(12) STANDARD PATENT (11) Application No. **AU 2006291835 B2**
(19) AUSTRALIAN PATENT OFFICE

(54) Title
A method for quantification of allergens

(51) International Patent Classification(s)
G01N 33/68 (2006.01)

(21) Application No: **2006291835** (22) Date of Filing: **2006.09.01**

(87) WIPO No: **WO07/031080**

(30) Priority Data

(31) Number	(32) Date	(33) Country
PA 2005 01293	**2005.09.15**	**DK**
60/717,942	**2005.09.16**	**US**

(43) Publication Date: **2007.03.22**
(44) Accepted Journal Date: **2012.04.05**

(71) Applicant(s)
ALK-Abello A/S

(72) Inventor(s)
Seppala, Ulla

(74) Agent / Attorney
FB Rice, Level 23 44 Market Street, Sydney, NSW, 2000

(56) Related Art
D6. van Ree R. Clin Exp Allergy. 2002:32(7):975-8.
D1. Kristiansson M.H. et al, Rapid Commun. Mass Spectrom. 2004: 18:1592-1598.
D3. Patton W.F. et al, Curr Opin Biotechnol. 2002:13(4):321-8.
D5. US 2004/0106146 A1 (PALOSUO et al) 3 June 2004
D4. Neverova I, et al, J Chromatogr B Analyt Technol Biomed Life Sci. 2005:815(1-2):51-63.
D2. Helsper J.P. et al, J Allergy Clin Immunol. 2002: 110(1):131-8.

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
22 March 2007 (22.03.2007)

PCT

(10) International Publication Number
WO 2007/031080 A1

(51) International Patent Classification:
G01N 33/68 (2006.01)

(21) International Application Number:
PCT/DK2006/000480

(22) International Filing Date:
1 September 2006 (01.09.2006)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:

PA 2005 01293	15 September 2005 (15.09.2005)	DK
60/717,942	16 September 2005 (16.09.2005)	US

(71) Applicant *(for all designated States except US)*: **ALK-ABELLÓ A/S** [DK/DK]; Bøge Allé 6-8, DK-2970 Hørsholm (DK).

(72) Inventor; and

(75) Inventor/Applicant *(for US only)*: **SEPPÄLÄ, Ulla** [FI/SE]; Östra Allén 9, SE-254 51 Helsingborg (SE).

(74) Agent: **INSPICOS A/S**; Bøge Allé 5, P.O. Box 45, DK-2970 Hørsholm (DK).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LV, LY, MA, MD, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, SV, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IS, IT, LT, LU, LV, MC, NL, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Declarations under Rule 4.17:

— *as to applicant's entitlement to apply for and be granted a patent (Rule 4.17(ii))*
— *as to the applicant's entitlement to claim the priority of the earlier application (Rule 4.17(iii))*
— *of inventorship (Rule 4.17(iv))*

Published:

— *with international search report*
— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: A METHOD FOR QUANTIFICATION OF ALLERGENS

(57) Abstract: The invention relates to method for quantification of the absolute amount of allergen in an allergen sample comprising : a) providing a known amount of one or more allergen calibration standard peptide(s) having a sequence of amino acids which is identical with, and optionally unique for, a sequence to be found in the allergen to be quantified and optionally labelling said allergen calibration standard peptide(s), b) degrading the allergen sample to obtain a mixture of peptides, and optionally labelling said peptides with one or more labelling agent(s), wherein at least the peptides in the degraded allergen sample or the calibration standard peptides are labelled, and if both the peptides in the degraded allergen sample and the allergen calibration standard peptide(s) are labelled, the labelling agent(s) used for labelling the allergen calibration standard peptide(s) are different from the labelling agent(s) used for labelling the peptides of the degraded allergen sample, c) quantifying the absolute amount of allergen by correlating the amount of the allergen calibration standard peptide(s) with the amount of the corresponding peptide(s) of the degraded allergen sample by mass analysis.

WO 2007/031080 A1

A METHOD FOR QUANTIFICATION OF ALLERGENS

TECHNICAL FIELD

This invention relates to the field of quantification of allergens.

BACKGROUND OF THE INVENTION

Allergens are antigenic molecules inducing allergic responses and production of IgE antibodies in humans. They are used both for diagnosis and for treatment of allergy i.e. allergen immunotherapy, the latter in the form of allergen vaccines. Allergenic source materials to which humans are exposed, such as foods, pollens or mite faecal particles occur naturally as complex mixture(s) of major and minor allergens. Major allergens are allergens to which a majority of patients, who are allergic to the source, react. It appears, however, that any protein is a potential allergen, since still more and more minor allergens are identified as knowledge increases.

Due to the complexity of the allergen sources, the amino acid sequences of several allergens were first deduced from cDNA-derived nucleotide sequences. Cloning of genes encoding allergens revealed that most allergens are heterogeneous and that they occur as mixtures of isoallergens and variants. Amino acid sequence alignment of homologous allergens and isoallergens demonstrated that they can be identified by and/or divided into constant and variable region sequences. Constant region amino acid sequences are unique to the species however variable region amino acid sequences are unique to each of the isoallergens.

Conventional allergen specific immunotherapy and diagnosis are currently performed by use of standardized natural allergen extracts which are further formulated to allergen vaccines. These aqueous vaccines are based on allergenic natural source materials, such as tree and grass pollen, dust mite cultures and animal hair and dander particles. The composition of these natural source materials are known to vary considerably depending on time and place of collection of the allergenic source materials. Commercial allergen vaccines can furthermore be formulated to mixtures of allergens using various species.

Knowledge of the composition of the extracts and the content of essential allergens is a prerequisite for reproducibility, safety and efficacy of the final product. A major challenge in the manufacture of allergen vaccines is standardisation, i.e. securing a constant potency from batch to batch. Since the raw material is of natural origin, variation is considerable and needs to be controlled by scientifically based measures. The composition of the extract should ideally reflect the composition of the water soluble components of the allergenic source

material as it is extracted on the mucosal surface of the airways and presented to the human immune system. All extracts, however, contain several allergens contributing to the total IgE-binding in different combinations for individual patients. Ideally, therefore, all components need to be controlled both qualitatively and quantitatively, but with the current technology this is not practically possible.

Standardisation is currently performed in many different ways, since each manufacturer has company specific standardisation procedures. Standardisation is performed by techniques such as SDS-PAGE, isoelectric focusing in addition to a variety of immunoelectrophoretic (QIE) and ELISA techniques using mono- and/or polyclonal antibodies and radio allergosorbent (RAST) or related techniques. An optimal batch-to-batch standardisation, such as the SQ-standardisation procedure, is essentially a three step procedure: 1) securing optimal composition and constant ratios between all components by semi-quantitative immuno-electrophoretic techniques, 2) determining major allergen components by quantitative immunoelectrophoresis, and 3) adjusting the overall IgE-binding potency as determined in Magic Lite® assays. In Europe, all standardisation is currently performed relative to an in-house company specific reference preparation, whereas in the U.S, the FDA issues standards to be used by all manufacturers. All quantitative aspects of these currently used techniques are dependent on antibodies as reagents and as such vulnerable to change over time.

Absolute quantification of specific vaccine components in complex mixtures of allergen is not straightforward and has yet not been established as a sensitive, routine high-through-put technique.

Also in the food industry routine high-through-put techniques for reliable detection and quantification of food allergens is necessary. Nuts may be found as a hidden part of a food because of accidental cross-contamination during manufacturing. Companies producing similar foods with and without e.g. nuts may have difficulty in cleaning production equipment in between making the different types of foods. Traces of previously produced foods such as nuts can remain on the equipment. The first batches of foods made without nuts that go through the same equipment will likely contain traces of nuts. Foods that may cause allergic reaction due to cross-contamination of nuts or peanuts are e.g. chocolate, candies, cookies, desserts, sweets, donuts, cereal, milkshakes, granola bars, muesli, pies, muffins, ice cream, barbecue sauce. Cows' milk may cause an allergic reaction to small amounts of milk protein from dairy products, from cows' milk, formula based on cows' milk or baby foods containing protein from milk. To avoid contamination of milk protein during production of baby foods or baby formula to milk allergic children a detection and quantification method for milk allergens is therefore needed. Reliable detection and quantification methods for food allergens are

necessary in order to ensure compliance with food labelling and to improve consumer protection. Physicochemical methods e.g. mass spectrometry, as well as immunological methods have been described . The usual criteria of sensitivity, specificity, reproducibility, precision and accuracy have to be fulfilled. Still, there remain problems of cross-reactivity, of matrix effects and of food processing. Biological activity may remain when the protein is denatured.

Biological mass spectrometry (MS) was first employed to assess molecular weight and identity of proteins and peptides. In recent years, advances in mass spectrometry have resulted in techniques which can be used for quantification of a variety of biomolecules from complex mixtures such as plasma, cell and tissue samples. Earlier quantification techniques have established only relative quantification of proteins whereas the more recent techniques assess absolute quantities of molecules of interest. The rapid development of quantification techniques is mainly due to progress in the field of proteomics, particularly in applications distinguishing, for instance, healthy and diseased states and identification of marker molecules for several diseases such as cancer, rheumatoid arthritis and Alzheimers disease. The major advantage of these quantification techniques by MS is the high sensitivity of the techniques ranging from 300 amol to 300 fmol of samples.

WO 2004/070352 discloses a method for quantification of peptides relative to an internal standard using isobaric labelling reagents or sets of isobaric labelling reagents.

US 6,872,575 discloses a method for identification of one or more proteins in complex sample mixtures without purifying the protein or obtaining its composite peptide signature.

US 6,864,089 discloses a method for quantification using differential isotopic labelling of peptide or protein samples.

Other methods for quantification of proteins using MS techniques are e.g. the AQUA technique using internal calibration peptides synthesized with incorporated stable isotopes (^{13}C, ^{15}N) to mimic native peptides formed by enzymatic digestion using e.*g.*,trypsin (Stemmann O et al. Cell 2001;107(6):715-26, Gerber SA et al. Proc Natl Acad Sci U S A 2003;100(12):6940-5).

Another method is the ICPL (Isotope Coded Protein Labelling) method described by Kellermann et al, Proteomics 5, 4-15, using e.g $^{12}C/^{13}C_6$-Nicotinic acid-succinimide as ICPL label.

Mass spectrometry was first introduced to the field of allergy research to characterize natural allergens including post translational modifications such as glycosylation patterns. It was further employed to characterize recombinant isoallergens and/or variants many of which have been expressed in various expression systems such as *Esherica Coli*, *Pichia Pastoris* and *Baculovirus* expression systems.

Johannes et al, J Allergy Clin Immunol, Vol. 110, No. 1 (2002), pages 131-138 describes the use of MS to study the actual expression of allergen isoforms identified by PCR cloning and in Swoboda et al., J. Biol Chem, Vol 270, No.6 (1995), pages 2607-2613, liquid chromatography, MS and cDNA cloning is used to analyze isoforms of the major birch pollen allergen, Bet v 1.

There still is a need for sensitive method, however, by which active components such as allergens from the same species or different species, and/or isoallergens e.g. in a vaccine may be quantified. A method using MS techniques and the species specific and allergen specific amino acid sequences provides a very sensitive method by which the content of specific allergens or groups of allergens (isoallergens or homologous allergens) may be quantified. The method according to the invention is useful e.g. in a release assay in order to ensure a safe and accurate amount of allergen during production of a vaccine, in the final product, and also during the various stages of storage of active ingredients and/or products. The method would also be beneficial in development of second generation allergen vaccines e.g. using recombinant allergens as active ingredients. Such a method would make it possible to optimize active ingredients in second generation allergen vaccines based on the knowledge and/or composition of the current vaccines.

SUMMARY OF THE INVENTION

In accordance with the present invention, a method for absolute quantification of allergens from a plurality of sources is provided.

According to one aspect the invention provides a method for quantification of the absolute amount of allergen in an allergen sample where the allergen consists of more than one isoallergen(s) or homologous allergen(s) comprising the following steps:

a) identifying a sequence of amino acids in the allergen to be quantified which is constant in the isoallergens or homologous allergens in the allergen by comparing the amino acid sequences of isoallergens or homologous allergens in the allergen and preparing a synthetic allergen calibration standard peptide having this constant sequence and labelling said allergen calibration standard peptide(s) with mass-modifying functionalities,

b) degrading the allergen sample to obtain a mixture or peptides, and optionally labeling said peptides with one or more labelling agent(s) with mass-modifying functionalities, wherein if both the peptides in the degraded allergen sample and the allergen calibration standard peptide(s) are labelled, the labelling agent(s) used for labelling the allergen calibration standard peptide(s) are different from the labelling agent(s) used for labelling the peptides of the degraded allergen sample,

c) quantifying the absolute amount of allergen by correlating the amount of the labeled allergen calibration standard peptide(s) with the amount of the corresponding peptide(s) of the degraded allergen sample by mass spectrometry.

Any discussion of documents, acts, materials, devices, articles or the like which has been included in the present specification is not to be taken as an admission that any or all of these matters form part of the prior art base or were common general knowledge in the field relevant to the present invention as it existed before the priority date of each claim of this application.

Throughout this specification the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated element, integer or step, or group of elements, integers or steps, but not the exclusion of any other element, integer or step, or group of elements, integers or steps.

In one embodiment of the invention the allergen to be quantified consist of more than one isoallergen, e.g. members of an allergen group from the same species with more than > 67 % amino acid sequence identity.

In another embodiment of the invention the allergen to be quantified consist of more than one homologous allergens.

2006291835 11 Jan 2012

According to another aspect, the invention provides a method for quantification of the absolute amount of allergens in an allergen sample comprising the following steps:

a) providing a known amount of one or more allergen calibration standard peptide(s) having a sequence of amino acids which is unique for a sequence to be found in the allergen or isoallergen to be quantified, and optionally labelling said allergen calibration standard peptide(s),

b) degrading the allergen sample to obtain a mixture of peptides, and optionally labelling said peptides with one or more labelling agent(s),

wherein that at least the peptides in the degraded sample or the calibration standard peptide(s) are labelled, and if both the peptides in the degraded sample and the allergen calibration standard peptide(s) are labelled, the labelling agent(s) used for the allergen calibration standard peptide(s) are different from the labelling agent(s) used for labelling the peptides of the degraded sample,

c) quantifying the absolute amount of allergen by correlating the amount of the allergen calibration standard peptide(s) with the amount of the corresponding peptide(s) from the degraded allergen sample by mass analysis.

In one aspect of the invention, use of a synthetic sequence of amino acids which sequence is identical with a constant sequence to be found within a group of isoallergens of an allergen or homologous allergens to be quantified as an allergen calibration standard peptide for absolute quantification of the allergen consisting of more than one isoallergen or homologous allergen by mass spectrometry, wherein said allergen calibration standard peptide is labelled with a mass-modifying functionality.

Preferably, the degradation in step b) results in a mixture of peptides where one of the peptides comprises the same amino acid sequence as the allergen calibration standard peptide.

In another aspect of the invention, the use of a sequence of amino acids which sequence is unique for the allergen or isoallergen to be quantified is provided as an allergen calibration standard peptide for absolute quantification of the allergen, and optionally identification. Preferably, the degradation in step b) results in a mixture of peptides where one of the peptides comprises the same amino acid sequence as the allergen calibration standard.

2006291835 11 Jan 2012

In a further aspect of the invention, a method is provided for obtaining a synthetic allergen calibration standard peptide as defined in the above aspects, wherein the allergen calibration standard peptide is obtained by:

identifying a constant sequence of amino acids within a group of isoallergens of an allergen or homologous allergens which are to be quantified by comparing amino acid sequences of isoallergens or homologous allergens, and preparing a synthetic allergen calibration standard peptide having this constant sequence.

In yet a further aspect of the invention, a method is provided for obtaining an allergen calibration standard peptide for use in quantification of an allergen or isoallergen wherein the allergen calibration standard peptide is obtained by:

identification of a variable sequence of amino acids which is unique for the allergen or isoallergen to be quantified by comparison of the sequence of the allergen or isoallergen with other isoallergens and/or homologous allergens, and preparation of a synthetic allergen calibration standard peptide having this unique sequence.

SHORT DESCRIPTION OF THE FIGURES

Fig.1. Amino acid sequence alignment of mite group 2 allergen species (fig 1a) and Bet v 1 (fig 1b), the major birch allergen by Vector NTI software (Invitrogen). The putative amino acid sequences that can be assessed as internal calibration standard peptides (useful for quantification of isoallergens) are highlighted as bold text.

Fig.2. Theoretical enzymatic cleavage by trypsin of house dust mite allergens, a) Der f 2 and b) Der p 2, c) Phl p 1, d) Phlp 5a e) Phl p 5b and f) Bet v 1 (GPMAW, Lighthouse data). The species specific peptides selected for the quantification assays are highlighted with bold text and gray color.

Fig.3. a) MALDI-TOF MS fingerprint analysis of a mixture of purified and trypsin digested natural Der f 2 and Der p 2 (1:1) and b) MALDI-TOF MS fingerprint analysis of a mixture of purified and trypsin digested recombinant Der f 2 and Der p 2 (1:1).

Fig. 4. SDS-PAGE analysis of of the HDM (House Dust Mite) allergen extract separated by use of hydrophobic interaction chromatography. The fractions the HDM proteins were divided into two major protein pools (I and II) and further subjected to quantification analysis.

Fig. 5. A strategy for labelling of samples when employing the ITRAQ™ (Applied Biosystems, Foster City, CA, USA) chemistry in quantification of allergens.

Fig. 6. The MS analyses of iTRAQ labelled a) Peptide 1, m/z 2353.44 (Der p 2, 32-48) b) Peptide 2, m/z 2326.35 (Der f 2, 32-48) c) trypsin digested and iTRAQ labelled nDer p 2 peptides and d) nDer f 2 peptides.

Fig.7. MS/MS fragmentation of the mixture of nDer f 2, nDer p 2, Peptide 1 and Peptide 2. The amount of isoallergens, nDer p 2 (114.10) and and Der f 2 (115.10), in the sample mixture were calculated as the ratio of the signal area of m/z 114 to area of m/z 116 (Peptide 1) and as the ratio of the area of m/z 115 to area of m/z 117 (Peptide 2).

Fig.8. Reversed phase analysis of the mixture of nDer f 2, nDer p 2, Peptide 1 and Peptide 2 from SCX chromatography. MS and MS/MS analyses were used to identify the peaks spotted on the MALDI-TOF target.

Fig.9. MS analysis of the mixture of trypsin digested nBet v 1 and the internal calibration standard (AQUA peptide). The mass difference of 6 Da between the native peptide and the calibration standard is demonstrated in the upper corner of the figure.

DETAILED DESCRIPTION OF THE INVENTION

In the present context the term, "allergen" refers to any naturally occurring protein, a modified protein, a recombinant protein, a recombinant mutant protein, or any protein

fragment thereof or mixtures of proteins that have been reported to induce allergic, i.e. IgE mediated reactions upon their repeated exposure to an individual.

Examples of naturally occurring allergens include pollen allergens (tree, weed, herb and grass pollen allergens), mite allergens (from e.g. house dust mites and storage mites), insect allergens (inhalant, saliva- and venom origin allergens), animal allergens from e.g. saliva, hair and dander from e.g. dog, cat, horse, rat, mouse, etc., fungi allergens and food allergens.

Important pollen allergens from trees, grasses and herbs are such originating from the taxonomic orders of Fagales, Oleales, Pinales and platanaceae including i.a. birch (Betula), alder (Alnus), hazel (Corylus), hornbeam (Carpinus), olive (Olea), cedar (Cryptomeria and Juniperus), Plane tree (Platanus), the order of Poales including i.a. grasses of the genera Lolium, Phleum, Poa, Cynodon, Dactylis, Holcus, Phalaris, Secale, and Sorghum and the orders of Asterales and Urticales including i.a. herbs of the genera Ambrosia, Artemisia, and Parietaria. Other important inhalation allergens are those from house dust mites of the genus Dermatophagoides and Euroglyphus, storage mite e.g Lepidoglyphys, Glycyphagus and Tyrophagus, those from cockroaches, midges and fleas e.g. Blatella, Periplaneta, Chironomus and Ctenocepphalides, and those from mammals such as cat, dog and horse, venom allergens including such originating from stinging or biting insects such as those from the taxonomic order of Hymenoptera including bees (superfamily Apidae), wasps (superfamily Vespidea), and ants (superfamily Formicoidae). Important inhalation allergens from fungi are i.a. such originating from the genera Alternaria, Cladosporium, Aspergillus and Penicillium.

Examples of food allergens are allergens from wheat (e.g. Tri a 18-19), crustacean food including shrimp (e.g. Met e 1, Pen a 1, Pen I 1, Pen m 1 and Pen m 2), prawn, crab and lobster, fish (e.g. Gad c 1 and Sal s 1), hen's eggs (e.g. Gal d 1, Gal d 2), peanut (e.g. Ara h 1-8), soy (Gly m 1-4), cows' milk (Bos d 4-8), nuts such as almond (Pru du 4), brazil nut (Ber e 1, Ber e 2), cashew nut (Ana o 1-3), hazelnut (e.g. Cor a 1.04, Cor a 2, Cor a 8) and walnut (e.g. Jug n 1-2, Jug r 1-3), celery (Api g 1, Api g 4, Api g 5), mustard (Sin a 1 and Bra j 1) and sesame seed (Ses i 1-6), and in particular allergens from wheat (e.g. Tri a 18-19), hen's eggs (e.g. Gal d 1, Gal d 2), peanut (e.g. Ara h 1-8), soy (Gly m 1-4), cows' milk (Bos d 4-8).

Examples of recombinant allergens include but are not limited to proteins/peptides from plant pollens, grass pollens, tree pollens, weed pollens, insect venom, dust and storage mite proteins, animal dander, saliva, fungal spores and food allergens (i.e., peanut, milk, gluten and egg) prepared using recombinant techniques. Recombinant allergens can be obtained

e.g. on a large scale by using microbial expression systems that may be grown on fermenters, produced by recombinant DNA techniques, or chemical precursors or other chemicals when synthesized chemically. In one embodiment of the invention, the allergen is rBet v 1, rAln g 1, rCor a 1, rCar b 1, rCry j 1, rCry j 2 , rOle e 1 , rAmb a 1, rArt v 1, rCyn d 1, rDac g 1, rLol p 1, rLol p 5, rPhl p 1, rPhl p 5, rPoa p 1, rPoa p 5, rSor h 1, rDer f 1 , rDer f 2, rDer p 1, rDer p 2, rEur m 1, rEur m 2, rGly d 1, rLep d 2, rBla g 1, rBla g 2, rFel d 1, rCan f 1, rCan f 2 , rBos d 2, rEqu c 1, rEqu c 2, rMus m 1, rApis m 1, rApi m 2 , rVes v 1, rVes v 2, rVes v 5, rDol m 1, rDol m 2, rDol m 5, rPol a 1, rPol a 2, rPol a 5, rAlt a 1 or rCla h 1 (r meaning recombinant).

A recombinant mutant allergen differs from the wild type in that the genes for the allergens have been modified by genetic manipulation methods such that the polypeptides which they encode exhibit substitutions, deletions and/or additions of individual or several amino acids as compared with the wild type. Examples of a recombinant mutant allergen include allergen substitution variants, addition variants, oligomers, fragments, deletion variants, hybrid molecules and other variants.

Examples of a modified allergen include allergens, which in naturally occurring form are associated with allergic disease conditions in sensitive subjects, wherein said modified recombinant allergen is altered compared to the naturally occurring allergen. Included are allergen variants containing a few amino acid exchanges, allergen mutants, oligomers, fragments, deletion variants, hybrid molecules, myristylated, glycosylated, palmitoylated and phosphorylated allergens and other variants. The modified allergen can be produced by any method suitable such as a site-directed mutagenesis method, a PCR method, chemical synthesis and a mixture of these methods.

In one embodiment of the invention, the allergen to be quantified is selected from one or more of the group of Bet v 1, Aln g 1, Cor a 1 and Car b 1, Que a 1, Cry j 1, Cry j 2 , Cup a 1, Cup s 1, Jun a 1, Jun a 2, Jun a 3, Ole e 1 , Lig v 1, Syr v 1, Pla l 1, Pla a 1, Pla a 2, Amb a 1, Amb a 2, Amb t 5, Art v 1, Art v 2, Art v 3, Par j 1 , Par j 2, Par j 3, Sal k 1, Ave e 1, Cyn d 1, Cyn d 7, Dac g 1, Fes p 1, Hol l 1, Lol p 1 and 5, Pha a 1, Pas n 1, Phl p 1, Phl p 2, Phl p 3, Phl p 4, Phl p 5, Phl p 6, Poa p 1, Poa p 5, Sec c 1, Sec c 5, Sor h 1, Der f 1 , Der f 2, Der f 3, Der f 7, Der p 1, Der p 2, Der p 3 , Der p 7, Der m 1, Eur m 1, Eur m 2, Gly d 1, Gly d 2, Lep d 1, Lep d 2, Blo t 1, Tyr p 2, Bla g 1, Bla g 2, Per a 1, Per a 3, Per a 7, Fel d 1, Fel d 2, Fel d 3 , Fel d 4, Can f 1, Can f 2 , Bos d 2, Equ c 1, Equ c 2, Equ c 3 , Mus m 1, Rat n 1, Apis m 1, Api m 1, Api m 2 , Ves v 1, Ves v 2, Ves v 5, Ves f 5, Ves g 5, Ves m 1, Ves m 2, Ves m 5, Ves p 5, Ves s 5, Ves vi 5, Dol m 1, Dol m 2, Dol m 5, Dol a 5, Pol a 1, Pol a 2, Pol a 5, Sol i 1, Sol i 2, Sol i 3 and Sol i 4, Alt a 1, Alt a 3, Alt a 4, Alt a 5, Alt a 6, Cla h 1, Cla h 2, Cla h

6 Asp f 1, Bos d 4, Mal d 1, Mal d 3, Gly m 1, Gly m 2, Gly m 3, Ara h 1, Ara h 2, Ara h 3, Ara h 4, Ara h 5 or hybrids of any of these.

In another embodiment of the invention, the allergen to be quantified is selected from one or more of the group of grass pollen allergens such as Phl p 1, Phl p 5, Phl p 6, Poa p 1, Poa p 5, Dac g 1, Fes p 1, Lol p 1 and Lol p 5, dust mite allergens such as Der f 1 , Der f 2, Der p 1 and Der p 2, venom allergens such as Api m 1, Api m 2 , Ves v 1, Ves v 2, Ves v 5, Dol m 1, Dol m 2, Dol m 5, Dol a 5, Pol a 1, Pol a 2, and Pol a 5, weed allergens such as Amb a 1, Amb a 2, Par j 1, Par o 1 and Par m 1, birch allergens such as Bet v 1, Japanese cedar allergens such as Cry j 1 and Cry j 2, cockroach allergens such as Per a 1 , olive pollen allergens such as Ole e 1, cat allergens such as Fel d 1 , dog allergens such as Can f 1 and Can f 2, horse allergens such as Equ c 1 and Equ c 2, mugworth allergens such as Art v 1, Art v 2, Art v 3, mold allergens such as Alt a 1, Alt a 3, Alt a 4, Alt a 5, Alt a 6, Cla h 1, Cla h 2 and Cla h 6 and fire ant allergens such as Sol i 2, Sol i 3 and Sol i 4.

In yet a further embodiment of the invention, the allergen to be quantified is selected from one or more of the group of grass pollen allergens such as Phl p 1, Phl p 5 and Phl p 6, olive pollen allergens such as Ole e 1, dust mite allergens such as Der f 1 , Der f 2, Der p 1 and Der p 2 , venom allergens such as Ves v 1, Ves v 2 and Ves v 5, weed allergens such as Amb a 1, Amb a 2, Par j 1, Par o 1 and Par m 1 and tree allergens such as Bet v 1, Cry j 1 and Cry j 2.

In a yet a further embodiment, the allergen to be quantified is one or more selected from the isoallergens of Der f 1 , Der p 1, Der f 2 and Der p 2.

In a yet a further embodiment the allergen to be quantified is one or more selected from Phl p 1, Phl p 5, Phl p 6, Poa p 1, Poa p 5, Dac g 1, Fes p 1, Lol p 1, Lol p 5.

In a yet a further embodiment the allergen to be quantified is one or more selected from the isoallergens of Amb a 1 and Amb a 2,

An allergen from a single species may consist of several closely similar molecules. These similar molecules are designated as isoallergens when they share the following common biochemical properties: a. similar molecular size; b. identical biological function, if known, e.g. enzymatic action; and c. > 67% identity of amino acid sequences. In the present context, the members of an allergen group which have > 67% amino acid sequence identity and are from the same species are designated as isoallergens. Each isoallergen may have multiple forms of closely similar sequences with only a few amino acids differing; these are designated as variants, and falls under the term "isoallergen" in the present context.

In the present context the term "homologous allergens" refers to allergens from different species, which are thought to share similar three-dimensional structures, molecular size, identical biological function, if known, e.g. enzymatic action and they may share structural epitopes for IgE antibodies. In a further embodiment according to the invention the homologous allergens has > 20% identity of amino acid sequences and share a commomn constant sequence of amino acids, preferably a sequence of at least 2-20 amino acids, more preferred 4-15 and most preferred 6-10.

As an example of homologous allergens can be mentioned e.g. Amp m 2 and Ves v 2, and Der f 2 and Der p 2.

In the present context, the expression "allergen extract" refers to any extract obtained by extraction of a biological allergen source material as generally described in "Allergenic extracts", H. Ipsen et al, chapter 20 in Allergy, principle and practice (Ed. S. Manning) 1993, Mosby-Year Book, St. Louis. Such extract may be obtained by aqueous extraction of water soluble material followed by purification steps like filtration to obtain the solution i.e. the extract. The extract may then be subjected to further purification and/or processing like freeze-drying removing substantially all the water. Generally, an allergen extract comprises a mixture of proteins and other molecules. Allergen proteins are often classified as a major allergen or, an intermediate allergen, a minor allergen or no classification. An allergen extract generally comprises both major and minor allergens. Major allergens will generally constitute approximately 5-15% of an average allergen extract, more often about 10%. Classification of an allergen is based on an assessment of the clinical importance of the particular allergen and is given below. Examples of important major allergens found in an extract include grass group 1 and 5 and 6 allergens (e.g. Phl p 1, 5, and 6), dust mite group 1 and 2 allergens (e.g. Der p 1 , Der p 2), tree pollen allergen 1 (Bet v 1), cedar pollen allergen 1 and 2 (e.g. Cry j 1, Cry j 2), ragweed pollen 1 and 2 (Amb a 1, Amb a 2), cat allergen 1 (i.e. Fel d1).

The expression "biological allergen source material" as used herein refers to any biological material comprising one or more allergens. Examples of such materials are acarids PMB (Pure Mite Body) or WMC (Whole Mite Culture), defatted or non-defatted pollens from e.g. grasses, herbs, weeds and trees, animal hair and dander, pelt, fungi mycelia and spores, insect bodies, venom or saliva and foods.

Biological allergen source materials may comprise contaminating materials, such as foreign pollen and plant and flower debris from an allergen pollen source material. The maximum level of accepted contamination with pollen from other species is 1%. It should also be devoid of flower and plant debris, with a limit of 5% by weight.

The term "allergen vaccine" as used in the present context comprises at least one allergen either originating from the same allergic source or originating from different allergenic sources e.g. grass group 1 and grass group 5 allergens or mite group 1 and group 2 allergens from different mite and grass species respectively, weed antigens like short and giant ragweed allergens, different fungi's allergens like alternaria and cladosporium, tree allergens like birch, hazel, hornbeam, oak and alder allergens, food allergens like peanut, soybean and milk allergens.

Preparation of vaccines is generally well known in the art. Vaccines are typically prepared as injectables either as liquid solutions or suspensions. Such vaccine may also be emulsified or formulated so as to enable nasal administration as well as oral, including buccal and sublingual, administration. The immunogenic component in question may suitably be mixed with excipients which are pharmaceutically acceptable and further compatible with the active ingredient. Examples of suitable excipients are water, saline, dextrose, glycerol and the like as well as combinations thereof. The vaccine may additionally contain other substances such as wetting agents, emulsifying agents, buffering agents or adjuvants enhancing the effectiveness of the vaccine.

According to an aspect of the invention, a method for quantification of the absolute amount of an allergen in an allergen sample is provided, comprising the following steps:

a) providing a known amount of one or more allergen calibration standard peptide(s) having a sequence of amino acids which is identical with a sequence to be found in the allergen to be quantified, and optionally labelling said allergen calibration standard peptide(s),

b) degrading the allergen sample to obtain a mixture of peptides, and optionally labelling said peptides with one or more labelling agent(s),

wherein at least the peptides in the degraded allergen sample or the calibration standard peptide(s) are labelled, and if both the peptides in the degraded allergen sample and the allergen calibration standard peptide(s) are labelled, the labelling agent(s) used for labelling the allergen calibration standard peptide(s) are different from the labelling agent(s) used for labelling the peptides of the degraded allergen sample,

c) quantifying the absolute amount of allergen by correlating the amount of the allergen calibration standard peptide(s) with the amount of the corresponding peptide(s) from the degraded allergen sample by mass analysis.

In one particular embodiment of the invention, both the calibration standard peptide(s) and peptides of the degraded sample are labelled but with different labelling agents.

In another embodiment of the invention, the calibration standard peptide(s) are labelled and peptides of the degraded sample are not labelled.

According to a further embodiment of the invention, the allergen calibration standard peptide(s) are not labelled whereas the peptides of the degraded allergen sample are labelled.

In a preferred embodiment of the invention as described above, one allergen calibration standard peptide is provided in step a). Thus, preferably, only one allergen calibration standard peptide is used for each allergen sample.

According to a further preferred embodiment of the invention the degraded sample in step b) is, when labelled, labelled with only one labelling agent.

Thus, preferably, only one allergen calibration standard peptide is provided in step a) and if labelled, the degraded sample in step b) is only labelled with one labelling agent.

Furthermore, when labelled, the calibration standard peptide is preferably labelled with only one labelling agent.

The mass analysis, e.g. the MS as such, may be carried out on mixtures of several pairs of an allergen sample and an allergen calibration standard peptide provided according to the invention, for that particular allergen sample.

In the present context, the term "allergen calibration standard peptide(s) having a sequence of amino acids which sequence is identical with a sequence to be found in the allergen(s) to be quantified" refers to an amino acid sequence region which is constant i.e. identical in the group of isoallergens of the allergen or in the homologous allergens to be quantified. According to a preferred embodiment of the invention, the allergen calibration standard peptide(s) is selected so that the degradation in step b) of the allergen (isoallergens or homolologous allergens) to be quantified results in a mixture of peptides where one of the peptides in the mixture comprises the same amino acid sequence as the allergen calibration standard peptide.

It is also possible according to the invention to quantify a specific isoallergen or allergen.

According to this aspect, the invention provides a method for quantification of a specific allergen or isoallergen in an allergen sample comprising the following steps:

a) providing a known amount of one or more allergen calibration standard peptide(s) having a sequence of amino acids which is unique for a sequence to be found in the allergen or isoallergen to be quantified, and optionally labelling said allergen calibration standard peptide(s),

b) degrading the allergen sample to obtain a mixture of peptides, and optionally labelling said peptides with one or more labelling agent(s),

wherein that at least the peptides in the degraded sample or the calibration standard peptide(s) are labelled, and if both the peptides in the degraded sample and the allergen calibration standard peptide(s) are labelled, the labelling agent(s) used for the allergen calibration standard peptide(s) are different from the labelling agent(s) used for labelling the peptides of the degraded sample,

c) quantifying the absolute amount of allergen by correlating the amount of the allergen calibration standard peptide(s) with the amount of the corresponding peptide(s) from the degraded allergen sample by mass analysis.

In one particular embodiment of the invention, both the calibration standard peptide(s) and peptides of the degraded sample are labelled but with different labelling agents.

In another embodiment of the invention, the calibration standard peptide(s) are labelled and the peptides of the degraded sample are not labelled.

According to a further embodiment of the invention, the allergen calibration standard peptide(s) are not labelled whereas the peptides of the degraded allergen sample are labelled.

In a preferred embodiment of the invention as described above, one allergen calibration standard peptide is provided in step a). Thus, preferably, only one allergen calibration standard peptide is used for each allergen sample.

According to a further preferred embodiment of the invention the degraded sample in step b) is, when labelled, labelled with only one labelling agent.

Thus, preferably, only one allergen calibration standard peptide is provided in step a) and if labelled, the degraded sample in step b) is only labelled with one labelling agent.

Furthermore, when labelled, the calibration standard peptide is preferably labelled with only one labelling agent.

The mass analysis, e.g. the MS as such, may be carried out on mixtures of several pairs of an allergen sample and an allergen calibration standard peptide provided according to the invention, for that particular allergen sample.

In the present context, the term "allergen calibration standard peptide(s) having a sequence of amino acids which sequence is unique for the allergen or the isoallergen to be quantified" refers to an amino acid sequence region which is variable i.e. unique for the isoallergen or the allergen which is to quantified. According to a preferred embodiment of the invention, the degradation in step b) of the allergen to be quantified results in a mixture of peptides where one of the peptides comprises the same amino acid sequence as the allergen calibration standard peptide.

The number of amino acids in the allergen calibration standard peptide is preferably in the range of 2-20 amino acids, more preferred in the range of 4-15 and most preferred in the range of 6-15. The number is dependent on the optimal enzymatic cleavage site found to match to the amino acid sequence within the sample i.e the constant or the variable region sequence when the sample is cleaved by an enzyme. Furthermore, the allergen calibration standard to be used according to the invention depends on the label and the quantification method to be used in order to give a detectable signal and fragmentation when analysed in a MS instrument.

In the present context the term "allergen sample" refers to a sample comprising one or more allergen(s).

In one embodiment of the invention, the allergen sample comprises an allergen extract, a naturally occurring purified allergen, a modified allergen, a recombinant allergen, a recombinant mutant allergen, any allergen fragment, a mixture of isoallergens, or mixtures of homologous allergens, or a combination thereof, and an allergen extract comprising artificial spiking with purified natural or recombinant allergens.

The allergen sample can be in the form of a final product such as an allergen vaccine in the form of a tablet or a solution, or a product/intermediate taken out during production such as after an extraction of a biological allergen source material or a raw material.

In a preferred embodiment of the invention, the allergen sample is in the form of an allergen extract, a final product in the form of a tablet or an intermediate product.

In one aspect of the invention, an allergen extract is provided which allergen extract is comprised of natural allergen and recombinant allergen and is obtained by quantifying the amount of allergen in a natural extract and adding recombinant allergen or natural purified allergen to the extract in order to obtain the required amount of allergen in the final extract such as a natural extract which has been artificial spiked with purified natural or recombinant allergens.

The method according to the invention makes is possible to quantify one or more allergen(s) present as isoallergens or homologous allergens and having a constant sequence of amino acids in common, from one or more species in an allergen sample simultaneously, or in one operation.

It is possible to quantify the isoallergens of a species in an allergen sample simultaneously, or in one procedure using the method according to the invention.

Depending on the sample, it can be necessary to use denaturants and buffer solutions in order to obtain an appropriate solution.

In case the sample contains substances such as thiols e.g. DTT or mercaptoethanol, high concentrations of detergent and/or denaturants such as SDS, octyl B-D-glucopyranoside and Triton® X-100 and/or active proteases or primary amines (other than the allergen of interests), which may interfere with the method according to the invention the sample preparation can involve various treatments e.g. precipitation with acetone. Recommended buffers and alternative detergents and/or denaturants and substances which may interfere with the method according to the invention are listed in e.g. Applied Biosystems iTRAQ™ Reagents Amine-Modifying Labeling Reagents for Multiplexed Relative and Absolute Protein Quantification Protocol from Applied Biosystems, Foster City, CA, USA.

Depending on the complexity of the sample, it might be beneficial to pre-fractionate the sample before degradation e.g. if there are molecules interfering with the detection of the allergen(s) of interest in the sample. The sample may also need to be separated/eluted from its formulation and/or any adjuvant e.g. aluminium hydroxide or calcium phosphate. In order to obtain a less complex mixture, the sample can be fractionated by use of various chromatography techniques such as hydrophobic interaction, ion exchange and/or immunoaffinity chromatography.

In one embodiment of the invention, the allergen sample is a fraction resulting from pre-fractionation e.g. an allergen extract fraction comprising one or more isoallergens.

In one embodiment of the invention, the allergen sample is a fraction from a pre-fractionation step where the sample has been fractionated according to size, solubility, electric charge and/or ligand specificity. In a further embodiment of the invention, the pre-fractionation is performed by chromatography, such as by hydrophobic interaction chromatography, reversed-phase chromatography, ion- exchange chromatography, size exclusion chromatography or affinity chromatography e.g. by hydrophobic interaction chromatography.

An example of pre-fractionation of an intermediate product containing HDM group 1 and 2 allergens by use of hydrophobic interaction chromatography is shown in Fig 4. The fractions containing respectively HDM group 1 and 2 allergens are separated by their physico-chemical properties and identified by immunopreciptiation. Both fractions can then be subjected to quantification studies.

In one embodiment of the invention, the allergen sample is desalted after chromatography.

In one embodiment of the invention, the allergen sample is reduced and any cysteine residue is blocked before degradation such as e.g. by alkylation.

According to the invention, the sample is degraded by treatment with one or more enzymes in order to obtain a mixture of peptides. The enzyme can be chosen to have a very predictable degradation pattern enabling that peptides are obtained that may be identified and quantified by comparison with the allergen calibration standard peptide. The enzyme can be one or more protease(s) such as e.g. two proteases or one or more other enzyme(s). Examples of proteolytic enzymes include trypsin, papain, pepsin, ArgC, LysC, V8 protease, AspN, pronase, chymotrypsin and carboxypeptidease C. For example, the proteolytic enzyme trypsin is a serine protease that cleaves peptide bonds between lysine or arginine and an unspecific amino acid to thereby produce peptides that comprise an amine terminus (N-terminus) and lysine or arginine carboxyl terminal amino acid (C- terminus). In this way, the peptides from the cleavage of the protein are predictable and their presence and/or quantity, in a sample from a trypsin digest, is indicative of the presence and/or quantity of the protein of their origin. Moreover, the free amine termini of a peptide can be a good nucleophile that facilitates its labelling. Because activity of the enzymes is predictable, the sequence of peptides that are produced from degradation of a protein of a known sequence can be predicted. With this information, "theoretical" peptide information can be generated. A determination of the "theoretical" peptide fragments in e.g. computer assisted analysis of

daughter fragment ions from mass spectrometry analysis of an actual sample can therefore be used to identify one or more peptides.

In one embodiment of the invention, the allergen sample is degraded before labelling by digestion of the sample with at least one enzyme to partially, or fully degrade the sample. In a further embodiment of the invention, the enzyme is a proteolytic enzyme such as trypsin, papain, pepsin, ArgC, LysC, V8 protease, AspN, pronase, chymotrypsin or carboxypeptidase C or a combination thereof, such as selected from the group of ArgC, LysC and trypsin or a combination thereof. In yet a further embodiment of the invention, the enzyme is trypsin.

The digested sample can be prepared before labelling by any of several methods if necessary.

For those skilled in the art, it will be obvious that there are numerous possibilities for labelling the sample and the allergen calibration standard peptides in order to introduce, in a predetermined manner, different mass-modifying functionalities that makes quantification of the allergen peptides possible. Labelling can e.g. be performed as described in WO 2004/070352, US 6,864,089, Stemmann O et al. Cell 2001; 107(6):715-26, and Gerber SA et al. Proc Natl Acad Sci U S A 2003;100(12):6940-5, which are incorporated herein by this reference.

In one embodiment of the invention, the labelling is with ITRAQ™ chemistry (Applied Biosystems, Foster City, CA, USA).

According to this embodiment of the invention, the labelling of the degraded allergen sample and/or the calibration standard peptides is performed by set of isomeric or isobaric labelling reagents such as iTRAQ™ Reagents (Applied Biosystems, Foster City, CA, USA). Each of these reagents contains a reactive group (RG) that reacts with the analyte and a unique reporter group (RP) that produces a unique "signature ion" in MS/MS analysis. These two groups are further linked together with a linker moiety (LK) using X and Y bonds. Labelling of the degraded allergen sample therefore yields an analyte named as RP-X-LK-Y-sample. The analysis of the analyte is performed by adjusting a mass spectrometer so that both X and Y bond fragment. Fragmentation of bond X releases the reporter from the analyte and the reporter can then be determined independently from the analyte. Fragmentation of Y bond releases RP-LK combination from the analyte. Hence, based on the fragmentation the presence and / or the amount of the reporter can be correlated with the presence and / or the amount of the analyte in the sample.

Labelling with e.g. 4 iTRAQ™ reagents, allows absolute quantification of different allergen samples simultaneously (the allergen samples are degraded and each peptide mixture is

labelled different iTRAQ™ reagents. The possibility of simultaneous analysis of different allergen samples enables the comparison of the labelled peptides, the sample(s), with the known amount of calibration standard peptide(s) and thereby makes quantification and identification by use of MS/MS in one step possible.

Labelling of the samples when using the ITRAQ™ and/or other labelling reagents can be performed according to manufacturer's procedure as shown in Fig. 5.

Another method for labelling in connection with quantification of proteins using MS techniques are e.g. the AQUA technique using internal calibration peptides synthesized with incorporated stable isotopes (^{13}C, ^{15}N) to mimic native peptides formed by enzymatic digestion using e.*g.*,trypsin (Stemmann O et al. Cell 2001;107(6):715-26, Gerber SA et al. Proc Natl Acad Sci U S A 2003;100(12):6940-5).

Another method is the ICPL (Isotope Coded Protein Labelling) method described by Kellermann et al, Proteomics 5, 4-15, using e.g $^{12}C/^{13}C_6$-Nicotinic acid-succinimide as ICPL label.

In one embodiment of the invention, the differently labelled peptide(s) and allergen calibration standard peptide(s) are labelled separately and mixed after labelling before quantification.

Depending on how the labelling of the allergen and the calibration standard peptides are performed, an appropriate way of identification may be selected.

In one embodiment of the invention, the allergen is further positively identified by comparing the labelled allergen peptide(s) and allergen calibration standard peptide(s) by peptide identification analysis.

Cation-exchange chromatography can also be used for separation of the peptides, in combination with reversed-phase chromatography as two-dimensional chromatography and for reducing the amount, if necessary, of any salts and organic compounds before MS analysis.

In one embodiment of the invention, the quantification is performed using mass spectrometry.

In a further embodiment of the invention, the identification of the allergen and/or isoallergens can be performed using tandem mass spectrometers and other mass spectrometers that are capable of selecting and fragmenting molecular ions. This is especially suitable when iTRAQ™ reagents are used for labelling.

Tandem mass spectrometers (and to a lesser degree single-stage mass spectrometers) have the ability to select and fragment molecular ions according to their mass-to-charge (m/z) ratio, and then record the resulting fragment (daughter) ion spectra. More specifically, daughter fragment ion spectra can be generated by subjecting selected ions to dissociative energy levels (e.g. collision-results in dissociation (CID)). For example, ions corresponding to labelled peptides of a particular m/z ratio can be selected from a first mass analysis, fragmented and reanalyzed in a second mass analysis. Representative instruments that can perform such tandem mass analysis include, but are not limited to, magnetic four-sector, tandem time-of flight, triple, quadrupole, ion-trap, and hybrid quadrupole time-of-flight (Q-TOF) mass spectrometers.

These types of mass spectrometers may be used in conjunction with a variety of ionization sources, including, but not limited to, electrospray ionization (ESI) and matrix assisted laser desorption ionization (MALDI). Ionization sources can be used to generate charged species for the first mass analysis where the analyses do not already possess a fixed charge. Additional mass spectrometry instruments and fragmentation methods include post source decay in MALDI-MS instruments and high-energy CID using MALDI-TOF (time of flight)-TOF MS. For a recent review of tandem mass spectrometers please see: R. Aebersold and D. Goodlett, Mass Spectrometry in Proteomics. Chem. Rev. 101: 269-295 (2001). Also see United States Patent No. 6,319,476, herein incorporated by reference, for a discussion of TOF TOF mass analysis techniques.

The allergen calibration standard peptide(s) (variable or constant sequence(s)) are chosen dependent on whether the quantification to be made is of an allergen or homologues allergens or specific allergens or isoallergens.

In one embodiment of the invention, absolute quantification of an allergen (absolute amount of isoallergens of the allergen) can be made.

When using the iTRAQ™ reagents the chosen allergen calibration standard peptide(s) is labelled with an isomeric or isobaric label of the set of labels, e.g. iTRAQ-114, iTRAQ-115, iTRAQ-116 or iTRAQ-117, used to label the allergen peptides. Once the relative amount of reporter for the calibration standard peptide, or standard peptides, is determined with relation to the relative amounts of the reporter for the differentially labelled peptides, it is

possible to calculate the absolute amount (often expressed in concentration and/or quantity) of all of the differentially labelled peptides in the sample mixture and thereby calculate the amount of allergen (e.g. absolute amount of isoallergen(s) from a species when the sample is an extract). The acquisition of MS and MS/MS from the ITRAQ™ labelled samples can be performed e.g. using 4700 Explorer™ software. In addition, the GPS Explorer software can be used to perform the database searches which would result in the definitive identification of the peptides from MS/MS. The obtained data can then be used for quantification based on the known amount of allergen calibration standard peptide i.e. ratio between the sample and the calibration standard peptide.

The method according to the invention is useful e.g. in a release assay in order to ensure a safe and predictable amount of allergen during production of a vaccine, and in the final product, and also during the various stages of storage of ingredients and/or products, and raw extracts. The method according to the invention is useful in development of second generation allergen vaccines e.g. using recombinant allergen as active ingredients by optimizing active ingredients in second generation allergen vaccines based on the knowledge and/or composition of the current vaccines. Current vaccines are often formulated using allergens from a number of allergen species, and the method would also be beneficial in determination of the composition of species specific allergens from these allergen mixtures. The method according to the invention can be used in cleaning validation, where trace amounts of allergen(s) are measured, release assays and analyses of intermediate and final products.

Peptides which can be used as calibration standard peptides can be made by using protein and/or nucleotide databases and cleavage analyses program(s) and/or perform *in vitro* mass fingerprinting experiment(s).

In the present context, the term "allergen calibration standard peptide" refers to an allergen calibration standard with an amino acid sequence identical with, either a variable or a constant sequence in a group of isoallergens or homologous allergens, depending on whether it is to be used for quantification of an allergen consisting of more than one isoallergens or homologous allergens or a specific allergen or an isoallergen. The allergen calibration peptide is preferably prepared by peptide synthesis.

In some cases, the sequence of the allergen of interest is already known. An official list of allergens can e.g. be found on the website (www.allergen.org) which is maintained by I.U.I.S Allergen Nomenclature Sub-committee. The known allergen sequences can be obtained from protein and nucleotide databases e.g. Uniprot Knowlegdebase. The protein and/or nucleotide sequences can be searched using e.g. Sequence Retrieval System (SRS), or by using

keywords e.g. submitting entry name (ID), description (DE), gene name (GN), species (OS) and/or organelle (OG). Further analysis of the protein/allergen of interest is performed by using e.g. Vector NTI software (Invitrogen) and/or by use of ExPASy (**Ex**pert **P**rotein **A**nalysis **Sy**stem) proteomics server of the Swiss Institute of Bioinformatics (SIB). Sequence alignment of existing allergen isoforms and allergen species is performed using e.g. Blast search which can be used to align homologous protein and/or nucleotide sequences. Sequence alignments are providing a way to compare e.g. novel sequences with previously characterized genes and/or protein(s). The sequence alignments of homologous allergens or isoallergens can be used to demonstrate the identical (constant) and variable sequences within and between allergen species as shown in Fig. 1.

In order to obtain optimal calibration standard peptides, the cleavage analysis for allergen of interest can be simulated using cleavage (degradation) analyses program(s). Allergen sequences can be subjected to e.g. GPMAW program (Lighthouse data, Odense, Denmark) which is created to support MS analyses. Cleavage (degradation) analysis of an allergen sequence can be deduced for several known proteases such as trypsin, Asp-N and Lys-C and/ or a combination of two or more of them. The resulting theoretical peptides (Fig. 2) are then used to verify the optimal degradation enzyme(s) and further to design the synthetic peptides which can be used as calibration standard peptides.

On the other hand, the calibration standard peptides can be deduced from *in vitro* mass fingerprinting experiment(s), where allergens are cleaved by enzyme and mixed. The species specific peptides can be detected by mass fingerprinting analyses and identified by database searches e.g. using Mascot Search engine, as described below:.

Purified natural (n) Der f 2 and nDer p 2 and recombinant (r) Der f 2 (A61501) and Der p 2 (BAA01241) can be dissolved in 25 mM Tris-Cl pH 7.5, 1.0 M Urea. An aliquot of recombinant molecules (rDer f 2 and rDer p 2) and natural (nDer f 2 and nDer p 2) molecules can be mixed (e.g. 1:1) or digested as individual allergens by trypsin and mixed after that. The digestions of mixed and/or individual allergens are desalted and evaluated by mass fingerprinting. The peptides that are species specific can be identified by mass fingerprinting analyses from the mixture of these two species.The digested individual HDM 2 allergens can be mixed after digestion with trypsin and the same species specific constant sequences can be demonstrated. Based on the species specific constant sequences synthetic peptides can be designed. The quantification and the verification of the peptide sequences can be performed by labelling the individual allergens and the calibration standard peptides e.g. with ITRAQ™ reagents and analysed by tandem mass spectrometry (MS/MS).

Natural allergen extracts e.g. intermediate product of two HDM species *Dermatophagoides farinae* and *Dermatophagoides pteronyssinus* (ALK-Abelló, Hørsholm, Denmark) (1.0 mg/ml dry weight) are dissolved. In order to remove the interfering component the samples can be precipitated with acetone. The precipitate containing proteins can then be resolved into the chosen buffer. For quantification purposes, the sample(s) and the chosen synthetic peptides from the two species used as calibration standard peptide can be labelled with e.g. ITRAQ™ reagents and analysed using MS/MS.

The final product e.g. house dust mite allergen tablet is dissolved into 20 mM Na-phosphate buffer pH 7.0. In order to remove the interfering components, the sample may need to be pre-fractionated and/or precipitated with acetone. The dissolved tablet and the chosen calibration standard peptides are labelled e.g. with ITRAQ™ reagents and analysed using tandem MS/MS.

Release study; the final product e.g. 5 grass mixture(s), including 5 grass species coupled with aluminiumhydroxide is dissolved into a chosen buffer which can elute bound allergens from the aluminium hydroxide. The unbound allergens can be further separated and / or desalted into a chosen digestion buffer and the chosen calibration standard peptides can be labelled e.g. with ITRAQ™ reagents and the quantification can be assessed using MS/MS.

All aspects or features mentioned in connection with any of the methods of the invention are naturally equally relevant to any of the other methods according to the invention.

EXAMPLES

Example 1

Identification of unique constant regions for natural Der f 2, Der p 2, Phl p 1, Phl p 5 and Bet v 1 and synthetic calibration standard peptides

The absolute quantification of the isoallergens using the unique constant region sequences i.e. signature peptides in natural Der f 2, Der p 2, Phl p 1, Phl p 5 and Bet v 1 was demonstrated by employing two different approaches. Two calibration standard peptides were synthesized to assess iTRAQ™ labelling technique (Applied Biosystems). In addition, four calibration standard peptides were synthesized to assess stable isotope labelling technique such as Protein-AQUA™ (Sigma-Aldrich).

Allergen calibration standard peptides having species specific sequences corresponding to the amino acid sequence of 32 – 48 in Der f 2 and in Der p 2, 149 - 158 in Phl p 1, 123 - 135 in

Phl p 5a, 115 - 127 in Phl p 5b a and 151 – 164 in Bet v 1 were designed based on the amino acid sequence alignments (Vector NTI) (Fig.1), cleavage analyses by GPMAW (Fig.2) and Blast database searches. The *in vitro* mass fingerprinting analyses of trypsin digested natural allergens Der f 2, Der p 2, Phl p 1. Phl p 5, Bet v 1 and mixed Der f 2 and Der p 2 were performed to demonstrate the occurrence of the species specific peptides. All the *in vitro* digested samples and mixture(s) were identified by use of the Mascot search engine (Matrix Science Inc., Boston, MA, USA).

Synthetic peptides corresponding to the amino acid sequence of 32 – 48 in Der f 2 and Der p 2 were be obtained from Sigma GENOSYS, Texas, US. The concentration of the peptides was determined by the amino acid analyses (Sigma GENOSYS, Texas, US). Stable isotope labelled synthetic peptides (Protein- AQUA™ peptides) corresponding to the amino acid sequence of 149-158 in Phl p 1 (Arg ^{13}C ^{15}N), 123-135 in Phl p 5a (Arg ^{13}C ^{15}N), 115-127 in Phl p 5b a (Arg ^{13}C ^{15}N) and 151 – 164 in Bet v 1 (Val ^{13}C ^{15}N) were obtained from Sigma GENOSYS, Texas, US (Table 1).

Example 2

Purification of natural and recombinant grass, birch and mite group 2 allergens

Natural Der f 2 and Der p 2 were purified from 100 mg of *Dermatophagoides farinae and Dermatophagoides pteronyssinus* extracts (ALK-Abelló,Horsholm, Denmark). Natural Phl p 1 and 5 were purified from 50 mg of *Phleum prantense* extract (ALK-Abelló) and natural Bet v 1 from 50 mg of *Betula verrucosa* extract (ALK-Abelló). The purification of the molecules was performed as described in the literature (Johannessen BR et al. FEBS Lett 2005;579:1208-12, Aasmul-Olsen S. et al. New Horizons in Allergy Immunotherapy, edit by Sehon et al. Plenum Press. New York 1996, p.261-65, Petersen A et al. Clin Exp Allergy. 1994 Mar;24(3):250-6, Ipsen H & Lowenstein H J Allergy Clin Immunol. 1983;72(2):150-59). Recombinant Der f 2 and Der p 2 were expressed in *Pichia pastoris* expression system and purified as described in the literature (Johannessen BR et al. FEBS Lett 2005;579:1208-12). The proteins were stored as freeze dried aliquots in -20°C.

The concentration of the purified allergens were measured using the extinction coefficient of one of the isoallergens at A_{280} and by use of Lambda 800 UV/VIS Spectrometer (Perkin Elmer Instruments, CA, USA).

Example 3

Pre-fractionation of HDM extracts and protein digestions

Hydrophobic interaction chromatography was used to pre-fractionate the *Dermatophagoides farinae (Der f) and Dermatophagoides pteronyssinus (Der p)* extracts. The fractionation of the mite extracts was performed with a 1.0 ml HiTrap Phenyl column (GE-Healthcare, Uppsala,Sweden). The column was equilibrated with 50 mM Na-phosphate buffer (Merck, Darmstadt, Germany), pH 7.0, 1.0 M ammonium sulphate (Fluka, Buchs, Switzerland) and the bound sample was eluted with 50 mM Na-phosphate buffer (Merck), pH 7.0 in 5 column volumes using a decreasing linear gradient. The chromatography was performed separately for each of the HDM extracts, *Der f* and *Der p*. The fractions were analysed by SDS-PAGE (Invitrogen, Carlsbad, CA, USA) and based on this analyses the HDM proteins were divided into two major protein pools (Fig 4). The *Der f* and *Der p* pools containing HDM 2 allergens were subjected to a dialysis step against 10 mM ammonium bicarbonate (BDH, Poole, England). The dialysed *Der f* and *Der p* pools were freeze dried, aliquated in ~5 mg (dry weight) vials and stored frozen at -20°C. The *Der f* and *Der p* aliquots containing HDM 2 allergens were further subjected to the absolute quantification studies.

Example 4

Enzymatic cleavage and iTRAQ labelling

iTRAQ™ labelling was employed from three samples sets:

a) 15 µg of natural Der f 2 and 15 µg of natural Der p 2

b) 15 µg recombinant Der f 2 and 15 µg of recombinant Der p 2 and

c) 100 µg of pre-fractionated *Der f* and *Der p* extracts.

The synthetic standard peptides, 15 µg Peptide 1 (Der p 2) and 15 µg Peptide 2 (Der f 2) were dissolved into a 100 mM Triethyl ammonium bicarbonate (TEAB) pH 8.5 and labelled to be used as internal calibration standards for each of the experiment sets as described below.

The blocking of free cysteine residues, the enzymatic cleavage using trypsin and the peptide labelling with iTRAQ™ reagents was performed according to the manufacturer's protocol:

Each of the six protein samples and the two internal calibration standards were dissolved into 20 µl of 100 mM TEAB, pH 8.5. The protein samples were denatured with 1.0 µl of 0.05% SDS and reduced by 2.0 µl of 4.8 mM TCEP Tris(2-carboxyethyl)phosphine at 60°C following blocking of the cysteine residues by 1.0 µl of 10 mM s-methyl methanethiosufonate (MMTS) at the room temperature. The protein samples were digested with 10% (w/w) Seq. grade modified trypsin (Promega, Madison, WI, USA) for 18 hours at 37°C.

The iTRAQ™ labelling of the trypsin digested samples and internal calibration standard peptides, Peptides 1 and 2 were performed at room temperature. Each iTRAQ labelling reagent from 114 to 117 was dissolved into 70 µl of 70% ethanol which was then applied on the sample(s). The final volumes of the reagent mixtures were 100 µl / sample. The labelled samples were stored at -20°C.

The trypsin digested peptides of natural Der p 2, recombinant Der p 2 *and Der p* extract were labelled with iTRAQ-114. The trypsin digested peptides of natural Der f 2, recombinant Der f 2 and *Der f* extract were labelled with iTRAQ-115. The synthetic peptides Peptide 1 (Der p 2) and Peptide 2 (Der f 2) were labelled with iTRAQ-116 (Der p 2) and iTRAQ-117 (Der f 2) (Fig 5). Each of the labelled samples were analysed by Voyager STR and/or 4700 Proteomic Analyser (Applied Biosystems) to demonstrate the labelling of the peptides. The MS/MS fragment analyses were performed with 4700 Proteomic Analyser (Applied Biosystems). The labelled samples were diluted 1:10 and 1.0 µl of the sample(s) was desalted by C18 Micro columns (ZipTips, Millipore) and/or handmade C18 micro columns (Poros R2, Applied Biosystems). The sample was eluted on the target by 1.0 µl 70% acetonitrile (ACN), 0.1% Trifluoro acetic acid (TFA) and 1.0 µl of alpha-cyano-4-hydroxy-cinnamic acid (CHCA) (Agilent Technologies, Böblingen, Germany) matrix was added on top of the sample. The sample was dried and subjected to MS analyses.

The MS analyses of the iTRAQ™ labelled internal calibration standards revealed masses corresponding to Peptide 1 at the m/z 2353.44 and Peptide 2 at the m/z 2326.35 (Fig. 6a and 6b).The results showed that the modification in the Der p 2 specific Peptide 1 and Der f 2 specific Peptide 2 corresponded to the modification by the iTRAQ™ reagent when bound to the in the amino-terminus and the C-terminal Lysine. The MS analyses of the iTRAQ™ labelled natural and recombinant Der p 2 peptides revealed iTRAQ™ modified massed at m/z 2353.29. Similarly, MS analyses of the labelled natural and recombinant Der f 2 peptides revealed the iTRAQ™ modified peptide at m/z 2326.29, respectively (Fig 6c and 6d). No missed cleavages by trypsin were observed. These results show that the iTRAQ labelled Peptides 1 and 2 can be used as internal calibration standards for absolute quantification assays of Der p 2 and Der f 2 isoallergens.

Example 5

Absolute quantification from the mixture of two different HDM species

The absolute quantification of the isoallergens of *Dermatophagoides farinae and Dermatophagoides pteronyssinus* species was first employed from the direct mixture of the purified natural allergens. The iTRAQ™ labelled natural tryptic Der p 2, Der f 2 and Peptides 1 and 2 were mixed in ration of 1:1:1:1, diluted 1:5 and 1:10 and desalted by handmade C18 micro columns (Poros R2, Applied Biosystems). The sample was eluted on the target by using 1.0 µl of 5 µg/µl of CHCA (Sigma) in 70% ACN (Sigma), 0.1% TFA (Fluka). The MS/MS fragment analyses was performed by 4700 Proteomic Analyser (Applied Biosystems).

The fragment analyses of m/z 2353.29 revealed signals at m/z 114 and m/z at 116 corresponding to the reporter ions for natural Der p 2 and Peptide 1. The amount of natural Der p 2 isoallergens in the sample was calculated as the ratio of the signal area of m/z 114 to area of m/z 116, the internal calibration standard (Fig 7a). The MS/MS fragment analyses of m/z 2326.29 revealed the signals at m/z 115 and m/z at 117 corresponding to the reporter ions for natural Der f 2 and Peptide 2.

The amount of natural Der f 2 isoallergens in the sample was calculated as the ratio of the signal area of m/z 115 to m/z of 117, the internal calibration standard (Fig 7b). In addition to the absolute quantification the fragment ion peak lists of m/z 2353.29 and m/z 2326.29 were submitted for database analyses by Mascot search engine (Matrix Science). The database analyses resulted identification of the peptides as 32-48 of *Dermatophagoides farinae and Dermatophagoides pteronyssinus* HDM allergens 2.

Example 6

Separation of the labelled peptide mixtures by two-dimentional chromatography

The absolute quantification of the mixture of recombinant Der f 2 and Der p 2 and the isoallergens in the complex mixture of *Der f and Der p* extracts was assessed by two-dimentional chromatography. Cation exchange chromatography (SCX) was assessed as the first dimension and reversed-phase chromatography as the second dimension separation step.

Recombinant Der p 2, Der f 2 and Peptides 1 and 2 were mixed in a ratio of 1:1:1:1, in a final volume of 50 µl. *Der f and Der p* extracts and Peptides 1 and 2 were mixed in ration 4:4:1:1, in a final volume of 50 µl.

Separation of the labelled peptide mixtures by cation exchange chromatography (both recombinant Der p 2 and Der f 2 and extracts of Der p 2 and Der f 2):

The sample mixtures were diluted 1:10 into 5% ACN (Sigma) 0.05% Formic acid (Merck) and subjected to SCX. The SCX was performed in 0.8x50 mm Zorbax BIO-SCX (3.5 µm) column (Agilent Technologies) in a SMART™ system (GE-HealthCare, Uppsala, Sweden). The column was equilibrated with 5% ACN (Sigma) 0.05% Formic acid (Merck) and the chromatography was performed with a increasing linear gradient from 0 to 100 % of 5% ACN (Sigma) 0.05% Formic acid (Merck) 0.5 M NaCl (Merck) in 30 min. The flow rate was 50 µl/min and the chromatography was monitored at 214 nm. The 50 µl fractions were collected and 1.0 µl of each fraction was analysed by Voyager-STR MS (Applied Biosystems) and/or 4700 Proteomic Analyser (Applied Biosystems) instruments. Peptides at m/z 2353.29 and m/z 2326.29 were identified eluting in the end of the gradient together with some other HDM peptides. The *Der f* and *Der p* mixture fractions (the extracts) containing the peptides at m/z 2353.29 and m/z 2326.29 were chosen to be further separated by reversed-phase chromatography, see below. However, the quantification of the mixture of rDer f 2 and rDer p 2 (recombinant) and the internal calibration standards was performed directly after SCX from the target plate.

The MS/MS fragment analyses of m/z 2353.29 revealed signals at m/z 114 and m/z at 116 corresponding to the reporter ions for Der p 2 and Peptide 1. The amount of Der p 2 clone the rDer p 2/rDer f 2 mixture was calculated as the ratio of the signal area of m/z 114 to area of m/z 116, the internal calibration standard. The MS/MS fragment analyses of m/z 2326.29 revealed the signals at m/z 115 and m/z at 117 corresponding to the reporter ions for Der f 2 and Peptide 2. The amount of Der f 2 clone in the in the rDer p 2/rDer f 2 mixture was calculated as the ratio of the signal area of m/z 115 to m/z of 117, the internal calibration standard. The fragment ion peak lists of m/z 2353.29 and m/z 2326.29 were submitted for database analyses by Mascot search engine (Matrix Science). The database analyses resulted identification of the peptides as 32-48 of *Dermatophagoides farinae and Dermatophagoides pteronyssinus* HDM allergens 2.

The peptides at m/z 2353.29 and m/z 2326.29 in both samples; the mixture of recombinant Der f 2 and Der p 2 and the *Der f* and *Der p* extract mixtures, eluted from the SCX column with similar retention times. This experiment showed that SCX can be used as a fractionation and desalting step for the more simple sample-mixtures before the quantification of the allergens. The experiments also showed that SCX can be employed as the first dimension fractionation step for analyses of more complex mixtures of allergens such as conventional allergen extracts used in immunotherapy.

Separation of labelled peptides (from extracts of Der p 2 and Der f 2) by SCX followed by reversed phase chromatography and followed by absolute quantification MALDI TOF-TOF MS.

The separation of the iTRAQ™ labelled HDM peptides from SCX fractionation as described above, was performed by C18 PepMap100 (3 µm) column (LC Packings Dionex, Sunny Vale, CA, USA). The column was equilibrated with 0.05% TFA (Fluka), 2 % ACN (Sigma). The peptides were eluted with 0.04% TFA (Fluka), 80% ACN in a gradient of 0 – 50%, in 80 min, 50 - 100%, in 120 min. The chromatography is performed with Ultimate3000 (LC Packings, Dionex) 2.0 µl/ min and monitored at 210 and 214 nm. 1.0 µl of SCX the fraction was injected to the column. The fractions were collected by spotting them directly on a MALDI-TOF target plate. The spotting was performed with the Probot (LC Packings, Dionex) instrument which was connected on-line to the Ultimate3000 instrument. The spotting was performed every 30 s mixing the HCCA matrix (Agilent Technologies) to the sample in a ratio of 1:1.

The spotted samples were analysed by MS and MS/MS using 4700 Proteomic Analyser (Applied Biosystems). Peptides at m/z 2353.29 and m/z 2326.29 were identified from the spots on target and they were shown to correspond to the signals at 214 nm in the chromatography (Fig.8). The MS/MS fragment analyses of m/z 2353.29 revealed signals at m/z 114 and m/z at 116 corresponding to the reporter ions for Der p 2 and Peptide 1. The amount of Der p 2 isoallergens in the *Der p/f* extract mixture was calculated as the ratio of the signal area of m/z 114 to area of m/z 116, the internal calibration standard. The MS/MS fragment analyses of m/z 2326.29 revealed the signals at m/z 115 and m/z at 117 corresponding to the reporter ions for Der f 2 and Peptide 2. The amount of Der f 2 isoallergens in the in the *Der p/f* extract mixture was calculated as the ratio of the signal area of m/z 115 to m/z of 117, the internal calibration standard. The fragment ion peak lists of m/z 2353.29 and m/z 2326.29 were submitted for database analyses by Mascot search engine (Matrix Science). The database analyses resulted identification of the peptides as 32-48 of *Dermatophagoides farinae and Dermatophagoides pteronyssinus* HDM allergens 2.

This experiment shows that reversed phase chromatography can be used as the second dimension in fractionation of the simple and/or complex mixtures of allergen extracts for quantification of isoallergens.

Example 7

Absolute quantification of the isoallergens employing the AQUA strategy

In the AQUA technique (Stemmann O et al. Cell 2001;107(6):715-26, Gerber SA et al. Proc Natl Acad Sci U S A 2003;100(12):6940-5.) the internal calibration peptides are synthesized with incorporated stable isotopes (^{13}C, ^{15}N) to mimic native peptides formed by enzymatic digestion using *e.g.*,trypsin. The incorporation of one isotope labelled amino acid residue changes the molecular masses of the peptides typically from 6 to 10 Da. Unlike in the iTRAQ technique either the samples or the internal calibration standard(s) needs to be modified by the labelling reagents. In quantification experiments e.g. when using a LC-MS/MS, the abundance of a specific fragment ion from both the native sample peptide and the synthesized internal calibration standard can be measured as a function of reverse-phase chromatography retention time. The absolute quantification is determined by comparing the abundance of the known internal standard with the native sample peptide.

The synthetic internal calibration standard peptides for natural Phl p 1, Phl p 5 a and b form, and Bet v 1 were designed as described above (Fig.2). The synthetic peptides are described in more detail in table 1.

Species	**Allergen**	**Amino acid sequence**	**Theoretical mass**	**Modification**	**Modified mass**
Betula verrucosa	Bet v 1	AVESYLLAHSDAYN	1552.73	(Val ^{13}C ^{15}N)	1558.64
Phleum prantense	Phl p 1	SAGEVEIQFR	1135.57	(Arg ^{13}C ^{15}N)	1145.27
Phleum prantense	Phl p 5a	YDAYVATLSEALR	1471.74	(Arg ^{13}C ^{15}N)	1481.66
Phleum prantense	Phl p 5b	FDSFVASLTEALR	1455.74	(Arg ^{13}C ^{15}N)	1465.66

Table 1: Labelling of the synthetic internal calibration standards

Natural Phl p 1, Phl p 5 and Bet v 1 were re-dissolved into a 25 mM Tris-Cl (Sigma), 1.0 M Urea (Fluka) pH 7.8 in a concentration of 2.5 pmol/µl. Internal calibration standards were mixed into the samples in a ratio of 1:1. The digestion was performed by 10% (w/w) Seq. grade Trypsin (Promega) in 37°C for 18 hours. The samples were stored in -20°C.

The trypsin digested Phl p 1. Phl p 5 and Bet v 1 were analysed by MS and MS/MS using the Voyager STR and/or 4700 Proteomic Analyser (Applied Biosystems). The all samples were diluted 1:10 in 0.1% TFA (Fluka) and 1.0 µl of each sample was desalted by hand made C18 micro columns (Poros R2 Applied Biosystems).

MS analyses of the trypsin digested natural Phl p 1 revealed the native peptide at m/z 1135.60 and the internal calibration standard peptide for Phl p 1 isoallergens at m/z 1145.61 (data not shown). The MS/MS analyses of the internal calibration peptide showed fragmentation pattern which was corresponding to the unique amino acid sequence of the natural Phl p 1 isoallergens.

MS analyses of the trypsin digested natural Phl p 5 revealed the native peptide of Phl p 5a at m/z 1471.81 and the native peptide of Phl p 5b at m/z 1455.77 (data not shown). The internal calibration standard peptides for Phl p 5a and Phl p 5b isoallergens were detected at m/z 1481.83 and at m/z 1465.78. The MS/MS analyses of the internal calibration peptides showed fragmentation patterns which were corresponding to the unique amino acid sequences of the natural Phl p 5a and Phl p 5b isoallergens.

MS analyses of the trypsin digested natural Bet v1 revealed the native peptide at m/z 1552.76 and the internal calibration standard peptide for Bet v 1 isoallergens at m/z 1558.77 (Fig.9) The MS/MS analyses of the internal calibration peptide showed fragmentation pattern which was corresponding to the unique amino acid sequence of the natural Bet v 1 isoallergens.

For the absolute quantification of Betv 1 Phl p 1, Phl p 5a and Phl p 5b isoallergens the samples can be subjected to analysis by *e.g.,* by LC coupled-MS/MS instruments such as LCQ DecaXP (ThermoFinnigan), QSTAR® Hybrid LC/MS/MS system, and 4000 Q TRAP LC/MS/MS system (Applied Biosystems).

The experiments with the AQUA peptides showed that the stable isotope labelled synthetic peptides mimicking the native isoallergen sequences can be used for absolute quantification of isoallergens in natural Phl p 1, Phl p 5 and Bet v 1. Furthermore, database analyses of the tryptic digests did not reveal any missed cleavages by trypsin. The two dimensional chromatography the combination of SCX and RP-HPLC as described for iTRAQ chemistry can be used in fractionation of the more complex allergen mixtures of chemically identical native and synthetic internal calibration standards.

2006291835 11 Jan 2012

The Claims Defining the Invention are as follows:

1. A method for quantification of the absolute amount of allergen in an allergen sample where the allergen consists of more than one isoallergen(s) or homologous allergen(s) comprising the following steps:

a) identifying a sequence of amino acids in the allergen to be quantified which is constant in the isoallergens or homologous allergens in the allergen by comparing the amino acid sequences of isoallergens or homologous allergens in the allergen and preparing a synthetic allergen calibration standard peptide having this constant sequence and labelling said allergen calibration standard peptide(s) with mass-modifying functionalities,

b) degrading the allergen sample to obtain a mixture or peptides, and optionally labeling said peptides with one or more labelling agent(s) with mass-modifying functionalities, wherein if both the peptides in the degraded allergen sample and the allergen calibration standard peptide(s) are labelled, the labelling agent(s) used for labelling the allergen calibration standard peptide(s) are different from the labelling agent(s) used for labelling the peptides of the degraded allergen sample,

c) quantifying the absolute amount of allergen by correlating the amount of the labeled allergen calibration standard peptide(s) with the amount of the corresponding peptide(s) of the degraded allergen sample by mass spectrometry.

2. The method according to claim 1 wherein the allergen sample is degraded to obtain a mixture of peptides, and labelled with one or more mass-modifying functionalities and the absolute amount of allergen is quantified by correlating the amount of the labeled allergen calibration standard peptide(s) with the amount of the corresponding labelled peptide(s) of the degraded allergen sample by mass spectrometry.

3. The method according to claim 1 wherein the allergen sample is degraded to obtain a mixture of peptides, and the absolute amount of allergen is quantified by correlating the amount of the labeled allergen calibration standard peptide(s) with the amount of the corresponding peptide(s) of the degraded allergen sample by mass spectrometry.

4. The method according to any one of claims 1-3, wherein the allergen calibration standard peptide(s) have a sequence of amino acids which is identical with a sequence or amino acids in a peptide obtained by degradation according to step b).

5. The method according to claims 1-4, wherein the allergen to be quantified consists of more than one isoallergen.

6. The method according to claims 1-4 wherein the allergen to be quantified consists of more than one homologous allergens.

7. The method according to any one of claims 1-6, wherein the allergen to be quantified is selected from the group consisting of Phl p 1, Phl p 5, Phl p 6, Poa p 1, Poa p 5, Dac g 1, Fes p 1, Lol p 1, Lol p 5, Der f 1, Der f 2, Der p 1, Der p *2,* Api m 1, Api m 2 , Ves v 1, Ves v 2, Ves v 5, Dol m 1, Dol m 2, Dol m S, Dol a 5, Pol a 1, Pol a 2, Pol a 5, Amb a 1, Amb a 2, Par j 1, Par o 1, Par m 1, Bet v 1, Cry j 1, Cry j 2, Per a 1, Ole e 1, Fel d 1, Can f 1, Can f 2, Equ c 1, Equ c 2, Art v 1, Art v 2, Art v 3, Alt a 1, Alt a 3, Alt a 4, Alt a 5, Alt a 6, Cla h 1, Cla h 2, Cla h 6, Sol i 2, Sol i 3 and Sol i 4.

8. The method according to claim 7, wherein the allergen to be quantified is selected *from* the group consisting of Phl p 1, Phl p 5, Phl p 6, Ole e 1, Der f 1, Der f 2, Der p 1, Der p 2, Ves v 1, Ves v 2, Ves v 5, Amb a 1, Amb a 2, Par j 1, Par o 1, Par m 1, Bet v 1, Cry j 1 and Cry j 2.

9. The method according to claim 8, wherein the allergen to be quantified is selected from the group consisting of Der f 1, Der p 1, Der f 2 and Der p 2.

10. The method according to claim 7, wherein the allergen to be quantified is selected from the group consisting of Phl p 1, Phl p 5, Phl p 6, Poa p 1, Poa p 5, Dac g 1, Fes p 1, Lol p 1, and Lol p 5.

11. The method according to claim 7, wherein the allergen to be quantified is selected from the group consisting of Amb a 1, and Amb a 2.

12. The method according to any one of above claims, wherein (i) the allergen to be

2006291835 11 Jan 2012

quantified is Der f 2 and the allergen standard calibration peptide comprises amino acids 32-48 of Der f 2, or (ii) the allergen to be quantified is Der p 2 and the allergen standard calibration peptide comprises amino acids 32-48 of Der p 2.

13. The method according to according to any one of above claims, wherein the allergen is positively identified by comparing the allergen peptide mixture and allergen calibration standard peptide(s) by peptide identification analysis.

14. The method according to according to any one of above claims, wherein the allergen sample is degraded by digestion with at least one proteolytic enzyme to partially, or fully degrade the sample.

15. The method according to claim 14, wherein the proteolytic enzyme is selected from the group *of* consisting of trypsin, papain, pepsin, ArgC, LysC, V8 protease, AspN, pronase, chymotrypsin and carboxypeptidase C, or a combination thereof.

16. The method according to claim 15, wherein the enzyme is trypsin.

17. Use of a synthetic sequence of amino acids which sequence is identical with a constant sequence to be found within a group of isoallergens of an allergen or homologous allergens to be quantified as an allergen calibration standard peptide for absolute quantification of the allergen consisting of more than one isoallergen or homologous allergen by mass spectrometry, wherein said allergen calibration standard peptide is labelled with a mass-modifying functionality.

18. The use according to claim 17, wherein the allergen to be quantified is selected from one or more of the group of grass pollen allergens.

19. The use according to claim 17, wherein the allergen to be quantified is selected from one or more of the group of dust mite allergens.

20. The use according to claim 17, wherein the allergen to be quantified is selected from one or more of the group of weed pollen allergens.

21. The use according to claim 17, wherein the allergen to be quantified is selected from one or more of the group of birch pollen allergens.

22. The use according to claim 17, wherein the allergen to be quantified is selected from one or more of the group of olive pollen allergens.

23. The use according to claim 17, wherein the allergen to be quantified is selected from one of more of the group consisting of Bet v 1, Aln g 1, Cor a 1 and Car b 1, Que a 1, Cry j 1, Cry j 2, Cup a 1, Cup s 1, Jun a 1, Jun a 2, Jun a 3, Ole e 1 , Lig v 1, Syr v 1, Pla I 1, Pla a 1, Pla a 2, Amb a 1, Amb a 2, Amb t 5, Art v 1, Art v 2, Art v 3, Par j 1, Par j 2, Par j 3, Sal k 1, Ave e 1, Cyn d 1, Cyn d 7, Dac g 1, Fes p 1, Hol I 1, Lol p 1 and 5, Pha a 1, Pas n 1, Phl p 1, Phl p 2, Phl p 3, Phl p 4, Phl p 5, Phl P 6, Poa p 1, Poa p 5, Sec c 1, Sec c 5, Sor h 1, Der f 1 , Der f 2, Der f 3, Der f 7, Der p 1, Der p 2, Der p 3 , Der p 7, Der m 1, Eur m 1, Eur m 2, Gly d 1, Gly d 2, Lep d 1, Lep d 2, Blo t 1, Tyr p 2, Bla g 1, Bla g 2, Per a 1, Per a 3, Per a 7, Fel d 1, Fel d 2, Fel d 3 , Fel d 4, Can f 1, Can f 2 , Bos d 2, Equ c 1, Equ c 2, Equ c 3 , Mus m 1, Rat n 1, Apis m 1, Api m 1, Api m 2 , Ves v 1, Ves v 2, Ves v 5, Ves f 5, Ves g 5, Ves m 1, Ves m 2, Ves m 5, Ves p 5, Ves s 5, Ves v 5, Dol m 1, Dol m 2, Dol m 5, Dol a 5, Pol a 1, Pol a 2, Pol a 5, Sol i 1, Sol i 2, Sol i 3 and Sol i 4, Alt a 1, Alt a 3, Alt a 4, Alt a 5, Alt a 6, Cla h 1, Cla h 2, Cla h6, Asp f 1, Bos d 4, Mal d 1, Mal d 3, Gly m 1, Gly m 2, Gly m 3, Ara h 1, Ara h 2, Ara h 3, Ara h 4, and Ara h 5.

24. The use according to claim 17, wherein the homologous allergens are selected from the group consisting of Amp m 2 and Ves v 2; and Der f 2 and Der p 2.

25. The use according to claim 17, wherein the allergen to be quantified is selected from the group consisting of Phl p 1, Phl p 5, Phl p 6, Poa p 1, Poa p 5, Dac g 1, Fes p 1, Lol p 1, Lol p 5, Der f 1 , Der f 2, Der p 1, Der p 2, Api m 1, Api m 2 , Ves v 1, Ves v 2, Ves v 5, Dol m 1, Dol m 2, Dol m 5, Dol a 5, Pol a 1, Pol a 2, Pol a 5, Amb a 1, Amb a 2, Par j 1, Par o 1, Par m 1, Bet v 1, Cry j 1, Cry j 2, Per a 1, Ole e 1, Fel d 1, Can f 1, Can f 2, Equ c 1, Equ c 2, Art v 1, Art v 2, Art v 3, Alt a 1, Alt a 3, Alt a 4, Alt a 5, Alt a 6, Cla h 1, Cla h 2, Cla h 6, Sol i 2, Sol i 3 and Sol i 4.

26. The use according to claim 17, wherein the allergen to be quantified is selected from the group consisting of Phl p 1, Phl p 5, Phl p 6, Ole e 1, Der f 1 , Der f 2, Der p 1, Der p 2, Ves v 1, Ves v 2, Ves v 5, Amb a 1, Amb a 2, Par j 1, Par o 1, Par m 1, Bet v 1, Cry j 1 and Cry j 2.

2006291835 11 Jan 2012

27. The use according to claim 17, wherein the allergen to be quantified is selected from the group consisting of Der f 1, Der p 1, Der f 2 and Der p 2.

28. The use according to claim 17, wherein the allergen to be quantified is selected from the group consisting of Phl p 1, Phl p 5, Phl p 6, Poa p 1, Poa p 5, Dac g 1, Fes p 1, Lol p 1, and Lol p 5.

29. The use according to claim 17, wherein the allergen to be quantified is selected from the group consisting of Amb a 1, and Amb a 2.

30. The use according to claim 17, wherein (i) the allergen to be quantified is Der f 2 and the allergen standard calibration peptide comprises amino acids 32-48 of Der f 2, or (ii) the allergen to be quantified is Der p 2 and the allergen standard calibration peptide comprises amino acids 32-48 of Der p 2.

31. A method for obtaining a synthetic allergen calibration standard peptide as defined in any of claims 1-16, wherein the allergen calibration standard peptide is obtained by: identifying a constant sequence of amino acids within a group of isoallergens of an allergen or homologous allergens which are to be quantified by comparing amino acid sequences of isoallergens or homologous allergens, and preparing a synthetic allergen calibration standard peptide having this constant sequence.

Figure 1a

```
                        1                                                          60

Der p 2 1A9V            -----------------SQVDVKDC-----------------------------ANHEIK
Der p 2 1KTJ_B          -----------------SEVDVKDC-----------------------------ANHEIK
Der p 2 AAF86462        -MYKILCLSLLVAAVARDQVDVKDC-----------------------------ANHEIK
Der p 2 P49278          MMYKILCLSLLVAAVARDQVDVKDC-----------------------------ANHEIK
Lep d 2 2118249A        -MMKFIALFALVAVASAGKMTFKDCG-----------------------------HGEVT
Lep d 2 CAA57160        ------------------------------------------------------------
Lep d 2 CAA61419        -MMKFIALFALVAVASAGKMTFKDCG-----------------------------HGEVT
Lep d 2 CAD32313        -MMKFIALFALVAVASAGKMTFKDCG-----------------------------HGEVT
Lep d 2 118249B         -MMKFIALFALVAVASAGKMTFKDCG-----------------------------HGEVT
Lep d 2 CAA58755        -MMKFIALFALVAVASAGKMTFKDCG-----------------------------HGEVT
Lep d 2 CAD32314        -MMKFIALFALVAVASAGKMTFKDCG-----------------------------HGEVT
Lep d 2 CAB76459        -----------------GKMKFKDCG-----------------------------KGEVT
Lep d 2 CAB59976        -----------------GKMNFTDCG-----------------------------HNEIK
Pso o 2 AAK61827        MMKTLVVLAITLAVVSAGKVKFQDCG-----------------------------KGEVE
Tyr p 2 CAA73221        --MKFLILFALVAVAAAGQVKFTDCG-----------------------------KKEIA
Eur m 2 AAC82349        -MYKILCLSLLVAAVAADQVDIKDC-----------------------------ANHEIK
Eur m 2 AAC82350        -----------VAAVAADQVDVKDC-----------------------------ANHEIK
Der f 2 CAI05848        MISKILCLSLLVAAVVADQVDVKDC-----------------------------ANHEIK
Der f 2 CAI05850        MISKILCLSLLVAAVVADQVDVKDC-----------------------------ANNEIK
Der f 2 CAI05849        MISKILCLSLLVAAVVADQVDVKDC-----------------------------ANNEIK
Der f 2 1WRF_A          -----------------DQVDVKDC-----------------------------ANNEIK
Der f 2 AAB30829        ----GTMVSLLVAAVVADQVDVKDC-----------------------------ANNEIK
Der f 2 A61241          --------SLLVAAVVADQVDVKDC-----------------------------ANNEIK
Der f 2 JU0394          -----------------DQVDVKDC-----------------------------ANNEIK
Der f 2 Q00855          MISKILCLSLLVAAVVADQVDVKDC-----------------------------ANNEIK
Der f 2 BAD74060        MISKILCLSLLVAAVVADQVDVKDC-----------------------------ANNEIK
Der f 2 B61241          --------SLLVAAVVADQVDVKDC-----------------------------ANNEIK
Der f 2 1XWV_B          -----------------DQVDVKDC-----------------------------ANNEIK
Der f 2 A61501          -----------------DQVDVKDC-----------------------------ANNEIK
Der f 2 AAL47677        -----------------DQVDVKDC-----------------------------ANNEIK

                        61                                                        120
Der p 2 1A9V            KVLVPGCHGSEPCIIHRGKPFQLEAVFEANQNTKTAKIEIKASIDG-LEVDVPGIDPNAC
Der p 2 1KTJ_B          KVLVPGCHGSEPCIIHRGKPFQLEAVFEANQNTKTAKIEIKASIDG-LEVDVPGIDPNAC
Der p 2 AAF86462        KVLVPGCHGSEPCIIHRGKPFQLEAVFEANQNTKTAKIEIKASIDG-LEVDVPGIDPNAC
Der p 2 P49278          KVLVPGCHGSEPCIIHRGKPFQLEAVFEANQNTKTAKIEIKASIDG-LEVDVPGIDPNAC
Lep d 2 2118249A        ELDITGCSG-DTCVIHRGEKMTLEAKFAANQDTAKVTIKVLAKVAG-TTIQVPGLETDGC
Lep d 2 CAA57160        --------------IHRGEKMTLEAKFAANQDTAKVTIKVLAKVAG-TTIQVPGLETDGC
Lep d 2 CAA61419        ELDITGCSG-DTCVIHRGEKMTLEAKFAANQDTAKVTIKVLAKVAG-TTIQVPGLETDGC
Lep d 2 CAD32313        ELDITGCSG-DTCVIHRGEKMTLEAKFAANQDTAKVTIKVLTKVAG-TTIQVPGLETDGC
Lep d 2 2118249B        ELDISGCSG-DTCVIHRGQKMTLDAKFAANQDTNKVTIKVLAKVAG-TTIQVPGLETDGC
Lep d 2 CAA58755        ELDISGCSG-DTCVIHRGQKMTLDAKFAANQDTNKVTIKVLAKVAG-TTIQVPGLETDGC
Lep d 2 CAD32314        ELDISGCSG-DTCVIHRGQKMTLDAKFAANQDTNKVTIKVLAKVAG-TTIQVPGLETDGC
Lep d 2 CAB76459        ELDITDCSG-DFCVIHRGKPLTLEAKFAANQDTTKATIKVLAKVAG-TPIQVPGLETDGC
Lep d 2 CAB59976        ELSVSNCTG-NYCVIHRGKPLTLDAKFDANQDTASVGLVLTAIEDGDIAIDIPGLETNAC
Pso o 2 AAK61827        SLEVEGCSG-DYCVIHKGKKLDLAISVTSNQDSANLKLDIVADING-VQIEVPGVDHDGC
Tyr p 2 CAA73221        SVAVDGCEG-DLCVIHKSKPVHVIAEFTANQDTCKIEVKVTGQLNG-LEVPIPGIETDGC
Eur m 2 AAC82349        KVMVPGCKGSEPCVIHRGTAFQLEAVFDANQNSNAAKIEIKATIDG-VEIDVPGIDNNLC
```

```
Eur m 2 AAC82350        KVMVPGCKGSEPCVIHRGTAFQLEAVFDANQNSNAAKIEIKATIDG-VEIDVPGIDNNLC
Der f 2 CAI05848        KVMVDGCHGSDPCIIHRGKPFNLEATFDANQNTKTAKIEIKANIDG-LEVDVPGIDTNAC
Der f 2 CAI05850        KVMVDGCHGSDPCIIHRGKPFTLEALFDANQNTKTAKIEIKANIDG-LEVDVPGIDTNAC
Der f 2 CAI05849        KVMVDGCHGSDPCIIHRGKPFTLEALFDANQNTKTAKIEIKANING-LEVDVPGIDTNAC
Der f 2 1WRF_A          KVMVDGCHGSDPCIIHRGKPFTLEALFDANQNTKTAKIEIKASIDG-LEIDVPGIDTNAC
Der f 2 AAB30829        KVMVDGCHGSDPCIIHRGKPFTLEALFDANQNTKTAKIEIKASLDG-LEIDVPGIDTNAC
Der f 2 A61241          KVMVDGCHGSDPCIIHRGKPFTLEALFDANQNTKTAKIEIKASLDG-LEIDVPGIDTNAC
Der f 2 JU0394          KVMVDGCHGSDPCIIHRGKPFTLEALFDANQNTKTAKIEIKASLDG-LEIDVPGIDTNAC
Der f 2 Q00855          KVMVDGCHGSDPCIIHRGKPFTLEALFDANQNTKTAKIEIKASLDG-LEIDVPGIDTNAC
Der f 2 BAD74060        KVMVDGCHGSDPCIIHRGKPFTLEALFDANQNTKTAKIEIKASLDG-LEIDVPGIDTNAC
Der f 2 B61241          KVMVDGCHGSDPCIIHRGKPFTLEALFDANQNTKTAKIEIKASLDG-LEIDVPGIDTNAC
Der f 2 1XWV_B          KVMVDGCHGSDPCIIHRGKPFTLEALFDANQNTKTAKIEIKASLDG-LEIDVPGIDTNAC
Der f 2 A61501          KVMVDGCHGSDPCIIHRGKPFTLEALFDANQNTKTAKTEIKASLDG-LEIDVPGIDTNAC
Der f 2 AAL47677        KVMVDGCHGSDPCIIHRGKPFTLEALFDANQNTKTAKIEIKASLDG-LEIDVPGIDTNAC

                                121                                     176
Der p 2 1A9V            HYMKCPLVKGQQYDIKYTWNVPKIAPKSENVVVTVKVMGDDGVLACAIATHAKIRD
Der p 2 1KTJ_B          HYMKCPLVKGQQYDIKYTWNVPKIAPKSENVVVTVKVMGDDGVLACAIATHAKIRD
Der p 2 AAF86462        HYMKCPLVKGQQYDIKYTWNVPKIAPKSENVVVTVKVMGDDGVLACAIATHAKIRD
Der p 2 P49278          HYMKCPLVKGQQYDIKYTWNVPKIAPKSENVVVTVKVMGDDGVLACAIATHAKIRD
Lep d 2 2118249A        KFIKCPVKKGEALDFIYSG-TIPAITPKVKADVTAELIGDHGVMACGTVHGVQE--
Lep d 2 CAA57160        KFIKCPVKKGEALDFIYSG-TIPAITPKVKADVTAELIGDHGVMACGTVHGQVE--
Lep d 2 CAA61419        KFIKCPVKKGEALDFIYSG-TIPAITPKVKADVTAELIGDHGVMACGTVHGQVE--
Lep d 2 CAD32313        KFIKCPVKKGEALDFIYSG-TIPAITPKVKADVTAELIGDHGVMACGTVHGQVE--
Lep d 2 2118249B        KVLKCPIKKGEALDFNYGM-TIPAITPKIKADVTAELVGDHGVMACGTIHGVQE--
Lep d 2 CAA58755        KVLKCPIKKGEALDFNYGM-TIPAITPKIKADVTAELVGDHGVMACGTIHGQVE--
Lep d 2 CAD32314        KVLKCPIKKGEALDFNYGM-TIPAITPKIKVDVTAELVGDHGVMACGTIHGQVE--
Lep d 2 CAB76459        KFVKCPIKKGDPIDFKYTT-TVPAILPKVKAEVTAELVGDHGVLACGRFGRQVE--
Lep d 2 CAB59976        KLMKCPIRKGEHQELIYNIGEIPDATPEIKAKVKAQLIGEHGVLACGWVDGEVQE-
Pso o 2 AAK61827        HYVKCPIKKGQHFDVKYTY-SIPAILPITKAKIIAKTIGDKGLGGGIVINGEIQD-
Tyr p 2 CAA73221        KVLKCPLKKGTKYTMNYSV-NVPSVVPNIKTVVKILATGEHGVLACGAVNTDVKP-
Eur m 2 AAC82349        HFMKCPLVKGQEYDIKYTWNVPRIAPKSENVVVTVKLLGDNGVLACAIATHAKIRD
Eur m 2 AAC82350        HFMKCPLVKGQEYDIKYTWNVPRIAPKSENVVVTVKLLGDNGVLACAIATHAKIRD
Der f 2 CAI05848        HYIKCPLVKGQQYDAKYTWNVPKIAPKSENVVVTVKLVGDNGVLACAIATHAKIRD
Der f 2 CAI05850        HYIKCPLVKGQQYDAKYTWNVPKIAPKSENVVVTVKLVGDNGVLACAIATHAKIRD
Der f 2 CAI05849        HYIKCPLVKGQQYDAKYTWNVPKIAPKSENVVVTVKLIGDNGVLACAIATHAKIRD
Der f 2 1WRF_A          HFVKCPLVKGQQYDIKYTWNVPKIAPKSENVVVTVKLIGDNGVLACAIATHGKIRD
Der f 2 AAB30829        HFVKCPLVKGQQYDIKYTWNVPKIAPKSENVVVTVKLIGDNGVLACAIATHGKIRD
Der f 2 A61241          HFVKCPLVKGQQYDIKYTWNVPKIAPKSENVVVTVKLIGDNGVLACAIATHGKIRD
Der f 2 JU0394          HFMKCPLVKGQQYDIKYTWNVPKIAPKSENVVVTVKLIGDNGVLACAIATHGKIRD
Der f 2 Q00855          HFMKCPLVKGQQYDIKYTWNVPKIAPKSENVVVTVKLIGDNGVLACAIATHGKIRD
Der f 2 BAD74060        HFMKCPLVKGQQYDIKYTWDVPKIAPKSENVVVTVKLVGDNGVLACAIATHGKIRD
Der f 2 B61241          HFMKCPLVKGQQYDAKYTWNVPKIAPKSENVVVTVKLVGDNGVLACAIATHAKIRD
Der f 2 1XWV_B          HFMKCPLVKGQQYDAKYTWNVPKIAPKSENVVVTVKLVGDNGVLACAIATHAKIRD
Der f 2 A61501          HFMKCPLVKGQQYDAKYTWNVPKIAPKSENVVVTVKLVGDNGVLACAIATHAKIRD
Der f 2 AAL47677        HFMKCPLVKGQQYDAKYTWNVPKIAPESENVVVTVKLVGDNGVLACAIATHAKIRD
```

Figure 1a (contd.)

Figure 1b

```
                          1                                                  60
Bet v 1 P15494 (1)  MGVFNYETETTSVIPAARLFKAFILDGDNLFPKVAPQAISSVENIEGNGGPGTIKKISFP
Bet v 1 P43177 (1)  MGVFNYEIETTSVIPAARLFKAFILDGDNLVPKVAPQAISSVENIEGNGGPGTIKKINFP
Bet v 1 P43180 (1)  MGVFNYESETTSVIPAARLFKAFILEGDNLIPKVAPQAISSVENIEGNGGPGTIKKINFP
Bet v 1 P43185 (1)  MGVFNYETEATSVIPAARMFKAFILDGDKLVPKVAPQAISSVENIEGNGGPGTIKKINFP
Bet v 1 P43176 (1)  MGVFNYESETTSVIPAARLFKAFILEGDTLIPKVAPQAISSVENIEGNGGPGTIKKITFP
Bet v 1 P43184 (1)  MGVFNYESETTSVIPAARLFKAFILEGDTLIPKVAPQAISSVENIEGNGGPGTIKKITFP
Bet v 1 P45431 (1)  MGVFNYETETTSVIPAARLFKAFILEGDTLIPKVAPQAISSVENIEGNGGPGTIKKITFP
Bet v 1 P43186 (1)  MGVFNYESETTSVIPAARLFKAFILDGDNLIPKVAPQAISSVENIEGNGGPGTIKKITFP
Bet v 1 P43178 (1)  MGVFNYETEATSVIPAARLFKAFILDGDNLFPKVAPQAISSVENIEGNGGPGTIKKISFP
Bet v 1 P43179 (1)  MGVFNYEIEATSVIPAARLFKAFILDGDNLFPKVAPQAISSVENIEGNGGPGTIKKISFP
Bet v 1 P43183 (1)  MGVFNYETEATSVIPAARLFKAFILDGDNLFPKVAPQAISSVENIEGNGGPGTIKKISFP

                          61                                                 120
Bet v 1 P15494(61)  EGFPFKYVKDRVDEVDHTNFKYNYSVIEGGPIGDTLEKISNEIKIVATPDGGSILKISNK
Bet v 1 P43177(61)  EGFPFKYVKDRVDEVDHTNFKYNYSVIEGGPVGDTLEKISNEIKIVATPDGGCVLKISNK
Bet v 1 P43180(61)  EGFPFKYVKDRVDEVDHTNFKYNYSVIEGGPVGDTLEKISNEIKIVATPDGGCVLKISNK
Bet v 1 P43185(61)  EGFPFKYVKDRVDEVDHTNFKYNYSVIEGGPVGDTLEKISNEIKIVATPDGGCVLKISNK
Bet v 1 P43176(61)  EGSPFKYVKERVDEVDHANFKYSYSMIEGGALGDTLEKICNEIKIVATPDGGSILKISNK
Bet v 1 P43184(61)  EGSPFKYVKERVDEVDHANFKYSYSMIEGGALGDTLEKICNEIKIVATPDGGSILKISNK
Bet v 1 P45431(61)  EGSPFKYVKERVDEVDHANFKYSYSMIEGGALGDTLEKICNEIKIVATPDGGSILKISNK
Bet v 1 P43186(61)  EGSPFKYVKERVDEVDHANFKYSYSMIEGGALGDTLEKICNEIKIVATPDGGSILKISNK
Bet v 1 P43178(61)  EGIPFKYVKGRVDEVDHTNFKYSYSVIEGGPVGDTLEKISNEIKIVATPNGGSILKINNK
Bet v 1 P43179(61)  EGFPFKYVKDRVDEVDHTNFKYSYSVIEGGPVGDTLEKISNEIKIVATPNGGSILKINNK
Bet v 1 P43183(61)  EGFPFKYVKDRVDEVDHTNFKYSYSVIEGGPVGDTLEKISNEIKIVATPNGGSILKINNK

                          121                                  160
Bet v 1 P15494 (121)  YHTKGDHEVKAEQVKASKEMGETLLRAVESYLLAHSDAYN
Bet v 1 P43177 (121)  YHTKGNHEVKAEQVKASKEMGETLLRAVESYLLAHSDAYN
Bet v 1 P43180 (121)  YHTKGNHEVKAEQVKASKEMGETLLRAVESYLLAHSDAYN
Bet v 1 P43185 (121)  YHTKGNHEVKAEQVKASKEMGETLLRAVESYLLAHSDAYN
Bet v 1 P43176 (121)  YHTKGDQEMKAEHMKAIKEKGEALLRAVESYLLAHSDAYN
Bet v 1 P43184 (121)  YHTKGDHEMKAEHMKAIKEKGEALLRAVESYLLAHSDAYN
Bet v 1 P45431 (121)  YHTKGDHEMKAEHMKAIKEKGEALLRAVESYLLAHSDAYN
Bet v 1 P43186 (121)  YHTKGDHEMKAEHMKAIKEKGEALLRAVESYLLAHSDAYN
Bet v 1 P43178 (121)  YHTKGDHEVKAEQIKASKEMGETLLRAVESYLLAHSDAYN
Bet v 1 P43179 (121)  YHTKGDHEVKAEQIKASKEMGETLLRAVESYLLAHSDAYN
Bet v 1 P43183 (121)  YHTKGDHEVKAEQIKASKEMGETLLRAVESYLLAHSDAYN
```

Figure 2

Theoretical cleavage analyses of calibration standard peptides for quantification by MS

a) Tryptic digestion of Der f 2 (A61501)

Peptide	From-To	MH+	pI	Sequence
1	1-6	703.36	3.92	DQVDVK
2	7-14	905.39	4.11	DCANNEIK
3	15-15	147.11	9.47	K
4	16-31	1736.77	6.00	VMVDGCHGSDPCIIHR
5	**32-48**	**1893.97**	**6.99**	**GKPFTLEALFDANQNTK**
6	49-51	319.20	9.80	TAK
7	52-55	490.29	6.97	TEIK
8	56-77	2345.10	4.00	ASLDGLEIDVPGIDTNACHFMK
9	78-82	558.32	9.30	CPLVK
10	83-89	809.38	6.71	GQQYDAK
11	90-96	907.47	9.49	YTWNVPK
12	97-100	428.29	10.15	IAPK
13	101-109	974.55	6.97	SENVVVTVK
14	110-126	1651.87	7.14	LVGDNGVLACAIATHAK
15	127-128	288.20	11.15	IR
16	129-129	134.04	2.98	D

b) Tryptic digestion of Der p 2 (BAA01241)

Peptide	From-To	MH+	pI	Sequence
1	1-6	703.36	3.92	DQVDVK
2	7-14	928.41	5.24	DCANHEIK
3	15-15	147.11	9.47	K
4	6-31	1714.85	7.15	VLVPGCHGSEPCIIHR
5	**32-48**	**1920.98**	**7.00**	**GKPFQLEAVFEANQNTK**
6	49-51	319.20	9.80	TAK
7	52-55	502.32	6.99	IEIK
8	56-77	2343.09	4.00	ASIDGLEVDVPGIDPNACHYMK
9	78-82	558.32	9.30	CPLVK
10	83-89	851.43	6.71	GQQYDIK
11	90-96	907.47	9.49	YTWNVPK
12	97-100	428.29	10.15	IAPK
13	101-109	974.55	6.97	SENVVVTVK
14	110-126	1670.81	5.12	VMGDDGVLACAIATHAK
15	127-128	288.20	11.15	IR
16	129-129	134.04	2.98	D

c) Tryptic digestion of Phl p 1 Q40967

Peptide	From-To	MH+	pI	Sequence
1	1-26	2601.44	9.90	MASSSSVLLVVALFAVFLGSAHGIPK
2	27-40	1429.73	6.68	VPPGPNITATYGDK
3	41-45	632.34	6.98	WLDAK
4	46-58	1363.70	10.12	STWYGKPTAAGPK
5	59-67	884.35	6.09	DNGGACGYK
6	68-87	2110.99	6.24	DVDKPPFSGMTGCGNTPIFK
7	88-90	319.17	10.85	SGR
8	91-99	943.40	6.15	GCGSCFEIK
9	100-140	4349.01	4.25	CTKPEACSGEPVVVHITDDNEEPIAAYHFDL SGIAFGSMAK
10	141-141	147.11	9.47	K
11	142-146	576.26	4.11	GDEQK
12	147-148	288.20	11.10	LR

13	**149-158**	**1135.57**	**4.31**	**SAGEVEIQFR**
14	159-159	175.11	10.76	R
15	160-161	246.18	10.10	VK
16	162-163	250.12	9.30	CK
17	164-169	694.34	6.67	YPEGTK
18	170-176	859.46	7.82	VTFHVEK
19	177-188	1288.72	9.80	GSNPNYLALLVK
20	189-202	1404.73	2.78	FVAGDGDVVAVDIK
21	203-204	276.15	6.99	EK
22	205-206	204.13	10.15	GK
23	207-208	262.13	6.99	DK
24	209-213	630.39	9.95	WIALK
25	214-221	1004.49	7.04	ESWGAIWR
26	222-229	914.51	4.11	IDTPEVLK
27	230-235	676.37	11.15	GPFTVR
28	236-243	856.40	6.67	YTTEGGTK
29	244-247	404.21	6.99	GEAK
30	248-255	943.48	4.11	DVIPEGWK
31	256-263	884.39	4.11	ADTAYESK

d) Tryptic digestion of Phl p 5a Q40962

Peptide	From-To	MH+	pI	Sequence
1	1-33	2910.42	3.92	ADLGYGPATPAAPAAGYTPATPAAPAGADAAGK
2	34-40	806.38	4.31	ATTEEQK
3	41-44	502.32	6.99	LIEK
4	45-50	649.36	10.15	INAGFK
5	51-63	1168.63	6.99	AALAGAGVQPADK
6	64-65	338.18	9.60	YR
7	66-77	1239.63	9.80	TFVATFGPASNK
8	78-88	1105.55	4.31	AFAEGLSGEPK
9	89-96	736.34	6.99	GAAESSSK
10	97-102	590.35	10.20	AALTSK
11	103-108	680.36	6.68	LDAAYK
12	109-112	494.29	9.76	LAYK
13	113-122	974.47	4.31	TAEGATPEAK
14	**123-135**	**1471.74**	**4.11**	**YDAYVATLSEALR**
15	136-154	1975.12	5.42	IIAGTLEVHAVKPAAEEVK
16	155-166	1295.75	4.31	VIPAGELQVIEK
17	167-172	650.35	6.98	VDAAFK
18	173-186	1284.65	6.98	VAATAANAAPANDK
19	187-199	1530.74	3.87	FTVFEAAFNDEIK
20	200-210	1133.51	6.90	ASTGGAYESYK
21	211-220	1058.62	6.97	FIPALEAAVK
22	221-234	1419.74	6.67	QAYAATVATAPEVK
23	235-243	1071.57	6.67	YTVFETALK
24	244-244	147.11	9.47	K
25	245-254	1049.52	6.99	AITAMSEAQK
26	255-285	2605.35	10.25	AKPAAAATATATAAVGAATGAATAATGGYK
27	286-286	118.08	6.00	V

e) Tryptic digestion of Phl p 5b Q40963

Peptide	From-To	MH+	pI	Sequence
1	1-7	655.38	11.20	AAAAVPR
2	8-8	175.11	10.76	R
3	9-11	329.19	11.15	GPR
4	12-16	443.23	11.15	GGPGR

Figure 2 (contd.)

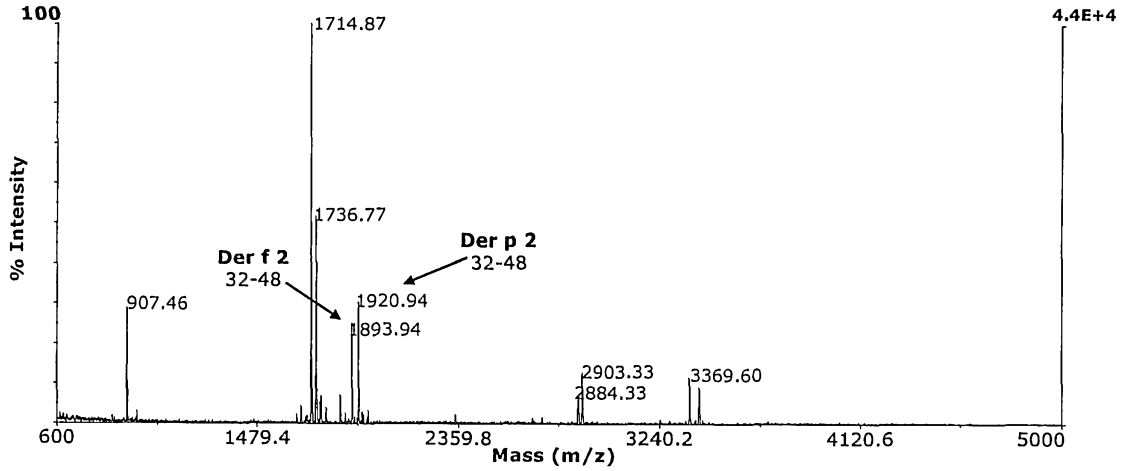
5	17-38	1952.93	6.49	SYTADAGYAPATPAAAGAAAGK
6	39-45	806.38	4.31	ATTEEQK
7	46-55	1147.63	4.11	LIEDINVGFK
8	56-69	1212.65	6.99	AAVAAAASVPAADK
9	70-71	294.18	9.85	FK
10	72-82	1175.55	6.97	TFEAAFTSSSK
11	83-87	431.26	10.20	AAAAK
12	88-94	681.42	10.20	APGLVPK
13	95-104	1100.56	6.66	LDAAYSVAYK
14	105-114	914.49	6.99	AAVGATPEAK
15	**115-127**	**1455.74**	**4.11**	**FDSFVASLTEALR**
16	128-149	2246.22	5.42	VIAGALEVHAVKPVTEEPGMAK
17	150-160	1196.68	4.11	IPAGELQIIDK
18	161-166	664.36	6.99	IDAAFK
19	167-180	1272.64	3.92	VAATAAATAPADDK
20	181-190	1173.59	6.97	FTVFEAAFNK
21	191-193	331.23	10.20	AIK
22	194-204	1191.51	4.11	ESTGGAYDTYK
23	205-214	1030.56	6.30	CIPSLEAAVK
24	215-228	1388.75	9.49	QAYAATVAAAPQVK
25	229-238	1112.59	6.67	YAVFEAALTK
26	239-248	1077.56	6.99	AITAMSEVQK
27	249-283	2919.48	9.76	VSQPATGAATVAAGAATTAA GAASGAATVAAGGYK
28	284-284	118.08	6.00	V

f) Tryptic digestion of Bet v 1

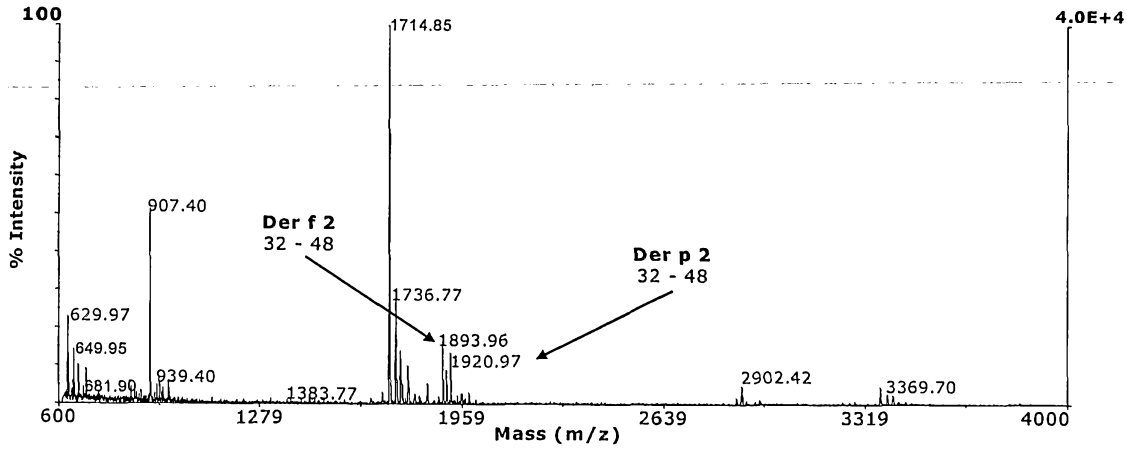
Peptide	From-To	MH+	pI	Sequence
1	1-18	1985.9637	4.31	MGVFNYETETTSVIPAAR
2	19-21	407.2653	10.10	LFK
3	22-33	1349.7100	3.92	AFILDGDNLFPK
4	34-57	2386.2249	4.31	VAPQAISSVENIFTEGNGGPGTIK
5	58-58	147.1128	9.47	K
6	59-68	1134.6194	6.99	ISFPEGLPFK
7	69-71	409.2445	9.49	YVK
8	72-73	290.1459	7.04	DR
9	74-83	1203.5640	4.34	VDEVDHTNFK
10	84-100	1854.9120	3.87	YNYSVIEGGPIGDTLEK
11	101-106	703.3985	6.99	ISNEIK
12	107-120	1418.7890	6.97	FTIVATPDGGSILK
13	121-124	461.2718	10.15	ISNK
14	125-128	548.2827	9.49	YHTK
15	129-134	684.3311	5.24	GDHEVK
16	135-139	574.3195	6.99	AEQVK
17	140-142	305.1819	10.20	ASK
18	143-150	948.4819	4.31	EMGETLLR
19	**151-164**	**1552.7278**	**4.10**	**AVESYLLAHSDAYN**

Figure 2 (contd.)

Figure 3


100
1714.87
4.4E+4
% Intensity
1736.77
Der f 2
32-48
Der p 2
32-48
907.46
1920.94
1893.94
2903.33
2884.33
3369.60
600
1479.4
2359.8
3240.2
4120.6
5000
Mass (m/z)

a)


100
1714.85
4.0E+4
% Intensity
907.40
Der f 2
32 - 48
Der p 2
32 - 48
1736.77
629.97
649.95
1893.96
1920.97
881.90
939.40
1383.77
2902.42
3369.70
600
1279
1959
2639
3319
4000
Mass (m/z)

b)

Figure 4

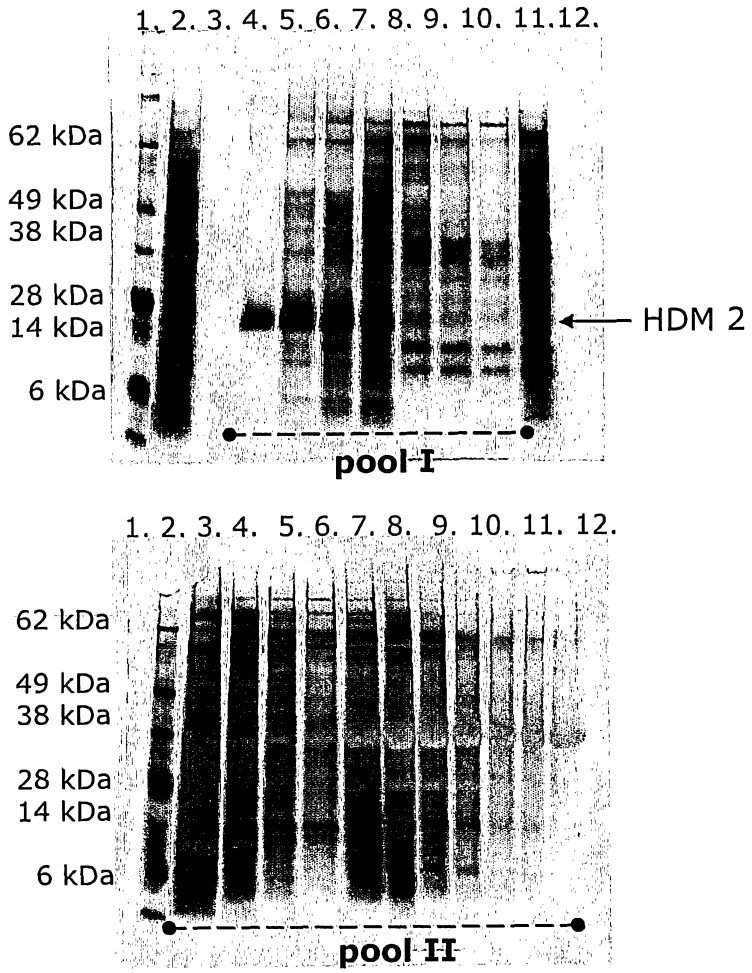

1. 2. 3. 4. 5. 6. 7. 8. 9. 10. 11.12.
62 kDa
49 kDa
38 kDa
28 kDa
14 kDa
6 kDa
HDM 2
pool I
1. 2. 3. 4. 5. 6. 7. 8. 9. 10. 11. 12.
62 kDa
49 kDa
38 kDa
28 kDa
14 kDa
6 kDa
pool II

Figure 5

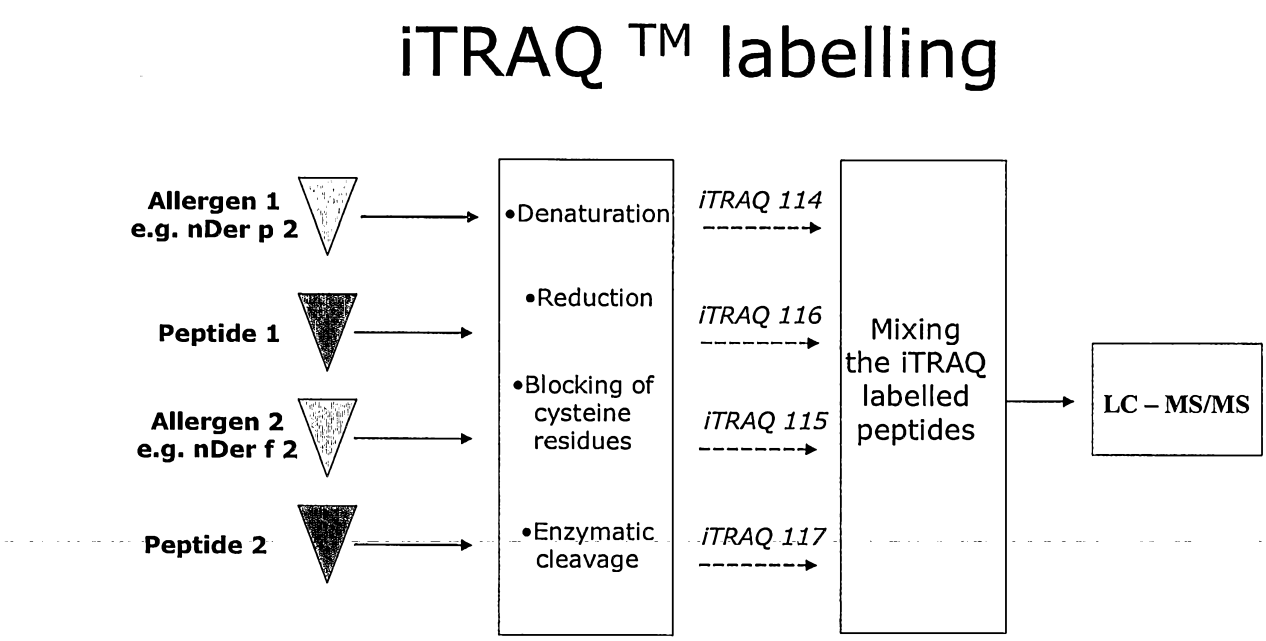
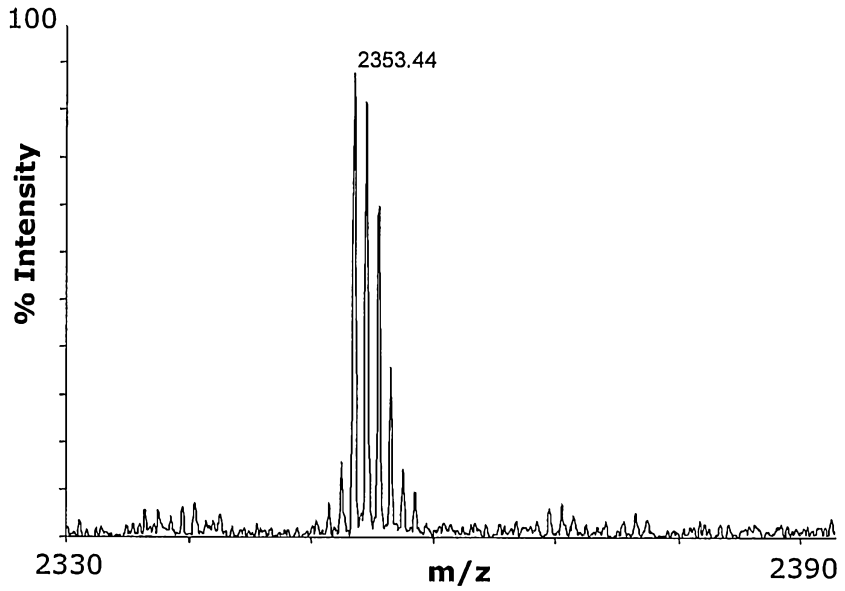
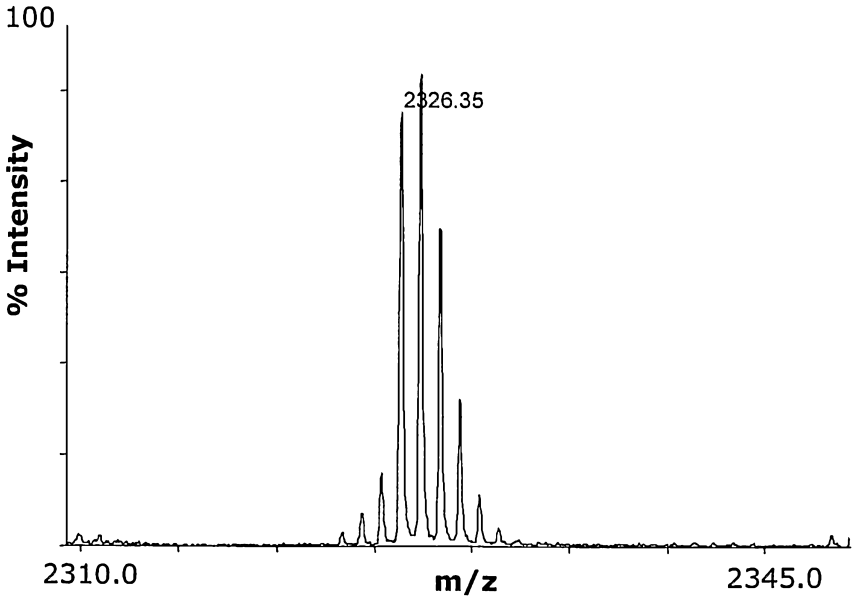

iTRAQ TM labelling
Allergen 1
e.g. nDer p 2
Peptide 1
Allergen 2
e.g. nDer f 2
Peptide 2
•Denaturation
•Reduction
•Blocking of cysteine residues
•Enzymatic cleavage
iTRAQ 114
iTRAQ 116
iTRAQ 115
iTRAQ 117
Mixing the iTRAQ labelled peptides
LC – MS/MS

Figure 6

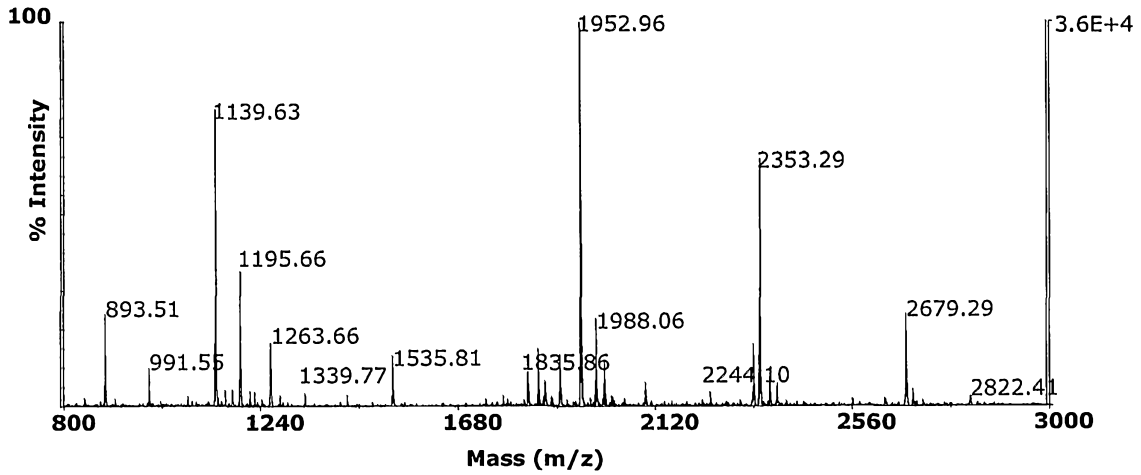

100
2353.44
% Intensity
2330
m/z
2390

a)

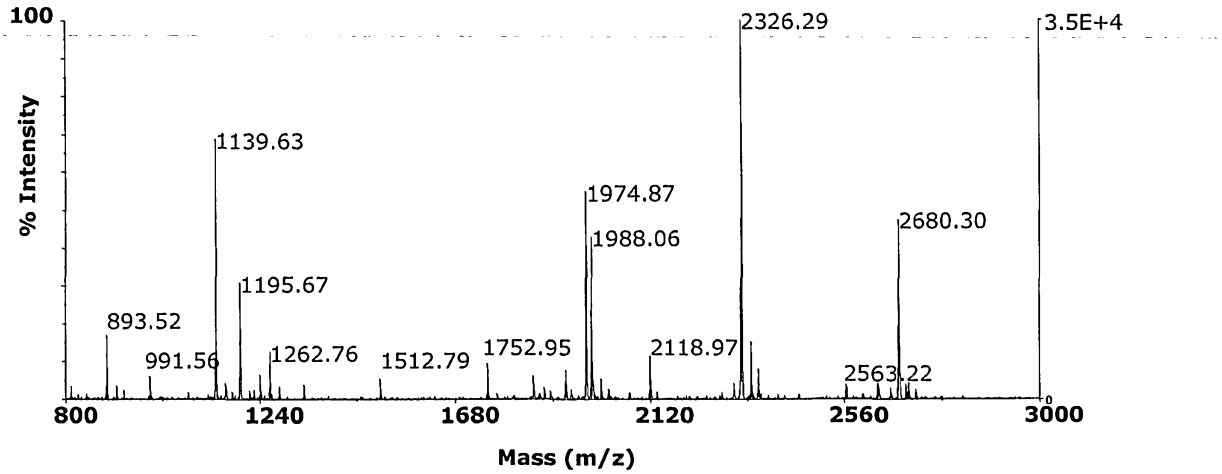

100
2326.35
% Intensity
2310.0
m/z
2345.0

b)

Figure 6 (cont.)

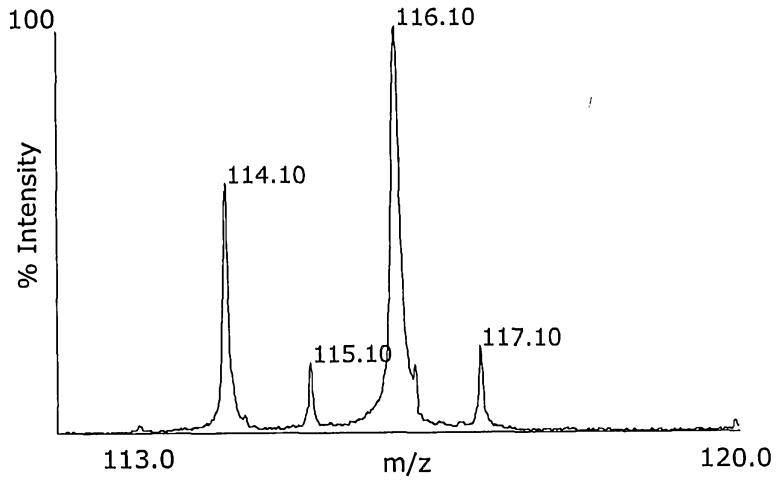


c)

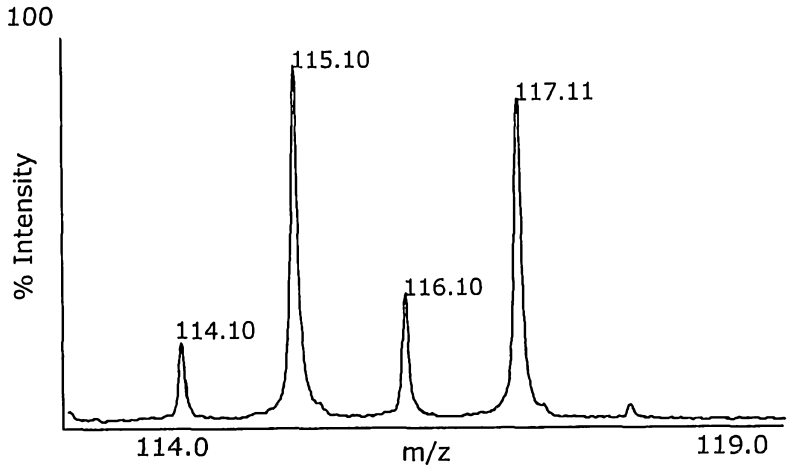


d)

Figure 7

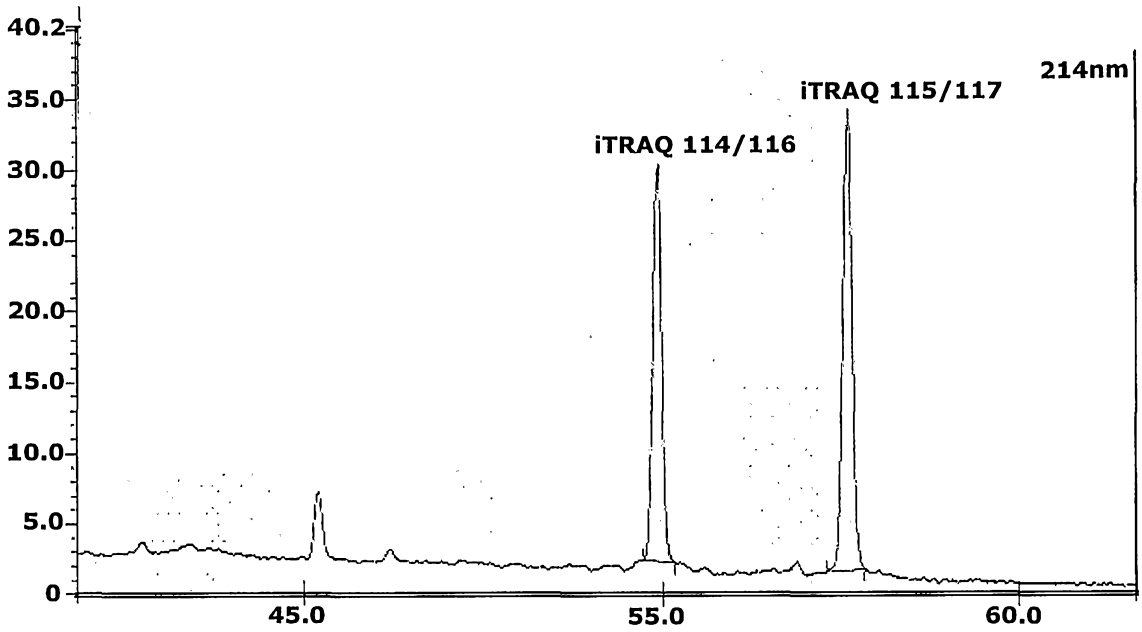

100
116.10
% Intensity
114.10
115.10
117.10
113.0
m/z
120.0

a)


100
115.10
117.11
% Intensity
116.10
114.10
114.0
m/z
119.0

b)

Figure 8


40.2
35.0
30.0
25.0
20.0
15.0
10.0
5.0
0
45.0
55.0
60.0
214nm
iTRAQ 115/117
iTRAQ 114/116

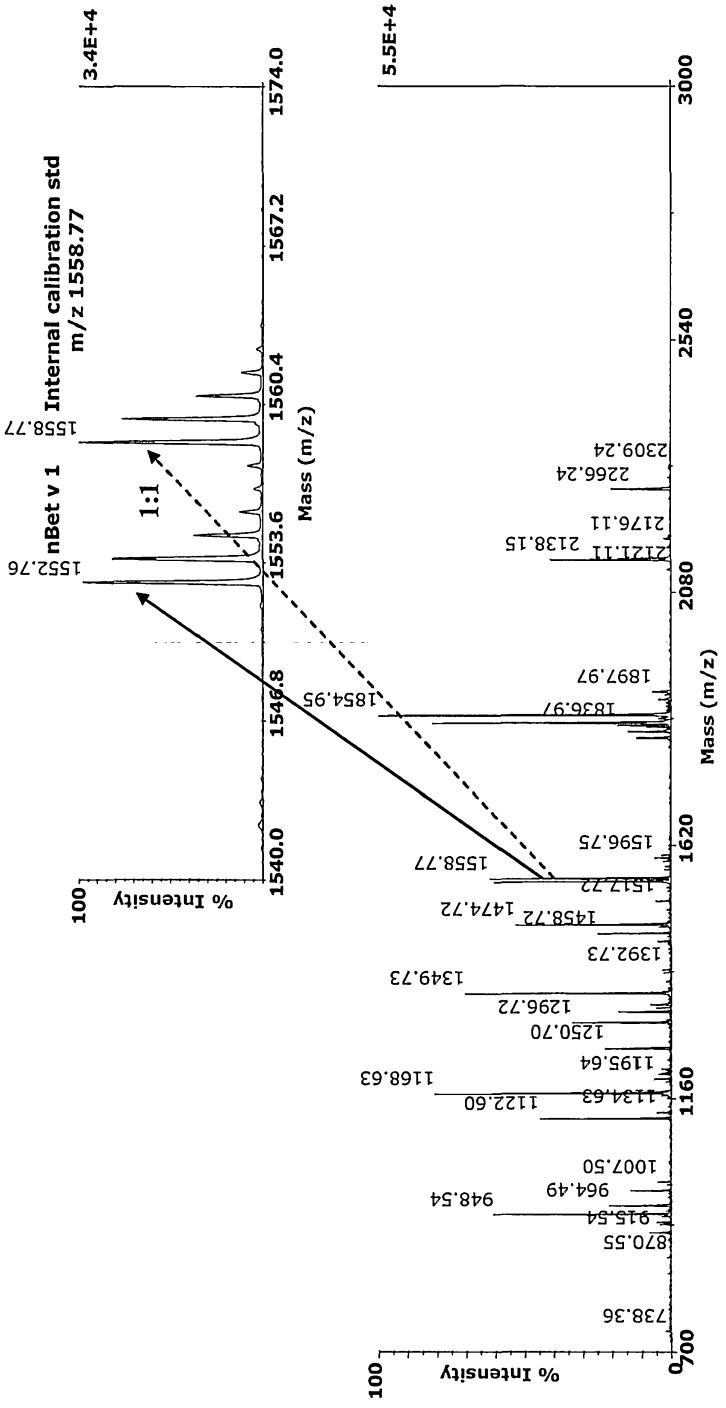

3.4E+4
Internal calibration std
m/z 1558.77
nBet v 1
1558.77
1552.76
1:1
1574.0
1567.2
1560.4
1553.6
1546.8
1540.0
Mass (m/z)
% Intensity
100
5.5E+4
3000
2540
2080
1620
1160
700
0
1897.97
1854.95
1836.97
1596.75
1558.77
1517.72
1474.72
1458.72
1392.73
1349.73
1296.72
1250.70
1195.64
1168.63
1134.63
1122.60
1007.50
964.49
948.54
915.54
870.55
738.36
2309.24
2266.24
2176.11
2138.15
2121.11
Mass (m/z)
% Intensity
100

Figure 9