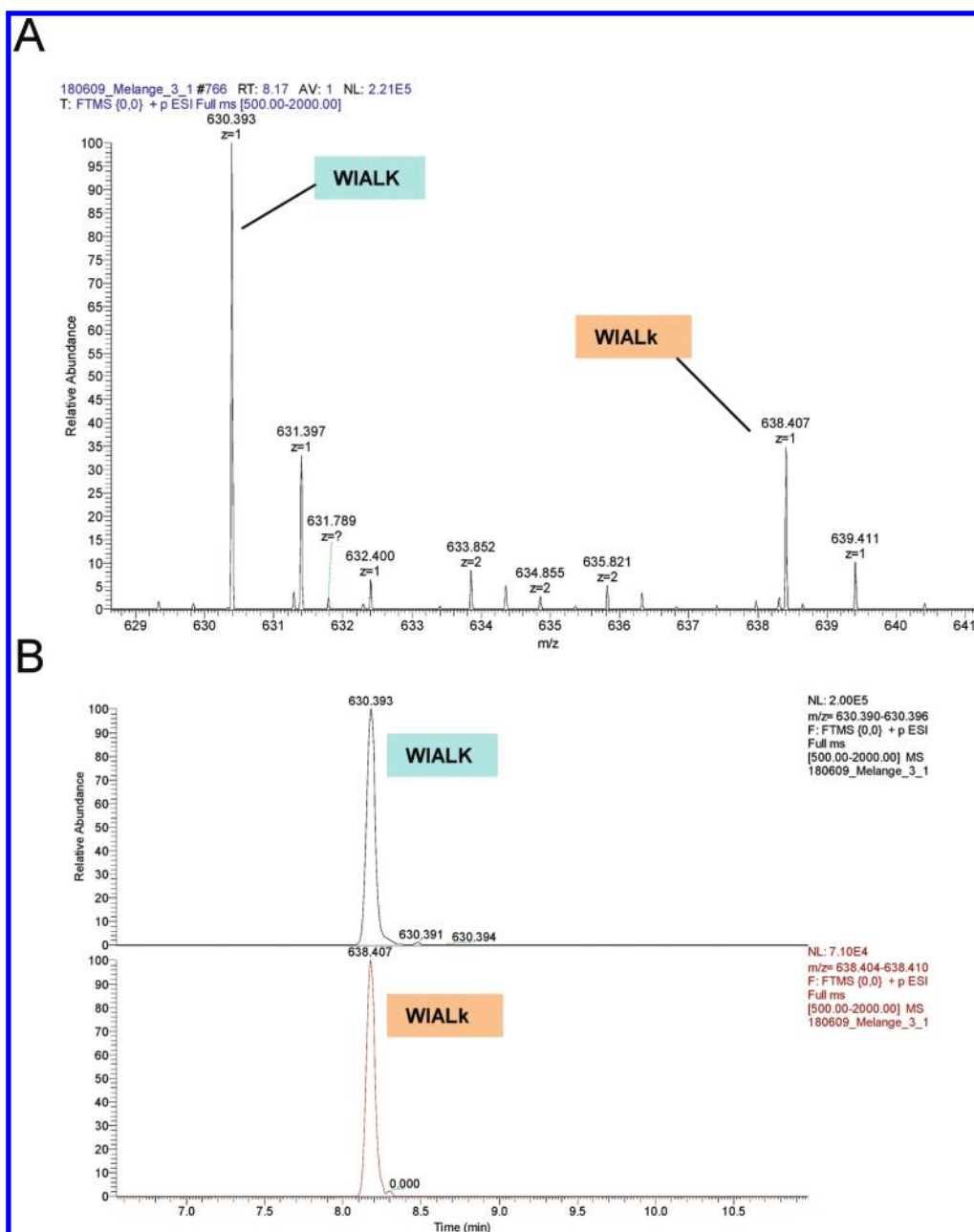


Figure 4. (A) The light (light blue) and heavy (orange) peptides of WIALK (Phl p 1a) were detected with (B) high resolution and high mass accuracy which allows accurate extraction of the signal of each respective peptide with mass tolerance of 5 ppm.

from natural allergen extract. Grass pollens are one of the most important allergen sources throughout the world. Pollens of all grass species are divided into allergen groups. Phl p 1 seems to be the most important *P. pratense* allergen followed by group 5 and 2/3.^{20,21,26} In this study, an HRAM MS method was assessed to quantify Phl p 1 and Phl p 5 from the crude allergen extract based on full scan MS ion signals. The sensitivity of this technique is dependent on the signal strength of the native, light peptides. Therefore, cautious preliminary analyses are needed to identify and to select the appropriate peptide sequence to be used for the absolute quantification. In addition to signal intensity and sequence specificity, other criteria such as the length of the peptide and the absence of potentially variably modified amino acids (such as methionine or cysteine) were also taken into account for the

selection of the peptides used for quantification. Peptide identity also needed to be confirmed by either MS/MS spectral library matching or database search. The condition and the amount of trypsin (3–10% w/w) were found suitable for the *P. pratense* extract and the allergens. However, the present conditions for the enzymatic cleavage do not apply for all the allergens and/or species. Optimal conditions and digestion parameters have to be determined individually for each allergen and/or allergen extract.^{22,23}

Purified nPhl p 1 and nPhl p 5 were chosen for the preliminary experiments to evaluate the signal strength of the peptides. Two major Phl p 1 and Phl p 5 isoallergens and/or variants were identified using MS/MS (Table 1). During the preliminary analyses of the allergen extract, a number of important *P. pratense* allergens and isoallergens were identified based on accurate mass

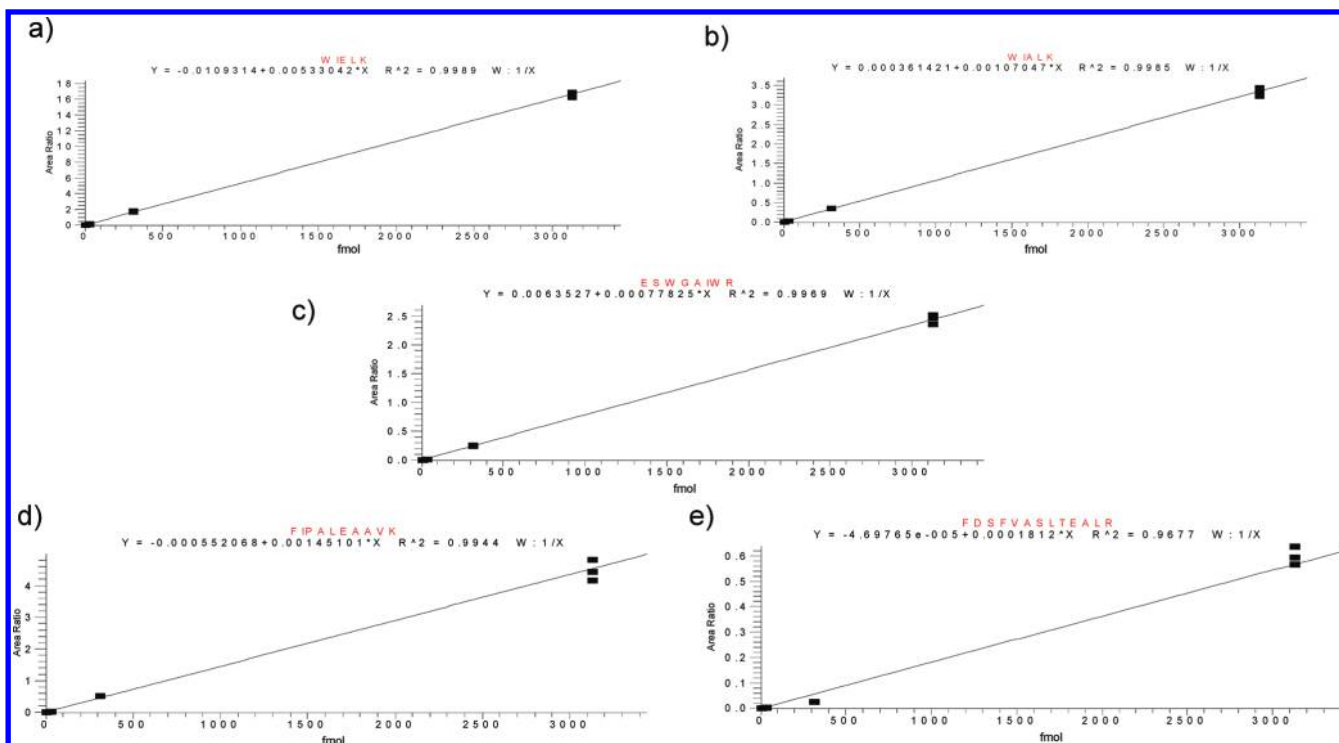


Figure 5. Calibration curves for each isotope labeled heavy peptide. Peak areas were integrated using QuanBrowser software (Thermo Fisher Scientific) and a reverse calibration curve was built after calculating the heavy/light (H/L) signal ratio for each of the isotope labeled heavy peptide concentration. Linearity was observed with a coefficient of correlation superior to 0.99 for 4 peptides and 0.97 for 1 peptide. Observed limits of quantification were 3–30 fmol depending on the peptide (RSD < 20%).

Table 3. Absolute Quantification of Phl p 1 and Phl p 5 Protein Variants^a

peptide sequence	protein name	LLOQ (fmol)	ratio L/H	Q heavy (fmol)	Q light (fmol)	conc light (fmol/uL)	RSD (%)
WIALK	PhlP1a	30	2.90	312	905	724	1
ESWGAIWR	PhlP1a, Phl P1b	30	4.13	312	1289	1031	3
WIELK	Phl P1b	3	0.60	312	187	150	3
FIPALEAAVK	Phl P5a	3	2.00	312	624	499	1
FDSFVASLTEALR	Phl P5b	30	1.76	3120	5491	4393	5

^a LLOQ, lower limit of quantification; Q, Quantity.

signal from the full scan LC–MS analysis (data not shown). These results show that HRAM MS could also be applied as a qualitative tool for monitoring the production of allergen vaccines.²⁴ One specific signature peptide of Phl p 1a and Phl p 1b was chosen to quantify each of the variants individually (Figure 1A). A common Phl p 1 peptide ESWGAIWR was also selected to monitor the concentration of the two known Phl p 1 variants. Finally, a signature peptide was selected for each of the Phl p 5 isoallergens Phl p 5a and Phl p 5b (Figure 1B). Interestingly, the preliminary experiments demonstrated that the signal intensity used for the quantification can be improved 1.5–22.6 times by selecting the correct peptide (Figure 2). It is to be noted that Phl p 1 and Phl p 5 have numerous isoforms which renders this selection of sensitive and specific proteotypic peptides challenging. For this study, peptides were selected for their uniqueness in the *P. pratense* extract sample. Different criteria would have been applied in the case of a mixture of different species.²⁶ Another peptide was selected and used for quantitation of Phl p 1a (SAGEVEIQFR) and proved to give very similar quantification results for Phl p 1a (data not shown).

The approach that we selected for the quantification is the single-point which is a common method used in many biomarker laboratories.^{12,13} This method consists in spiking in a known amount of heavy peptide into the sample. On the basis of the ratio of light to heavy peptide, the amount of light peptide can be determined. The advantage of single-point quantification is that it only requires one run per sample, which maximizes efficient use of instrument time and minimizes sample consumption. However, a disadvantage is that with this method it cannot be determined with certainty whether or not the sample lies on the linear portion of the concentration curve. For this reason, it was decided to build a calibration curve for each of the peptides. The FDA guidelines indicate that “A calibration curve should be prepared in the same biological matrix as the samples in the intended study by spiking the matrix with known concentrations of the analyte.”²⁷ In the case of peptide quantification, there is no equivalent matrix substitute for a biological matrix and as a result, peptide calibration curves have endogenous peptides present in them. Methods and post acquisition data analysis have to take into account the endogenous peptide that is present in the

background matrix and this can lead to difficulties especially if the endogenous amount of light peptide is higher than the concentration of spiked standards. For this reason, we decided to build the calibration curve based on varying amounts of heavy peptide while using the constant endogenous amount of light peptide as an internal standard. This way we could determine the LLOQ for each of the peptides and use a concentration of heavy isotope labeled peptide close to the endogenous amount for the single point quantification.

The lack of blank matrix sample also renders difficult the evaluation of the selectivity of the assay. Here the method selectivity is given by the co-elution of the endogenous light peptide and its isotope labeled heavy version in addition to the very high mass accuracy (below 3 ppm) of the orbitrap mass analyzer. In addition, the high resolving power of the mass spectrometer allows elimination of closely related co-eluting species from the peak integration.

Reverse calibration curves showed that quantification by HRAM exhibits good to excellent linearity and sensitivity at the low femtomole level. Beside the peptide selection for quantification, little development was required to produce a fast (20 min) assay and achieve this performance. A column diameter of 1 mm was selected to increase sensitivity (compared to traditional 2.1 mm column) while a particle size of 1.9 μ m diameter allowed improvement of chromatographic resolution with average FWHM (full width at half-maximum) of 4 s, producing more than 16 points to define a chromatographic peak. In contrast to SRM methods performed with a triple quadrupole MS, no lists of masses and transitions with potentially lengthy development time were required for this assay since the mass spectrometer was set to only acquire full scan mass spectra. Another benefit from this acquisition mode is the possibility to return to existing LC-MS data if information is needed on peptides that were not targeted for analysis in the first place. For SRM-based triple quadrupole MS, a sample would need to be reanalyzed to observe new peptides of interest. This would be especially useful in the case of allergen analysis if in a few months or years a new variant of Phl p 1 or Phl p 5 proteins were to be discovered and need to be quantified.

While, the quantification of allergen extracts is currently performed by antibody based techniques,^{1,3–6} the present study shows that a quantitative proteomics approach by HRAM LC-MS is a robust technique with intra day precision $\leq 5\%$ ($n = 3$). In addition to robustness, HRAM MS showed the advantageous ability to multiplex assays compared with current ELISA assays without the cost of developing antibodies. Furthermore, ELISA assays typically employ monoclonal antibodies, which are able to identify single epitopes and/or single isoallergens only.^{6,28} In the present study, the quantity and the ratios of specific isoallergens in *P. pratense* extract were measured accurately, directly from the extract (Table 3). For example, Phl p 5b was shown to be 9 times more abundant than Phl p 5a (Table 3). Interestingly, however, when Phl p 5 was quantified from *P. pratense* extract by use of polyclonal antibodies against purified Phl p 5, the quantity of Phl p 5 was found to be in good agreement with the HRAM MS quantification results (data not shown). The common peptide to Phl p 1 isoforms exhibited a concentration which was slightly higher than the summation of the respective concentration of Phl p 1a and Phl p 1b. This result could indicate the presence of other yet unidentified isoallergens of group 1 in the Phl p extract. Alternatively, one of the light peptides could have a post-translational modification which was not observed in the preliminary

analyses of the purified allergens and/or crude Phl p allergen extract. These are issues which can be resolved and investigated by the improvement of the LC-MS/MS method. Quantification is one of the key elements in the standardization of allergen extracts, and therefore, our results suggest that rigorous studies exploring pros and cons between HRAM MS and mono- and polyclonal antibody-based quantification techniques are needed. The present study has demonstrated that HRAM MS is a robust quantitative and qualitative analytical tool which gives in-depth information on allergen extracts. Moreover, it is an essential step for future product standardization and development of next generation allergen vaccines.

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Author Contributions

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ABBREVIATIONS:

ELISA, enzyme-linked immunosorbent assay; HRAM, high-resolution accurate mass; LC-MS, liquid chromatography-mass spectrometry; MS, mass spectrometry; MS/MS, tandem mass spectrometry; Phl p 1a, Phl p 1.0101; Phl p 1b, Phl p 1.0102; Phl p 5a, Phl p 5.01; Phl p 5b, Phl p 5.02; RSD, relative standard deviation; SRM, selective reaction monitoring

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