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(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).

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(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.



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## A METHOD FOR QUANTIFICATION OF ALLERGENS

### TECHNICAL FIELD

This invention relates to the field of quantification of allergens.

### BACKGROUND OF THE INVENTION

5 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  
10 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  
15 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.

20 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  
25 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  
30 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 radioallergosorbent (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 ( $^{13}\text{C}$ ,  $^{15}\text{N}$ ) to mimic native peptides formed by enzymatic digestion using e.g., trypsin (Stemann 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  $^{12}\text{C}/^{13}\text{C}_6$ -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  
5 *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  
10 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  
15 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  
20 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  
25 from a plurality of sources is provided.

According to one aspect the invention provides a method for quantification of the absolute amount of an allergen 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 identical with a sequence to be found in the allergen to be  
30 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 peptide(s) 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 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.

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, the use of a sequence of amino acids which is identical with a sequence to be found in the isoallergens or homologous allergen(s) to be quantified is provided as an allergen calibration standard peptide for absolute quantification of the allergen(s), 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 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.

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

identification of a sequence of amino acids which is constant in the isoallergens or homologous allergens which are to be quantified, by comparison of the sequences of the isoallergens or homologous allergens and preparation of 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.

- 5 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).

10 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.

15 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).

20 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.

25 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.

5 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.

10 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  
15 *Dermatophagoides* and *Euroglyphus*, storage mite e.g. *Lepidoglyphus*, *Glycyphagus* and *Tyrophagus*, those from cockroaches, midges and fleas e.g. *Blatella*, *Periplaneta*, *Chironomus* and *Ctenocephalides*, 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  
20 (superfamily Vespidae), and ants (superfamily Formicoidea). Important inhalation allergens from fungi are i.a. such originating from the genera *Alternaria*, *Cladosporium*, *Aspergillus* and *Penicillium*.

25 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-  
30 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).

35 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).

10 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  
15 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,  
20 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  
25 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,  
30 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,  
35 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, mugwort 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 common 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  
5 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  
10 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  
15 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  
20 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  
25 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  
30 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.

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

10 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.

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

20 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.

25 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.

30 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 homologous 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.

35 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:

- 5 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),

10 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,

- 15 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.

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

25 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.

30 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.

5 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.

10 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 be 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  
15 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  
20 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  
25 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  
30 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  
35 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 it 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,  
5 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.

10 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 immunoprecipitation. Both fractions can then be subjected to quantification studies.

15 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  
20 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 carboxypeptidase C. For example, the proteolytic enzyme  
25 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  
30 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  
5 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.

10 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  
15 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).

20 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  
25 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  
30 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.

- 5 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 (<sup>13</sup>C, <sup>15</sup>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. <sup>12</sup>C/<sup>13</sup>C<sub>6</sub>-Nicotinic acid-succinimide as ICPL label.

- 15 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.

- 20 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.

- 5 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
- 10 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.
- 15 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
- 20 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.
- 25 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.

- 30 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.

10 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  
15 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  
20 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).

25 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  
30 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](http://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 Knowledgebase. The protein and/or nucleotide  
35 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 (**Expert Protein Analysis System**) proteomics server of the Swiss Institute of Bioinformatics (SIB). Sequence  
5 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  
10 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. GPMW program (Lighthouse data, Odense, Denmark) which is created to support MS analyses. Cleavage (degradation) analysis of an allergen  
15 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  
20 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  
25 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  
30 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.

## 20 **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).

30 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 GPMW (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 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 <sup>13</sup>C <sup>15</sup>N), 123-135 in Phl p 5a (Arg <sup>13</sup>C <sup>15</sup>N), 115-127 in Phl p 5b a (Arg <sup>13</sup>C <sup>15</sup>N) and 151 - 164 in Bet v 1 (Val <sup>13</sup>C <sup>15</sup>N) were obtained from Sigma GENOSYS, Texas, US (Table 1).

15 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 pratense* 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).

25 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<sub>280</sub> and by use of Lambda 800 UV/VIS Spectrometer (Perkin Elmer Instruments, CA, USA).

30

## 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  
5 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  
10 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  
15 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:

- 20 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  
25 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  $\mu$ l of 100 mM TEAB, pH 8.5. The protein samples were denatured with 1.0  $\mu$ l of 0.05% SDS and reduced by 2.0  $\mu$ l of 4.8 mM TCEP Tris(2-carboxyethyl)phosphine at 60°C following blocking of the cysteine residues by 1.0  $\mu$ l of 10 mM s-methyl methanethiosulfonate (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  $\mu$ l of 70% ethanol which was then applied on the sample(s). The final volumes of the reagent mixtures were 100  $\mu$ l / 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  $\mu$ l 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  $\mu$ l 70% acetonitrile (ACN), 0.1% Trifluoro acetic acid (TFA) and 1.0  $\mu$ l 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).

10 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.

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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.

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## 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.

25

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.

30

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  $\mu$ m) column  
5 (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  $\mu$ l/min and the chromatography was monitored at 214 nm. The 50  $\mu$ l fractions were collected and 1.0  $\mu$ l of  
10 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.  
15 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  
20 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  
25 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  
30 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 ( $^{13}\text{C}$ ,  $^{15}\text{N}$ ) 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 $^{13}\text{C}$ $^{15}\text{N}$ )	1558.64
Phleum prantense	Phl p 1	SAGEVEIQFR	1135.57	(Arg $^{13}\text{C}$ $^{15}\text{N}$ )	1145.27
Phleum prantense	Phl p 5a	YDAYVATLSEALR	1471.74	(Arg $^{13}\text{C}$ $^{15}\text{N}$ )	1481.66
Phleum prantense	Phl p 5b	FDSFVASLTEALR	1455.74	(Arg $^{13}\text{C}$ $^{15}\text{N}$ )	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/ $\mu\text{l}$ . 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).

5 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.

10 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  
15 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  
20 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).

25 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  
30 be used in fractionation of the more complex allergen mixtures of chemically identical native and synthetic internal calibration standards.

## Claims:

1. A 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 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) of the degraded allergen sample by mass analysis.
2. The method according to claim 1 for quantification of the absolute amount of allergen in an allergen sample, comprising the following steps:
  - a) labelling 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 with a labelling agent(s) which is different from the labelling agent(s) used in step b),
  - b) degrading the allergen sample to obtain a mixture of peptides, and labelling the obtained peptides with one or more labelling agent(s),
  - c) quantifying the absolute amount of allergen by correlating the amount of the labelled allergen calibration standard peptide(s) with the amount of the corresponding labelled peptide(s) of the degraded allergen sample by mass analysis.
3. The method according to claim 1 for quantification of the absolute amount of allergen in an allergen sample, comprising the following steps:

- a) labelling 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 with a labelling agent(s),
- 5 b) degrading the allergen sample to obtain a mixture of peptides,
- c) quantifying the absolute amount of allergen by correlating the amount of the labelled allergen calibration standard peptide(s) with the amount of the corresponding peptide(s) of the degraded allergen sample by mass analysis.
- 10 4. The method according to claim 1 for quantification of the absolute amount of allergen 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 identical with a sequence to be found in the allergen to be  
15 quantified,
- b) degrading the allergen sample to obtain a mixture of peptides, and labelling the obtained peptides with one or more labelling agent(s),
- c) quantifying the absolute amount of allergen by correlating the amount of the allergen calibration standard peptide(s) with the amount of the corresponding labelled peptide(s) of  
20 the degraded allergen sample by mass analysis.
5. The method according to any of claims 1-4 wherein the allergen calibration standard peptide(s) have a sequence of amino acids which is identical with a sequence of amino acids in a peptide obtained by degradation according to step b).
- 25 6. The method according to claims 1-5, where in the allergen to be quantified consist of more than one isoallergen.
7. The method according to claims 1-5 wherein the allergen to be quantified consist of more than one homologous allergens.
- 30 8. The method according to any one of above claims 1-7, wherein the quantification in step c) is performed using mass spectrometry.

9. The method according to any one of above claims 1- 8, wherein the allergen to be quantified is one or more isoallergens 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.
10. The method according to claim 9, wherein the allergen to be quantified is one or more isoallergens 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.
11. The method according to claim 10, wherein the allergen to be quantified is one or more isoallergens selected from the group consisting of Der f 1, Der p 1, Der f 2 and Der p 2.
12. The method according to claim 9, wherein the allergen to be quantified is one or more isoallergens 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,
13. The method according to claim 9, wherein the allergen to be quantified is one or more isoallergens selected from the group consisting of Amb a 1, and Amb a 2,
14. The method according to any one of the above claims, wherein the labelling is with ITRAQ™ chemistry.
15. The method according to claim 14, wherein the labelling is with ITRAQ-114, ITRAQ 115, ITRAQ 116 and/or ITRAQ 117.
16. The method according to any one of above claims, 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.
17. 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.

18. The method according to according to any one of above claims, wherein the allergen sample is degraded by digestion with at least one enzyme to partially, or fully degrade the sample.
19. The method according to claim 18, wherein the enzyme is a proteolytic enzyme.
- 5 20. The method according to claim 19, 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.
21. The method according to claim 20, wherein the enzyme is selected from the group consisting of ArgC, LysC and trypsin, or a combination thereof.
- 10 22. The method according to claim 21, wherein the enzyme is trypsin.
23. The method according to any one of above claims, wherein the allergen sample is reduced and any cysteine residue is blocked before degradation.
24. The method according to any one of above claims, wherein the allergen sample is a fraction from a pre-fractionation step.
- 15 25. The method according to claim 24, wherein the allergen sample is a fraction from a pre-fractionation step comprising one or more isoallergens of the allergen.
26. The method according to claims 24-25, wherein the pre-fractionation is performed by chromatography.
27. The method according to claim 26, wherein the chromatography fractionation is by  
20 hydrophobic interaction chromatography, reversed-phase chromatography, ion-exchange chromatography, size exclusion chromatography or affinity chromatography.
28. The method according to claim 27, wherein the chromatography fractionation is by hydrophobic interaction chromatography.
29. The method according to claim 24-28, wherein the allergen sample is desalted after  
25 chromatography.

30. The method according to any of the above claims where the degraded and optionally labelled mixture of peptides and the calibration standard peptide(s), is subjected to fractionation to separate the peptides corresponding to the calibration standard peptide(s) from other peptides in the mixture before mass analysis.

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31. The method according to claim 30 wherein the fractionation is by chromatography.

32. The method according to claim 31 wherein the fractionation is by cation exchange chromatography or cation exchange chromatography followed by reversed phase  
10 chromatography.

33. A method for quantification of a specific allergen or isoallergen from an allergen sample comprising:

15 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),

20 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  
25 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) of the degraded sample by mass analysis.

30 34. A method according to claim 33 for quantification of the absolute amount of an allergen in an allergen sample, comprising the following steps:

35 a) labelling 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 with labelling agent(s) which is different from the labelling agent(s) used in step b),

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

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

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35. A method according to claim 33 for quantification of the absolute amount of an allergen in an allergen sample, comprising the following steps:

a) labelling 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 to be quantified, with a labelling agent(s),

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b) degrading the sample to obtain a mixture of peptides,

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

15

36. A method according to claim 33 for quantification of the absolute amount of an allergen 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 to be quantified,

20

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

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

25

37. The method according to any of claims 33-36 wherein the allergen calibration standard peptide(s) have a sequence of amino acids which is identical with a sequence of amino acids in a peptide obtained by degradation according to step b).

38. The method according to claim 33-37 wherein the allergen is an isoallergen.

39. The method according to claim 33-38, wherein the allergen is an isoallergen of an allergen selected from the group 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.

40. The method according to claim 39, wherein the allergen is an isoallergen of an allergen selected from the group 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.

41. The method according to any one of claims 33-40 wherein the quantification in step c) is performed using mass spectrometry.

42. The method according to any one of claims 33-41, wherein the labelling is with ITRAQ™ chemistry.

43. The method according to any one of claims 33-42, wherein the isoallergen is positively identified by comparing the labelled allergen peptide mixture and allergen calibration standard peptide(s) by peptide identification analysis.

44. The method according to any one of claims 33-43, wherein the allergen sample is degraded by digestion with at least one enzyme to partially, or fully degrade the sample.

45. The method according to claim 44, wherein the enzyme is a proteolytic enzyme.

46. The method according to claim 45, wherein the proteolytic enzyme is selected from the group of trypsin, papain, pepsin, ArgC, LysC, V8 protease, AspN, pronase, chymotrypsin and carboxypeptidase C, or a combination thereof.

47. The method according to claim 46, wherein the enzyme is selected from the group of ArgC, LysC and trypsin, or a combination thereof.

48. The method according to claim 47, wherein the enzyme is trypsin.

49. The method according to any one of claims 33-48, wherein the allergen sample is reduced and any cysteine residue is blocked before degradation.
50. A method according to any one of claims 33-49, wherein the allergen sample is a fraction from a pre-fractionation step.
- 5 51. The method according to claim 50, wherein the allergen sample is a fraction from a pre-fractionation step comprising an isoallergen.
52. The method according to claim to any one of claims 50-51, wherein the pre-fractionation is performed by chromatography.
53. The method according to any one of claims 52, wherein the chromatography fractionation  
10 is performed by hydrophobic interaction chromatography, reversed-phase chromatography, ion-exchange chromatography, size exclusion chromatography or affinity chromatography.
54. The method according to claim 53, wherein the chromatography fractionation is by hydrophobic interaction chromatography.
55. The method according to any one of claims 33-54 wherein the allergen sample is desalted  
15 after chromatography.
56. The method according to claims 33-52 where the degraded and optionally labelled mixture of peptides and the calibration standard peptide(s) is subjected to fractionation to separate peptides corresponding to the calibration standard peptide(s) from other peptides in the mixture before mass analysis.  
20
57. The method according to claim 56 wherein the fractionation is by chromatography.
58. The method according to claim 57 wherein the fractionation is by cation exchange chromatography or cation exchange chromatography followed by reversed phase  
25 chromatography.
59. The method according to any one of above claims wherein the number of amino acids in the allergen calibration standard peptide(s) is in the range of 2-20 amino acids, more preferred in the range of 4-15 and most preferred in the range of 6-10.
- 30 60. A method for obtaining an allergen calibration standard peptide for use in quantification of an allergen wherein the allergen calibration standard peptide is obtained by:

identifying a constant sequence of amino acids in the 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.

61. The method according to claim 60 wherein the allergen is isoallergens of an allergen.

5 62. Use of a sequence of amino acids which sequence is identical with a sequence to be found in the allergen to be quantified as an allergen calibration standard peptide for absolute quantification of the allergen, and optionally identification.

10 63. Use of a sequence of amino acids which sequence is identical with a sequence to be found in the isoallergen(s) of an allergen to be quantified as an allergen calibration standard peptide for absolute quantification of the allergen, and optionally identification.

64. A method for obtaining an allergen calibration standard peptide for use in quantification of an allergen wherein the allergen calibration standard peptide is obtained by:

15 identifying a sequence of amino acids which sequence is unique for the allergen to be quantified by comparing of isoallergens or homologous allergens, and preparing of a synthetic allergen calibration standard peptide having this sequence.

65. The method according to claim 64 wherein the allergen is an isoallergen.

66. The use of a sequence of amino acids which sequence is unique for the allergen to be quantified as an allergen calibration standard peptide for absolute quantification of the allergen, and optionally identification.

20 67. The use of a sequence of amino acids which sequence is unique for the isoallergen to be quantified as an allergen calibration standard peptide for absolute quantification of the isoallergen, and optionally identification.

Figure 1a

1

60

Der p 2 1A9V -----SQVDVKDC-----ANHEIK  
 Der p 2 1KTJ\_B -----SEVDVKDC-----ANHEIK  
 Der p 2 AAF86462 -MYKICLSLLLVAAVARDQVDVKDC-----ANHEIK  
 Der p 2 P49278 MMYKICLSLLLVAAVARDQVDVKDC-----ANHEIK  
 Lep d 2 2118249A -MMKFTALFALVAVASAGKMTFKDCG-----HGEMT  
 Lep d 2 CAA57160 -----  
 Lep d 2 CAA61419 -MMKFTALFALVAVASAGKMTFKDCG-----HGEMT  
 Lep d 2 CAD32313 -MMKFTALFALVAVASAGKMTFKDCG-----HGEMT  
 Lep d 2 118249B -MMKFTALFALVAVASAGKMTFKDCG-----HGEMT  
 Lep d 2 CAA58755 -MMKFTALFALVAVASAGKMTFKDCG-----HGEMT  
 Lep d 2 CAD32314 -MMKFTALFALVAVASAGKMTFKDCG-----HGEMT  
 Lep d 2 CAB76459 -----GKMTFKDCG-----KGEMT  
 Lep d 2 CAB59976 -----GKMTNFTDCG-----HNEIK  
 Pso o 2 AAK61827 MMKTLVLAITAVVSAGKVKFQDCG-----KGEVE  
 Tyr p 2 CAA73221 --MKFLLFALVAVAAAGQVKFTDCG-----KKEIA  
 Eur m 2 AAC82349 -MYKICLSLLLVAAVAADQVDIKKDC-----ANHEIK  
 Eur m 2 AAC82350 -----VAAVAADQVDVKDC-----ANHEIK  
 Der f 2 CAI05848 MISKICLSLLLVAAVVADQVDVKDC-----ANHEIK  
 Der f 2 CAI05850 MISKICLSLLLVAAVVADQVDVKDC-----ANNEIK  
 Der f 2 CAI05849 MISKICLSLLLVAAVVADQVDVKDC-----ANNEIK  
 Der f 2 1WRF\_A -----DQVDVKDC-----ANNEIK  
 Der f 2 AAB30829 ----GTMMTSLLLVAAVVADQVDVKDC-----ANNEIK  
 Der f 2 A61241 -----SLLVAAVVADQVDVKDC-----ANNEIK  
 Der f 2 JU0394 -----DQVDVKDC-----ANNEIK  
 Der f 2 Q00855 MISKICLSLLLVAAVVADQVDVKDC-----ANNEIK  
 Der f 2 BAD74060 MISKICLSLLLVAAVVADQVDVKDC-----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 KVLVPGCHGSEPCITHRGKPFQLEAVFEANONTKTAKIEIKASTDG-LEVDVPGIDPNAC  
 Der p 2 1KTJ\_B KVLVPGCHGSEPCITHRGKPFQLEAVFEANONTKTAKIEIKASTDG-LEVDVPGIDPNAC  
 Der p 2 AAF86462 KVLVPGCHGSEPCITHRGKPFQLEAVFEANONTKTAKIEIKASTDG-LEVDVPGIDPNAC  
 Der p 2 P49278 KVLVPGCHGSEPCITHRGKPFQLEAVFEANONTKTAKIEIKASTDG-LEVDVPGIDPNAC  
 Lep d 2 2118249A ELDTSGCSG-DTCVTHRGEKMTLEAKFAANQDTAKVTIKVLAKVAG-TTIQVPGLETDG  
 Lep d 2 CAA57160 -----THRGEKMTLEAKFAANQDTAKVTIKVLAKVAG-TTIQVPGLETDG  
 Lep d 2 CAA61419 ELDTSGCSG-DTCVTHRGEKMTLEAKFAANQDTAKVTIKVLAKVAG-TTIQVPGLETDG  
 Lep d 2 CAD32313 ELDTSGCSG-DTCVTHRGEKMTLEAKFAANQDTAKVTIKVLAKVAG-TTIQVPGLETDG  
 Lep d 2 2118249B ELDTSGCSG-DTCVTHRGQKMTLDAKFAANQDTNKVTIKVLAKVAG-TTIQVPGLETDG  
 Lep d 2 CAA58755 ELDTSGCSG-DTCVTHRGQKMTLDAKFAANQDTNKVTIKVLAKVAG-TTIQVPGLETDG  
 Lep d 2 CAD32314 ELDTSGCSG-DTCVTHRGQKMTLDAKFAANQDTNKVTIKVLAKVAG-TTIQVPGLETDG  
 Lep d 2 CAB76459 ELDTDCSG-DFCVTHRGKPLTLEAKFAANQDTTKATIKVLAKVAG-TPIQVPGLETDG  
 Lep d 2 CAB59976 ELSVSNCTG-NYCVTHRGKPLTLDAKFDANQDTASVGLVITAIDGDIAIDIPGLETNAC  
 Pso o 2 AAK61827 SLEVEGCSG-DYCVTHKGKLDLAISVTSNQDSANLKIDIVADING-VQIEVPGVDHDDG  
 Tyr p 2 CAA73221 SVAVDGCEG-DLCVTHSKPVHIAEFTANQDTCKIEVKVTGOING-LEVPIPGLETDG  
 Eur m 2 AAC82349 KVMVPGCKGSEPCITHRGTAFQLEAVFDANQSNAAKIEIKATTDG-VEIDVPGIDNNLC

Eur m 2 AAC82350 KVMVPGCKGSEPCV<sup>1</sup>IHRGTAFQLEAV<sup>1</sup>FDANQNSNAAKIEIKATIDG<sup>1</sup>-VEIDVPGIDNNLC<sup>1</sup>  
Der f 2 CAI05848 K<sup>1</sup>VMVDGCHGSDPCI<sup>1</sup>IHR<sup>1</sup>GKPF<sup>1</sup>NLEA<sup>1</sup>IFDANQNTKTAKIEIKANIDG<sup>1</sup>-LEVDVPGIDTNA<sup>1</sup>C<sup>1</sup>  
Der f 2 CAI05850 K<sup>1</sup>VMVDGCHGSDPCI<sup>1</sup>IHR<sup>1</sup>GKPF<sup>1</sup>TLEA<sup>1</sup>IFDANQNTKTAKIEIKANIDG<sup>1</sup>-LEVDVPGIDTNA<sup>1</sup>C<sup>1</sup>  
Der f 2 CAI05849 K<sup>1</sup>VMVDGCHGSDPCI<sup>1</sup>IHR<sup>1</sup>GKPF<sup>1</sup>TLEA<sup>1</sup>IFDANQNTKTAKIEIKANING<sup>1</sup>-LEVDVPGIDTNA<sup>1</sup>C<sup>1</sup>  
Der f 2 1WRF\_A K<sup>1</sup>VMVDGCHGSDPCI<sup>1</sup>IHR<sup>1</sup>GKPF<sup>1</sup>TLEA<sup>1</sup>IFDANQNTKTAKIEIKASLDG<sup>1</sup>-LEIDVPGIDTNA<sup>1</sup>C<sup>1</sup>  
Der f 2 AAB30829 K<sup>1</sup>VMVDGCHGSDPCI<sup>1</sup>IHR<sup>1</sup>GKPF<sup>1</sup>TLEA<sup>1</sup>IFDANQNTKTAKIEIKASLDG<sup>1</sup>-LEIDVPGIDTNA<sup>1</sup>C<sup>1</sup>  
Der f 2 A61241 K<sup>1</sup>VMVDGCHGSDPCI<sup>1</sup>IHR<sup>1</sup>GKPF<sup>1</sup>TLEA<sup>1</sup>IFDANQNTKTAKIEIKASLDG<sup>1</sup>-LEIDVPGIDTNA<sup>1</sup>C<sup>1</sup>  
Der f 2 JU0394 K<sup>1</sup>VMVDGCHGSDPCI<sup>1</sup>IHR<sup>1</sup>GKPF<sup>1</sup>TLEA<sup>1</sup>IFDANQNTKTAKIEIKASLDG<sup>1</sup>-LEIDVPGIDTNA<sup>1</sup>C<sup>1</sup>  
Der f 2 Q00855 K<sup>1</sup>VMVDGCHGSDPCI<sup>1</sup>IHR<sup>1</sup>GKPF<sup>1</sup>TLEA<sup>1</sup>IFDANQNTKTAKIEIKASLDG<sup>1</sup>-LEIDVPGIDTNA<sup>1</sup>C<sup>1</sup>  
Der f 2 BAD74060 K<sup>1</sup>VMVDGCHGSDPCI<sup>1</sup>IHR<sup>1</sup>GKPF<sup>1</sup>TLEA<sup>1</sup>IFDANQNTKTAKIEIKASLDG<sup>1</sup>-LEIDVPGIDTNA<sup>1</sup>C<sup>1</sup>  
Der f 2 B61241 K<sup>1</sup>VMVDGCHGSDPCI<sup>1</sup>IHR<sup>1</sup>GKPF<sup>1</sup>TLEA<sup>1</sup>IFDANQNTKTAKIEIKASLDG<sup>1</sup>-LEIDVPGIDTNA<sup>1</sup>C<sup>1</sup>  
Der f 2 1XWV\_B K<sup>1</sup>VMVDGCHGSDPCI<sup>1</sup>IHR<sup>1</sup>GKPF<sup>1</sup>TLEA<sup>1</sup>IFDANQNTKTAKIEIKASLDG<sup>1</sup>-LEIDVPGIDTNA<sup>1</sup>C<sup>1</sup>  
Der f 2 A61501 K<sup>1</sup>VMVDGCHGSDPCI<sup>1</sup>IHR<sup>1</sup>GKPF<sup>1</sup>TLEA<sup>1</sup>IFDANQNTKTAKTEIKASLDG<sup>1</sup>-LEIDVPGIDTNA<sup>1</sup>C<sup>1</sup>  
Der f 2 AAL47677 K<sup>1</sup>VMVDGCHGSDPCI<sup>1</sup>IHR<sup>1</sup>GKPF<sup>1</sup>TLEA<sup>1</sup>IFDANQNTKTAKIEIKASLDG<sup>1</sup>-LEIDVPGIDTNA<sup>1</sup>C<sup>1</sup>

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Der p 2 1A9V HYMKCELVKGGQOYDI<sup>1</sup>KY<sup>1</sup>TWNVPKIAPKSENVVVTVKVMGDDGVLA<sup>1</sup>CAIATHAKIRD<sup>1</sup>  
Der p 2 1KTJ\_B HYMKCELVKGGQOYDI<sup>1</sup>KY<sup>1</sup>TWNVPKIAPKSENVVVTVKVMGDDGVLA<sup>1</sup>CAIATHAKIRD<sup>1</sup>  
Der p 2 AAF86462 HYMKCELVKGGQOYDI<sup>1</sup>KY<sup>1</sup>TWNVPKIAPKSENVVVTVKVMGDDGVLA<sup>1</sup>CAIATHAKIRD<sup>1</sup>  
Der p 2 P49278 HYMKCELVKGGQOYDI<sup>1</sup>KY<sup>1</sup>TWNVPKIAPKSENVVVTVKVMGDDGVLA<sup>1</sup>CAIATHAKIRD<sup>1</sup>  
Lep d 2 2118249A KFI<sup>1</sup>KCPVKKGEALDFI<sup>1</sup>YSG<sup>1</sup>-TIPAITPKVKADVTAELIGDHGVMA<sup>1</sup>CGTVHGQVE--  
Lep d 2 CAA57160 KFI<sup>1</sup>KCPVKKGEALDFI<sup>1</sup>YSG<sup>1</sup>-TIPAITPKVKADVTAELIGDHGVMA<sup>1</sup>CGTVHGQVE--  
Lep d 2 CAA61419 KFI<sup>1</sup>KCPVKKGEALDFI<sup>1</sup>YSG<sup>1</sup>-TIPAITPKVKADVTAELIGDHGVMA<sup>1</sup>CGTVHGQVE--  
Lep d 2 CAD32313 KFI<sup>1</sup>KCPVKKGEALDFI<sup>1</sup>YSG<sup>1</sup>-TIPAITPKVKADVTAELIGDHGVMA<sup>1</sup>CGTVHGQVE--  
Lep d 2 2118249B KVL<sup>1</sup>KCPVKKGEALDFN<sup>1</sup>YGM<sup>1</sup>-TIPAITPKIKADVTAELVGDHGVMA<sup>1</sup>CGTIIHGQVE--  
Lep d 2 CAA58755 KVL<sup>1</sup>KCPVKKGEALDFN<sup>1</sup>YGM<sup>1</sup>-TIPAITPKIKADVTAELVGDHGVMA<sup>1</sup>CGTIIHGQVE--  
Lep d 2 CAD32314 KVL<sup>1</sup>KCPVKKGEALDFN<sup>1</sup>YGM<sup>1</sup>-TIPAITPKIKADVTAELVGDHGVMA<sup>1</sup>CGTIIHGQVE--  
Lep d 2 CAB76459 KFI<sup>1</sup>KCPVKKGEALDFI<sup>1</sup>YSG<sup>1</sup>-TIPAITPKVKADVTAELVGDHGVMA<sup>1</sup>CGTVHGQVE--  
Lep d 2 CAB59976 KLMKCELVKGGQOYDI<sup>1</sup>KY<sup>1</sup>TWNVPKIAPKSENVVVTVKVMGDDGVLA<sup>1</sup>CAIATHAKIRD<sup>1</sup>  
Pso o 2 AAK61827 HYVKCELVKGGQOYDI<sup>1</sup>KY<sup>1</sup>TWNVPKIAPKSENVVVTVKVMGDDGVLA<sup>1</sup>CAIATHAKIRD<sup>1</sup>  
Tyr p 2 CAA73221 KVL<sup>1</sup>KCPVKKGEALDFI<sup>1</sup>YSG<sup>1</sup>-TIPAITPKVKADVTAELVGDHGVMA<sup>1</sup>CGTVHGQVE--  
Eur m 2 AAC82349 HFMKCELVKGGQOYDI<sup>1</sup>KY<sup>1</sup>TWNVPKIAPKSENVVVTVKVMGDDGVLA<sup>1</sup>CAIATHAKIRD<sup>1</sup>  
Eur m 2 AAC82350 HFMKCELVKGGQOYDI<sup>1</sup>KY<sup>1</sup>TWNVPKIAPKSENVVVTVKVMGDDGVLA<sup>1</sup>CAIATHAKIRD<sup>1</sup>  
Der f 2 CAI05848 HYIKCELVKGGQOYDAKY<sup>1</sup>TWNVPKIAPKSENVVVTVKVMGDDGVLA<sup>1</sup>CAIATHAKIRD<sup>1</sup>  
Der f 2 CAI05850 HYIKCELVKGGQOYDAKY<sup>1</sup>TWNVPKIAPKSENVVVTVKVMGDDGVLA<sup>1</sup>CAIATHAKIRD<sup>1</sup>  
Der f 2 CAI05849 HYIKCELVKGGQOYDAKY<sup>1</sup>TWNVPKIAPKSENVVVTVKVMGDDGVLA<sup>1</sup>CAIATHAKIRD<sup>1</sup>  
Der f 2 1WRF\_A HFV<sup>1</sup>KCELVKGGQOYDI<sup>1</sup>KY<sup>1</sup>TWNVPKIAPKSENVVVTVKVMGDDGVLA<sup>1</sup>CAIATHAKIRD<sup>1</sup>  
Der f 2 AAB30829 HFV<sup>1</sup>KCELVKGGQOYDI<sup>1</sup>KY<sup>1</sup>TWNVPKIAPKSENVVVTVKVMGDDGVLA<sup>1</sup>CAIATHAKIRD<sup>1</sup>  
Der f 2 A61241 HFV<sup>1</sup>KCELVKGGQOYDI<sup>1</sup>KY<sup>1</sup>TWNVPKIAPKSENVVVTVKVMGDDGVLA<sup>1</sup>CAIATHAKIRD<sup>1</sup>  
Der f 2 JU0394 HFMKCELVKGGQOYDI<sup>1</sup>KY<sup>1</sup>TWNVPKIAPKSENVVVTVKVMGDDGVLA<sup>1</sup>CAIATHAKIRD<sup>1</sup>  
Der f 2 Q00855 HFMKCELVKGGQOYDI<sup>1</sup>KY<sup>1</sup>TWNVPKIAPKSENVVVTVKVMGDDGVLA<sup>1</sup>CAIATHAKIRD<sup>1</sup>  
Der f 2 BAD74060 HFMKCELVKGGQOYDI<sup>1</sup>KY<sup>1</sup>TWNVPKIAPKSENVVVTVKVMGDDGVLA<sup>1</sup>CAIATHAKIRD<sup>1</sup>  
Der f 2 B61241 HFMKCELVKGGQOYDAKY<sup>1</sup>TWNVPKIAPKSENVVVTVKVMGDDGVLA<sup>1</sup>CAIATHAKIRD<sup>1</sup>  
Der f 2 1XWV\_B HFMKCELVKGGQOYDAKY<sup>1</sup>TWNVPKIAPKSENVVVTVKVMGDDGVLA<sup>1</sup>CAIATHAKIRD<sup>1</sup>  
Der f 2 A61501 HFMKCELVKGGQOYDAKY<sup>1</sup>TWNVPKIAPKSENVVVTVKVMGDDGVLA<sup>1</sup>CAIATHAKIRD<sup>1</sup>  
Der f 2 AAL47677 HFMKCELVKGGQOYDAKY<sup>1</sup>TWNVPKIAPSENVVVTVKVMGDDGVLA<sup>1</sup>CAIATHAKIRD<sup>1</sup>

Figure 1a (contd.)

Figure 1b

1 60

Bet v 1 P15494 (1) MGVFNYETETTSVIPAAARLFKAFILDGDNLFPKVAPQAISSVENIEGNGGPGTIKKISFF

Bet v 1 P43177 (1) MGVFNYEIEETTSVIPAAARLFKAFILDGDNLVPRKVAPQAISSVENIEGNGGPGTIKKINFF

Bet v 1 P43180 (1) MGVFNYBSETTSVIPAAARLFKAFILEGDNLIPKVAPQAISSVENIEGNGGPGTIKKINFF

Bet v 1 P43185 (1) MGVFNYETEATSVIPAARMFKAFILDGDNLVPRKVAPQAISSVENIEGNGGPGTIKKINFF

Bet v 1 P43176 (1) MGVFNYBSETTSVIPAAARLFKAFILEGDTLIPKVAPQAISSVENIEGNGGPGTIKKITFF

Bet v 1 P43184 (1) MGVFNYBSETTSVIPAAARLFKAFILEGDTLIPKVAPQAISSVENIEGNGGPGTIKKITFF

Bet v 1 P45431 (1) MGVFNYETETTSVIPAAARLFKAFILEGDTLIPKVAPQAISSVENIEGNGGPGTIKKITFF

Bet v 1 P43186 (1) MGVFNYBSETTSVIPAAARLFKAFILDGDNLIPKVAPQAISSVENIEGNGGPGTIKKITFF

Bet v 1 P43178 (1) MGVFNYETEATSVIPAARLFKAFILDGDNLFPKVAPQAISSVENIEGNGGPGTIKKISFF

Bet v 1 P43179 (1) MGVFNYEIEATSVIPAARLFKAFILDGDNLFPKVAPQAISSVENIEGNGGPGTIKKISFF

Bet v 1 P43183 (1) MGVFNYETEATSVIPAARLFKAFILDGDNLFPKVAPQAISSVENIEGNGGPGTIKKISFF

61 120

Bet v 1 P15494 (61) EGFPFKYVKDRVDEVDHTNFKYNYSVIEGGPVGDTLEKTSNEIKIVATPDGGSILKTSNK

Bet v 1 P43177 (61) EGFPFKYVKDRVDEVDHTNFKYNYSVIEGGPVGDTLEKTSNEIKIVATPDGGSILKTSNK

Bet v 1 P43180 (61) EGFPFKYVKDRVDEVDHTNFKYNYSVIEGGPVGDTLEKTSNEIKIVATPDGGSILKTSNK

Bet v 1 P43185 (61) EGFPFKYVKDRVDEVDHTNFKYNYSVIEGGPVGDTLEKTSNEIKIVATPDGGSILKTSNK

Bet v 1 P43176 (61) EGSPFKYVKERVDEVDHANFKYSYSMIEGGAIGDTLEKTCNEIKIVATPDGGSILKTSNK

Bet v 1 P43184 (61) EGSPFKYVKERVDEVDHANFKYSYSMIEGGAIGDTLEKTCNEIKIVATPDGGSILKTSNK

Bet v 1 P45431 (61) EGSPFKYVKERVDEVDHANFKYSYSMIEGGAIGDTLEKTCNEIKIVATPDGGSILKTSNK

Bet v 1 P43186 (61) EGSPFKYVKERVDEVDHANFKYSYSMIEGGAIGDTLEKTCNEIKIVATPDGGSILKTSNK

Bet v 1 P43178 (61) EGIPFKYVKGRVDEVDHTNFKYSYSVIEGGPVGDTLEKTSNEIKIVATPDGGSILKTSNK

Bet v 1 P43179 (61) EGFPFKYVKDRVDEVDHTNFKYSYSVIEGGPVGDTLEKTSNEIKIVATPDGGSILKTSNK

Bet v 1 P43183 (61) EGFPFKYVKDRVDEVDHTNFKYSYSVIEGGPVGDTLEKTSNEIKIVATPDGGSILKTSNK

121 160

Bet v 1 P15494 (121) YHTKGDHEVKAEQVKASKEMGETLLRAVESYLLAHS DAYN

Bet v 1 P43177 (121) YHTKGNHEVKAEQVKASKEMGETLLRAVESYLLAHS DAYN

Bet v 1 P43180 (121) YHTKGNHEVKAEQVKASKEMGETLLRAVESYLLAHS DAYN

Bet v 1 P43185 (121) YHTKGNHEVKAEQVKASKEMGETLLRAVESYLLAHS DAYN

Bet v 1 P43176 (121) YHTKGDQEMKAEHMKAIKEKGEALLRAVESYLLAHS DAYN

Bet v 1 P43184 (121) YHTKGDHEMKAHEHMKAIKEKGEALLRAVESYLLAHS DAYN

Bet v 1 P45431 (121) YHTKGDHEMKAHEHMKAIKEKGEALLRAVESYLLAHS DAYN

Bet v 1 P43186 (121) YHTKGDHEMKAHEHMKAIKEKGEALLRAVESYLLAHS DAYN

Bet v 1 P43178 (121) YHTKGDHEVKAEQIKASKEMGETLLRAVESYLLAHS DAYN

Bet v 1 P43179 (121) YHTKGDHEVKAEQIKASKEMGETLLRAVESYLLAHS DAYN

Bet v 1 P43183 (121) YHTKGDHEVKAEQIKASKEMGETLLRAVESYLLAHS DAYN

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	VMVDGCHGSDPCIHR
<b>5</b>	<b>32-48</b>	<b>1893.97</b>	<b>6.99</b>	<b>GKPFTLEALFDANQNTK</b>
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	YTNWVVK
12	97-100	428.29	10.15	IAPK
13	101-109	974.55	6.97	SENVVTVK
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	VLVPGCHGSEPCIHR
<b>5</b>	<b>32-48</b>	<b>1920.98</b>	<b>7.00</b>	<b>GKPFQLEAVFEANQNTK</b>
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	YTNWVVK
12	97-100	428.29	10.15	IAPK
13	101-109	974.55	6.97	SENVVTVK
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	VPPGNITATYGDK
3	41-45	632.34	6.98	WLDK
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
				SGIAFGSMK
10	141-141	147.11	9.47	K
11	142-146	576.26	4.11	GDEQK
12	147-148	288.20	11.10	LR

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<b>13</b>	<b>149-158</b>	<b>1135.57</b>	<b>4.31</b>	<b>SAGEVEIQFR</b>
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	ADLGYGPATPAAPAAAGYTPATPAAPAGADAAGK
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	GAAESSK
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
<b>14</b>	<b>123-135</b>	<b>1471.74</b>	<b>4.11</b>	<b>YDAYVATLSEALR</b>
15	136-154	1975.12	5.42	IIAGTLEVHAVKPAEEVK
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	FTVFEEAFNDEIK
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	GPGPR

Figure 2 (contd.)

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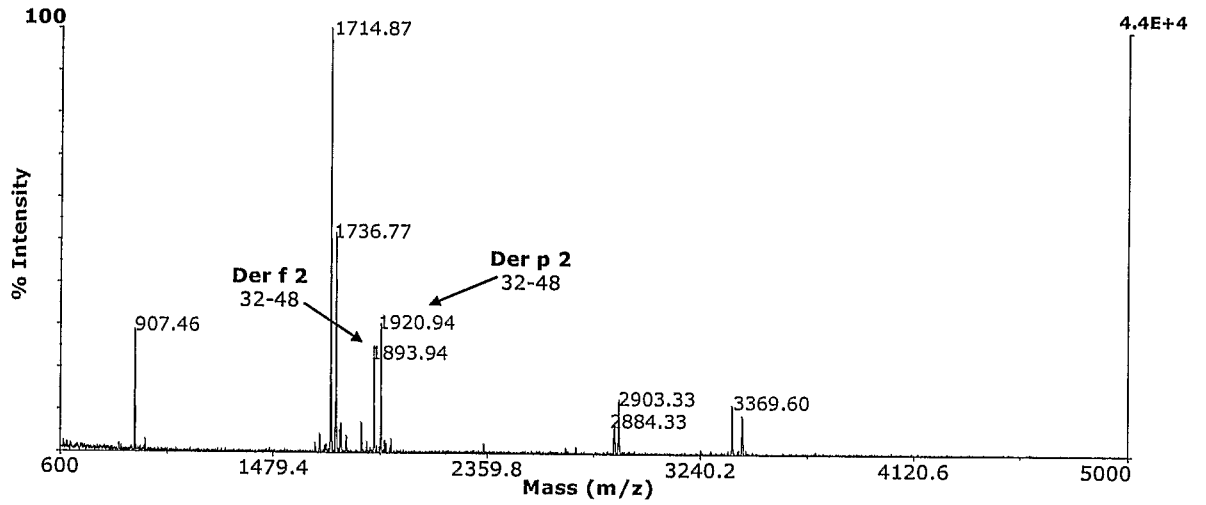
5	17-38	1952.93	6.49	SYTADAGYAPATPAAAGAAAGK
6	39-45	806.38	4.31	ATTEEQK
7	46-55	1147.63	4.11	LIEDINVGFK
8	56-69	1212.65	6.99	AAVAAAASVPAADK
9	70-71	294.18	9.85	FK
10	72-82	1175.55	6.97	TFEAFTSSSK
11	83-87	431.26	10.20	AAAAK
12	88-94	681.42	10.20	APGLVPK
13	95-104	1100.56	6.66	LDAAYSVAYK
14	105-114	914.49	6.99	AAVGATPEAK
<b>15</b>	<b>115-127</b>	<b>1455.74</b>	<b>4.11</b>	<b>FDSFVASLTEALR</b>
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	FTVFEEAFNK
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	YAVFEAALK
26	239-248	1077.56	6.99	AITAMSEVQK
27	249-283	2919.48	9.76	VSQPATGAATVAAGAATTA GAASGAATVAAGGYK
28	284-284	118.08	6.00	V

**f) Tryptic digestion of Bet v 1**

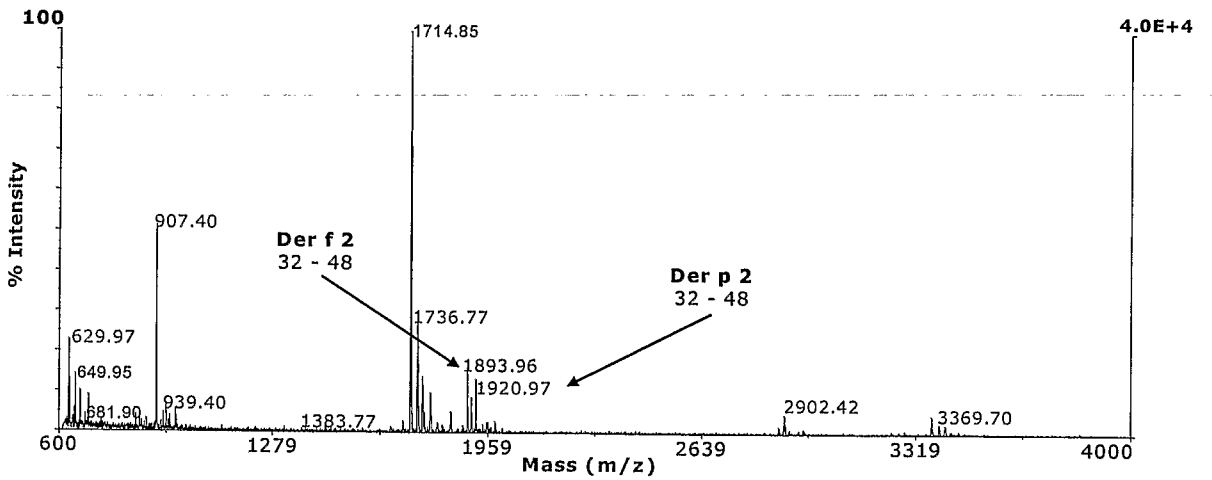
Peptide	From-To	MH+	pI	Sequence
1	1-18	1985.9637	4.31	MGVFNJETETTSVIPAAR
2	19-21	407.2653	10.10	LFK
3	22-33	1349.7100	3.92	AFILDGDNLFPK
4	34-57	2386.2249	4.31	VAPQAISSVENIFTEGNGGPGTIK
5	58-58	147.1128	9.47	K
6	59-68	1134.6194	6.99	ISFPEGLPFK
7	69-71	409.2445	9.49	YVK
8	72-73	290.1459	7.04	DR
9	74-83	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
<b>19</b>	<b>151-164</b>	<b>1552.7278</b>	<b>4.10</b>	<b>AVESYLLAHSDAYN</b>

Figure 2 (contd.)

Figure 3



a)



b)

Figure 4

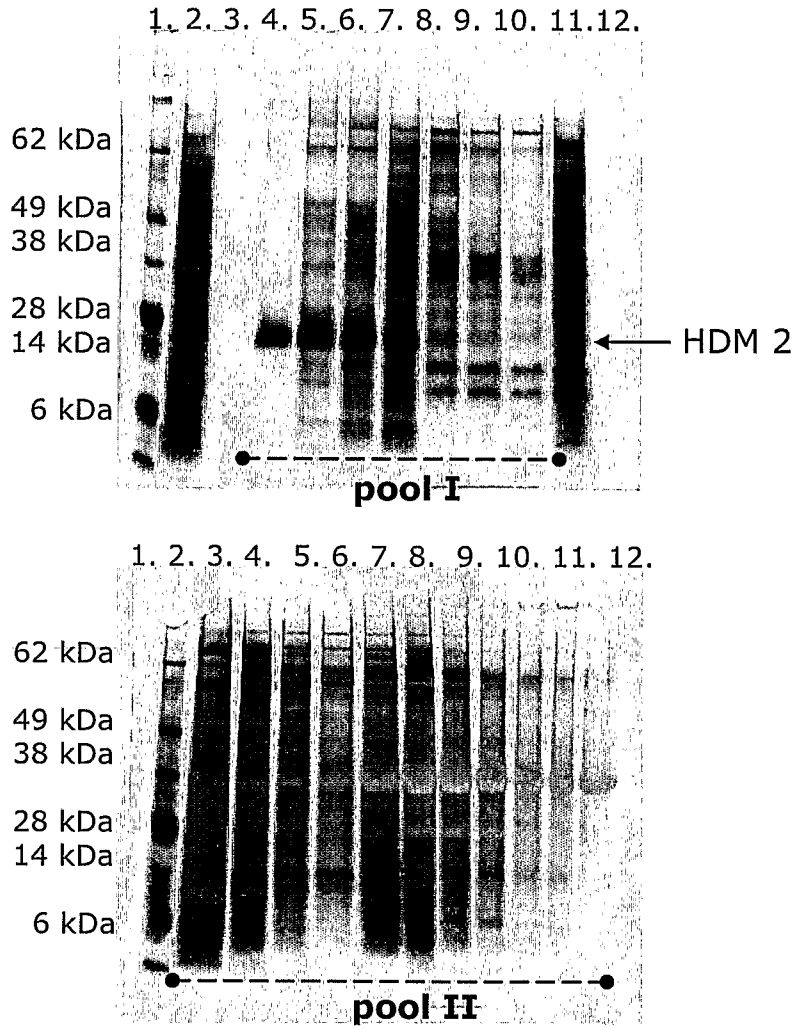
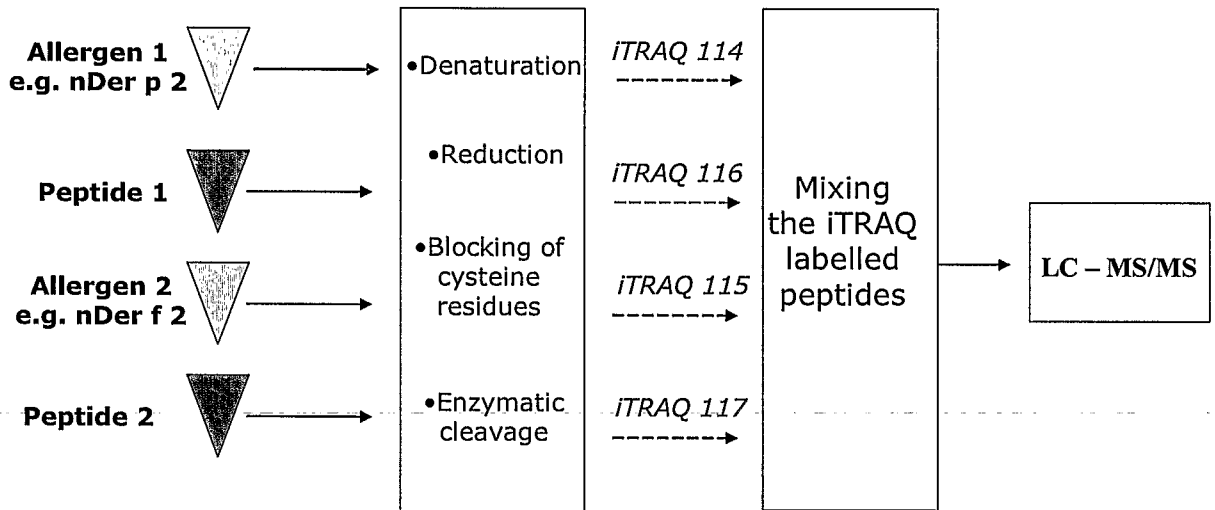


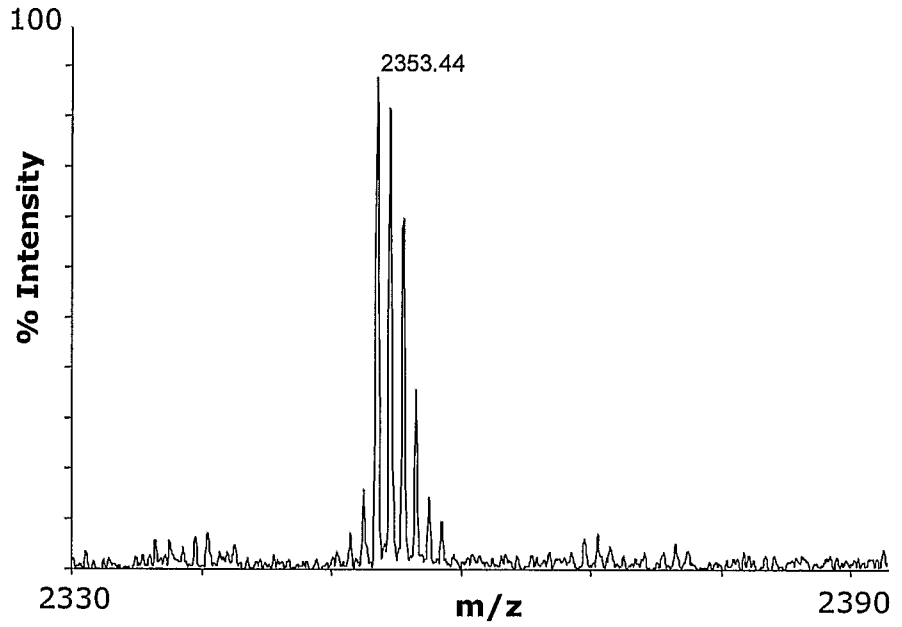
Figure 5

# iTRAQ™ labelling

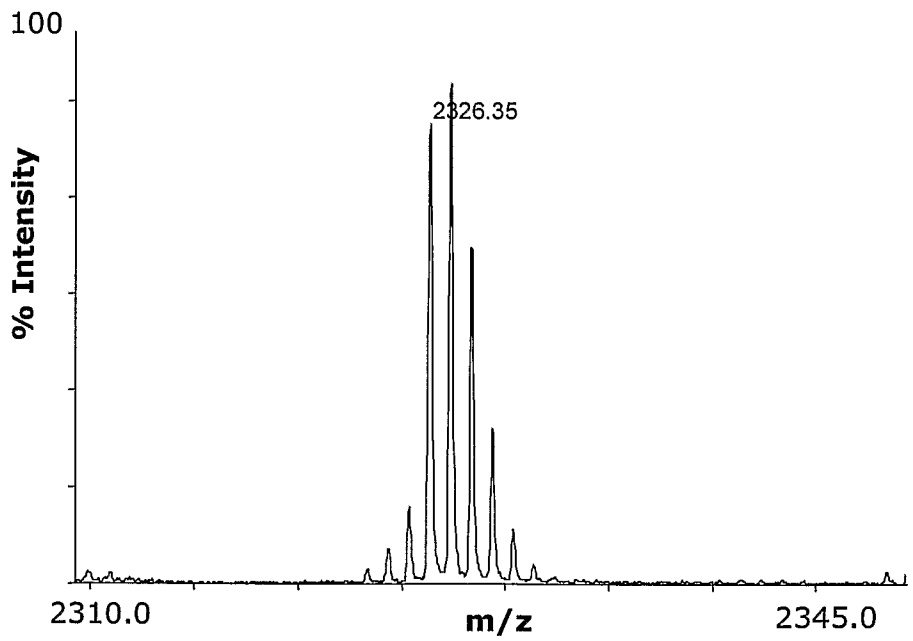


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Figure 6



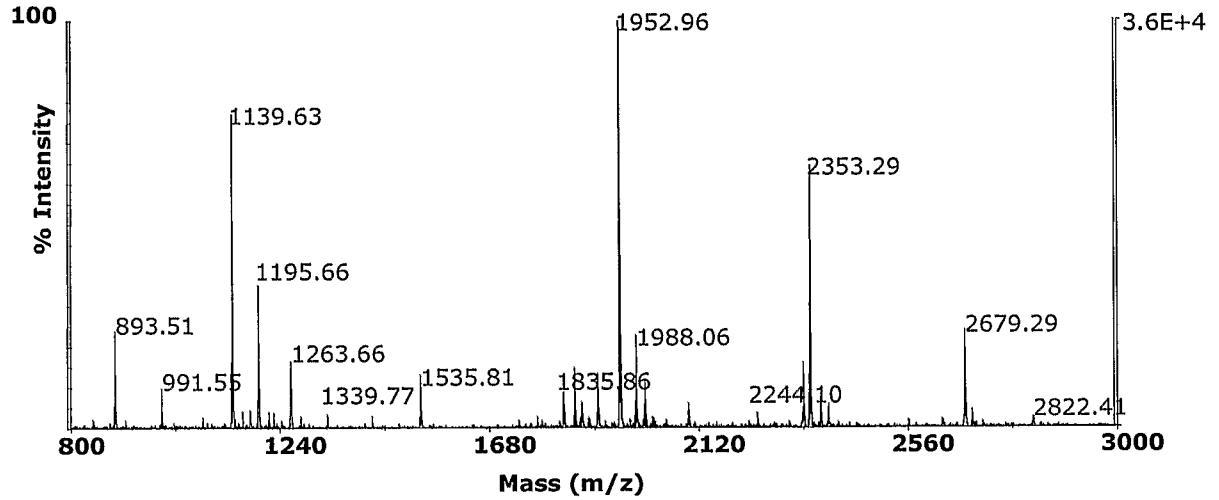
a)



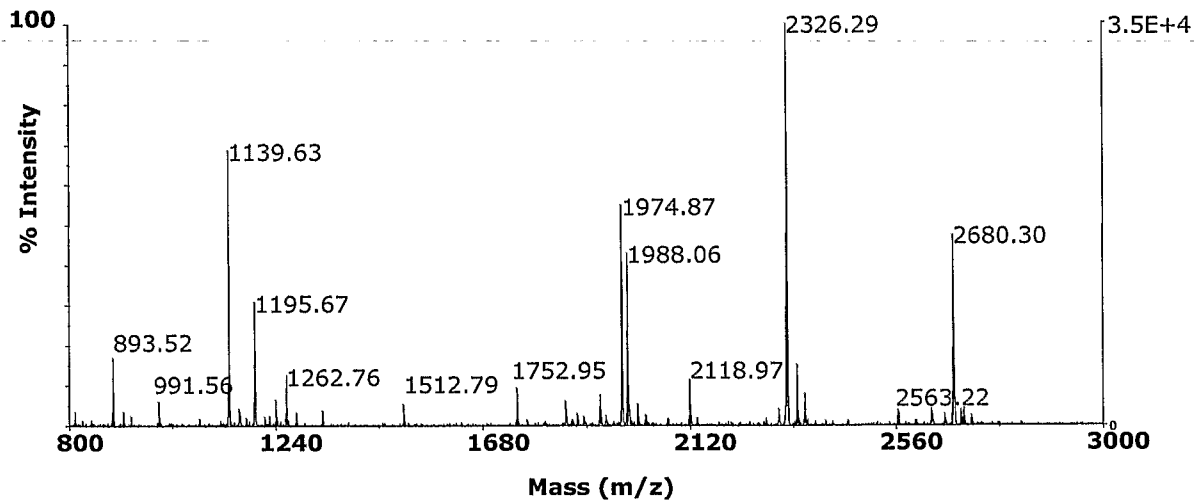
b)

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Figure 6 (cont.)



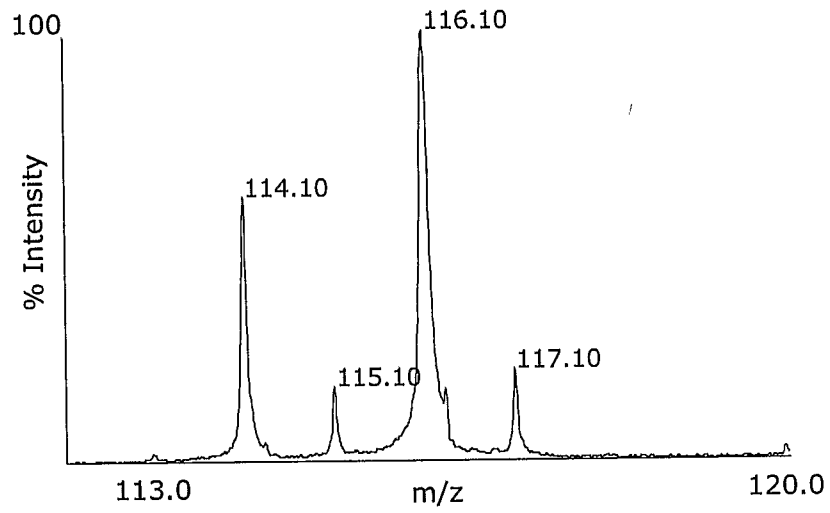
c)



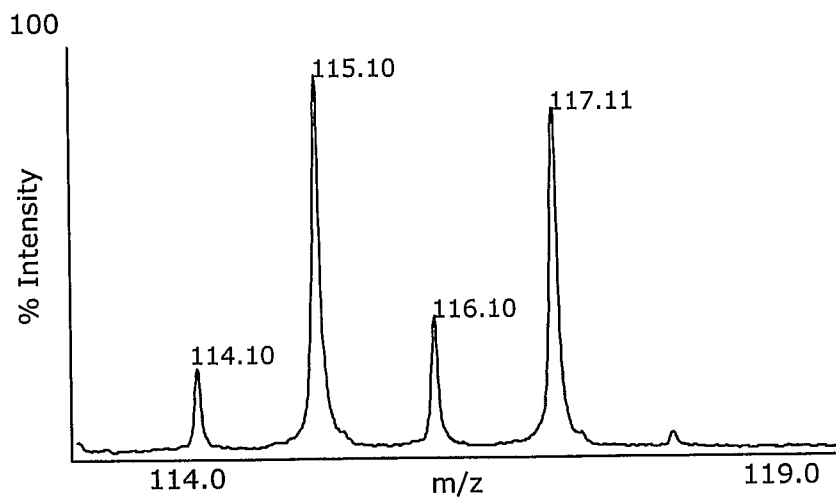
d)

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Figure 7



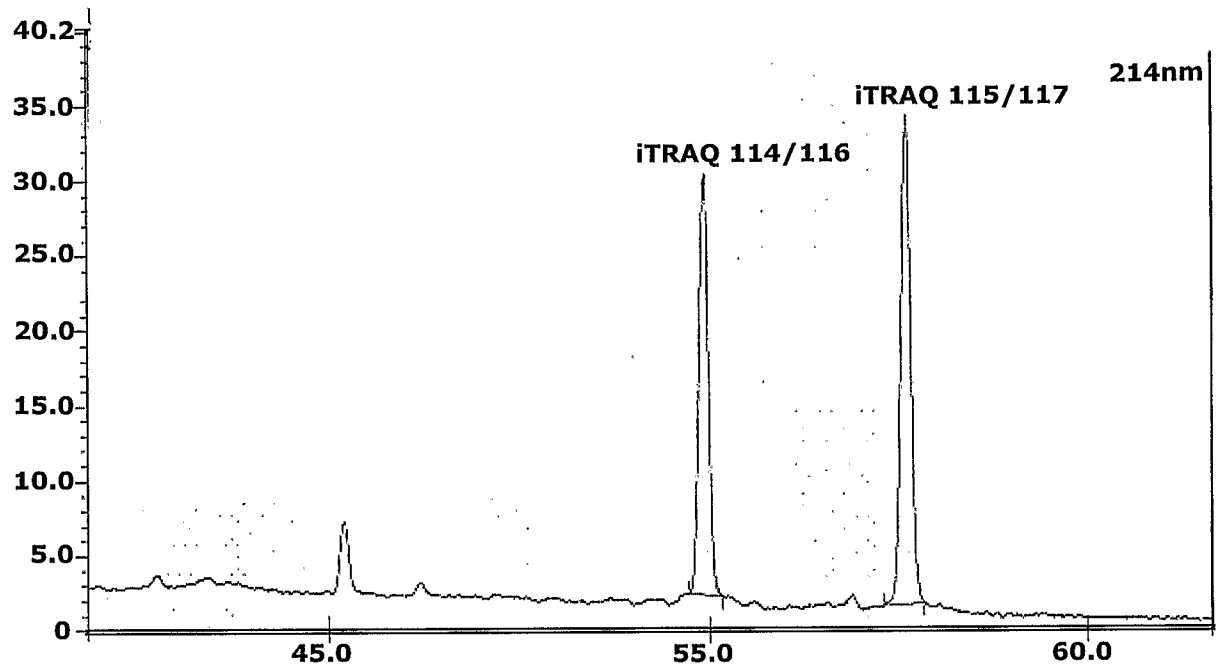
a)



b)

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Figure 8



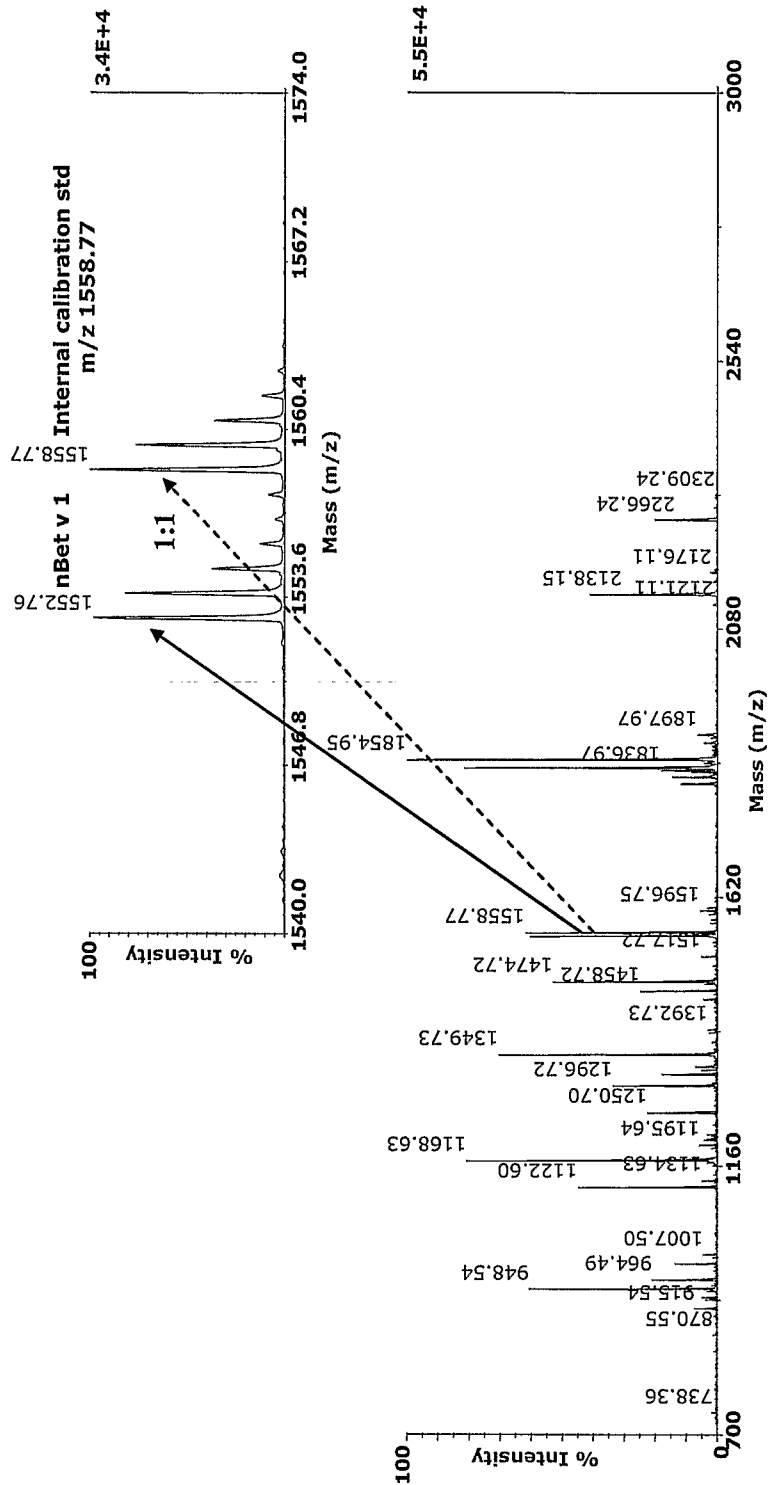


Figure 9

INTERNATIONAL SEARCH REPORT

International application No  
PCT/DK2006/000480

A. CLASSIFICATION OF SUBJECT MATTER  
INV. G01N33/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)  
G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, BIOSIS, EMBASE, WPI Data, PAJ

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	KRISTIANSSON MONICA H ET AL: "Correlations between air levels of hexahydrophthalic anhydride (HHPA) and HHPA-adducted albumin tryptic peptides in nasal lavage fluid from experimentally exposed volunteers" RAPID COMMUNICATIONS IN MASS SPECTROMETRY, vol. 18, no. 14, 2004, pages 1592-1598, XP002374465 ISSN: 0951-4198 the whole document page 1593, column 2 tables 1,3  -----  -/--	1-32

Further documents are listed in the continuation of Box C.

See patent family annex.

\* Special categories of cited documents :

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- "&" document member of the same patent family

Date of the actual completion of the international search  
  
15 November 2006

Date of mailing of the international search report  
  
02.02.2007

Name and mailing address of the ISA/  
European Patent Office, P.B. 5818 Patentlaan 2  
NL - 2280 HV Rijswijk  
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,  
Fax: (+31-70) 340-3016

Authorized officer  
  
Jenkins, Gareth

## INTERNATIONAL SEARCH REPORT

International application No

PCT/DK2006/000480

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>HELSPER JOHANNES P F G ET AL: "Quadrupole time-of-flight mass spectrometry: A method to study the actual expression of allergen isoforms identified by PCR cloning"            JOURNAL OF ALLERGY AND CLINICAL IMMUNOLOGY,            vol. 110, no. 1, July 2002 (2002-07),            pages 131-138, XP002374466            ISSN: 0091-6749            the whole document            figure 4</p>	1-32
A	<p>PATTON WAYNE F ET AL: "Two-dimensional gel electrophoresis; better than a poke in the ICAT?"            CURRENT OPINION IN BIOTECHNOLOGY,            vol. 13, no. 4, August 2002 (2002-08),            pages 321-328, XP002374467            ISSN: 0958-1669            the whole document            page 321, column 1, paragraph 1</p>	1-32
A	<p>NEVEROVA I ET AL: "Role of chromatographic techniques in proteomic analysis"            JOURNAL OF CHROMATOGRAPHY B: BIOMEDICAL SCIENCES &amp; APPLICATIONS, ELSEVIER, AMSTERDAM, NL,            vol. 815, no. 1-2,            5 February 2005 (2005-02-05), pages 51-63,            XP004713818            ISSN: 1570-0232            the whole document            page 62, paragraph 2</p>	1-32
A	<p>US 2004/106146 A1 (PALOSUO TIMO ET AL)            3 June 2004 (2004-06-03)            the whole document            paragraph [0103]</p>	1-32
A	<p>VAN REE RONALD: "Isoallergens: A clinically, relevant phenomenon or just a product of cloning?"            CLINICAL AND EXPERIMENTAL ALLERGY,            vol. 32, no. 7, July 2002 (2002-07), pages 975-978, XP002407470            ISSN: 0954-7894            the whole document</p>	1-32

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## INTERNATIONAL SEARCH REPORT

International application No

PCT/DK2006/000480

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,A	<p>SEPPALA ET AL: "Analysis of N-Glycosylation in Vespula Vulgaris Hyaluronidase Ves v 2" JOURNAL OF ALLERGY AND CLINICAL IMMUNOLOGY, MOSBY - YEARLY BOOK, INC, US, vol. 117, no. 2, February 2006 (2006-02), page S119, XP005359867 ISSN: 0091-6749 the whole document -----</p>	1-32

# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/DK2006/000480

## Box II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1.  Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:
  
2.  Claims Nos.:  
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
  
3.  Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1.  As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
  
2.  As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
  
3.  As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
  
4.  No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1-32

### Remark on Protest

- The additional search fees were accompanied by the applicant's protest.
- No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. claims: 1-32

Method of independent claim 1 relating to quantification of the absolute amount of an allergen in a sample using mass analysis.

---

2. claims: 33-59

Method of independent claim 33 relating to quantification of a specific allergen or isoallergen in a sample using mass analysis.

---

3. claims: 60-61

Method of independent claim 60 relating to obtaining an allergen calibration standard peptide by identifying constant sequences.

---

4. claim: 62

Use of independent claim 62 relating to absolute quantification of an allergen using allergen sequences as an allergen calibration standard.

---

5. claim: 63

Use of independent claim 63 relating to absolute quantification of an allergen using isoallergen sequences as an allergen calibration standard.

---

6. claims: 64-67

Method and uses relating to an allergen calibration standard peptide having a unique sequence.

---

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/DK2006/000480

Patent document cited in search report	Publication date	Patent family member(s)	Publication date	
US 2004106146	A1	03-06-2004	AU 3747101 A	27-08-2001
			EP 1269186 A2	02-01-2003
			WO 0161305 A2	23-08-2001
-----				